MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo — *La Coopération au coeur de la Méditerranée*

Progetto PYRGI
Strategia d'impresa in settori di nicchia per l'economia agroindustriale del Mediterraneo

COMPONENTE 5
COMUNICAZIONE

Prodotto 26. Articoli e Poster scientifici

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

REGIONE LIGURIA

REGIONE TOSCANA

Collectivité Territoriale de CORSE
Cullettività Territuriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

DESCRIZIONE DEL PRODOTTO

All'interno della componente 5, azione 5.1, sono stati pubblicati 12 articoli scientifici.
Se ne riporta il titolo, gli autori, la rivista di pubblicazione, l'anno e l'abstract riassuntivo (in lingua inglese) relativo a ciascun articolo pubblicato negli anni 2010, 2011, 2012 e 2013.

DESCRIPTION DU PRODUIT

Dans la section 5, action 5.1, 12 articles scientifiques ont été publiés. On présente ci-après le titre, les auteurs, la revue de publication, l'année et un résumé en anglais de chaque article paru dans les années 2010, 2011, 2013 et 2013.

ANNO 2010

1) Poiroux-Gonord F., Bidel L.P.R., Fanciullino A.L., Gautier H., Lauri-Lopez F., Urban L., 2010. Health benefits of vitamins and secondary metabolites of fruits and vegetables and prospects to increase their concentrations by agronomic approaches. Journal of Agricultural and Food Chemistry, 58, 12065–12082; DOI:10.1021/jf1037745 (prodotto 26a.1)

ABSTRACT

Fruits and vegetables (FAVs) are an important part of the human diet and a major source of biologically active substances such as vitamins and secondary metabolites. The consumption of FAVs remains globally insufficient, so it should be encouraged, and it may be useful to propose to consumers FAVs with enhanced concentrations in vitamins and secondary metabolites. There are basically two ways to reach this target: the genetic approach or the environmental approach. This paper provides a comprehensive review of the results that have been obtained so far through purely agronomic approaches and brings them into perspective by comparing them with the achievements of genetic approaches. Although agronomic approaches offer very good perspectives, the existence of variability of responses suggests that the current understanding of the way regulatory and metabolic pathways are controlled needs to be increased. For this purpose, more in-depth study of the interactions existing between factors (light and temperature, for instance, genetic factors × environmental factors), between processes (primary metabolism and ontogeny, for example), and between organs (as there is some evidence that photooxidative stress in leaves affects antioxidant metabolism in fruits) is proposed.

ANNO 2011

1) Poiroux-Gonord F., Fanciullino A.L., Berti L., Urban L., 2011. Effect of fruit load on maturity and carotenoid content of clementine (*Citrus clementina* Hort. ex Tan.) fruits. Wileyonlinelibrary.com; DOI 10.1002/jsfa.5584 (Prodotto 26a.2)

ABSTRACT

Citrus fruits contain many secondary metabolites displaying valuable health properties. There is a lot of interest in enhancing citrus quality traits, especially carotenoid contents, by agronomic approaches. In this study the influence of carbohydrate availability on maturity and quality criteria was investigated in clementine fruits during ripening. Fruiting branches were girdled and defoliated after fruit set to obtain three levels of fruit load:

high (five leaves per fruit), medium (15) and low (30).
Considering the soluble solid content/titratable acidity (SSC/TA) ratio, it was found that fruits of the high and medium fruit load treatments reached maturity 1.5 months later than fruits of the control. At the time of maturity the SSC/TA ratio of fruits of all treatments was about 13.6. At harvest, fruits were 23% smaller and total sugar concentration of the endocarp was 12.6% lower in the high fruit load treatment than in the control. In contrast, the concentrations of organic acids and total carotenoids were 55.4 and 93.0% higher respectively. Total carotenoids were not positively correlated with either soluble sugars or total carbohydrates.

Taken together, the results do not support the common view that carbohydrate availability directly determines carotenoid synthesis by influencing precursor availability.

2) Zappettini S. , Mura E., Grilli M., Preda S., Salamone A., Olivero G., Govoni S., Marchi M., 2011. Different presynaptic nicotinic receptor subtypes modulate in vivo and in vitro the release of glycine in the rat hippocampus. Neurochemistry International 59 (2011) 729–738 (prodotto 26a.3).

ABSTRACT

In the present study, using an in vivo approach (a microdialysis technique associated to HPLC with fluorimetric detection) and in vitro purified hippocampal synaptosomes in superfusion, we investigated the glycinergic transmission in the hippocampus, focusing on the nicotinic control of glycine (GLY) release.
The acute administration of nicotine in vivo was able to evoke endogenous GLY release in the rat hippocampus.
The specific nicotinic agonists PHA-543613 hydrochloride (PHA543613) selective for the α7 nicotinic receptor subtype administered in vivo also elicited GLY release in a similar extent, while the $\alpha4\beta2$ agonist 5-IA85380 dihydrochloride (5IA85380) was less effective. Nicotine elicited GLY overflow also from hippocampal synaptosomes in vitro. This overflow was Ca2+-dependent and inhibited by methyllycaconitine (MLA), but was not modified by dihydro-beta-erythroidine (DHbE, 1 lM). Choline(Ch)-evoked GLY overflow was Ca^{2+} dependent, unaltered in presence of DHbE and blocked by methyllycaconitine (MLA). Additionally, 5IA85380 elicited a GLY overflow, which in turn was Ca^{2+} dependent, was significantly inhibited by DHβE but was unaffected by MLA. The GLY overflow produced by these nicotinic agonists quantitatively resembles that evoked by 9 mM KCl. The effects of a high concentration of 5IA85380 (1 mM), in the presence of 2 μM DHβE, on the release of GLY was also studied comparatively to that on glutamate and aspartate release. The nicotinic agonist 5IA85380 tested at high concentration (1 mM) was able to produce a stimulatory effect of endogenous release of the three amino acids, even in the presence of 2 μM DHβE, indicating the existence of a DHβE resistant, $\alpha4\beta2$ nAChR subtype with a functional role in the modulation of GLY, ASP, and GLU release. Our results show that in the rat hippocampus the release of GLY is, at least in part, of neuronal origin and is modulated by the activation of both α7 and $\alpha4\beta2$ (low and high affinity) nAChR subtypes.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

ANNO 2012

1) Mura E., Zappettini S., Preda S., Biundo F., Lanni C., Grilli M., Cavallero A., Olivero,G., Salamone A., Govoni S., Marchi M., 2012. Dual effect of beta-amyloid on α7 and α4β2 nicotinic receptors controlling the release of glutamate, aspartate and GABA in rat hippocampus. PLoS ONE 7(1): e29661. doi:10.1371/journal.pone.0029661 (Prodotto 26a.4)

ABSTRACT

We previously showed that beta-amyloid (Aβ), a peptide considered as relevant to Alzheimer's Disease, is able to act as a neuromodulator affecting neurotransmitter release in absence of evident sign of neurotoxicity in two different rat
brain areas. In this paper we focused on the hippocampus, a brain area which is sensitive to Alzheimer's Disease pathology, evaluating the effect of Aβ (at different concentrations) on the neurotransmitter release stimulated by the activation of presynaptic cholinergic nicotinic receptors (nAChRs, α4β2 and α7 subtypes). Particularly, we focused on some neurotransmitters that are usually involved in learning and memory: glutamate, aspartate and GABA.
We used a dual approach: in vivo experiments (microdialysis technique on freely moving rats) in parallel to in vitro experiments (isolated nerve endings derived from rat hippocampus). Both *in vivo* and *in vitro* the administration of nicotine stimulated an overflow of aspartate, glutamate and GABA. This effect was greatly inhibited by the highest concentrations of Aβ considered (10 μM *in vivo* and 100 nM *in vitro*). In vivo administration of 100 nM Aβ (the lowest concentration considered) potentiated the GABA overflow evoked by nicotine. All these effects were specific for Ab and for nicotinic secretory stimuli. The in vitro administration of either choline or 5-Iodo-A-85380 dihydrochloride (a7 and a4b2 nAChRs selective agonists, respectively) elicited the hippocampal release of aspartate, glutamate, and GABA. High Aβ concentrations (100 nM) inhibited the overflow of all three neurotransmitters evoked by both choline and 5-Iodo-A-85380 dihydrochloride. On the contrary, low Aβ concentrations (1 nM and 100 pM) selectively acted on α7 subtypes potentiating the choline-induced release of both aspartate and glutamate, but not the one of GABA.
The results reinforce the concept that Ab has relevant neuromodulatory effects, which may span from facilitation to inhibition of stimulated release depending upon the concentration used.

2) Marchi, M., Zappettini, S., Olivero, G., Pittaluga, A., Grilli, M., 2012. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochemistry International, 60, 622-630 (Prodotto 26a.5)

ABSTRACT
The effect of chronic nicotine treatment on the release of endogenous glutamate (GLU), aspartate (ASP) and GABA evoked in vitro by KCl, 4-aminopyridine (4AP) and nicotinic agonists in synaptosomes of rat hippocampus was investigated. Rats were chronically administered with nicotine bitartrate or saline vehicle each for 14 days using osmotic mini-pumps. Hippocampal synaptosomes were stimulated with KCl, 4AP, nicotine or with choline (Ch) and 5-iodo-A-85380 dihydrochloride (5IA85380). The GLU and ASP overflow evoked by Ch, nicotine, KCl and 4AP were increased in treated animals while the nicotine-evoked GABA overflow was reduced and that evoked by Ch, KCl and 4AP was unaffected. The 5IA85380-evoked overflow of the three aminoacids (AAs) was always reduced. The increase of ASP and GLU overflow evoked by KCl, 4AP or Ch was blocked by DL-threo-b-benzyloxyaspartic acid (DL-TBOA), a carrier transporter inhibitor, and by inhibitors of the Na^+/Ca^{2+} exchangers 2-[[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester (SN-6) and 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R7943).
In conclusion long-term nicotine treatment may selectively increase GLU and ASP overflow elicited by KCl, 4AP and Ch through the activation of a carrier-mediated release mechanism and completely abolished the stimulatory effects of a4b2 nAChRs which modulate the release of all the three AA.

3) Mulas, M. 2012. The myrtle (*Myrtus communis* l.) case: from a wild shrub to a new fruit crop. Acta Hort. (ISHS) 948: 235-242. ISBN: 978-90-66052-56-7 (prodotto 26a.6).

ABSTRACT
Myrtle is an aromatic shrub spontaneously growing in the Mediterranean region. The edible fruits of this plant are widely used to produce a typical liqueur by hydroalcoholic infusion. The myrtle liqueur industry was completely based on the supply of the fruit harvest from wild plants with a consequent risk of selective pressure on the spontaneous species, low quality standardization and difficulties to assure every year an increasing quantity of raw material. In order to avoid the erosion of natural genetic resources a domestication process of the species was carried out starting in 1995. A mass selection of about 130 mother plants was the first step of a research completely performed on the Sardinia island, where the myrtle is a part of the Mediterranean maquis. Accessions were described for the main plant characters and morphology following a special *descriptor list* and only the pure clonal lines obtained by agamic propagation (softwood cuttings) were planted in a repository located in the experimental farm of the University of Sassari in Oristano (Central Western Sardinia). Yield quantity, plant vigor, rooting ability and resistance to nursery management were the main characters evaluated in the first stage of selection. The field observation of candidate selections produced a list of about 40 cultivars completely described and newly tested for fruit production and quality in different localities. Cultivars were compared also for fruit chemical composition and quality of the corresponding liqueurs, as well as for tolerance to the main transplant pathogens and phytoplasms. In vitro propagation of the better cultivars was the strategy adopted to provide nurseries of a great number of plants of few cultivars in the space of 4-5 years. The result was the plantation of about 200 ha that today may mitigate the effects of the harvest of wild fruits with a better quality standardization and yield security, with respect to seasonal meteoric events.

4) Grilli M., Summa M., Salamone A., Olivero G., Zappettini S., Di Prisco S., Feligioni M., Usai C., Pittaluga A., Marchi M., 2012. *In vitro* exposure to nicotine induces endocytosis of presynaptic AMPA receptors modulating dopamine release in rat nucleus accumbens nerve terminals. Neuropharmacology, 63, 916-926 (Prodotto 26a.7).

ABSTRACT

Here we provide functional and immunocytochemical evidence supporting the presence on Nucleus Accumbens (NAc) dopaminergic terminals of cyclothiazide-sensitive, alfa-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA) receptors, which activation causes Ca^{2+}-dependent [^{3}H]dopamine ([^{3}H]DA) exocytosis. These AMPA receptors cross-talk with co-localized nicotinic receptors (nAChRs), as suggested by the finding that in vitro short-term pre-exposure of synaptosomes to 30 μM nicotine caused a significant reduction of both the 30 μM nicotine and the 100 μM AMPA-evoked [3H]DA overflow.

Entrapping pep2-SVKI, a peptide known to compete for the binding of GluA2 subunit to scaffolding proteins involved in AMPA receptor endocytosis, in NAC synaptosomes prevented the nicotine-induced reduction of AMPA-mediated [^{3}H]DA exocytosis, while pep2-SVKE, used as negative control, was inefficacious.

Immunocytochemical studies showed that a significant percentage of NAc terminals were dopaminergic and that most of these terminals also possess GluA2 receptor subunits. Western blot analysis of GluA2 immunoreactivity showed that presynaptic GluA2 proteins in NAc terminals were reduced in nicotine-pretreated synaptosomes when compared to the control. The nACh-AMPA receptor ereceptor interaction was not limited to dopaminergic terminals since nicotine pre-exposure also affected the presynaptic AMPA receptors controlling hippocampal noradrenaline release, but not the presynaptic AMPA receptors controlling GABA and acetylcholine release. These observations could be relevant to the comprehension of the molecular mechanisms at the basis of nicotine rewarding.

5) Zappettini S., Grilli M., Olivero G., Mura E., Preda S., Govoni S., Salamone A., Marchi M., 2012. Beta amyloid differently nicotinic and muscarinic receptor subtypes which stimulate *in vitro* and *in vivo* the release of glycine in the rat hippocampus. Frontiers in Pharmacology, 3, article 146 (Prodotto 27a.8)

ABSTRACT

Using both *in vitro* (hippocampal synaptosomes in superfusion) and *in vivo* (microdialysis) approaches we investigated whether and to what extent β amyloid peptide 1–40 (Aβ 1–40) interferes with the cholinergic modulation of the release of glycine (GLY) in the rat hippocampus.

The nicotine-evoked overflow of endogenous GLY in hippocampal synaptosomes in superfusion was significantly inhibited by Aβ 1–40 (10 nM) while increasing the concentration to 100 nM the inhibitory effect did not further increase. Both the Choline (Ch; α7 agonist; 1mM) and the 5-Iodo-A-85380 dihydrochloride (5IA85380, α4β2 agonist; 10 nM)-evoked GLY overflow were inhibited by Aβ 1–40 at 100 nM but not at 10 nM concentrations. The KCl evoked [H]GLY and [H]Acetylcholine (ACh) overflow were strongly inhibited in presence of oxotremorine; however this inhibitory muscarinic effect was not affected by Aβ 1–40. The effects of Aβ 1–40 on the administration of nicotine, veratridine, 5IA85380, and PHA543613 hydrochloride (PHA543613; a selective agonist of α7 subtypes) on hippocampal endogenous GLY release *in vivo* were also studied. Aβ 1–40 significantly reduced (at 10 μM but not at 1 μM) the nicotine-evoked *in vivo* release of

GLY. Aβ 1–40 (at 10 μM but not at 1 μM) significantly inhibited the PHA543613 (1mM)-elicited GLY overflow while was ineffective on the GLY overflow evoked by 5IA85380 (1mM). Aβ 40–1 (10 μM) did not produce any inhibitory effect on nicotine-evoked GLY overflow both in the *in vitro* and *in vivo* experiments. Our results indicate that (a) the cholinergic modulation of the release of GLY occurs by the activation of both α7 and α4β2 nicotinic ACh receptors (nAChRs) as well as by the activation of inhibitory muscarinic ACh receptors (mAChRs) and (b) Aβ 1–40 can modulate cholinergic evoked GLY release exclusively through the interaction with α7 and the α4β2 nAChR nicotinic receptors but not through mAChR subtypes.

ANNO 2013

1) Pittaluga A., Olivero G., Di Prisco S., Merega E., Bisio A., Romussi G., Grilli M., Marchi, M., 2013. Effects of the neoclerodane Hardwickiic acid on the presynaptic opioid receptors which modulate noradrenaline and dopamine release in mouse central nervous system. Neurochemistry International, 62, 354-359 (Prodotto 26a.9).

ABSTRACT

We have comparatively investigated the effects of Hardwickiic acid and Salvinorin A on the K+-evoked overflow of [^{3}H]noradrenaline ([^{3}H]NA) and [3H]dopamine ([^{3}H]DA) from mouse hippocampal and striatal nerve terminals, respectively. The K^+-evoked overflow of [^{3}H]DA was inhibited in presence of Salvinorin A (100 nM) but not in presence of Hardwickiic acid (100 nM). Hardwickiic acid (100 nM) mimicked Salvinorin A (100 nM) in facilitating K+-evoked hippocampal [^{3}H]NA overflow and the two compounds were almost equipotent. Facilitation of [^{3}H]NA overflow caused by 100 nM Hardwickiic acid was prevented by the κ-opioid receptor (KOR) antagonist norbinaltorphimine (norBNI, 100 nM) and by the selective δ-opioid receptor (DOR) antagonist naltrindole (100 nM), but was not altered by 100 nM D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), a selective l-opioid receptor (MOR) antagonist. We conclude that Hardwickiic acid modulates hippocampal [^{3}H]NA overflow evoked by a mild depolarizing stimulus by acting at presynaptic opioid receptor subtypes.

2) Mulas M., Fadda A., Angioni A., 2013. Effect of maturation and cold storage on the organic acid composition of myrtle fruits. Journal of the Science of Food and Agriculture, 93, 37-44. DOI 10.1002/jsfa.5724 (Prodotto 26a.10)

ABSTRACT

The effect of maturation and senescence on the chemical composition of two myrtle cultivars was studied in mature, overripe and cold-stored fruits in order to find the most appropriate harvesting period and best storage technology for industrial purposes.
After cold storage at 10 °C for 15 days, berry weight loss ranged from 12.5 to 18.4%, with the highest losses in less mature fruits. Titratable acidity decreased during maturation and cold storage in both cultivars. Reducing and total sugars increased during maturation. Anthocyanin concentration increased during maturation but decreased in overripe berries. The major organic acids in myrtle fruits were quinic, malic and gluconic acids. In fresh and cold-stored fruits, malic acid rose to 3 g kg(-1) and decreased thereafter. Quinic acid peaked at 90 or 120 days after bloom and decreased thereafter to reach low concentrations in mature fruits.

Cold storage for 15 days at 10 °C does not affect myrtle fruit quality for liqueur production. Anthocyanin concentration is the best indicator of harvest time for industrial

purposes. Gluconic acid concentration is high in mature, overripe and cold-stored berries. This parameter can be used as a marker of the onset of fruit senescence. Copyright © 2012 Society of Chemical Industry.

3) Summa M., Di Prisco S., Grilli M., Usai C., Marchi M., Pittaluga A., 2013. Presynaptic mGlu7 receptors control GABA release in mouse hippocampus. Neuropharmacology, 66, 215-224 (Prodotto 26a.11).

ABSTRACT

The functional role of presynaptic release-regulating metabotropic glutamate type 7 (mGlu7) receptors in hippocampal GABAergic terminals was investigated. Mouse hippocampal synaptosomes were preloaded with [^{3}H]D-γ-aminobutyric acid ([^{3}H]GABA) and then exposed in superfusion to 12 mM KCl. The K^+-evoked [^{3}H]GABA release was inhibited by the mGlu7 allosteric agonist N,N'-dibenzyhydryl-ethane-1,2-diamine dihydrochloride (AMN082, 0.001e10 mM), as well as by the group III mGlu receptor agonist L-(þ)-2-amino-4-phosphonobutyric acid [(L)-AP4, 0.01e1 mM]. The mGlu8 receptor agonist (S)-3,4-dicarboxyphenylglycine [(S)-3,4-DCPG, 10e100 nM] was ineffective. AMN082 and (L)-AP4-induced effects were recovered by the mGlu7 negative allosteric modulator (NAM) 6-(4-methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one hydrochloride (MMPIP). AMN082 also inhibited in a MMPIP-sensitive manner the Kþ-evoked release of endogenous GABA. AMN082 and the adenylyl cyclase (AC) inhibitor MDL-12,330A reduced [3H]GABA exocytosis in a 8-Br-cAMP-sensitive. AMN082- inhibitory effect was additive to that caused by (☐)baclofen, but insensitive to the GABAB antagonist 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl) phosphinic acid (CGP52432). Conversely, (☐)baclofen-induced inhibition of GABA exocytosiswas insensitive to MMPIP. Finally, the forskolin-evoked [3H]GABA release was reduced by AMN082 or (☐)baclofen but abolished when the two agonists were added concomitantly. Mouse hippocampal synaptosomal plasmamembranes posses mGlu7 receptor proteins; confocal microscopy analysis unveiled that mGlu7 proteins colocalize with syntaxin-1A (Stx-1A), with vesicular GABA transporter (VGAT)-proteins and with GABAB receptor subunit proteins. We propose that presynaptic inhibitory mGlu7 heteroreceptors, negatively coupled to AC-dependent intraterminal pathway, exist in mouse hippocampal GABA-containing terminals, where they colocalize, but do not functionally cross-talk, with GABAB autoreceptors.

4) Pistelli La., Noccioli C., D'Angiolillo F., Pistelli Lu., 2013. Composition of volatile in micropropagated and field grown aromatic plants from Tuscany Islands. Acta Biochimica Polonica, 60, 43-50 (Prodotto 26.a12).

ABSTRACT

Aromatic plant species present in the natural Park of Tuscany Archipelago are used as flavoring agents and spices, as dietary supplements and in cosmetics and aromatherapy. The plants are usually collected from wild stands, inducing a depletion of the natural habitat. Therefore, micropropagation of these aromatic plants can play a role in the protection of the natural ecosystem, can guarantee a massive sustainable production and can provide standardized plant materials for diverse economical purposes. The aim of this study is to compare the volatile organic compounds produced by the wild plants with those from in vitro plantlets using headspace solid phase micro-extraction (HS-SPME) followed by capillary gas-chromatography coupled to mass spectrometry (GC-MS).

Typical plants of this natural area selected for this work were *Calamintha nepeta* L., *Crithmum maritimum* L., *Lavandula angustifolia* L., *Myrtus communis* L., *Rosmarinus officinalis* L., *Salvia officinalis* L. and *Satureja hortensis* L. Different explants were used: microcuttings with vegetative apical parts, axillary buds and internodes. Sterilization percentage, multiplication rate and shoot length, as well as root formation were measured. The volatile aromatic profiles produced from in vitro plantlets were compared with those of the wild plants, in particular for *C. maritimum*, *R. officinalis*, *S. officinalis* and *S. hortensis*. This study indicated that the micropropagation technique can represent a valid alternative to produce massive and sterile plant material characterised by the same aromatic flavour as in the wild grown plants.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a
ARTICOLI SCIENTIFICI

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

REGIONE LIGURIA

REGIONE TOSCANA

Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale

Programme cofinancé par le Fonds Européen de Développement Régional

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.1

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

REGIONE LIGURIA

REGIONE TOSCANA

Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale

Programme cofinancé par le Fonds Européen de Développement Régional

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
REVIEW

J. Agric. Food Chem. 2010, 58, 12065–12082 12065
DOI:10.1021/jf1037745

Health Benefits of Vitamins and Secondary Metabolites of Fruits and Vegetables and Prospects To Increase Their Concentrations by Agronomic Approaches

Florine Poiroux-Gonord,[†] Luc P. R. Bidel,[‡] Anne-Laure Fanciullino,[†] Hélène Gautier,[#] Félicie Lauri-Lopez,[§] and Laurent Urban*,[§]

[†]INRA − Centre de Corse, Unité "Génétique et Ecophysiologie de la Qualité des Agrumes", F-20230 San Giuliano, France, [‡]Laboratoire de Biochimie et Physiologie Végétale (LBPV), Université de Montpellier II, CC024 - Bât 15 − Porte 329, Place Eugène Bataillon, F-34095 Montpellier Cedex 5, France, [#]INRA − Centre d'Avignon, Unité "Plantes et Systèmes Horticoles (PSH)", Site agroparc − Domaine Saint Paul, F-84914 Avignon Cedex 9, France, and [§]Laboratoire de Physiologie des Fruits et Légumes, Université d'Avignon et des Pays du Vaucluse, Bât Agrosciences, 301 rue Baruch de Spinoza, B.P. 21239, F-84916 Avignon Cedex 9, France

Fruits and vegetables (FAVs) are an important part of the human diet and a major source of biologically active substances such as vitamins and secondary metabolites. The consumption of FAVs remains globally insufficient, so it should be encouraged, and it may be useful to propose to consumers FAVs with enhanced concentrations in vitamins and secondary metabolites. There are basically two ways to reach this target: the genetic approach or the environmental approach. This paper provides a comprehensive review of the results that have been obtained so far through purely agronomic approaches and brings them into perspective by comparing them with the achievements of genetic approaches. Although agronomic approaches offer very good perspectives, the existence of variability of responses suggests that the current understanding of the way regulatory and metabolic pathways are controlled needs to be increased. For this purpose, more in-depth study of the interactions existing between factors (light and temperature, for instance, genetic factors × environmental factors), between processes (primary metabolism and ontogeny, for example), and between organs (as there is some evidence that photooxidative stress in leaves affects antioxidant metabolism in fruits) is proposed.

KEYWORDS: Carbohydrates; carotenoids; glucosinolates; maturity; nitrogen; organic farming; phenolic compounds; stress; vitamin C

INTRODUCTION

Plants produce a very diverse set of organic molecules, some of which are traditionally considered not to participate directly in the major processes involved in growth and development. These substances are called secondary metabolites, an arguable term if one considers the importance of, for instance, lignin for all vascular plants or salicylic acid, a hormone. According to the nomenclature adopted by the British Nutrition Foundation, plant secondary metabolites can be divided into four major groups: terpenoids (about 25000 compounds), alkaloids (about 12000 compounds), phenolic compounds (about 8000 compounds), and sulfur-containing compounds (*1*). Unlike primary metabolites, so-called secondary metabolites are often unevenly distributed among taxonomic groups within the plant kingdom. Many secondary metabolites have positive effects on human health. Some of them are even essential to life, as are vitamins (such as tocopherols and tocotrienols, alias vitamin E). However, all vitamins are not considered to be secondary metabolites, like ascorbate, the major antioxidant in plants and for humans. A few other secondary metabolites are provitamins, that is, compounds that are converted into vitamins in animal bodies (such as β-cryptoxanthin, a carotenoid found in *Citrus* fruits, or β-carotene, found in several fruits and vegetables endowed with provitamin A properties). The vitamins and secondary metabolites this paper discusses are referred to by the generic term "phytochemicals" hereafter.

It is well established that fruits and vegetables (FAVs) represent the major source of phytochemicals and other useful compounds such as amino acids and fatty acids (*2*). Indeed, the much praised health benefits of FAVs are, at least partially, attributable to their high concentrations in phytochemicals. Even though no final evidence has been found that FAVs protect against cancer, there is vast consensus with regard to the positive role they play in preventing or controlling particular diseases or disorders (*3*), and it can be globally recommended to most people to substantially increase their consumption of FAVs.

In a report released jointly by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) in 2003, statistics are provided showing that, in 2001, chronic

*Author to whom correspondence should be addressed [phone +33(0)490842214; fax +33(0)490842201; e-mail laurent.urban@univ-avignon.fr].

Published on Web 11/10/2010

diseases contributed approximately 59% of the 56.5 million total reported deaths in the world and 46% of the global burden of disease (*4*). The report commissioned by WHO and FAO recommends increasing the amounts of fresh fruits and vegetables in the diet. At least 400 g, ideally 800 g, of fruits and vegetables should be consumed daily. Although the nutritional benefits of fresh FAVs have been well established for a long time, their consumption remains insufficient. In some developed countries, such as France, despite the numerous campaigns of information organized by the Ministries of Health or growers' organizations, quantities of FAVs bought by consumers even decreased over recent years. In developed countries, it may be tempting for the fruit and vegetable industry to propose to consumers FAVs with increased or guaranteed amounts of micronutrients. This could greatly help the cause of public health while contributing to the competitiveness of the industry in a more and more challenging global market. Similarly, FAVs with high concentrations in micronutrients could potentially help the cause of public health in developing countries where intake is generally in the range of 20−50% of the minimum recommended level, largely due to poverty and food insecurity, lack of nutritional knowledge, and some unfavorable food habits (*4*,*5*). In these countries, women and children, as well as the elderly and those infected with human immunodeficiency virus (HIV), suffer disproportionately because of their relatively higher need for vitamins and minerals while often being discriminated against in terms of food supply and availability. The situation is particularly critical in sub-Saharan Africa (*6*).

With regard to the issue of FAVs with increased concentrations in phytochemicals, several questions need to be answered. Can targets in terms of concentrations be defined, knowing that precise dietary recommendations cannot be currently made in the absence of sufficient understanding about the bioavailability of bioactive compounds, their interactions, their dynamic after ingestion and metabolization, and the variability of responses due to the existence of genetic profiles? If not, does it all the same make sense to try to produce FAVs with increased concentrations in phytochemicals? If the answer to the latter question is a positive one, what are the perspectives of genetic and agronomic approaches, respectively? Can genetic and environmental factors be considered as realistic levers? Is it realistic to imagine that innovative plant materials and growing techniques can supply FAVs with consistently increased concentrations in phytochemicals, without harming other important cropping objectives such as yield? What are the stumbling blocks, and what research needs to be done?

The first objective of this paper is to briefly review the dietary effects of phytochemicals. We shall argue that, even though no targets in terms of concentrations can be set, it is desirable to try to increase them within reasonable limits. We shall then consider the achievements and prospects of genetic approaches, before reviewing extensively the agronomic data accumulated about the effects of environmental factors on the concentrations of phytochemicals in FAVs. The prospects and challenges of agronomic approaches are eventually discussed in the light of some elements about interactions between factors, between processes, and between organs.

This review is focused mainly on vitamins C and E, phenolic compounds, carotenoids, and glucosinolates.

DIETARY EFFECTS OF PHYTOCHEMICALS CURRENTLY FOUND IN FAVs

Unlike carbohydrates, lipids, and proteins, which are hydrolyzed into small assimilable molecules upon ingestion by humans, most vitamins and phenolics are taken up directly and are subject to very weak biochemical modifications. Humans do not have the enzymatic arsenal to substantially modify these molecules after ingestion, which means that they can benefit in turn from the protective properties they have in the plants that synthesize them. On the contrary, glucosinolates and provitamin A carotenoids must be converted in the intestinal tract, into isothiocyanate and vitamin A respectively, before becoming active. Secondary metabolites and vitamins have positive dietary effects as this brief review tends to demonstrate. It must, however, be kept in mind that they may occasionally prove toxic or interact in an antagonistic way. For instance, an antagonistic effect of some flavonoids was observed on ascorbate uptake. The sodium-dependent vitamin C transporter 1 (SVCT1) is inhibited by flavonoids largely found in foods such as quercetin, fisetin, rutin, apigenin, and genistein (*7*,*8*).

Vitamins. Vitamin C, also known as ascorbate, is a vital micronutrient for humans. A lack of vitamin C hampers the activity of a range of enzymes and may lead to scurvy in humans (*9*). Unlike most animals, humans are unable to synthesize their own vitamin C, and they must therefore find it in plants, in particular, fruits and vegetables. In addition to its involvement in the production of collagen, ascorbic acid serves as a cofactor in several vital enzymatic reactions, including those involved in the synthesis of catecholamines, carnitine, and cholesterol, and in the regulation of transcription factors controlling the expression of important genes of the metabolism (*10*). Ascorbic acid is present in three forms: ascorbate, monodehydroascorbate (MDHA), and dehydroascorbate (DHA), which corresponds to the oxidized form of ascorbate. In most cellular functions, ascorbate acts as an electron donor, but it may also act directly to scavenge reactive oxygen species (ROS) generated by cellular metabolism. Due to the role of ascorbate in protecting cells against oxidative stress and the involvement of ROS in neurodegenerative disorders (Alzheimer's and Parkinson's diseases) or inflammatory response (arteriosclerosis), it is strongly suggested that vitamin C could prevent heart, chronic inflammatory, and neurodegenerative diseases (*11*).

Vitamin E (tocopherols and tocotrienols) is present in all cell membranes and plasma lipoproteins, especially in red blood cells of the human body. As the major lipid-soluble chain-breaking antioxidants in humans, vitamin E protects DNA, low-density lipoproteins, and polyunsaturated fatty acids from oxidative damage. It moreover plays a role in hemoglobin biosynthesis, modulation of immune response, and stabilization of the structure of membranes (*12*).

Vitamin K_1 is a liposoluble vitamin, synthesized from phylloquinone by bacteria in the intestinal tract. It plays a positive role in the control of blood clotting, bone formation, and repair. Deficiency of vitamin K_1 may result in hemorrhagic disease in newborn babies, as well as postoperative bleeding, muscle hematomas, and intercranial hemorrhages in adults (*13*). Vitamin K_3 menadione was shown to exhibit cytotoxic activity and inhibit growth of tumors in humans (*14*).

Phenolic Compounds. The specific action of each plant phenolic compound is not easy to assess because only a very small part of them is really absorbed (*15*) and because they moreover potentially undergo transformations. Enterocyte and epathocyte can cleave glycoside moieties. They are responsible for glucuronidation, methylation, and sulfation of flavonoids. First, they protect some major cellular components from oxidation. Many dietary phenolics are antioxidants capable of quenching ROS and toxic free radicals formed from the peroxidation of lipids and, therefore, have anti-inflammatory and antioxidant properties at the body level. Several hydroxycinnamic acid derivatives, for instance, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, and sinapic acid, present strong antioxidant activities by inhibiting lipid oxidation and scavenging ROS (*16*). Flavonoids are known to prevent production of free radicals by chelating iron and copper ions to directly scavenge ROS and toxic free radicals

and to inhibit lipid peroxidation. Production of peroxides and free radicals, which may damage DNA, lipids, and proteins, has been linked to aging, atherosclerosis, cancer, inflammation, and neurodegenerative diseases such as Alzheimer's and Parkinson's. Flavonoids were also demonstrated to protect low-density lipoprotein (LDL) cholesterol from being oxidized, thus preventing the formation of atherosclerotic plaques at the level of the arterial wall. Many dietary flavonoids and hydroxycinnamic acids bind to human serum albumin, the main protein involved in lipid transportation within blood. This cotransport provides an efficient antioxidant protection to lipids. Additionally, when tannins form bond with enzymes, they inhibit part of the lipoxygenase and peroxidase activity, therefore exhibiting antioxidant effects. Chlorogenic acid and caffeic acid inhibit N-nitrosation reaction and prevent the formation of mutagenic and carcinogenic *N*-nitroso compounds (*17*). Without entering into details, it may be said that the range of activities of phenolic compounds encompasses protection against coronary heart diseases, anti-inflammatory effects, and inhibition of the development of cancer cells.

Some phenolics are known to modulate other enzymes of the metabolism (see the review in ref *18*). In particular, some flavonoids, such as quercetin, myricetin, and fisetin, inhibit the intestinal glucose transporter isoform 2 (GLUT2), thus exerting an antiglycemic effect (*7*, *19*). This inhibition is reversible and noncompetitive as far as quercetin is concerned. Chicoric acid has also an antidiabetic effect, but its cellular target has not yet been discovered. Flavonoid fractions of numerous plant extracts have been found to have a hypoglycemic effect that remains to be elucidated. Hydrosoluble tannins such as gallic acid esters and condensed tannins such as catechin polymers and many flavonoids accumulating in plant organs are known to exert an antifeeding effect by binding to the enzymes and other proteins of defoliating insects and other pests.

Flavonoids modulate signaling pathways (*20*). Among signaling roles, isoflavones such as daidzein and genistein have received considerable attention due to their ability to bind to mammalian estrogen receptors (α and β) and mimic estrogen and anti-estrogen actions. They modulate the endocrine system and may exert a preventive role against breast cancer and osteoporosis.

Middleton et al. (*18*) stated that dietary flavonoids, such as quercetin, affect each immune cell line specifically (T cells, B cells, macrophages, NK cells, basophils, mast cells, neutrophils, eosinophils, and platelets). In particular, dietary flavonoids have an antihistaminic action. During allergic reactions, when IgE binds to its specific receptors on the plasma membrane of mast cells and basophils, these cells are induced to produce histamine. Both quercetin and apigenin inhibit anti-IgE-induced histamine release (*21*). Many flavonoids that are inhibitors of histamine release are also good lipoxygenase inhibitors.

Many flavonoids protect plants against their pathogenic bacteria and fungi. Some phenolic acids such as benzoic acid, hydrobenzoic acid, caffeic acid, and vanillic acid possess antifungal and antimicrobial properties (*22*). These properties are useful during postharvest storage and are conserved after assimilation. They may also exhibit antiviral properties by limiting the multiplication of viruses.

Carotenoids. Carotenoids endowed with provitamin A activity are vital components of the human diet. Vitamin A is implicated in hormone synthesis, immune responses, and the regulation of cell growth and differentiation (*13*). It can be produced within certain tissues from carotenoids such as β-cryptoxanthin present in *Citrus* fruits, β-carotene present in carrots, spinach, and sweet potatoes, and α-carotene found in carrots, pumpkin, and red and yellow peppers (*23*). A carotenoid-deficient diet can lead to night blindness and premature death. Carotenoid-rich diets are correlated with a significant reduction in the risk for certain cancers, coronary heart disease, and several degenerative diseases.

Carotenoids have demonstrated anticancer and antimutagenic properties (*24*). Underlying mechanisms are not well understood, but the dietary importance of carotenoids is discussed, at least in part, in terms of antioxidant properties (*13*, *25*). Carotenoids are known for their capacity to efficiently quench $^1O_2$ singlet oxygen by energy transfer (*26*). $^1O_2$ is a particularly active ROS, capable of damaging DNA (*27*) and provoking genetic mutations (*28*). Eventually, $^1O_2$ can damage lipids and membranes (*29*).

Beutner et al. have classified carotenoids on the basis of three criteria: the dependence on the partial pressure of molecular oxygen, the potential for inhibiting the formation of peroxide, and the potential for quenching of $^1O_2$ (*25*). Astaxanthin, a xanthophyll produced by some algae, is an excellent antioxidant capable of quenching free radicals in either their standard or excited form. β-Carotene and lycopene are efficient antioxidants, capable of inhibiting strongly the formation of peroxide. They are prone to degradation after ingestion, but their breakdown product seems to have interesting properties that may explain the cancer preventive activity of these carotenoids (*30*). ζ-Carotene is a poor antioxidant.

When lipophilic antioxidants such as lutein or lycopene are associated with hydrophilic antioxidants such as rutin, a supra-additive protection of low-density lipoprotein occurs (*31*). When rutin is associated with ascorbic acid, a synergetic protection also occurs.

Phytosterols. Phytosterols are found in high amounts in broccoli, Brussels sprouts, cauliflower, and spinach (*32*). They regulate the fluidity and permeability of the phospholipid bilayers of plant membranes (*33*). Certain phytosterols are precursors of brassinosteroids, plant hormones involved in cell division, embryonic development, fertility, and plant growth (*34*). Some sterols are provitamins: upon skin exposure to UV radiation, they may give rise to calciferol, also known as vitamin D_2, which is involved in the absorption of calcium and bone growth. Plant sterols possess, morevoer, cholesterol-lowering properties and play a positive role by decreasing the incidence of cardiovascular diseases. Being structurally similar to cholesterol, they can compete with cholesterol, thus limiting its absorption from fat matrices into the intestinal tract (*35*). Plant sterols have been hypothesized to have anticancer, antiatherosclerosis, anti-inflammation, and antioxidant activities (*36*).

Saponins. Saponins are attributed with cardioprotective, immunomodulatory, antifatigue, and hepatoprotective physiological and pharmacological properties (*37*). Antifungal activity is generally ascribed to the ability of saponins to complex with sterols in fungal membranes, thus causing pore formation and loss of membrane integrity (*38*). They also affect membrane fluidity (*39*). Dietary saponins have been observed to reduce blood cholesterol, stimulate the immune system, and inhibit the growth of cancer cells (*40*). Saponins inhibit active transport by increasing the general permeability of the enterocytes (*41*). Saponins can also form insoluble complexes with minerals such as zinc and iron (*42*).

Glucosinolates. A reduction in the prevalence of certain forms of cancer has been attributed to the anticarcinogenic properties of certain glucosinolates and their breakdown products (*43*). Glucosinolates act by activating enzymes involved in the detoxification of carcinogens and by providing protection against oxidative damage (*44*) [see also the recent review of Traka and Mithen (*45*)]. Certain glucosinolates have been observed to inhibit enzymes involved in the metabolism of steroid hormones.

INCREASING THE CONCENTRATION OF VITAMINS AND SECONDARY METABOLITES OF FAVs

Although there is compelling evidence that vitamins and secondary metabolites are essential for human health, many questions

remain unresolved. Assessing the nutritional benefits of food with enhanced concentrations of specific biologically active substances is not an easy task, which, in turn, makes it impossible to define precise targets in terms of concentrations. Biologically active substances found in FAVs always come as part of a mixture in the diet. In a mixture, metabolites may have potentiating, antagonizing, or synergistic effects (*46*). Moreover, health benefits may be influenced by other ingredients such as dietary fibers, monounsaturated fatty acids, agents stimulating the immune system, minerals, and even ethanol (*47*). Then there is the issue of the so-called bioavailability of biologically active substances, which is affected by several factors such as tannin and lignin concentrations that differ greatly from one species to another. Tannins have antifeeding effects, due to their protein-binding properties, whereas lignin decreases the digestibility of plant material. The bioavailability of β-carotene, for instance, ranges from 47% in kiwi fruits to 2% in red grapefruits (*48*). Besides, it is now established that not all individuals respond identically to bioactive food components because of the existence of genetic profiles that modulate the responses. Finally, very little is known about the dynamics of food components after they are ingested and then metabolized in the body. A major challenge for researchers in the future will consist of working out the best combinations of beneficial components of FAVs according to existing genetic profiles while minimizing antagonistic interactions and determining the duration of exposure and timing. Meanwhile, it is very clear that no precise recommendations in terms of concentrations in phytochemicals can be formulated.

The issue of target concentrations for phytochemicals in FAVs is made even more complicated by the existence of some secondary metabolites that can be toxicants. Not all secondary metabolites are micronutrients. Some are natural food toxicants, such as furanocoumarins, which are found in grapefruit juice. Psoralens can generate hazardous drug interactions (*49*). Moreover, psoralens are recognized as skin photocarcinogens (*50*). It has been hypothesized that the increase in cutaneous melanoma incidence may be attributed to the increase in consumption of grapefruit and orange juices in developed countries (*50*). Similarly, some saponins, such as sapotoxin, can be toxic to humans by causing irritations of the skin and membranes (*40*). Eventually, extreme overconsumption of glucosinolate-rich food can cause inflammation of the mucous membranes of the stomach and disrupt synthesis of the thyroid hormone (*51*).

At this stage, one may conclude, rather hastily, that promoting the consumption of bioactive compounds of fruits and vegetables may be dangerous or, at least, beside the point. Several attitudes may be adopted. The first one consists of waiting for dieticians to come up with more precise recommendations in terms of doses or combinations of doses of bioactive food compounds. This will take some time, perhaps even a lot of time, considering the importance of the scientific challenges created by the issues of the bioavailability, the dynamics and the interactions of bioactive compounds, and the genetic diversity of consumers' responses. Clearly the issue of human health requires urgent measures to be taken. A more down-to-earth attitude consists of considering that enough evidence has been accumulated respectively through epidemiologic and clinical studies, first about the global benefits of FAVs in the human diet and second about the dietary effects of their bioactive compounds, especially vitamins and secondary metabolites. On the basis of such undisputed evidence, even in the absence of precise recommendations, it makes sense to encourage people to consume more FAVs. Unfortunately, the five-a-day campaigns in developed countries to persuade people to eat at least five portions of FAVs every day have proven to be a relative failure so far, and the situation is no better in developing countries. Taking these facts into account, it appears reasonable to try to improve the current situation by encouraging, besides the consumption of FAVs, the consumption of foods and food supplements with enhanced concentrations in phytochemicals, or, as we may say in the case of phenolic compounds at least, restored concentrations when compared to the concentrations that prevailed in the FAVs before centuries of breeding to obtain bigger, less lignified, less astringent, and less indigestible edible plant parts resulted in strongly impoverishing food. Within this view, the proposition to produce FAVs with increased concentrations in useful bioactive compounds makes sense. Prudence demands that potentially toxic secondary metabolites should not be included in studies aiming at designing innovative plant materials or techniques to increase the concentration in phytochemicals of FAVs, or at least that they are not given the priority. Moreover, it may be argued that prudence also demands that targets in terms of concentrations remain in the range of those observed as the consequence of natural genetic variability or of the influence of not-too-extreme variations or levels of environmental factors (note that such a strategy probably excludes nutraceuticals). To throw light on both the issues of the potential of genetic and agronomic approaches (does it work?) and the limits to keep in mind when trying to enhance the concentrations in bioactive compounds (how much is too much?), it is necessary to review the achievements of genetic and agronomic approaches and discuss their respective prospects.

ACHIEVEMENTS AND PROSPECTS OF CONVENTIONAL BREEDING AND METABOLIC ENGINEERING

Our objective is not to review all genetic approaches but to focus on FAVs. See the review of Newell-McGloughlin (*52*) for a broader approach to the issue of nutritionally improved crops. The reader can also refer to the AGBIOS crop database (http://www.agbios.com/dbase.php?action=ShowForm). Moreover, we shall not consider here the suppression of toxic compounds by metabolic engineering (*53*).

There are two basic approaches to modifying a biosynthetic pathway with the objective of increasing the amounts of desirable compounds. It may be tempting either to manipulate the pathway flux or to introduce novel biosynthetic activities from other organisms. Increasing, preventing, or redirecting the flux into or within the pathway may rely on such methods as increasing the levels of identified or suspected rate-limiting biosynthetic enzymes, inhibiting the activity of genes that code for enzymes competing for limited substrate supply, and up- or down-regulation of regulatory factors (*54*).

The experience gained with regard to the flavonoid pathway demonstrates that all of the above-mentioned approaches can be applied successfully to modify the production of plant metabolites (*55*−*57*). More specifically, up-regulation of the flavonoid pathway has been obtained by using transgenes for biosynthetic enzymes as well as for transcription factors.

Ascorbate. Ascorbate (AsA) is essential not only to humans but also to the plants that synthesize it. Indeed, AsA plays an important role in many plant physiological processes, acting as a regulator of growth and plant development and as an electron donor in such essential adaptative processes as nonphotochemical quenching. AsA is also an major antioxidant, playing a pivotal role in the maintenance of the redox status of cells. AsA is abundantly found in fruits and vegetables. Its concentration depends on the type of tissue (leaves, fruits, and roots) and the age of organs. It also varies greatly as a function of species and cultivar, besides environmental conditions (light, drought, ozone, ...). Considering genetic factors, Johnston et al. (*58*) reported that AsA ranges from 20 to 300 mg/kg in apple and from 300 to 500 mg/kg in orange and reaches up to 17.5 g/kg in *Acerola*. In kiwi, AsA concentration

ranges from 290 to 800 mg/kg, depending on cultivar. It was suggested that genotypes with very high potential could be used for breeding objectives to reach very high concentrations, up to 21 g/kg of fresh weight in *Actinidia latifolia* (*59*). In tomato, the highest concentrations were observed in the smaller fruit varieties (*60*) and in wild species. There is up to 5 times more AsA in *Solanum penneli* compared to *Solanum lycopersicum* (*61*).

The AsA concentration in plant cell depends on biosynthesis, recycling (*62*), and degradation (*63*). The different biosynthetic pathways of vitamin C have been elucidated recently and the genes involved identified (*64*). The genetic molecular (GM) approaches have helped us to identify the limiting steps in the AsA pathway. Two important regulatory steps were identified: GDP-mannose epimerase (GME) (*65*, *66*) and GDP-L-galactose phosphorylase (GGP) (*67*), with two genes coding for the latter enzyme, vtc2 and vtc5. A 4-fold increase in AsA was observed when the kiwifruit GGP was overexpressed in *Arabidopsis*, and an up to 7-fold increase in AsA was observed when both the GGP and the GME genes were overexpressed (*66*). Similarly, the overexpression of the GGP gene from *Actinidia chinensis* in tobacco resulted in a 3-fold increase in foliar AsA (*67*). Stevens et al. (*61*) revealed that the gene coding for GME is present in the quantative trait loci (QTLs) responsible for high levels of AsA in tomato. Another promising way to manipulate AsA content might be achieved via regulatory genes of this pathway. It was observed that an ascorbic acid mannose pathway regulor mutant (AMR1 mutant) (it was found that the AMR1 gene regulated the Smirnoff−Wheeler pathway) with reduced expression of AMR1 had 2−3-fold higher foliar AsA concentrations due to increased expression of limiting-step enzymes such as GME, GPP, and vitamin C defective 2 (VTC2) (*68*). Other attempts to manipulate AsA concentration via up-regulation of plants' AsA recycling also proved to be efficient. For example, GM approaches showed that overexpression of wheat dehydroascorbate reductase (DHAR) increased AsA content in tobacco or wheat chloroplast up to 3.9-fold (*69*).

Synthesis, transport, and accumulation of AsA in the different cell and tissue compartments appear tightly regulated. Moreover, the pivotal role played by AsA in the cell balance suggests that increasing AsA concentration in plant parts of interest represents a difficult task ahead. Due to the complexity of AsA regulation in plants, producing FAVs with increased concentrations in AsA will clearly require a deeper understanding of AsA metabolism (*70*) .

Carotenoids. Several surveys and studies have revealed the potential of conventional breeding to increase the concentration in carotenoids of carrot, spinach (*71*), and tomatoes (*72*). Increases of 120, 30, and 50%, respectively, have been reported. Results from breeding programs at the Asian Vegetable Research and Development Center and the U.S. Department of Agriculture suggest that it is possible to obtain lines of tomatoes with 10−25 times the concentration in β-carotene of conventional varieties (*73*, *74*).

There have been numerous attempts to engineer carotenoid biosynthesis (*75*), but they have not been very successful so far. The carotenoid biosynthetic pathway is well-known, and carotenogenic genes have been isolated from a variety of organisms, which facilitates manipulation of this pathway (*76*). Consequently, most attempts consisted of overexpressing one or more specific genes, selected for coding for enzymes thought to catalyze key controlling steps of the biosynthetic pathway. They generally did not result in the accumulation of the targeted carotenoid or produced detrimental collateral effects.

The majority of the plants of the first transgenic tomato line, generated with the tomato phytoene desaturase (PSY) under the control of the constitutive cauliflower mosaic virus 35S promoter, showed a dwarf genotype (*77*). It was probably due to a competition between carotenoids and gibberellins for geranylgeranyl diphosphate (GGPP). Interferences with other processes address the question of a tissue-specific or constitutive promoter for constructions used to generate transgenic plants and underscore the lack of information on metabolic cross-talk between carotenoid and other pathways. The problems encountered originate from the complexity of the regulation of biosynthesis of isoprenoids in plant cells, at both the gene and enzyme levels, and the poor understanding we have of the existing mechanisms (*78*, *79*). It has been observed, for instance, that overexpression of PSY, which is believed to exert the greatest control over pathway flux, in genetically modified tomato plants obtained by inserting homologous bacterial genes reduced the control exerted by this enzyme on the flux, eventually shifting it from one step of the metabolic pathway to another (*80*). Therefore, a modest (almost 2-fold) increase in lycopene content was achieved with bacterial *psy* transgene under a fruit-specific promoter (*80*). Higher increases in carotenoid levels have been seen for plant tissues with low carotenoid levels or for plants with carotenoid-free tissues. It was reported that overexpression of a bacterial *psy* gene under a seed-specific promoter results in a significant increase in total carotenoid and β-carotene contents in canola seed (*81*). More recently, tissue-specific coexpression of *psy* and phytoene désaturase (*crtI*) led to Golden Rice (*82*) and to Golden Potato (*83*) with β-carotene enhancement of up to 23 times in Golden Rice 2.

The central role of carotenoids in plant development and adaptation suggests that their synthesis is coordinated with development processes such as plastid differentiation and fruit development (*78*). Only a few regulatory genes involved in carotenoid biosynthesis have been isolated so far (*84*). It was shown that a transcription factor, AtRAP2.2, a member of the APETALA2 (AP2)/ethylene responsive element-binding protein transcription factor family, binds to a regulatory region of psy promoter and modestly regulates *psy* and *pds* expression (*85*). Some genes involved in the light signal pathway (*de-etiolated1* (*DDB1*) and *UV-damage DNA-binding protein 1* (*DET1*) or in chromoplast differentiation (*orange* (*Or*)) were reported to control carotenoid metabolism (*84*). There are many forms of control and many controlling points, presumably at each branch point of the isoprenoid pathway. The dominant form of control is thought to be at the transcriptional level (*78*, *86*), but others probably exist. For instance, Marty et al. (*87*) attributed the decorrelation they observed between β-carotene accumulation and expression of ζ-carotene desaturase (ZDS) in the white apricot variety 'Moniqui' to a post-transcriptional modification of the ZDS, which may have resulted in an inactive form (*87*). Post-transcriptional regulation over key steps of the biosynthetic pathway may also involve redox status and external signals, such as light. Eventually, observations also suggest that feedback control mechanisms and metabolic channeling between each branch of the isoprenoid pathway play a key role and may be behind unexplained hindering of endproduct formation or unwanted side effects. The organization of carotenoid enzymes into metabolons may explain such observations (*80*), and it has been argued that understanding the interactions within the enzymatic complexes catalyzing biosynthesis of carotenoids and their conversion is as important as understanding the regulation at the level of gene expression (*88*). In addition to transcriptional and post-transcriptional regulations, a certain form of regulation may be exerted by the sequestration of carotenoids within the cell. Observations made on tomato and cauliflower suggest that the accumulation of carotenoids depends on genes involved in sequestration (*79*, *89*). Preventing the degradation of carotenoids and exploiting pleiotrophic collateral effects by interfering in the light signal transduction pathway (*90*) have been suggested as promising strategies that could allow the difficulties arising

from the complexity of the regulation of the biosynthetic pathway of carotenoids to be bypassed (*91*). For instance, the expression of the *Or* gene in potato tuber causes high levels of β-carotene accumulation and a 6-fold increase in total carotenoids (*92*).

Glucosinolates. The potential of conventional breeding to increase the concentration in glucosinolates in *Brassica* seems to be considerable. Kushad et al. (*93*) observed huge variability among the 50 cultivars of broccoli they screened. In this study, the concentration in glucosinolates was 20 times higher in the best performing cultivar compared to the least one.

There have been several attempts to breed broccoli for enhanced concentrations of glucosinolates (*94*, *95*). Cultivars have been developed by introgression of two genomic segments from *Brassica villosa* that present a 4-fold increase in 3-methylsulfinylpropyl and 4-methylsulfinylbutyl glucosinolates, as well as an increased conversion of glucosinolates to isothiocyanates associated with a reduction in nitrile production (*95*). It has been hypothesized that enhanced glucosinolate synthesis is associated with allelic forms of the methylalkylmalate synthase genes in these genomic segments which are involved in the control of chain elongation of methionine-derived glucosinolates and appear to be associated with QTLs involved in the total amount of glucosinolates in both *Brassica* (*96*) and *Arabidopsis* (*97*). Although there have been no attempts so far to engineer commercial *Brassica* cultivars with enhanced concentrations in glucosinolates, metabolic engineering looks promising when considering what has been achieved on *Arabidopsis* and the way our understanding of glucosinolate molecular genetics, particularly of the role played by transcription factors, has progressed (*98*). See also Desjardins for a review (*91*). According to Traka and Mithen, metabolic engineering should focus on 3-methylsulfinylpropyl and 4-methylsulfinylbutyl glucosinolates because of their biological activity and because the isothiocyanates that derive from them, iberin and sulforaphane, respectively, are not volatile and thus do not contribute to the unpleasant flavor of *Brassica* foods, unlike those deriving from most other glucosinolates (*45*).

Phenolic Compounds. Anthocyanin content has been an important target of FAV breeding for long time. Generally speaking, conventional breeding looks promising as far as polyphenols are concerned. Kalt et al. (*99*), for instance, found in the some 250 blueberry genotypes they surveyed that there was a 1.2−1.6-fold difference in the total phenolic and anthocyanin contents, respectively, between the 10th and the 90th percentiles. Anttonen and Karjailanen (*100*) observed a 2-fold difference in total phenolics and a 3-fold difference in quercetin and ellagic acid in the 17 cultivars of raspberry they analyzed. In studies conducted on strawberry, 2−3-fold differences in the anthocyanin content were reported by Wang and Lin (*101*) and Cordenunsi et al. (*102*), respectively. Atkinson et al. (*103*) reported a 5-fold difference in concentration in ellagic acid among the 45 strawberry cultivars they studied. Several breeding programs exist that aim at releasing cultivars with enhanced concentrations of phenolic compounds. Successful improvements have been registered in cranberry, strawberry, peach, and plum (*104*). We are certainly entitled to expect breeding programs to yield interesting results in the coming years.

There have been several attempts to exploit studies of the biosynthetic pathway of flavonoids in flowers, dating back to the early 1990s, to engineer tomatoes with higher concentrations in phenolic compounds. Target genes identified during these early studies belong to two categories: those involved in the biosynthetic pathway itself, such as chalcone synthase (CHS), chalcone flavanone isomerase (CHI), flavanone-3 hydroxylase (F3OH), and flavonol synthase (FLS), and those involved in the control of the pathway (*56*). Concomitant expression of CHS, CHI, F3OH, and FLS increases dramatically the level of quercetin glycosides in the peel of tomato (*105*). Tomatoes transformed with a heterologous double gene construct from a *Petunia* chalcone isomerase and a *Gerbera hybrida* flavone gene exhibited an 18-fold increase in flavonol quercetin-3-rutinoside and a >36-fold increase in kaempferol-rutoside when compared to the wild type (*106*). Several regulatory genes of flavonoid biosynthesis have been identified. Most of them belong to the MYB and MYC families. Overexpression of transcription factors of the MYB and MYC families was found to result in a 60-fold increase in flavonoid and, especially kaempferol biosynthesis in tomato (*107*). Other examples of metabolic engineering include the introduction of a stilbene synthase gene from grapevine in tomato and the subsequent accumulation of not only *trans*-resveratol and glycosylated forms of stilbene but also ascorbate and glutathione (*108*). Down-regulation of cinnamoyl-CoA reductase (CCR), the first committed enzyme of the lignin biosynthesis pathway, resulted in an increase in the availability of the coumaroyl-CoA precursors of kaempferol rutinoside and actually in the accumulation of the expected endproduct (*109*). But the CCR transformants exhibited altered phenotypes, demonstrating that rerouting of a metabolic flow may come at a price (*110*). See also the review of Chopra et al. (*111*).

Besides numerous attempts to increase the concentration in flavonoids in FAVs through metabolic engineering, there have been some very interesting achievements with hydroxycinnamic acids. Overexpression of hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase in tomato was found to cause plants to accumulate more chlorogenic acid with no side effects in terms of concentrations of other soluble phenolic compounds (*55*).

Technically, the potential of metabolic engineering and conventional breeding appears to be huge, even though, at this stage, we are not capable of quantifying what this potential will be when the other usual breeding criteria are taken into account. Then there is the issue of the commercial potential. Metabolic engineering and conventional breeding probably do not have the same perspectives. European consumers have expressed repeatedly that they do not trust genetically modified food. On the contrary, the same consumers should not express reluctance to accept new cultivars with enhanced concentrations in phytochemicals obtained by conventional breeding or by marker-assisted breeding for that matter (*112*). The problem is that breeding is a time-consuming process. Coming up with a new cultivar endowed with enhanced desirable traits takes years, which means that the impact of breeding will not express itself quickly and remain strictly restricted to only cultivars that were designed with the objective of increasing the concentration in phytochemicals. Breeders of FAVs should certainly be encouraged to find new cultivars meeting this objective. Meanwhile, it seems useful to evaluate the other major option, namely, the environmental one. From the farmer's point of view, the question is to assess whether it is technically and economically feasible to increase the content in phytochemical of FAVs by relying on cropping techniques.

ACHIEVEMENTS AND PROSPECTS OF AGRONOMIC APPROACHES

The idea that it is possible to increase the content in secondary metabolites of edible plant parts is a relatively ancient one. It has been demonstrated since the late 1980s that deficit irrigation improves grape quality by increasing the concentration of phenolic compounds and, more specifically, anthocyanins (see ref *113* for a review). More recent studies have started to focus on the effect of climatic factors on the content in micronutrients of FAVs. To date, there are more than 100 papers dealing with this issue. Convincing evidence has been collected which proves clearly that the environment can be manipulated to substantially increase the

Table 1. Effect of Environmental Factors, Light, Temperature, Carbon Supply, Drought, Salinity, and Nitrogen Fertilization, on Concentration, Expressed on a Fresh Matter Basis, If Not Indicated Otherwise, in Ascorbic Acid[a]

environmental factor	crop	effect	ref
high mean daily temperature	several crops	−	*159*
high light intensity combined with low mean daily temperature	*Pisum sativum* L.	+	*180*
low mean temperature	*Brassica oleracea* L. var. *italica* Plenck	++	*114*
increased fruit temperature	*Solanum lycopersicum* L.	−	*181*
high daily sum of light	*Brassica oleracea* L. var. *italica* Plenck	+	*114*
high light intensity	*Fortunella crassifolia* Swingle	+	*182*
high light intensity	*Spinacia oleracea* L. cv. Carambola	+++	*124*
high light exposure	*Malus domestica* Borkh.	++	*123*
high light exposure	*Solanum lycopersicum* L.	+ ?	*125*
high light exposure	several fruit species	+ ?	*183*
high light exposure	*Citrus aurantium* L. (leaves)	+++ (DW basis)	*184*
high light exposure	*Malus domestica* Borkh. cv. Gala (peel)	+++	*185*
high UV-B radiation	*Spinacia oleracea* Mill.	+++	*139*
high UV-B radiation	*Spinacia oleracea* Mill.	+++	*141*
long vs short days	*Fragaria* × *ananassa* Duch.	unclear	*186*
elevated CO_2	*Fragaria* × *ananassa* Duch.	+ (AA) − − (DHA)	*132*
elevated CO_2	*Citrus aurantium* L.	+	*187*
elevated CO_2	*Citrus aurantium* L. (leaves)	0	*184*
high leaf to fruit ratio	*Fortunella crassifolia* Swingle	+	*182*
drought	*Solanum lycopersicum* L.	+	*144*
drought	*Solanum lycopersicum* L.	depending on cv.	*188*
drought	*Spinacia oleracea* L.	+ to ++ depending on cv.	*155*
drought/high salinity	*Solanum lycopersicum* L.	− ?	*150*
high salinity	*Solanum lycopersicum* L.	+	*149*
high salinity	*Solanum lycopersicum* L.	0	*158*
high salinity	*Solanum lycopersicum* L.	variable	*188*
high salinity	*Solanum lycopersicum* L.	+ depending on cv.	*145*
high salinity	*Capsicum annuum* L.	−	*147*
high salinity	*Fragaria* × *ananassa* Duch.	−/− −	*148*
high salinity (fall−winter season)	*Solanum lycopersicum* L.	++ (AA) ++ (DHA)	*189*
high salinity (spring−summer season)	*Solanum lycopersicum* L.	+± (AA depending on cv. −(DHA)	*189*
high salinity	*Pisum sativum* cv. Puget	+++	*190*
high EC	*Cucurbita pepo* L.	+	*191*
low nitrogen	several crops	+	*159*
low nitrogen	citrus, potato, tomato	+	*160*
low nitrogen	*Capsicum annuum* L.	0	*163*
low nitrogen	*Solanum lycopersicum* L.	+	*144*
low nitrogen	*Solanum lycopersicum* L.	+ strong seasonal impact	*165*
boron stress	*Solanum lycopersicum* L.	+++	*192*
boron stress	*Citrus reshni* Hort ex Tan	+++	*193*

[a] EC, electrical conductivity; +, up to +30%; ++, +30 to +100%; +++, >+100%; −, up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

concentrations of vitamins and secondary metabolites in a large array of FAVs. The global picture we can draw is the following (**Tables 1**−**5**).

Low temperatures during the growth period are generally very favorable to the accumulation of ascorbic acid, phenolic compounds, carotenoids, and glucosinolates. However, all compounds are not affected to the same extent. Ascorbic acid seems to be the less sensitive, with a maximal potential gain of +60% found in broccoli when the mean daily temperature is decreased from 15/20 to 7/12 °C, whereas lutein has the potential to be increased up to 150% (*114*) and anthocyanins up to +240%, as observed in certain cultivars of strawberry when temperatures were decreased from 30 to 18 °C (*115*). The positive effect of low temperatures on ascorbic acid and glucoraphanin seems to be enhanced in broccoli in the presence of high light (*114*), which suggests that photooxidative stress is behind the effect of low temperatures (see below). Interestingly, a positive effect of low temperatures was also observed after harvest for carotenoids in tomatoes (*116*, *117*) and for anthocyanins in apples, the former submitted to UV-B radiation (*118*).

The effect of high temperatures after harvest has been investigated in several species of FAVs. The global picture is the following: with the exception of the skin of mango fruits (*119*), high temperatures have an effect ranging from insignificant to negative on the concentration in total ascorbic acid of tomatoes (*120*−*122*). A similar effect, ranging from insignificant to negative, was observed on the concentrations of carotenoids and α-tocopherol in tomatoes (*121*, *122*). On the contrary, positive effects of high temperatures have been reported on the concentration in total phenolics of tomatoes (*120*) and the skin of mango and banana fruits (*119*).

Generally, good exposure or high light intensity is a positive factor for the accumulation of ascorbic acid as observed in apple (*123*), broccoli (*114*), spinach (*124*), and tomatoes (*125*). A similar positive effect was observed on phenolic compounds in apple (*126*), lettuce (*127*), and tomatoes (*128*), for instance, and also on carotenoids [ca. +70% in well-exposed mangoes (*129*)] and glucosinolates [+200% at low temperature in broccoli (*114*)]. Differences between the different forms of phenolic compounds were observed by Ju et al. (*130*): flavonoids were not affected by high light

Table 2. Effect of Environmental Factors, Light, Temperature, Carbon Supply, Drought, Salinity, and Nitrogen Fertilization, on the Concentration, Expressed on a Fresh Matter Basis, If Not Indicated Otherwise, in Phenolic Compounds[a]

environmental factor	crop	compound	effect	ref
heat shock or short period of cold	*Lactuca sativa* L.	total phenolics	+++	*127*
cooling irrigation	*Malus domestica* Borkh.	anthocyanins	+++	*194*
low mean day temperatures	*Fragaria* × *ananassa* Duch.	anthocyanins and *p*-coumaroylglucose	+++	*115*
high light intensity	*Lactuca sativa* L.	total phenolics	+++	*127*
high light intensity	*Solanum lycopersicum* L.	soluble phenolics	+++	*128*
high light intensity	*Malus domestica* Borkh.	anthocyanins and flavonoids	+++	*126*
high light intensity	*Solanum lycopersicum* L.	quercetin	++	*195*
high light intensity	*Fortunella crassifolia* Swingle	hesperidin, naringin	+++/++	*182*
high light exposure	*Fragaria* × *ananassa* Duch.	total phenolics	0	*131*
high light exposure	*Malus domestica* Borkh.	anthocyanins	++	*130*
high light exposure	*Punica granatum* L.	anthocyanins	+ (peel) − (juice)	*196*
high light exposure	*Malus domestica* Borkh.	flavonoids	0	*130*
high light exposure	*Malus domestica* Borkh. cv. Gala (peel)	anthocyanins	+++	*185*
supplemental light (visible + UV-B)	*Malus domestica* Borkh.	anthocyanins, chlorogenic acid, quercetin and phloretin glycosides	+	*142*
red light	*Solanum lycopersicum* L.	anthocyanins	+	*197*
red light	*Vaccinium macrocarpon* Ait.	anthocyanins	++	*198*
elevated CO_2	*Fragaria* × *ananassa* Duch.	anthocyanins, *p*-coumaroylglucose, quercetin and kaempferol	+/+++	*132*
high leaf to fruit ratio	*Fortunella crassifolia* Swingle	hesperidin, naringin	+	*182*
drought	*Cynara scolymus* L.	total phenolics	0 to ++ according to harvest date	*156*
high salinity	*Capsicum annuum* L.	total phenolics	0	*147*
high salinity	*Fragaria* × *ananassa* Duch.	total phenolics	+	*148*
high salinity	*Lactuca sativa* L.	total phenolics	−	*146*
high salinity	*Solanum lycopersicum* L.	total phenolics	+	*149*
high EC	*Fragaria* × *ananassa* Duch.	ellagic acid, quercetin, kaempferol	+/++	*131*
high nitrogen	*Malus domestica* Borkh.	total phenolics	0	*199*
high nitrogen	*Capsicum annuum* L.	total phenolics	0	*163*
high nitrogen	*Prunus armeniaca* L.	total phenolics	+/++	*162*
high nitrogen	*Malus domestica* Borkh.	anthocyanins	−	*161*
high nitrogen	*Solanum lycopersicum L*	quercetin, kaempferol	0	*164*
high nitrogen	*Solanum lycopersicum* L.	caffeic acid derivates	− strong seasonal impact	*165*
high nitrogen	*Brassica oleracea* L. var. *italica*	quercetin, kaempferol	− − (DW basis)	*200*
selenium treatment	*Brassica oleracea* L.	caffeic acid, sinapic acid, ferulic acid	+++/++ (DW basis)	*201*
boron stress	*Solanum lycopersicum* L.	total phenolics	− −	*192*

[a] EC, electrical conductivity; +, up to +30%; ++, +30 to +100%; +++, >+100%; −, up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

exposure, whereas anthocyanins responded very positively. The highest positive response to light intensity (ca. +200%) was found for total phenolics in lettuce submitted to $Q = 800$ μmol of photons m^{-2} s^{-1} for 1 day (*127*). At the other end of the response scale of phenolic compounds to light, one finds strawberry, a typical shade plant that does not respond positively to increasing light intensity (*131*). At any rate, the effect of high light exposure or intensity on the concentration of ascorbate, phenolic compounds, carotenoids, and glucosinolates of FAVs can generally be rated as very positive. This positive effect may be attributed either to enhanced photooxidative stress, as the synergic effect of combined high light intensity and low temperature would suggest (see above), or to increased photosynthesis, considering the positive effect of increased carbon supply on the concentration in phenolic compounds of strawberry (*132*), in total carotenoids of mango (*129*), and in glucoraphanin of broccoli (*133*).

After harvest, the effect of high intensity on tomatoes appears very positive for phenolic compounds (*117*, *134*). The highest effect (+123%) was found for tomatoes exposed for 5 h to high solar radiation (*134*). The picture is more contrasted for carotenoids with reports of effects either positive (*117*) or negative (*134*). Similarly, the effect of high light intensity ranges from negative (*134*, *135*) to positive (*117*) for ascorbic acid. At any rate, the effect of high light intensity seems to be highly mediated by temperature (*117*).

Strong positive effects of specific wavelength were observed before and after harvest (**Tables 1** and **2**). Lycopene content increased in tomatoes with blue light (*136*, *137*), but the effect of red light was even more marked (+133%) for carotenoids (*138*). UV irradiation either before or after harvest may have a very dramatic effect: +170% ascorbic acid in spinach leaves before harvest (*139*), +400% anthocyanins in apples after harvest (*118*), +400% anthocyanins in figs after harvest (*140*), and +700% α-tocopherol in leaves of spinach and lettuce before harvest (*141*). Carotenoids in tomatoes either before (*142*) or after (*143*) harvest seem, however, to be less responsive. Globally, it may be said that postharvest irradiation is very promising as far as ascorbic acid, phenolic compounds, and α-tocopherol are concerned, especially using UV radiation, but the lack of references clearly begs for more studies.

The picture becomes more complex when one considers the effect of drought and high salinity (**Table 1**). For ascorbic acid there is a global positive trend (*144*), probably strongly dependent on genetic × environmental interactions, as observed for tomato (*145*), whereas there are conflicting responses, that is, ranging from negative (*146*) to not significant (*147*) to positive (*131*, *147*−*149*), with regard to phenolic compounds. When observable, the positive effect to be expected is <+40%. Similarly, responses ranging from negative (*150*, *151*) to not significant (*152*) to positive (*146*, *147*, *149*, *153*−*155*) were observed for carotenoids.

Table 3. Effect of Environmental Factors, Light, Temperature, Carbon Supply, Drought, Salinity, and Nitrogen Fertilization, on the Concentration, Expressed on a Fresh Matter Basis, If Not Indicated Otherwise, in Carotenoids[a]

environmental factor	crop	compound	effect	ref
low mean daily temperature	*Solanum lycopersicum* L.	total carotenoids	+++	*202*
low mean daily temperature	*Solanum lycopersicum* L.	phytoene, phytofluene, β-carotene and lycopene	+/+++ with the exception of carotene (− −)	*203*
increased fruit temperature	*Solanum lycopersicum* L.	total carotenoids	−	*181*
high light exposure	*Malus domestica* Borkh.	total carotenoids	0	*123*
high light exposure	*Citrus aurantium* L. (leaves)	total carotenoids	++ (DW basis)	*184*
high light exposure	*Malus domestica* Borkh. cv. Gala (peel)	total carotenoids	+	*185*
high light exposure	*Citrus clementina* Tanaka	total carotenoids	+	*204*
light spectrum (glass or plastic vs open field conditions	*Solanum lycopersicum* L.	lycopene	−/− −	*205*
light spectrum	*Solanum lycopersicum* L.	lycopene and β-carotene	+ with blue light	*136*
high UV-B radiation	*Malus domestica* Borkh.	β-carotene	+	*142*
elevated CO_2	*Solanum lycopersicum* L.	lycopene and β-carotene	0	*152*
elevated CO_2	*Citrus aurantium* L. (leaves)	total carotenoids	− −	*184*
high leaf to fruit ratio	*Mangifera indica* L.	total carotenoids	++	*129*
high leaf to fruit ratio	*Citrus clementina* Tanaka	total carotenoids	++	*204*
high leaf to fruit ratio	*Citrus clementina* Tanaka	total carotenoids	− − − (DW basis)	*204*
high leaf to fruit ratio	*Citrus unshiu* [Mak.] Marc.	total carotenoids	++	*168*
high leaf to fruit ratio	*Fortunella crassifolia* Swingle	β-cryptoxanthine	+++	*182*
drought	*Solanum lycopersicum* L.	total carotenoids	+ according to cv.	*206*
drought	*Solanum lycopersicum* L.	lycopene, β-carotene, and xanthophylls	0	*188*
drought	*Solanum lycopersicum* L.	lycopene and β-carotene	−	*151*
drought	*Spinacia oleracea* L.	β-carotene, lutein, neoxanthin, and violaxanthin	+	*155*
drought/high salinity	*Solanum lycopersicum* L.	total carotenoids	−	*150*
high salinity	*Lactuca sativa* L.	total carotenoids	++/+++	*146*
high salinity	*Capsicum annuum* L.	lycopene	+	*147*
high salinity	*Solanum lycopersicum* L.	lycopene and β-carotene	++	*153*
high salinity	*Solanum lycopersicum* L.	lycopene and β-carotene	+/++	*149*
high EC	*Solanum lycopersicum* L.	lycopene and β-carotene	0	*152*
high EC	*Solanum lycopersicum* L.	carotene	++	*154*
high nitrogen	*Brassica oleracea* L.	lutein and β-carotene	0 (++ on a DW basis)	*166*
high nitrogen	*Capsicum annuum* L.	lycopene and β-carotene	+/++	*163*
high nitrogen	*Solanum lycopersicum* L.	lycopene	+/++	*167*
high nitrogen	citrus, potato, tomato	carotenes	+	reviewed in *160*
high nitrogen	*Citrus unshiu* [Mak.] Marc.	color index	−	*168*
boron stress	*Solanum lycopersicum* L.	lycopene	+++	*192*

[a] EC, electrical conductivity; +, up to +30%; ++, +30 to +100%; +++, >+100%; −, up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

Potential positive responses may be >150%. Results may depend on the stage of application of drought as suggested by observations made on phenolic compounds in artichoke (*156*). It may be speculated that the contradictory observations are due to the fact that drought and high salinity, because they induce stomatal conductance and photosynthesis to decrease, are responsible for depleted carbon supply (arguably a negative factor regarding synthesis of secondary metabolites; see above) while they exacerbate photooxidative stress, thus providing a positive stimulus for their synthesis. Moreover, the effect of high salinity is not totally assimilable to water stress. This may explain why drought has a negative effect on the concentration of glucoraphanin in *Lepidium campestre* L., whereas high salinity on the contrary may increase its concentration up to 67% (*157*). There are not many studies about the effect of drought or high salinity on the accumulation of α-tocopherol in FAVs. Recent observations suggest that the positive effect of high salinity depends on cultivar, as observed in strawberry (*148*), and on the stage of application of high salinity as suggested by observations made on tomato (*158*).

The effect of nitrogen depletion can generally be considered as positive with regard to the concentration in ascorbic acid of many FAVs (*144*, *159*, *160*) and in phenolic compounds of such fruits as apple (*161*) and peach (*162*). An increase in total phenolics of about 60% was observed in peaches, for instance, as the consequence of a reduction of nitrogen fertilization from 150 to 80 kg of N ha^{-1} (*162*). However, there are also less positive reports: it was reported, for instance, that nitrogen fertilization in a 4−20 mol of NO_3^- m^{-3} range does not affect the concentration in ascorbic acid and phenolic compounds of pepper (*163*). Similarly, nitrogen fertilization in a 79−405 μg of N g^{-1} range does not affect significantly the concentrations in quercetin and kaempferol of tomatoes (*164*). Even a negative effect of nitrogen depletion on accumulation of phenolic compounds in tomatoes has been reported recently (*165*). This negative effect was associated with a slightly positive effect on the concentration in ascorbic acid. The picture is somewhat different for carotenoids, with reports ranging from negative effects of nitrogen depletion on pepper (*163*), citrus, potato, and tomato (*160*) to the absence of effect on kale (*166*) to positive effects on tomato (*167*) and citrus (*168*). The highest positive effect of nitrogen depletion was observed in kale, with an increase of about 100% in β-carotene expressed on a dry weight basis. Besides the usual considerations about the interactions between environmental and genetic factors (*166*), it may be put forward that the effect of the timing of application of nitrogen depletion is crucial. When applied too early, nitrogen depletion arguably affects negatively photosynthetic capacity and thus carbon supply, a

Table 4. Effect of Environmental Factors, UV-B and Salinity, on the Concentration, Expressed on a Fresh Matter Basis, in Tocopherols[a]

environmental factor	crop	compound	effect	ref
high UV-B radiation	*Lactuca sativa* L. and *Spinacia oleracea* L.	α-tocopherol	+++	*141*
high salinity	*Solanum lycopersicum* L.	α-tocopherol and β-tocopherol	+/+++	*158*
high salinity	*Fragaria* × *ananassa* Duch.	α-tocopherol	0/++	*148*

[a] +, up to +30%; ++, +30 to +100%; +++, >+100%; −, up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

Table 5. Effect of Environmental Factors, Light, Temperature, Carbon Supply, Drought, Salinity, and Nitrogen Fertilization, on the Concentration, Expressed on a Fresh Matter Basis, If Not Indicated Otherwise, in Glucosinolates[a]

environmental factor	crop	compound	effect	references
high mean daily temperature	*Brassica oleracea* L. var. *italica* Plenck	total glucosinolates	+ (low light) +++ (high light)	*114*
high mean daily temperature	*Brassica oleracea* L. var. *italica* Plenck	alkenyl glucosinolates	0	*114*
high vs low mean daily temperature	*Brassica oleracea* L. var. *italica* Plenck	glucoraphanin	+ (low light) +++ (high light)	*114*
high sum of light	*Brassica oleracea* L. var. *italica* Plenck	total glucosinolates	+++ (low temperature) − − (high temperature)	*114*
high sum of light	*Brassica oleracea* L. var. *italica* Plenck	alkenyl glucosinolates	0	*114*
high sum of light	*Brassica oleracea* L. var. *italica* Plenck	glucoraphanin	+++ (low temperature) − − (high temperature)	*114*
elevated CO_2	*Brassica oleracea* L. var. *italica* Plenck	total glucosinolates	+	*133*
elevated CO_2	*Brassica oleracea* L. var. *italica* Plenck	glucoraphanin	++	*133*
drought	*Brassica oleracea* L. var. *italica* Plenck	total glucosinolates	+++	*207*
drought	*Lepidium campestre* L.	glucoraphanin	−	*157*
high salinity	*Lepidium campestre* L.	glucoraphanin	++	*157*
low nitrogen	*Brassica rapa* ssp. *Rapifera* L.	total glucosinolates	0	*169*
low nitrogen	*Brassica oleracea* L. var. *italica* Plenck	total glucosinolates	+	*170*
high nitrogen	*Brassica oleracea* L. var. *italica*	glucobrassin, glucoraphin	+ ± − (DW basis)	*200*
selenium treatment	*Brassica oleracea* L.	total glucosinolates	− (DW basis)	*201*

[a] EC, electrical conductivity; +, up to +30%; ++, +30 to +100%; +++, >+100%; −, up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

negative factor when it comes to synthesis of secondary metabolites. We found no references about the effect of nitrogen fertilization on vitamin E synthesis in FAVs. Whereas nitrogen fertilization (in contrast to sulfur) does not affect total glucosinolate concentration of turnip, it increases the proportion of N-containing tryptophan-derived indole glucosinolates (*169*). In contrast, N depletion results in an increase of 17% in the total glucosinolate concentration in broccoli (*170*).

We dealt mainly with such conventional environmental factors as light and temperature here. After harvest, the composition of the atmosphere of FAVs may also be modified. It is not possible to draw a clear picture of what might be achieved by either increasing or decreasing the concentration in O_2, for instance, considering the general lack of references (**Table 6**). However, as suggested by the observations of increased concentration in α-tocopherol made on kiwi fruits stored in NO conditions, modified atmospheres after harvest may be exploited to increase the concentrations in phytochemicals of FAVs (*171*).

Increases of 10−25-fold in carotenoids (*73*), 20-fold in glucosinolates (*93*), and 36-fold in kaempferol-rutoside (*106*) have been observed as the consequence of natural genetic variability, conventional breeding, and metabolic engineering. When compared to genetic factors, the potential of environmental factors clearly appears to be less. However, because it may not be useful, and even hazardous, to increase exceedingly the concentration in phytochemicals of food, it may be argued that the prospects provided by agronomic approaches, a ca. 2-fold increase, represent arguably the perfect balance between effectiveness and safety.

Of course, some of the questions raised by the prospects of genetic approaches apply to agronomic approaches as well. It is quite obvious that increasing the concentration in phytochemicals of FAVs must not come at the price of an exaggeratedly lowered yield. There are not many studies assessing the impact of the tested growing techniques on yield and quality criteria other than the concentration in vitamins and secondary metabolites, but there are some. Not too surprisingly, low nitrogen (*162*), as well as drought and high salinity, seems to reduce yield (*150*, *151*, *155*, *156*), whereas high carbon supply has the opposite effect. Interestingly, brief stress treatments seem to be especially promising because they apparently do not negatively affect yield (*127*). Similarly, the potential of postharvest treatments appears to be very promising because the latter do not affect yield at all (**Table 5**). At any rate, the negative side effects of agronomic techniques aiming at increasing the concentration in phytochemicals of FAVs do not seem to exceed the ones observed with genetic approaches.

UPCOMING CHALLENGES FOR AGRONOMIC APPROACHES

At this stage, we can state with confidence that agronomic approaches are credible. The most serious cause for concern lies in the variability of the responses observed because poor control of the processes involved may impair our capacity to design realistic and reliable cropping techniques, making it possible to produce FAVs with increased and controlled concentrations in phytochemicals. The variability in responses hints at the existence of uncontrolled interactions between factors, between processes, and between organs.

Let us consider factors first. It is quite clear that increasing light intensity will increase temperature as well, whereas drought or high salinity will have the same effect through their negative effect on stomatal conductance. The existence of responses varying as a function of genetic factors suggests that interactions between genetic and environmental factors also play an important role.

The issue of interactions between processes can be illustrated by the observations made on *Citrus* fruits in ref *168*. Iglesias et al. (*168*) observed that maturity and biosynthesis of carotenoids

Table 6. Effect of Postharvest Factors, Light and Temperature, on the Concentrations of Ascorbic Acid, Phenolic Compounds, Carotenoids, and Glucosinolates in Several FAVs[a]

compound	environmental factor	crop	effect	references
total ascorbic acid	high light	*Solanum lycopersicum* L.	−	*135*
total ascorbic acid	high light for a brief period	*Solanum lycopersicum* L.	−	*134*
total ascorbic acid	high light	*Solanum lycopersicum* L.	+	recalcd from *117*
total ascorbic acid	UV-C	*Solanum lycopersicum* L.	++	*143*
total ascorbic acid	high temperature during a brief period	*Solanum lycopersicum* L.	0	*122*
total ascorbic acid	high temperature during a brief period	*Solanum lycopersicum* L.	− − −	*121*
total ascorbic acid	high temperature	*Solanum lycopersicum* L.	0	*122*
total ascorbic acid	high temperature	*Mangifera indica* L. (skin)	++	*119*
total ascorbic acid	high temperature	*Solanum lycopersicum* L.	− − −	*120*
total ascorbic acid	nitric oxide 1 μmol L^{-1}	*Actinidia chinensis* Planch. cv. Xuxiang	+	*171*
dehydroascorbate	high nitric oxide	*Prunus persica* L.	−	*208*
total ascorbic acid	2% O_2, 5% CO_2	*Pyrus communis*	− −	*209*
total ascorbic acid	irradiation stress	*Mangifera indica* L.	−	*210*
total ascorbic acid	dehydratation process	*Capsicum annuum* L.	− − − (DW basis)	*211*
anthocyanins	low mean daily temperature	*Malus domestica* Borkh.	+++	*118*
total phenolics	high temperature	*Mangifera indica* L. (skin)	++	*119*
total phenolics	high temperature	*Musa* spp. (skin)	++	*119*
total phenolics	high temperature	*Solanum lycopersicum* L.	++	*120*
caffeic acid derivates	high light	*Solanum lycopersicum* L.	+ (DW basis)	*117*
flavonoids	high light	*Solanum lycopersicum* L.	+++	*134*
rutin	high light	*Solanum lycopersicum* L.	++	*117*
anthocyanins	pulsed visible and UV light	*Ficus carica* L.	+++	*140*
anthocyanins	UV-B	*Malus domestica* Borkh.	+++	*118*
quercetin	UV-C	*Allium cepa* L.	++	*140*
total phenolics	UV-C	*Solanum lycopersicum* L.	+ (DW basis)	*143*
total phenolics, anthocyanins	100% O_2	*Myrica rubra* Sieb. & Zuce	++/+	*212*
total carotenoids mainly lycopene	relatively low mean daily temperature	*Solanum lycopersicum* L.	+ (depending on light conditions/DW basis)	*117*
lycopene and β-carotene	low mean daily temperature	*Solanum lycopersicum* L.	++	*116*
lycopene	high temperature	*Solanum lycopersicum* L.	++	*120*
lycopene	high temperature	*Solanum lycopersicum* L.	++	*213*
total carotenoids mainly lycopene	high light	*Solanum lycopersicum* L.	++	*117*
lycopene	UV-C	*Solanum lycopersicum* L.	0 (DW basis)	*143*
total carotenoids	high light for a brief period	*Solanum lycopersicum* L.	−	*134*
lycopene	red light	*Solanum lycopersicum* L.	+++	*138*
lycopene and β-carotene	high temperature during a brief period	*Solanum lycopersicum* L.	−/0	*122*
lycopene	high temperature during a brief period	*Solanum lycopersicum L*	− − −	*121*
lycopene and β-carotene	high temperature	*Solanum lycopersicum* L.	+++	*122*
total carotenoids	high nitric oxide	*Prunus persica* L.	0	*208*
total carotenoids	irradiation stress	*Mangifera indica* L.	− − −	*210*
β-carotene, zeaxanthin, antheraxanthin/violaxanthin, β-cryptoxanthin	dehydratation process	*Capsicum annuumm* L.	− − −/− − (DW basis)	*211*
α-tocopherol	high temperature during a brief period	*Solanum lycopersicum* L.	−	*122*
α-tocopherol	high temperature	*Solanum lycopersicum* L.	+	*122*
α-tocopherol	nitric oxide 1 μmol L^{-1}	*Actinidia chinensis* Planch. cv. Xuxiang	++	*171*

[a] +, up to +30%; ++, +30 to +100%; +++, >+100%; −. up to −30%; − −, −30 to −100%; − − −, <−100%; 0, no significant effect.

both depend strongly on sucrose supply. It is generally admitted that the carbon status influences the biosynthesis of vitamins and secondary metabolites, although the way it works is not so clear. It has been said that precursor availability is essential for biogenesis of secondary metabolites (*172*). Within this view, ecologists trying to predict how plants will allocate resources over a broad range between differentiation-related processes (including production of secondary metabolites) and growth-related processes have proposed theories such as the growth differentiation−balance hypothesis (*173*). Besides deterministic theories from ecologists, there are more mechanistic ones based on sugar signaling (*174*). The currently emerging view of physiologists is one of a modulating rather than a conditioning role of carbohydrates concerning biogenesis of secondary metabolites. It may be inferred from this literature that the positive effect of sucrose feeding on carotenoid concentration of *Citrus* fruits reported in ref *168* originates from a direct effect on the synthesis pathway of carotenoids. At the same time, it must remembered that biosynthesis of many secondary metabolites and, to a lesser extent, ascorbate is strongly regulated during ontogenesis of organs, especially in fruits. This is the case of

carotenoids, which always accumulate during ontogenesis. High carbon supply accelerates maturity in fruits (*129*), so it is eventually unclear whether high sucrose has a positive effect on synthesis of carotenoids in *Citrus* fruits directly (the carbon supply/sucrose control theory) or indirectly through its stimulating effect on maturity, or both.

The example of tomato and orange will help us to illustrate the issue of interactions between organs. It is now well established that most stresses result in oxidative stress (*175*). All factors that increase the imbalance between incoming energy and its utilization by photochemistry, such as drought, high electrical conductivity and salinity, high light, and, even better, the combination of high light and low temperature, will increase the risk of producing ROS. ROS and hormones have been demonstrated to systematically interact in signaling pathways controlling adaptative responses (*176*). Moreover, direct implication of either ROS or variations in redox status has been evidenced in the carotenoid biosynthesis pathway (*177*). Similarly, there is some evidence that synthesis of phenolic compounds is redox controlled (*178*). The role played by oxidative stress per se or the associated variations in redox status in the synthesis of phytochemicals raises a very interesting question. If we consider that the redox status or the concentration in ROS plays a key role in controlling biosynthesis of phytochemicals in the pulp of fruits, as suggested by several studies, then where do the ROS come from? They cannot originate from photosynthesis because the pulp has lost its photosynthetic machinery during maturation, when chloroplasts were converted into chromoplasts. Do ROS originate from NADPH oxidase, the respiratory electron transport chain of mitochondria of the pulp, or do they originate from the photosynthetic electron transport chain of the chloroplasts of the peel of fruits or of leaves close to them? Observations made on tomato (*179*) as well as our own observations on orange (unpublished data) clearly demonstrate that stressed leaves induce stress responses in nearby unstressed fruits, in other words, that signals are transmitted from the leaves to the fruits.

CONCLUSION AND PERSPECTIVES

Agronomic approaches offer very good perspectives to increase the concentrations in secondary metabolites of FAVs and, to a lesser extent ascorbic acid, and this probably without risking attaining undesirable levels. It is reasonable to expect agronomic approaches to have a more global reach than genetic approaches, at least in the short term. However, the response variability hints that we lack true control over metabolic and regulatory pathways. To improve the current situation, in addition to fundamental studies, it seems highly recommendable to develop quantified and integrated views of the way environmental factors affect the biosynthesis of phytochemicals. More specifically, we consider it desirable (i) to quantify more precisely the effects of environmental factors, (ii) to include response curves in mathematical models of production capable of dealing with interactions between environmental factors (light and temperature for instance), between processes (carbon metabolism, ontogeny of organs, and response to oxidative stress, as they are known to be intertwined), and between organs (because there is some evidence that photooxidative stress in leaves affects antioxidant metabolism in fruits), and (iii) to build models of genetic × ecophysiological interactions (i.e., models in which parameters of ecophysiological models are included in genetic models). It must be emphasized that such studies will not only help the cause of agronomic approaches but also be useful to genetic approaches. Moreover, what is at stake here in the long term is the capacity to design innovative cropping techniques or even combinations of varieties/terroirs/cropping techniques bringing to FAVs a clear added value in terms of nutritional benefits. In the meantime, it is desirable to explore more systematically the genetic variability of the concentrations in phytochemicals of a large range of FAVs, whereas it seems tempting to test simple and practical ideas, such as modifying the environment of harvested organs or imposing to plants stressing conditions limited in time and intensity at the end of the cropping cycle, maybe just by withholding irrigation for a while, at a time when no harm to yield is likely.

LITERATURE CITED

(1) Goldberg, G. *Plants: Diet and Health*; report of a British Nutrition Foundation Task Force; Blackwell Publishing: Oxford, U.K., 2003.

(2) Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables − the millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36* (7), 703−725.

(3) UNESCO. Fruit and Vegetable Summit, Paris, May 27−30, 2008.

(4) FAO. *Diet, Nutrition and the Prevention of Chronic Diseases*; WHO Technical Report; Geneva, Switzerland, 2003.

(5) Ganry, J. Fruits and vegetables for healthy diet in developing countries. In *1st International Symposium on Human Health Effects of Fruits and Vegetables*, Quebec City, Canada, Aug 17−20; Desjardins, Y., Ed.; International Society for Horticultural Science: Leuven, Belgium, 2007; pp 55−60.

(6) (a) Lopriore, C.; Muehlhoff, E. Food security and nutrition trends in West Africa − challenges and the way forward. In *International Workshop on Food-based Approaches for a Healthy Nutrition*, 2nd ed.; Ouagadougou; FAO: Rome, Italy, 2003. (b) Ruel, M.; Minot, N.; Smith, L. Patterns and determinants of fruit and vegetable consumption in sub-Saharan Africa: a multicountry comparison. In *Joint FAO/WHO Workshop on Fruit and Vegetables for Health*, Kobe; WHO: Geneva, Switzerland, 2004. (c) Ganry, J. Current status of fruits and vegetables production and consumption in francophone african countries. Potential impact on health. In *International Symposium on Human Health Effects of Fruits and Vegetables*, Houston, TX; International Society for Horticultural Science: Leuven, Belgium, 2009.

(7) Song, J.; Kwon, O.; Chen, S. L.; Daruwala, R.; Eck, P.; Park, J. B.; Levine, M. Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and glucose. *J. Biol. Chem.* **2002**, *277* (18), 15252−15260.

(8) Park, J. B.; Levine, M. Intracellular accumulation of ascorbic acid is inhibited by flavonoids via blocking of dehydroascorbic acid and ascorbic acid uptakes in HL-60, U937 and Jurkat cells. *J. Nutr.* **2000**, *130* (5), 1297−1302.

(9) Olmedo, J. M.; Yiannias, J. A.; Windgassen, E. B.; Gornet, N. K. Scurvy: a disease almost forgotten. *Int, J. Dermatol.* **2006**, *45* (8), 909−913.

(10) Arrigoni, O.; De Tullio, M. C. Ascorbic acid: much more than just an antioxidant. *Biochim. Biophys. Acta: Gen. Subj.* **2002**, *1569* (1−3), 1−9.

(11) Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (17), 7915−7922.

(12) Brigelius-Flohe, R.; Traber, M. G. Vitamin E: function and metabolism. *FASEB J.* **1999**, *13* (10), 1145−1155.

(13) (a) Combs, G. F. *The Vitamins: Fundamental Aspects in Nutrition and Health*; Academic Press: San Diego, CA, 1995. (b) Bender, D. A. *Nutritional Biochemistry of the Vitamins*; Cambridge University Press: Cambridge, U.K., 2003; p 512.

(14) Taper, H. S.; Jamison, J. M.; Gilloteaux, J.; Summers, J. L.; Calderon, P. B. Inhibition of the development of metastases by dietary vitamin C: K-3 combination. *Life Sci.* **2004**, *75* (8), 955−967.

(15) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1), 230S−242S.

(16) (a) Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol.* **2003**, *41* (6), 753−758. (b) Cheng, J. C.; Dai, F.; Zhou, B.; Yang, L.; Liu, Z. L. Antioxidant activity of hydroxycinnamic acid derivatives in human low density lipoprotein: mechanism and structure−activity relationship. *Food Chem.* **2007**, *104* (1), 132−139.

(17) Kono, Y.; Shibata, H.; Kodama, Y.; Sawa, Y. The suppression of the N-nitrosating reaction by chlorogenic acid. *Biochem. J.* **1995**, *312*, 947−953.
(18) Middleton, E.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52* (4), 673−751.
(19) Park, C.; Kim, J. R.; Shim, J. K.; Kang, B. S.; Park, Y. G.; Nam, K. S.; Lee, Y. C.; Kim, C. H. Inhibitory effects of streptozotocin, tumor necrosis factor-α, and interleukin-1β on glucokinase activity in pancreatic islets and gene expression of GLUT2 and glucokinase. *Arch. Biochem. Biophys.* **1999**, *362* (2), 217−224.
(20) (a) Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid antioxidants: chemistry, metabolism and structure−activity relationships. *J. Nutr. Biochem.* **2002**, *13* (10), 572−584. (b) Rice-Evans, C. A.; Packer, L. *Flavonoids in Health and Disease*; Dekker: New York, 2003; p 504.
(21) Middleton, E.; Drzewiecki, G.; Krishnarao, D. Quercetin − an inhibitor of antigen-induced human basophil histamine-release. *J. Immunol.* **1981**, *127* (2), 546−550.
(22) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12* (4), 564−582.
(23) Bureau, J. L.; Bushway, R. J. HPLC determination of carotenoids in fruits and vegetables in the United States. *J. Food Sci.* **1986**, *51* (1), 128−130.
(24) (a) Krinsky, N. I. Carotenoids as chemopreventive agents. *Prev. Med.* **1989**, *18* (5), 592−602. (b) Black, H. S.; Mathewsroth, M. M. Protective role of butylated hydroxytoluene and certain carotenoids in photocarcinogenesis. *Photochem. Photobiol.* **1991**, *53* (5), 707−716.
(25) Beutner, S.; Bloedorn, B.; Frixel, S.; Blanco, I. H.; Hoffmann, T.; Martin, H. D.; Mayer, B.; Noack, P.; Ruck, C.; Schmidt, M.; Schulke, I.; Sell, S.; Ernst, H.; Haremza, S.; Seybold, G.; Sies, H.; Stahl, W.; Walsh, R. Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions. *J. Sci. Food Agric.* **2001**, *81* (6), 559−568.
(26) Baltschun, D.; Beutner, S.; Briviba, K.; Martin, H. D.; Paust, J.; Peters, M.; Rover, S.; Sies, H.; Stahl, W.; Steigel, A.; Stenhorst, F. Singlet oxygen quenching abilities of carotenoids. *Liebigs Ann.−Recl.* **1997**, *9*, 1887−1893.
(27) Sies, H.; Menck, C. F. M. Singlet oxygen induced DNA damage. *Mutat. Res.* **1992**, *275* (3−6), 367−375.
(28) Devasagayam, T. P. A.; Steenken, S.; Obendorf, M. S. W.; Schulz, W. A.; Sies, H. Formation of 8-hydroxy(deoxy)guanosine and generation of strand breaks at guanine residues in DNA by singlet oxygen. *Biochemistry* **1991**, *30* (25), 6283−6289.
(29) Kalyanaraman, B.; Feix, J. B.; Sieber, F.; Thomas, J. P.; Girotti, A. W. Photodynamic-action of merocyanine-540 on artificial and natural cell-membranes-involvement of singlet molecular-oxygen. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84* (9), 2999−3003.
(30) Linnewiel, K.; Ernst, H.; Caris-Veyrat, C.; Ben-Dor, A.; Kampf, A.; Salman, H.; Danilenko, M.; Levy, J.; Sharoni, Y. Structure activity relationship of carotenoid derivatives in activation of the electrophile/antioxidant response element transcription system. *Free Radical Biol. Med.* **2009**, *47* (5), 659−667.
(31) Mildel, J.; Elstner, E. F.; Grabmann, J. Synergistic effects of phenolics and carotenoids on human low-density lipoprotein oxidation. *Mol. Nutr. Food Res.* **2007**, *51* (8), 956−961.
(32) Piironen, V.; Syvaoja, E. L.; Varo, P.; Salminen, K.; Koivistoinen, P. Tocopherols and tocotrienols in finnish foods − vegetables, fruits, and berrries. *J. Agric. Food Chem.* **1986**, *34* (4), 742−746.
(33) Hartmann, M. A. Plant sterols and the membrane environment. *Trends Plant Sci.* **1998**, *3* (5), 170−175.
(34) Clouse, S. D.; Sasse, J. M. Brassinosteroids: essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 427−451.
(35) Devaraj, S.; Jialal, I. The role of dietary supplementation with plant sterols and stanols in the prevention of cardiovascular disease. *Nutr. Rev.* **2006**, *64* (7), 348−354.
(36) (a) Awad, A. B.; Fink, C. S. Phytosterols as anticancer dietary components: evidence and mechanism of action. *J. Nutr.* **2000**, *130* (9), 2127−2130. (b) Ostlund, R. E. Cholesterol absorption. *Curr. Opin. Gastroenterol.* **2002**, *18* (2), 254−258. (c) Dutta, P. C. *Phytosterols as Functional Food Components and Nutraceuticals*; Dekker: New York, 2003; p 450.
(37) Attele, A. S.; Wu, J. A.; Yuan, C. S. Ginseng pharmacology − multiple constituents and multiple actions. *Biochem. Pharmacol.* **1999**, *58* (11), 1685−1693.
(38) Morrissey, J. P.; Osbourn, A. E. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* **1999**, *63* (3), 708−724.
(39) Armah, C. N.; Mackie, A. R.; Roy, C.; Price, K.; Osbourn, A. E.; Bowyer, P.; Ladha, S. The membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. *Biophys. J.* **1999**, *76* (1), 281−290.
(40) Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K. The biological action of saponins in animal systems: a review. *Br. J. Nutr.* **2002**, *88* (6), 587−605.
(41) Johnson, I. T.; Gee, J. M.; Price, K.; Curl, C.; Fenwick, G. R. Influence of saponins on gut permeability and active nutrient transport in vitro. *J. Nutr.* **1986**, *116* (11), 2270−2277.
(42) West, L. G.; Greger, J. L.; White, A.; Nonnamaker, B. J. In vitro studies on saponin-mineral complexation. *J. Food Sci.* **1978**, *43* (4), 1342−1343.
(43) Das, S.; Tyagi, A. K.; Kaur, H. Cancer modulation by glucosinolates: a review. *Curr. Sci.* **2000**, *79* (12), 1665−1671.
(44) Johnson, I. T. Glucosinolates: Bioavailability and Importance to Health. In *4th International Bioavailability Symposium on Bioavailability of Micronutrients in Relation to Human Health*, Interlaken, Switzerland, May 30−June 1, 2001; Verlag Hans Huber: Bern, Switzerland, 2002; pp 26−31.
(45) Traka, M.; Mithen, R. Glucosinolates, isothiocyanates and human health. *Phytochem. Rev.* **2009**, *8* (1), 269−282.
(46) Raskin, I.; Ripoll, C. Can an apple a day keep the doctor away? *Curr. Pharm. Des.* **2004**, *10* (27), 3419−3429.
(47) Halliwell, B. Dietary polyphenols: good, bad, or indifferent for your health? *Cardiovasc. Res.* **2007**, *73* (2), 341−347.
(48) (a) O'Connell, O. F.; Ryan, L.; O'Brien, N. M. Xanthophyll carotenoids are more bioaccessible from fruits than dark green vegetables. *Nutr. Res. (N.Y.)* **2007**, *27* (5), 258−264. (b) Tyssandier, V.; Reboul, E.; Dumas, J. F.; Bougteloup-Demange, C.; Armand, M.; Marcand, J.; Sallas, M.; Borel, P. Processing of vegetable-borne carotenoids in the human stomach and duodenum. *Am. J. Physiol.−Gastrointest. Liver Physiol.* **2003**, *284* (6), G913−G923.
(49) Row, E. C.; Brown, S. A.; Stachulski, A. V.; Lennard, M. S. Synthesis of 8-geranyloxypsoralen analogues and their evaluation as inhibitors of CYP3A4. *Bioorg. Med. Chem.* **2006**, *14* (11), 3865−3871.
(50) Sayre, R. M.; Dowdy, J. C. The increase in melanoma: are dietary furocoumarins responsible? *Med. Hypotheses* **2008**, *70* (4), 855−859.
(51) Fenwick, G. R.; Heaney, R. K. Glucosinolates and their breakdown products in cruciferous crops, food and feedingstuffs. *Food Chem.* **1983**, *11* (4), 249−271.
(52) Newell-McGloughlin, M. Nutritionally improved agricultural crops. *Plant Physiol.* **2008**, *147* (3), 939−953.
(53) (a) Lukaszewicz, M.; Matysiak-Kata, I.; Skala, J.; Fecka, I.; Cisowski, W.; Szopa, J. Antioxidant capacity manipulation in transgenic potato tuber by changes in phenolic compounds content. *J. Agric. Food Chem.* **2004**, *52* (6), 1526−1533. (b) Bout, S.; Vermerris, W. A candidate-gene approach to clone the sorghum Brown midrib gene encoding caffeic acid *O*-methyltransferase. *Mol. Genet. Genomics* **2003**, *269* (2), 205−214.(c) O'Neill, G. Ployolesins: close sesame. NZ researchers have developed a potential biological solutions to prevent lipids being transformed into trans fats. *Australian Life Scientist*, http://www.biotechnews.com.au/index.php/id;866694817;fp;4;fpid;2 (June 17, 2008).
(54) Davies, K. M. Genetic modification of plant metabolism for human health benefits. *Mutat. Res.−Fundam. Mol. Mech. Mutag.* **2007**, *622* (1−2), 122−137.
(55) Niggeweg, R.; Michael, A. J.; Martin, C. Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nat. Biotechnol.* **2004**, *22* (6), 746−754.
(56) Schijlen, E. G. W.; de Vos, C. H. R.; van Tunen, A. J.; Bovy, A. G. Modification of flavonoid biosynthesis in crop plants. *Phytochemistry* **2004**, *65* (19), 2631−2648.

(57) Dixon, R. A.; Xie, D. Y.; Sharma, S. B. Proanthocyanidins − a final frontier in flavonoid research? *New Phytol.* **2005**, *165* (1), 9−28.
(58) Johnston, C. S.; Ordman, A. B.; Levine, M.; Daruwala, R.; Wang, Y.; Park, J.; Rumsey, S. C. Recommendations for vitamin C intake. *JAMA, J. Am. Med. Assoc.* **1999**, *282* (22), 2118−2119.
(59) Huang, H. W.; Wang, Y.; Zhang, Z. H.; Jiang, Z. W.; Wang, S. M. *Actinidia* germplasm resources and kiwifruit industry in China. *HortScience* **2004**, *39* (6), 1165−1172.
(60) Stevens, M. Inheritance of tomato fruit quality components. *Plant Breed. Rev.* **1986**, *4*, 273−311.
(61) Stevens, R.; Buret, M.; Duffe, P.; Garchery, C.; Baldet, P.; Rothan, C.; Causse, M. Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. *Plant Physiol.* **2007**, *143* (4), 1943−1953.
(62) (a) Noctor, G.; Foyer, C. H. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249−279. (b) Pallanca, J. E.; Smirnoff, N. The control of ascorbic acid synthesis and turnover in pea seedlings. *J. Exp. Bot.* **2000**, *51* (345), 669−674.
(63) Green, M. A.; Fry, S. C. Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-*O*-oxalyl-l-threonate. *Lett. Nat.* **2005**, *433* (7021), 83−87.
(64) (a) Agius, F.; Gonzalez-Lamothe, R.; Caballero, J. L.; Munoz-Blanco, J.; Botella, M. A.; Valpuesta, V. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nature* **2003**, *21* (2), 177−181; (b) Wheeler, G. L.; Jones, M. A.; Smirnoff, N. The biosynthetic pathway of vitamin C in higher plants. *Nature* **1998**, *393* (6683), 365−369. (c) Lorence, A.; Chevone, B. I.; Mendes, P.; Nessler, C. L. *myo*-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol.* **2004**, *134* (3), 1200−1205. (d) Wolucka, B. A.; Van Montagu, M. GDP-mannose 3′,5′-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. *J. Biol. Chem.* **2003**, *278* (48), 47483−47490.
(65) Gilbert, L.; Alhagdow, M.; Nunes-Nesi, A.; Quemener, B.; Guillon, F.; Bouchet, B.; Faurobert, M.; Gouble, B.; Page, D.; Garcia, V.; Petit, J.; Stevens, R.; Causse, M.; Fernie, A. R.; Lahaye, M.; Rothan, C.; Baldet, P. GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *Plant J.* **2009**, *60* (3), 499−508.
(66) Bulley, S. M.; Rassam, M.; Hoser, D.; Otto, W.; Schunemann, N.; Wright, M.; MacRae, E.; Gleave, A.; Laing, W. Gene expression studies in kiwifruit and gene over-expression in *Arabidopsis* indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. *J. Exp. Bot.* **2009**, *60* (3), 765−778.
(67) Laing, W. A.; Wright, M. A.; Cooney, J.; Bulley, S. M. The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (22), 9534−9539.
(68) Zhang, W.; Lorence, A.; Gruszewski, H. A.; Chevone, B. I.; Nessler, C. L. AMR1, an *Arabidopsis* gene that coordinately and negatively regulates the mannose/L-galactose ascorbic acid biosynthetic pathway. *Plant Physiol.* **2009**, *150* (2), 942−950.
(69) Chen, Z.; Young, T. E.; Ling, J.; Chang, S. C.; Gallie, D. R. Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (6), 3525−3530.
(70) Ishikawa, T.; Dowdle, J.; Smirnoff, N. Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. *Physiol. Plant.* **2006**, *126* (3), 343−355.
(71) Kidmose, U.; Knuthsen, P.; Edelenbos, M.; Justesen, U.; Hegelund, E. In Carotenoids and flavonoids in organically grown spinach (*Spinacia oleracea* L.) genotypes after deep frozen storage. *International Conference on the Nutrional Enhancement of Plant Foods*, Norwich, England, Sept 6−9, 2000; Wiley: Chichester, U. K., 2001; pp 918−923.
(72) Abushita, A. A.; Daood, H. G.; Biacs, P. A. Change in carotenoids and antioxidant vitamins in tomato as a function of varietal and technological factors. *J. Agric. Food Chem.* **2000**, *48* (6), 2075−2081.
(73) Yang, R. Y.; Hanson, P. M.; Lumpkin, T. A. Better health through horticulture − AVRDC's approach to improved nutrition of the poor. In *1st International Symposium on Human Health Effects of Fruits and Vegetables*, Quebec City, Canada, Aug 17−20, 2005; Desjardins, Y., Ed.; International Society for Horticultural Science: Leuven, Belgium, 2007; pp 71−77.
(74) Stommel, J. R. USDA 97L63, 97L66, and 97L97: tomato breeding lines with high fruit β-carotene content. *HortScience* **2001**, *36* (2), 387−388.
(75) Sandmann, G.; Romer, S.; Fraser, P. D. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng.* **2006**, *8* (4), 291−302.
(76) Hirschberg, J. Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **2001**, *4* (3), 210−218.
(77) Fray, R. G.; Wallace, A.; Fraser, P. D.; Valero, D.; Hedden, P.; Bramley, P. M.; Grierson, D. Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J.* **1995**, *8* (5), 693−701.
(78) Fraser, P. D.; Bramley, P. M. The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* **2004**, *43* (3), 228−265.
(79) Bouvier, F.; Rahier, A.; Camara, B. Biogenesis, molecular regulation and function of plant isoprenoids. *Prog. Lipid Res.* **2005**, *44* (6), 357−429.
(80) Fraser, P. D.; Romer, S.; Shipton, C. A.; Mills, P. B.; Kiano, J. W.; Misawa, N.; Drake, R. G.; Schuch, W.; Bramley, P. M. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (2), 1092−1097.
(81) Shewmaker, C. K.; Sheehy, J. A.; Daley, M.; Colburn, S.; Ke, D. Y. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* **1999**, *20* (4), 401−412.
(82) Paine, J. A.; Shipton, C. A.; Chaggar, S.; Howells, R. M.; Kennedy, M. J.; Vernon, G.; Wright, S. Y.; Hinchliffe, E.; Adams, J. L.; Silverstone, A. L.; Drake, R. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **2005**, *23* (4), 482−487.
(83) Ducreux, L. J. M.; Morris, W. L.; Hedley, P. E.; Shepherd, T.; Davies, H. V.; Millam, S.; Taylor, M. A. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *J. Exp. Bot.* **2005**, *56* (409), 81−89.
(84) Lu, S.; Li, L. Carotenoid metabolism: biosynthesis, regulation, and beyond. *J. Integr. Plant Biol.* **2008**, *50* (7), 778−785.
(85) Welsch, R.; Maass, D.; Voegel, T.; DellaPenna, D.; Beyer, P. Transcription factor RAP2.2 and its interacting partner SINAT2: stable elements in the carotenogenesis of *Arabidopsis* leaves. *Plant Physiol.* **2007**, *145* (3), 1073−1085.
(86) Bramley, P. M. Regulation of carotenoid formation during tomato fruit ripening and development. *J. Exp. Bot.* **2002**, *53* (377), 2107−2113.
(87) Marty, I.; Bureau, S.; Sarkissian, G.; Gouble, B.; Audergon, J. M.; Albagnac, G. Ethylene regulation of carotenoid accumulation and carotenogenic gene expression in colour-contrasted apricot varieties (*Prunus armeniaca*). *J. Exp. Bot.* **2005**, *56* (417), 1877−1886.
(88) (a) Bonk, M.; Hoffmann, B.; VonLintig, J.; Schledz, M.; AlBabili, S.; Hobeika, E.; Kleinig, H.; Beyer, P. Chloroplast import of four carotenoid biosynthetic enzymes in vitro reveals differential fates prior to membrane binding and oligomeric assembly. *Eur. J. Biochem.* **1997**, *247* (3), 942−950. (b) Winkel, B. S. J. Metabolic channeling in plants. *Annu. Rev. Plant Biol.* **2004**, *55*, 85−107.
(89) Taylor, M.; Ramsay, G. Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality. *Physiol. Plant.* **2005**, *124* (2), 143−151.
(90) Liu, Y. S.; Roof, S.; Ye, Z. B.; Barry, C.; van Tuinen, A.; Vrebalov, J.; Bowler, C.; Giovannoni, J. Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. In *International Congress in the Wake of the Double Helix*, May 27−31, 2003, Bologna, Italy; National Academy of Sciences: Washington, DC, 2004; pp 9897−9902.
(91) Desjardins, Y. Physiological and ecological functions and biosynthesis of health-promoting compounds in fruit and vegetables. In *Improving the Health-Promoting Properties of Fruit and Vegetable Products*; Tomás-Barberán, F. A., Gil, M. A., Eds.; Woodhead Publishing: Cambridge, U.K., 2008.
(92) Lu, S.; Van Eck, J.; Zhou, X.; Lopez, A. B.; O'Halloran, D. M.; Cosman, K. M.; Conlin, B. J.; Paolillo, D. J.; Garvin, D. F.;

Vrebalov, J.; Kochian, L. V.; Kupper, H.; Earle, E. D.; Cao, J.; Li, L. The cauliflower or gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. *Plant Cell* **2006**, *18* (12), 3594−3605.

(93) Kushad, M. M.; Brown, A. F.; Kurilich, A. C.; Juvik, J. A.; Klein, B. P.; Wallig, M. A.; Jeffery, E. H. Variation of glucosinolates in vegetable crops of Brassica oleracea. *J. Agric. Food Chem.* **1999**, *47* (4), 1541−1548.

(94) (a) Faulkner, K.; Mithen, R.; Williamson, G. Selective increase of the potential anticarcinogen 4-methylsulphinylbutyl glucosinolate in broccoli. *Carcinogenesis* **1998**, *19* (4), 605−609. (b) Sarikamis, G.; Marquez, J.; MacCormack, R.; Bennett, R. N.; Roberts, J.; Mithen, R. High glucosinolate broccoli: a delivery system for sulforaphane. *Mol. Breed.* **2006**, *18* (3), 219−228.

(95) Mithen, R.; Faulkner, K.; Magrath, R.; Rose, P.; Williamson, G.; Marquez, J. Development of isothiocyanate-enriched broccoli, and its enhanced ability to induce phase 2 detoxification enzymes in mammalian cells. *Theor. Appl. Genet.* **2003**, *106* (4), 727−734.

(96) Toroser, D.; Thormann, C. E.; Osborn, T. C.; Mithen, R. RFLP mapping of quantitative trait loci controlling seed aliphatic-glucosinolate content in oilseed rape (*Brassica napus* L.). *Theor. Appl. Genet.* **1995**, *91* (5), 802−808.

(97) (a) Kliebenstein, D. J.; Kroymann, J.; Brown, P.; Figuth, A.; Pedersen, D.; Gershenzon, J.; Mitchell-Olds, T. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol.* **2001**, *126* (2), 811−825. (b) Kroymann, J.; Textor, S.; Tokuhisa, J. G.; Falk, K. L.; Bartram, S.; Gershenzon, J.; Mitchell-Olds, T. A gene controlling variation in arabidopsis glucosinolate composition is part of the methionine chain elongation pathway. *Plant Physiol.* **2001**, *127* (3), 1077−1088. (c) Heidel, A. J.; Clauss, M. J.; Kroymann, J.; Savolainen, O.; Mitchell-Olds, T. Natural variation in MAM within and between populations of *Arabidopsis lyrata* determines glucosinolate phenotype. *Genetics* **2006**, *173* (3), 1629−1636.

(98) (a) Sonderby, I. E.; Hansen, B. G.; Bjarnholt, N.; Ticconi, C.; Halkier, B. A.; Kliebenstein, D. J. A Systems biology approach identifies a R2R3MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. *PLoS One* **2007**, *2*, 12. (b) Wentzell, A. M.; Rowe, H. C.; Hansen, B. G.; Ticconi, C.; Halkier, B. A.; Kliebenstein, D. J. Linking metabolic QTLs with network and cis-eQTLs controlling biosynthetic pathways. *PLoS Genet.* **2007**, *3* (9), 1687−1701.

(99) Kalt, W.; Ryan, D. A. J.; Duy, J. C.; Prior, R. L.; Ehlenfeldt, M. K.; Vander Kloet, S. P. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (*Vaccinium* section *cyanococcus* spp.). *J. Agric. Food Chem.* **2001**, *49* (10), 4761−4767.

(100) Anttonen, M. J.; Karjalainen, R. O. Environmental and genetic variation of phenolic compounds in red raspberry. *J. Food Compos. Anal.* **2005**, *18* (8), 759−769.

(101) Wang, S. Y.; Lin, H. S. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* **2000**, *48* (2), 140−146.

(102) Cordenunsi, B. R.; do Nascimento, J. R. O.; Genovese, M. I.; Lajolo, F. M. Influence of cultivar on quality parameters and chemical composition of strawberry fruits grown in Brazil. *J. Agric. Food Chem.* **2002**, *50* (9), 2581−2586.

(103) Atkinson, C. J.; Dodds, P. A. A.; Ford, Y. Y.; Le Miere, J.; Taylor, J. M.; Blake, P. S.; Paul, N. Effects of cultivar, fruit number and reflected photosynthetically active radiation on *Fragaria* × *ananassa* productivity and fruit ellagic acid and ascorbic acid concentrations. *Ann. Bot.* **2006**, *97* (3), 429−441.

(104) (a) McCown, B. H.; Zeldin, E. L. 'HyRed', an early, high fruit color cranberry hybrid. *HortScience* **2003**, *38* (2), 304−305. (b) Khanizadeh, S.; Ehsani-Moghaddam, B.; Levasseur, A. Antioxidant capacity in June-bearing and day-neutral strawberry. In *Annual Conference of the Canadian Society of Agronomy/Canadian Society for Horticultural Science/Canadian Society of Animal Science*, Halifax, Canada, Aug 1−4, 2006; Agricultural Institute: Ottawa, Canada, 2006; pp 1387−1390. (c) Cevallos-Casals, B. A.; Byrne, D.; Okie, W. R.; Cisneros-Zevallos, L. Selecting new peach and plum genotypes rich in phenolic compounds and enhanced functional properties. *Food Chem.* **2006**, *96* (2), 273−280.

(105) Verhoeyen, M. E.; Bovy, A.; Collins, G.; Muir, S.; Robinson, S.; de Vos, C. H. R.; Colliver, S. Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. *J. Exp. Bot.* **2002**, *53* (377), 2099−2106.

(106) Schijlen, E.; de Vos, C. H. R.; Jonker, H.; van den Broeck, H.; Molthoff, J.; van Tunen, A.; Martens, S.; Bovy, A. Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. *Plant Biotechnol. J.* **2006**, *4* (4), 433−444.

(107) Bovy, A.; de Vos, R.; Kemper, M.; Schijlen, E.; Pertejo, M. A.; Muir, S.; Collins, G.; Robinson, S.; Verhoeyen, M.; Hughes, S.; Santos-Buelga, C.; van Tunen, A. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. *Plant Cell* **2002**, *14* (10), 2509−2526.

(108) Giovinazzo, G.; D'Amico, L.; Paradiso, A.; Bollini, R.; Sparvoli, F.; DeGara, L. Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. *Plant Biotechnol. J.* **2005**, *3* (1), 57−69.

(109) van der Rest, B.; Danoun, S.; Boudet, A. M.; Rochange, S. F. Down-regulation of cinnamoyl-CoA reductase in tomato (*Solanum lycopersicum* L.) induces dramatic changes in soluble phenolic pools. *J. Exp. Bot.* **2006**, *57* (6), 1399−1411.

(110) Quattrocchio, F.; Verweij, W.; Kroon, A.; Spelt, C.; Mol, J.; Koes, R. PH4 of petunia is an R2R3MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. *Plant Cell* **2006**, *18* (5), 1274−1291.

(111) Chopra, S. Flavonoïd pigments as tools in molecular genetics. In *The Science of Flavonoids*; Grotewold, E., Ed.; Springer: Columbus, OH, 2006; pp 147−174.

(112) Margolis, M. A truce in the crop wars. *Newsweek* **2009** (June 27).

(113) Esteban, M. A.; Villanueva, M. J.; Lissarrague, J. R. Effect of irrigation on changes in the anthocyanin composition of the skin of cv Tempranillo (*Vitis vinifera* L) grape berries during ripening. *J. Sci. Food Agric.* **2001**, *81* (4), 409−420.

(114) Schonhof, I.; Klaring, H. P.; Krumbein, A.; Claussen, W.; Schreiner, M. Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli. *Agric. Ecosyst. Environ.* **2007**, *119* (1−2), 103−111.

(115) Wang, S. Y.; Zheng, W. Effect of plant growth temperature on antioxidant capacity in strawberry. *J. Agric. Food Chem.* **2001**, *49* (10), 4977−4982.

(116) Ishida, B. K.; Mahoney, N. E.; Ling, L. C. Increased lycopene and flavor volatile production in tomato calyces and fruit cultured in vitro and the effect of 2-(4-chlorophenylthio)triethylamine. *J. Agric. Food Chem.* **1998**, *46* (11), 4577−4582.

(117) Gautier, H.; Diakou-Verdin, V.; Benard, C.; Reich, M.; Buret, M.; Bourgaud, F.; Poessel, J. L.; Caris-Veyrat, C.; Genard, M. How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance? *J. Agric. Food Chem.* **2008**, *56* (4), 1241−1250.

(118) Ubi, B. E.; Honda, C.; Bessho, H.; Kondo, S.; Wada, M.; Kobayashi, S.; Moriguchi, T. Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. *Plant Sci.* **2006**, *170* (3), 571−578.

(119) Kondo, S.; Kittikorn, M.; Kanlayanarat, S. Preharvest antioxidant activities of tropical fruit and the effect of low temperature storage on antioxidants and jasmonates. *Postharvest Biol. Technol.* **2005**, *36* (3), 309−318.

(120) Odriozola-Serrano, I.; Soliva-Fortuny, R.; Martin-Belloso, O. Antioxidant properties and shelf-life extension of fresh-cut tomatoes stored at different temperatures. *J. Sci. Food Agric.* **2008**, *88* (15), 2606−2614.

(121) Soto-Zamora, G.; Yahia, E. M.; Brecht, J. K.; Gardea, A. Effects of postharvest hot air treatments on the quality and antioxidant levels in tomato fruit. *LWT−Food Sci. Technol.* **2005**, *38* (6), 657−663.

(122) Yahia, E. M.; Soto-Zamora, G.; Brecht, J. K.; Gardea, A. Postharvest hot air treatment effects on the antioxidant system in stored mature-green tomatoes. *Postharvest Biol. Technol.* **2007**, *44* (2), 107−115.

(123) Ma, F. W.; Cheng, L. L. The sun-exposed peel of apple fruit has higher xanthophyll cycle-dependent thermal dissipation and

antioxidants of the ascorbate-glutathione pathway than the shaded peel. *Plant Science* **2003**, *165* (4), 819−827.
(124) Eskling, M.; Akerlund, H. E. Changes in the quantities of violaxanthin de-epoxidase, xanthophylls and ascorbate in spinach upon shift from low to high light. *Photosynth. Res.* **1998**, *57* (1), 41−50.
(125) El-Gizawy, A. M.; Abdallah, M. M. F.; Gomaa, H. M.; Mohamed, S. S. Effect of different shading levels on tomato plants. 2. Yield and fruit quality. *Acta Hortic.* **1993**, *323*, 349−354.
(126) Merzlyak, M. N.; Solovchenko, A. E.; Chivkunova, O. B. Patterns of pigment changes in apple fruits during adaptation to high sunlight and sunscald development. *Plant Physiol. Biochem.* **2002**, *40* (6−8), 679−684.
(127) Myung-Min, O.; Carey, E. E.; Rajashekar, C. B. Mild environmental stresses induce phytochemicals in lettuce. In *Second International Symposium on Human Health Effects of Fruits and Vegetables*, Houston, TX; International Society for Horticultural Science: Leuven, Belgium, 2009.
(128) Wilkens, R. T.; Spoerke, J. M.; Stamp, N. E. Differential responses of growth and two soluble phenolics of tomato to resource availability. *Ecology* **1996**, *77* (1), 247−258.
(129) Joas, J. Incidence de l'état physiologique de la mangue en cours de conservation; Université d'Avignon et Pays du Vaucluse, Avignon, France, 2008; 186 pp.
(130) Ju, Z. Q.; Duan, Y. S.; Ju, Z. G. Effects of covering the orchard floor with reflecting films on pigment accumulation and fruit coloration in "Fuji" apples. *Sci. Hortic.* **1999**, *82* (1−2), 47−56.
(131) Anttonen, M. J.; Hoppula, K. I.; Nestby, R.; Verheul, M. J.; Karjalainen, R. O. Influence of fertilization, mulch color, early forcing, fruit order, planting date, shading, growing environment, and genotype on the contents of selected phenolics in strawberry (*Fragaria* × *ananassa* Duch.) fruits. *J. Agric. Food Chem.* **2006**, *54* (7), 2614−2620.
(132) Wang, S. Y.; Bunce, J. A.; Maas, J. L. Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. *J. Agric. Food Chem.* **2003**, *51* (15), 4315−4320.
(133) Schonhof, I.; Klaring, H. P.; Krumbein, A.; Schreiner, M. Interaction between atmospheric CO_2 and glucosinolates in broccoli. *J. Chem. Ecol.* **2007**, *33* (1), 105−114.
(134) Torres, C. A.; Andrews, P. K.; Davies, N. M. Physiological and biochemical responses of fruit exocarp of tomato (*Lycopersicon esculentum* Mill.) mutants to natural photo-oxidative conditions. *J. Exp. Bot.* **2006**, *57* (9), 1933−1947.
(135) Adegoroye, A. S.; Jolliffe, P. A. Some inhibitory effects of radiation stress on tomato fruit ripening. *J. Sci. Food Agric.* **1987**, *39* (4), 297−302.
(136) Gautier, H.; Rocci, A.; Buret, M.; Grasselly, D.; Dumas, Y.; Causse, M. Effect of photoselective filters on the physical and chemical traits of vine-ripened tomato fruits. *Can. J. Plant Sci.* **2005**, *85* (2), 439−446.
(137) Ruiz-Hidalgo, M. J.; Benito, E. P.; Sandmann, G.; Eslava, A. P. The phytoene dehydrogenase gene of *Phycomyces*: regulation of its expression by blue light and vitamin A. *Mol. Gen. Genet.* **1997**, *253* (6), 734−744.
(138) Alba, R.; Cordonnier-Pratt, M. M.; Pratt, L. H. Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. *Plant Physiol.* **2000**, *123* (1), 363−370.
(139) Heuberger, H.; Praeger, U.; Georgi, M.; Schitrrmacher, J.; Grassmann, J.; Schnitzler, W. H. Precision stressing by UV-B radiation to improve quality of spinach under protected cultivation. *Acta Hortic.* **2004**, *659*, 6.
(140) Rodov, V.; Vinokur, Y.; Tietel, T.; Horev, B. Post-harvest stimulation of bioactie compounds production in fruits and vegetables by photobiological treatmens. In *Second International Symposium on Human Health Effects of Fruits and Vegetables*, Houston, TX; International Society for Horticultural Science: Leuven, Belgium, 2009.
(141) Jansen, M. A. K.; Hectors, K.; O'Brien, N. M.; Guisez, Y.; Potters, G. Plant stress and human health: do human consumers benefit from UV-B acclimated crops? *Plant Sci.* **2008**, *175* (4), 449−458.
(142) Rudell, D. R.; Mattheis, J. P.; Fan, X.; Fellman, J. K. Methyl jasmonate enhances anthocyanin accumulation and modifies production of phenolics and pigments in "Fuji" apples. *J. Am. Soc. Hortic. Sci.* **2002**, *127* (3), 435−441.
(143) Kim, H. J.; Fonseca, J. M.; Kubota, C.; Kroggel, M.; Choi, J. H. Quality of fresh-cut tomatoes as affected by salt content in irrigation water and post-processing ultraviolet-C treatment. *J. Sci. Food Agric.* **2008**, *88* (11), 1969−1974.
(144) Dumas, Y.; Dadomo, M.; Di Lucca, G.; Grolier, P. Effects of environmental factors and agricultural techniques on antioxidant content of tomatoes. *J. Sci. Food Agric.* **2003**, *83* (5), 369−382.
(145) Gautier, H.; Lopez-Lauri, F; Massot, C.; Murshed, R.; Marty, I.; Grasselly, D.; Keller, C.; Sallanon, H.; Génard, M. Impact of ripening and salinity on tomato fruit ascorbate content and enzymatic activities related to ascorbate recycling. *Funct. Plant Sci. Biotechnol.* **2009**, *4* (1), 66−75.
(146) Kim, H. J.; Fonseca, J. M.; Choi, J. H.; Kubota, C.; Kwon, D. Y. Salt in irrigation water affects the nutritional and visual properties of romaine lettuce (*Lactuca sativa* L.). *J. Agric. Food Chem.* **2008**, *56* (10), 3772−3776.
(147) Navarro, J. M.; Flores, P.; Garrido, C.; Martinez, V. Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chem.* **2006**, *96* (1), 66−73.
(148) Keutgen, A. J.; Pawelzik, E. Modifications of strawberry fruit antioxidant pools and fruit quality under NaCl stress. *J. Agric. Food Chem.* **2007**, *55* (10), 4066−4072.
(149) Krauss, S.; Schnitzler, W. H.; Grassmann, J.; Woitke, M. The influence of different electrical conductivity values in a simplified recirculating soilless system on inner and outer fruit quality characteristics of tomato. *J. Agric. Food Chem.* **2006**, *54* (2), 441−448.
(150) De Pascale, S.; Martino, A.; Raimondi, G.; Maggio, A. Comparative analysis of water and salt stress-induced modifications of quality parameters in cherry tomatoes. *J. Hortic. Sci. Biotechnol.* **2007**, *82* (2), 283−289.
(151) Riggi, E.; Patane, C.; Ruberto, G. Content of carotenoids at different ripening stages in processing tomato in relation to soil water availability. *Aust. J. Agric. Res.* **2008**, *59* (4), 348−353.
(152) Krumbein, A.; Schwarz, D.; Klaring, H. P. Effects of environmental factors on carotenoid content in tomato (*Lycopersicon esculentum* (L.) Mill.) grown in a greenhouse. *J. Appl. Bot. Food Qual.* (*Angew. Bot.*) **2006**, *80* (2), 160−164.
(153) De Pascale, S.; Maggio, A.; Fogliano, V.; Ambrosino, P.; Ritieni, A. Irrigation with saline water improves carotenoids content and antioxidant activity of tomato. *J. Hortic. Sci. Biotechnol.* **2001**, *76* (4), 447−453.
(154) Wu, M.; Buck, J. S.; Kubota, C. Effects of nutrient solution EC, plant microclimate and cultivars on fruit quality and yield of hydroponic tomatoes (*Lycopersicon esculentum*). In *7th International Symposium on Protected Cultivation in Mild Winter Climates − Production, Pest Management and Global Competition*, Kissimmee, FL, Mar 23−27; Cantliffe, D. J., Stoffella, P. J., Shaw, N. L., Eds.; International Society for Horticultural Science: Leuven, Belgium, 2004; pp 541−547.
(155) Leskovar, D. I.; Agehara, S.; Piccinni, G.; Yoo, K. Impact of deficit irrigation and plant population on yield, leaf quality and carotenoids content of spinach. In *Second International Symposium on Human Health Effects of Fruits and Vegetables*, Houston, TX; International Society for Horticultural Science: Leuven, Belgium, 2009.
(156) Shinohara, T.; Agehara, S.; Yoo, K. S.; Bae, H. J.; Leskovar, D. Irrigation and nitrogen impact on artichoke yield, head quality, and phenolics. *HortScience* **2007**, *42* (4), 879−879.
(157) Bandara, M.; Savidov, N.; Driedger, D. The impact of selected abiotic stresses on glucoraphanin content in field pepperweed (*Lepidium campestre* L.). In *Second International Symposium on Human Health Effects of Fruits and Vegetables*, Houston, TX; International Society for Horticultural Science: Leuven, Belgium, 2009.
(158) Sgherri, C.; Navari-Izzo, F.; Pardossi, A.; Soressi, G. P.; Izzo, R. The influence of diluted seawater and ripening stage on the content of antioxidants in fruits of different tomato genotypes. *J. Agric. Food Chem.* **2007**, *55* (6), 2452−2458.
(159) Dorais, M.; Ehret, D. L. Agronomy and the nutritional quality of fruit. In *Improving the Health-Promoting Properties of Fruit and*

Vegetable Products; Tomas-Barberan, F. A., Gil, M. I., Eds.; CRC Press, Woodhead Publishing: Cambridge, U.K., 2008; pp 346−381.

(160) Mozafar, A. Nitrogen fertilizers and the amount of vitamins in plants − a review. *J. Plant Nutr.* **1993**, *16* (12), 2479−2506.

(161) Saure, M. C. External control of anthocyanin formation in apple. *Sci. Hortic.* **1990**, *42* (3), 181−218.

(162) Radi, M.; Mahrouza, M.; Jaouad, A.; Amiot, M. J. Influence of mineral fertilization (NPK) on the quality of apricot fruit (cv. Canino). The effect of the mode of nitrogen supply. *Agronomie* **2003**, *23* (8), 737−745.

(163) Flores, P.; Navarro, J. M.; Garrido, C.; Rubio, J. S.; Martinez, V. Influence of Ca^{2+}, K^+ and NO_3-fertilisation on nutritional quality of pepper. *J. Sci. Food Agric.* **2004**, *84* (6), 569−574.

(164) Stewart, A. J.; Chapman, W.; Jenkins, G. I.; Graham, I.; Martin, T.; Crozier, A. The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant Cell Environ.* **2001**, *24* (11), 1189−1197.

(165) Benard, C.; Gautier, H.; Bourgaud, F.; Grasselly, D.; Navez, B.; Caris-Veyrat, C.; Weiss, M.; Genard, M. Effects of low nitrogen supply on tomato (*Solanum lycopersicum*) fruit yield and quality with special emphasis on sugars, acids, ascorbate, carotenoids, and phenolic compounds. *J. Agric. Food Chem.* **2009**, *57* (10), 4112−4123.

(166) Kopsell, D. A.; Kopsell, D. E.; Curran-Celentano, J. Carotenoid pigments in kale are influenced by nitrogen concentration and form. *J. Sci. Food Agric.* **2007**, *87* (5), 900−907.

(167) Aziz, A. B. A. Seasonal changes in the physical and chemical composition of tomato fruits as affected by nitrogen levels; University Wageningen, Meded Landbouwhogeschool; Wageningen, The Netherlands, 1968.

(168) Iglesias, D. J.; Tadeo, F. R.; Legaz, F.; Primo-Millo, E.; Talon, M. In vivo sucrose stimulation of colour change in citrus fruit epicarps: interactions between nutritional and hormonal signals. *Physiol. Plant.* **2001**, *112* (2), 244−250.

(169) Li, S. M.; Schonhof, I.; Krumbein, A.; Li, L.; Stutzel, H.; Schreiner, M. Glucosinolate concentration in turnip (*Brassica rapa* ssp. *rapifera* L.) roots as affected by nitrogen and sulfur supply. *J. Agric. Food Chem.* **2007**, *55* (21), 8452−8457.

(170) Schonhof, I.; Blankenburg, D.; Muller, S.; Krumbein, A. Sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. *J. Plant Nutr. Soil Sci. (Z. Pflanzenernaehr. Bodenkd.)* **2007**, *170* (1), 65−72.

(171) Zhu, S.; Sun, L.; Liu, M.; Zhou, J. Effect of nitric oxide on reactive oxygen species and antioxidant enzymes in kiwifruit during storage. *J. Sci. Food Agric.* **2008**, *88* (13), 2324−2331.

(172) Cunningham, F. X. Regulation of carotenoid synthesis and accumulation in plants. *Pure Appl. Chem.* **2002**, *74* (8), 1409−1417.

(173) Herms, D. A.; Mattson, W. J. The dilemma of plants − to grow or defend. *Q. Rev. Biol.* **1992**, *67* (3), 283−335.

(174) (a) Jang, J. C.; Leon, P.; Zhou, L.; Sheen, J. Hexokinase as a sugar sensor in higher plants. *Plant Cell* **1997**, *9* (1), 5−19. (b) Pego, J. V.; Kortstee, A. J.; Huijser, G.; Smeekens, S. G. M. Photosynthesis, sugars and the regulation of gene expression. In *Genetic Manipulation of Photosynthesis Session at the Annual Meeting of the Society for Experimental Biology*, Edinburgh, Scotland, March 22−24, **1999**; pp 407−416. (c) Smeekens, S. Sugar-induced signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2000**, *51*, 49−81. (d) Rolland, F.; Moore, B.; Sheen, J. Sugar sensing and signaling in plants. *Plant Cell* **2002**, *14*, S185−S205. (e) Rolland, F.; Winderickx, J.; Thevelein, J. M. Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem. Sci.* **2001**, *26* (5), 310−317. (f) Gibson, S. I. Control of plant development and gene expression by sugar signaling. *Curr. Opin. Plant Biol.* **2005**, *8* (1), 93−102.

(175) Grassmann, J.; Hippeli, S.; Elstner, E. F. Plant's defence and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiol. Biochem.* **2002**, *40* (6−8), 471−478.

(176) Fujita, M.; Fujita, Y.; Noutoshi, Y.; Takahashi, F.; Narusaka, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.* **2006**, *9* (4), 436−442.

(177) (a) Bouvier, F.; Backhaus, R. A.; Camara, B. Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.* **1998**, *273* (46), 30651−30659. (b) Kuntz, M.; Chen, H. C.; Simkin, A. J.; Romer, S.; Shipton, C. A.; Drake, R.; Schuch, W.; Bramley, P. M. Upregulation of two ripening-related genes from a nonclimacteric plant (pepper) in a transgenic climacteric plant (tomato). *Plant J.* **1998**, *13* (3), 351−361.

(178) (a) Wingate, V. P. M.; Lawton, M. A.; Lamb, C. J. Glutathione causes a massive and selective indution of plant defense genes. *Plant Physiol.* **1988**, *87* (1), 206−210. (b) Lillo, C.; Lea, U. S.; Ruoff, P. Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant Cell Environ.* **2008**, *31* (5), 587−601.

(179) Murshed, R. Etude de l'expression des gènes impliqués dans le recyclage de la vitamine C dans les fruits de la tomate en réponse à divers stress abiotiques; Université d'Avignon et Pays du Vaucluse, Avignon, France, 2009; 248 pp.

(180) Streb, P.; Aubert, S.; Gout, E.; Bligny, R. Cold- and light-induced changes of metabolite and antioxidant levels in two high mountain plant species *Soldanella alpina* and *Ranunculus glacialis* and a lowland species *Pisum sativum*. *Physiol. Plant.* **2003**, *118* (1), 96−104.

(181) Gautier, H.; Rocci, A.; Buret, M.; Grasselly, D.; Causse, M. Fruit load or fruit position alters response to temperature and subsequently cherry tomato quality. *J. Sci. Food Agric.* **2005**, *85* (6), 1009−1016.

(182) Kondo, S.; Katayama, R.; Uchino, K. Antioxidant activity in meiwa kumquat as affected by environmental and growing factors. *Environ. Exp. Bot.* **2005**, *54* (1), 60−68.

(183) Lee, S. K.; Kader, A. A. Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biol. Technol.* **2000**, *20* (3), 207−220.

(184) Schwanz, P.; Kimball, B. A.; Idso, S. B.; Hendrix, D. L.; Polle, A. Antioxidants in sun and shade leaves of sour orange trees (*Citrus aurantium*) after long-term acclimation to elevated CO_2. *J. Exp. Bot.* **1996**, *47* (305), 1941−1950.

(185) Li, P. M.; Cheng, L. L. The shaded side of apple fruit becomes more sensitive to photoinhibition with fruit development. *Physiol. Plant.* **2008**, *134* (2), 282−292.

(186) Tafazoli, E.; Shaybany, B. Effets of short-day treatments on 2nd crop summer-fruiting strawberries. *Exp. Agric.* **1978**, *14* (3), 217−221.

(187) Idso, S. B.; Idso, K. E. Effects of atmospheric CO_2 enrichment on plant constituents related to animal and human health. *Environ. Exp. Bot.* **2001**, *45* (2), 179−199.

(188) Zushi, K.; Matsuzoe, N. Effect of soil water deficit on vitamin C, sugar, organic acid, amino acid and carotene contents of large-fruited tomatoes. *J. Jpn. Soc. Hortic. Sci.* **1998**, *67* (6), 927−933.

(189) Zushi, K.; Matsuzoe, N. Seasonal and cultivar differences in salt-induced change in ascorbic acid and dehydroascorbic acid contents of tomato fruit. *Environ. Control Biol.* **2007**, *45* (3), 165−171.

(190) Hernandez, J.; Campillo, A.; Jimenez, A.; Alarcon, J. J.; Sevilla, F. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. *New Phytol.* **1999**, *141* (2), 241−251.

(191) Rouphael, Y.; Cardarelli, M.; Rea, E.; Battistelli, A.; Colla, G. Comparison of the subirrigation and drip-irrigation systems for greenhouse zucchini squash production using saline and non-saline nutrient solutions. *Agric. Water Manag.* **2006**, *82* (1−2), 99−117.

(192) Pratima, S.; Dube, B. K.; Singh, M. V.; Chatterjee, C. Effect of boron stress on yield, biochemical parameters and quality of tomato. *Indian J. Hortic.* **2006**, *63* (1), 39−43.

(193) Arbona, V.; Hossain, Z.; Lopez-Climent, M. F.; Perez-Clemente, R. M.; Gomez-Cadenas, A. Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. *Physiol. Plant.* **2008**, *132* (4), 452−466.

(194) Iglesias, I.; Salvia, J.; Torguet, L.; Montserrat, R. The evaporative cooling effects of overtree microsprinkler irrigation on "Mondial Gala" apples. *Sci. Hortic.* **2005**, *103* (3), 267−287.

(195) Hermann, K. Flavonols and flavones in food plants: a review. *J. Food Technol.* **1976**, *11*, 433−448.

(196) Gil, M. I.; Garciaviguera, C.; Artes, F.; Tomasbarberan, F. A. Changes in pomegranate juice pigmentation during ripening. *J. Sci. Food Agric.* **1995**, *68* (1), 77−81.

(197) Mancinelli, A. L. Light-dependent anthocyanin synthesis − a model system for the study of plant photomorphogenesis. *Bot. Rev.* **1985**, *51* (1), 107−157.

(198) Zhou, Y.; Singh, B. R. Red light stimulates flowering and anthocyanin biosynthesis in American cranberry. *Plant Growth Regul.* **2002**, *38* (2), 165−171.

(199) Unuk, T.; Tojnko, S.; Cmelik, Z.; Stopar, M. Polyphenol content in apple fruits as affected by crop load and rate of applied nitrogen. In *5th International Symposium on Mineral Nutrition of Fruit Plants*, Talca, Chile, Jan 16−21, 2005; Retamales, J. B., Ed.; International Society for Horticultural Science: Leuven, Belgium, 2009; pp 173−176.

(200) Jones, R. B.; Imsic, M.; Franz, P.; Hale, G.; Tomkins, R. B. High nitrogen during growth reduced glucoraphanin and flavonol content in broccoli (*Brassica oleracea* var. *italica*) heads. *Aust. J. Exp. Agric.* **2007**, *47*, 1498−1505.

(201) Robbins, R. J.; Keck, A. S.; Banuelos, G.; Finley, J. W. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. *J. Med. Food* **2005**, *8* (2), 204−214.

(202) Baqar, M. R.; Lee, T. H. Interaction of CPTA and high-temperature on carotenoid synthesis in tomato fruit fruit. *Z. Pflanzenphysiol.* **1978**, *88* (5), 431−435.

(203) Koskitalo, L. H.; Ormrod, D. P. Effects of sub-optimal ripening temperatures on the colour quality and pigment composition of tomato fruit. *J. Food Sci.* **1972**, *37*, 56−59.

(204) Gonord, F.; Fanciullino, A.-L.; Berti, L.; Urban, L. It is possible to increase the carotenoid concentration of *Citrus* fruits by acting on environmental factors. Presented at the *Third International Symposium on Human Health Effects of Fruits and Vegetables*, Avignon, Oct 18−21, 2009.

(205) Cabibel, M.; Ferry, P. Evolution de la teneur en caroténoïdes de la tomate en fonction de la maturation et des conditions de culture. *Ann. Technol. Agric.* **1980**, *29*, 27−46.

(206) Matsuzoe, N.; Zushi, K.; Johjima, T. Effect of soil water deficit on coloring and carotene formation in fruits of red, pink, and yellow type cherry tomatoes. *J. Jpn. Soc. Hortic. Sci.* **1998**, *67* (4), 600−606.

(207) Martinez-Ballesta, M.; Lopez-Perez, L.; Hernandez, M.; López-Berenguer, C.; Fernandez-Garcia, N.; Carvajal, M. Agricultural practices for enhanced human health. *Phytochem. Rev.* **2008**, *7* (2), 251−260.

(208) Flores, F. B.; Sanchez-Bel, P.; Valdenegro, M.; Romojaro, F.; Martinez-Madrid, M. C.; Egea, M. I. Effects of a pretreatment with nitric oxide on peach (*Prunus persica* L.) storage at room temperature. *Eur. Food Res. Technol.* **2008**, *227* (6), 1599−1611.

(209) Larrigaudiere, C.; Lentheric, I.; Pinto, E.; Vendrell, M. Short-term effects of air and controlled atmosphere storage on antioxidant metabolism in conference pears. *J. Plant Physiol.* **2001**, *158* (8), 1015−1022.

(210) Reyes, L. F.; Cisneros-Zevallos, L. Electron-beam ionizing radiation stress effects on mango fruit (*Mangifera indica* L.) antioxidant constituents before and during postharvest storage. *J. Agric. Food Chem.* **2007**, *55* (15), 6132−6139.

(211) Perez-Galvez, A.; Hornero-Mendez, D.; Minguez-Mosquera, M. I. Changes in the carotenoid metabolism of *Capsicum* fruits during application of modelized slow drying process for paprika production. *J. Agric. Food Chem.* **2004**, *52* (3), 518−522.

(212) Yang, Z.; Zheng, Y.; Cao, S. Effect of high oxygen atmosphere storage on quality, antioxidant enzymes, and DPPH-radical scavenging activity of Chinese bayberry fruit. *J. Agric. Food Chem.* **2009**, *57* (1), 176−181.

(213) Javanmardi, J.; Kubota, C. Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. *Postharvest Biol. Technol.* **2006**, *41* (2), 151−155.

Received for review August 16, 2010. Accepted October 20, 2010. This work was partially supported by the 2007−2013 Interreg IVA program "France−Italie maritime" on the PYRGI Project (Project B5H10000000006).

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.2

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

REGIONE LIGURIA

REGIONE TOSCANA

Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale

Programme cofinancé par le Fonds Européen de Développement Régional

Research Article

Received: 3 May 2011 Revised: 29 September 2011 Accepted: 4 December 2011 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.5584

Effect of fruit load on maturity and carotenoid content of clementine (*Citrus clementina* Hort. ex Tan.) fruits

Florine Poiroux-Gonord,[a] Anne-Laure Fanciullino,[a] Liliane Berti[b] and Laurent Urban[c]*

Abstract

BACKGROUND: Citrus fruits contain many secondary metabolites displaying valuable health properties. There is a lot of interest in enhancing citrus quality traits, especially carotenoid contents, by agronomic approaches. In this study the influence of carbohydrate availability on maturity and quality criteria was investigated in clementine fruits during ripening. Fruiting branches were girdled and defoliated after fruit set to obtain three levels of fruit load: high (five leaves per fruit), medium (15) and low (30).

RESULTS: Considering the soluble solid content/titratable acidity (SSC/TA) ratio, it was found that fruits of the high and medium fruit load treatments reached maturity 1.5 months later than fruits of the control. At the time of maturity the SSC/TA ratio of fruits of all treatments was about 13.6. At harvest, fruits were 23% smaller and total sugar concentration of the endocarp was 12.6% lower in the high fruit load treatment than in the control. In contrast, the concentrations of organic acids and total carotenoids were 55.4 and 93.0% higher respectively. Total carotenoids were not positively correlated with either soluble sugars or total carbohydrates.

CONCLUSION: Taken together, the results do not support the common view that carbohydrate availability directly determines carotenoid synthesis by influencing precursor availability.

Keywords: carbohydrate; carbon status; carotenoid; clementine fruit; maturity

INTRODUCTION

Carotenoids endowed with provitamin A activity in particular are important components of the human diet.[1,2] Vitamin A is implicated in hormone synthesis, immune responses and the regulation of cell growth and differentiation.[3,4] It can be produced within certain tissues from carotenoids such as β-cryptoxanthin present, for instance, in citrus fruits, β-carotene present in carrot, spinach and sweet potato and α-carotene found in carrot, pumpkin and red and yellow peppers.[5] Humans do not synthesise carotenoids but can accumulate them, thus relying on plant parts in their diet. A carotenoid-deficient diet can lead to night blindness and premature death. Carotenoid-rich diets are correlated with a significant reduction in the risk of certain cancers, coronary heart disease and several degenerative diseases.

Although the nutritional benefits of fresh fruits and vegetables as a source of carotenoids and other bioactive compounds have been well established for a long time, their consumption remains insufficient in developed as well as in developing countries. Given their dietary importance and the lack of consumption of fruits and vegetables, it seems useful to propose to consumers fruits and vegetables with enhanced levels of bioactive compounds. Carotenoid enhancement of vegetal crops represents a first step in diet improvement, and such efforts have to be combined with research on their bioavailability. Several surveys and studies have revealed the potential of conventional breeding to augment the concentration of carotenoids in carrot,[6] spinach[7] and tomato,[6] with increases of 120, 30 and 50% respectively being reported. In addition, there have been numerous attempts to engineer carotenoid biosynthesis in the recent past.[8] The agronomic/environmental approach also offers very good prospects, even in the short term. Convincing evidence has been collected proving clearly that the environment can be manipulated to substantially increase the concentrations of carotenoids in a large array of fruits and vegetables.[9] The most serious cause for concern lies in the variability of the responses

* *Correspondence to: Laurent Urban, Laboratoire de Physiologie des Fruits et Légumes, Université d'Avignon et des Pays du Vaucluse, Bât. Agrosciences, 301 Rue Baruch de Spinoza, BP 21239, F-84916 Avignon Cedex 9, France. E-mail: laurent.urban@univ-avignon.fr*

a *INRA – UR 1103, Génétique et Ecophysiologie de la Qualité des Agrumes, F-20230 San Giuliano, Corsica, France*

b *UMR CNRS 6134, Laboratoire Biochimie et Biologie Moléculaire du Végétal, Quartier Grossetti, BP 52, F-20250 Corte, Corsica, France*

c *Laboratoire de Physiologie des Fruits et Légumes, Université d'Avignon et des Pays du Vaucluse, Bât. Agrosciences, 301 Rue Baruch de Spinoza, BP 21239, F-84916 Avignon Cedex 9, France*

observed, because poor control of the processes involved may impair our capacity to design realistic and reliable cropping techniques making it possible to produce fruits and vegetables with increased and controlled levels of carotenoids.[9]

Among the various factors influencing the biosynthesis of secondary metabolites in general and carotenoids in particular, carbon status/carbohydrate concentration occupies a pre-eminent position.[9] The idea that one may influence secondary metabolism through the plant carbon balance has a lot of appeal, especially to agronomists who try to improve the nutritional status of plant products and who have at their disposal several levers such as irrigation deficit to limit carbon gains or fruit thinning to increase the source/sink ratio. But then, in which direction should action be taken? Precursor availability has been said to be essential for the biogenesis of secondary metabolites,[10] which can be seen as an argument in favour of cultural practices maximising carbon gains. In contrast, ecological theories such as the much-debated growth differentiation balance hypothesis (GDBH) predict that, at high levels of resource availability, plants decrease their relative investment in the so-called differentiation processes, including secondary metabolism.[11–13]

The objective of the present study was to address the issue of the effect of fruit load on the quality of citrus fruits in open field conditions, especially the concentration of carotenoids. The study was conducted on clementine, which has been demonstrated to be an exceptional source of β-cryptoxanthin, a xanthophyll endowed with provitamin A activity.[14] Girdling (the removal of a ring of phloem) was used as a methodological tool to create fruiting branches with controlled levels of fruit load. Girdling is a common horticultural practice used to manipulate tree growth and development in a variety of fruit species. Its most immediate effect is to stop the basipetal movement of assimilates through the phloem, which results in an accumulation of carbohydrates above the girdle.[15–17] Girdled branches with different leaf/fruit ratios may provide an excellent system to study the effects of changes in carbon availability on fruit growth and metabolite accumulation.[18–21] Because carbon supply potentially influences the biosynthesis of carotenoids in an indirect way, namely by influencing the maturation process,[22] we did not rely on such indicators of maturity as days after anthesis (DAA) or days after full bloom (DAFB) but rather calculated the maturity index as the soluble solid content/titratable acidity ratio.

MATERIALS AND METHODS

Plant material and experimental design

Measurements were performed on fruits of 18-year-old clementine (*Citrus clementina* Hort. ex Tan.) trees growing under identical conditions. The trees, grafted on Carrizo-citrange, were randomly selected within an experimental orchard near San Giuliano in Corsica (42° 18′ 55″ N, 9° 29′ 29″ E; 51 m a.s.l.). Trees were about 2.5 m high and were spaced at 4 m × 6 m. Water was supplied every day on the basis of 100% replacement of actual evapotranspiration estimated from the Penman–Monteith equation.[23] Fertilisers were supplied and insects and diseases controlled according to the recommendations of the local Department of Agriculture.

Twenty-seven branches composed of shoots less than 1 year old were selected among the experimental trees for being similar in light exposure and initial stem diameter (∼1 cm). All branches had the same eastern orientation and were a similar height above ground (∼1.5 m). Full bloom was observed in mid-April 2007 and the selected branches were girdled after fruit set in mid-July. Girdling consisted in removing a 10–15 mm wide band of bark in the middle of the main stem of each selected branch to prevent any movement of assimilates between the branch and the rest of the tree. We checked that no phloem connection formed again after girdling. Leaves were all fully developed at the time of girdling. Treatments were randomly allocated among the ten selected trees of the trial (two or three fruiting branches per tree). For the control we removed fruits and leaves, if needed, to obtain 30 leaves per fruit (low fruit load). This high ratio was chosen so as to ensure that carbon supply was truly non-limiting. Previous observations had shown that fruits from girdled fruiting branches with 25 leaves or more per fruit behaved in a similar manner to fruits from non-girdled branches. For the second treatment we left 15 leaves per fruit (medium fruit load). For the third treatment we left five leaves per fruit (high fruit load). Vegetative flushing after girdling was exceptional and new buds were removed. Moreover, no fruit drop was observed during the course of the trial.

Table 1. Climatic data from July 2007 to January 2008 at San Giuliano location (Corsica, France)

Month	T_{min} (°C)	T_{max} (°C)	T_{mean} (°C)	Rainfall (mm)	Global radiation (J cm^{-2})
July	17.9	29.6	23.8	0	2719.4
August	18.4	29.3	23.4	2	2152.4
September	15.3	25.7	20.1	12.5	1712.4
October	12.5	21.3	16.5	354.3	1108.2
November	8.6	16.6	11.9	49	711.9
December	6.1	15.5	9.6	165	564.8
January	6.2	14.8	9.9	63.2	625.1

T_{min}, monthly minimum temperature; T_{max}, monthly maximum temperature; T_{mean}, monthly mean temperature.

Three samples per treatment were harvested at each of the three harvesting dates selected to cover the ripening process: (i) time I, 25 October 2007; (ii) time II, 22 November 2007; (iii) time III, 9 January 2008. Citrus fruits are remarkable in that they can stay harvestable on the tree for a relatively long time, up to 2 months in the case of mandarins.[24] There were three fruits, i.e. samples, each made of one fruit per treatment and date. In addition, three high-performance liquid chromatography (HPLC) analyses were performed on each sample. Analysis precision was checked from three consecutive extractions/injections of one sample (corresponding to one fruit), and coefficients of variation were 5% or less. Fruit maturity was estimated using the usual maturity indicators, i.e. soluble solid content (SSC), titratable acidity (TA) and the so-called maturity index (SSC/TA). Immediately after harvest, fruits were peeled and the endocarp (pulp) of fruit samples was ground to a fine powder in liquid nitrogen, placed in sealed amber vials under nitrogen and kept frozen at −80 °C prior to analysis. A fraction of this powder was weighed and then lyophilised to obtain the dry matter of the fruit. Lyophilised powder was used for sugar and organic acid assays.

During the experiment, climatic data (temperature, rainfall and global radiation) were collected (Table 1).

Maturity index

TA was determined by titration to pH 8.2 with 0.1 mol L^{-1} NaOH using an automated titration system (Mettler DL 25,

Mettler-Toledo, Viroflay, France) and expressed as % anhydrous citric acid. SSC was determined with a refractometer (Atago 0–32% mas sacch ,VWR, West Chester, PA, USA.) The maturity index was evaluated as the SSC/TA ratio.

Reagents and standards

Extraction solvents were methanol and chloroform for sugars and RPE (analytical grade reagent) grade hexane, ethanol and dichloromethane for carotenoids, all from VWR (West Chester, PA, USA). Analytical solvents were HPLC-grade acetonitrile from VWR for sugars and organic acids and HPLC-grade methanol from VWR and methyl *tert*-butyl ether (MTBE) from Sigma-Aldrich (Steinheim, Germany) for carotenoids. Reagents for analyses were pure-grade polyvinylpyrrolidone (PVP) from Sigma-Aldrich for sugars, pure-grade potassium buffer from VWR for organic acids and pure-grade sodium chloride (NaCl), anhydrous sodium sulfate (Na_2SO_4) and potassium hydroxide (KOH) from VWR and magnesium carbonate ($MgCO_3$) from Sigma-Aldrich for carotenoids. Standards for sucrose, glucose, fructose, ascorbic acid, oxalic acid, malic acid, citric acid and succinic acid were from Sigma-Aldrich. Standards for β-carotene, lutein, β-cryptoxanthin, lycopene and zeaxanthin were from Extrasynthese (Genay, France).

Sugar assay

Sugar extraction was carried out according to the method of Albertini *et al*.[25] A 100 mg portion of lyophilised powder was homogenised in 10 mL of methanol/water (50 : 50 v/v) and 5 mL of chloroform, stirred for 30 min and centrifuged at 5500 × *g* for 15 min. The supernatant was dried overnight and redissolved in 3 mL of water and 1 mL of 50 g L^{-1} PVP. After stirring, the mixture was left at room temperature for 30 min, then centrifuged at 5500 × *g* for 10 min, filtered through a cellulose acetate membrane (25 mm, 22 µm; VWR) and placed in a vial for HPLC analysis.

Sugars were analysed by HPLC using a PerkinElmer Series 200 refractometer (Waltham, MA, USA) according to Albertini *et al*.[25] They were separated on a reverse phase C_{16} column of silica with amine groups (high-performance carbohydrates, 250 mm × 4.6 mm, 4 µm; Waters, Milford, MA, USA). Data were collected, stored and integrated using Total Chrom™ Version 6.2 (PerkinElmer Instruments, Shelton, WA, USA). Sugars were identified by their refractive index and co-injection with authentic standards. Quantification of sugars was achieved using calibration curves with six concentrations. The concentration of each sugar was expressed on a dry weight basis.

Organic acid assay

Organic acid extraction was carried out according to Albertini *et al*.[25] A 100 mg portion of lyophilised powder was homogenised in 5 mL of water, stirred and centrifuged at 400 × *g* for 10 min. The supernatant was filtered through a cellulose acetate membrane (25 mm, 22 µm; VWR) and placed in a vial for HPLC analysis.

Organic acids were analysed by HPLC using a PerkinElmer Series 200 UV detector according to Albertini *et al*.[25] They were separated on a reverse phase C_{18} ion exchange column (Spheri-5 RP-18, 220 mm × 4.6 mm, 5 µm; PerkinElmer). The absorbance at 210 nm was measured using a PerkinElmer Series 200 UV detector. Chromatographic data and UV spectra were collected, stored and integrated using Total Chrom™ Version 6.2 (PerkinElmer Instruments). Organic acids were identified by their retention time and co-injection with authentic standards. Quantification of organic acids was achieved using calibration curves with six concentrations. The concentration of each organic acid was expressed on a dry weight basis.

Carotenoid assay

Carotenoid extraction, identification and quantification were carried out according to the method of Fanciullino *et al*.[26] A 2.5 g portion of freeze-ground juice sac material was stirred with 120 mg of $MgCO_3$ and 35 mL of ethanol/hexane (4 : 3 v/v, containing 1 g L^{-1} ter-butylhydroxytoluene (BHT) as antioxidant). Lycopene (300 µL of solution, equivalent to 60 µg) was added as an internal standard. The residue was separated from the liquid phase by filtration with a filter funnel (porosity 2), then re-extracted with 35 mL of ethanol/hexane and finally 30 mL of ethanol and 30 mL of hexane. The organic phases were transferred to a separatory funnel and successively washed with 2 × 50 mL of 100 g L^{-1} NaCl and 3 × 50 mL of distilled water. The hexanic phase was dried, redissolved in 20 mL of hexane and saponified with an equal volume of 100 g L^{-1} methanolic KOH overnight at room temperature in the dark. The sample was then transferred to a separatory funnel, to which 50 mL of distilled water was added to separate the layers. The hexanic layer was washed with distilled water until free of alkali. The methanolic KOH layer was extracted with 3 × 10 mL of dichloromethane. The extracts were pooled, washed to remove alkali, dried using anhydrous Na_2SO_4, filtered and evaporated in a rotary evaporator. The residue was dissolved in 500 µL of dichloromethane and 500 µL of MTBE/methanol (80 : 20 v/v) and placed in amber vials before HPLC analysis.

Carotenoids were analysed by HPLC using a Hitachi L-2455 system (Tokyo, Japan). They were separated on a C_{30} column (250 mm × 4.6 mm, 5 µm; YMC Europ GmbH, Dinslaken, Germany) using the following mobile phases: water as eluent A, methanol as eluent B and MTBE as eluent C. The flow rate was fixed at 1 mL min^{-1}, the column temperature was set at 25 °C and the injection volume was 20 µL. The absorbance at 290, 350, 400, 450 and 470 nm was monitored using a Hitachi L-2455 photodiode array detector (DAD). Chromatographic data and UV–visible spectra were collected, stored and integrated using EZchrome Version 6.8 (VWR). Identification was carried out by HPLC-DAD through the combined use of retention times, UV–visible spectral data and co-injection with authentic standards (Table 2). Quantification of carotenoids was achieved using a calibration curve with β-carotene at five concentration levels (correlation coefficient of 0.997). Limits of detection (LOD) and quantification (LOQ) were calculated for β-carotene as LOD = 0.002 µg and LOQ = 0.007 µg. All carotenoids were quantified as β-carotene, with concentrations being expressed on both a dry and a fresh weight basis. The total content of carotenoids was calculated by summing the concentrations of individual compounds.

Statistical analysis

All results are expressed as mean ± standard error (SE). Analysis of variance, followed by multiple comparison of means, was performed using R statistical software (http.R-project.org). Means were compared using the Kruskal–Wallis test at $\alpha = 0.05$.

RESULTS

Fruit size and mass, and maturity indicators

The results presented in this paper correspond to the last stages of fruit development. No significant increase in either diameter (Fig. 1A) or fresh mass (Fig. 1B) was observed from time I to time

Table 2. Chromatographic and spectral characteristics of carotenoids found in pulp of clementine fruits

No.	Retention time (min)	Tentative identification		λ_{max} (nm) observed					λ_{max} (nm) literature			
				Peak I	Peak II	Peak III	%III/II		Peak I	Peak II	Peak III	%III/II
1	21.89	*cis*-Violaxanthin	Cis328	413	436	464	86	Cis328	412	436	464	81
2	23.67	Lutein[a]		419	444	472	33.3		422	444	472	48
3	25.20	Zeaxanthin[a]		421	450	475	6		426	450	476	17
4	26.39	*cis*-Antheraxanthin	Cis330	419	441	469	55.5	Cis330	417	440	468	47
5	28.94	*cis*-β-Cryptoxanthin	Cis335	422	442	469		Cis338	420	444	470	
6	30.85	Phytoene		276	286	297			276	286	298	
7	31.67	β-Cryptoxanthin[a]		428	452	478	25		427	450	477	20
8	34.38	Phytofluene		332	348	367	84		331	348	368	68
9	38.93	β-Carotene[a]			452	477	13			452	477	12
10	39.92	ζ-Carotene		380	401	426			379	400	420	90

[a] Identified using authentic standards. Other pigments were tentatively identified using spectral characteristics reported in Refs 40 and 41. Solvents and gradient programmes used were the same as in Ref. 26.

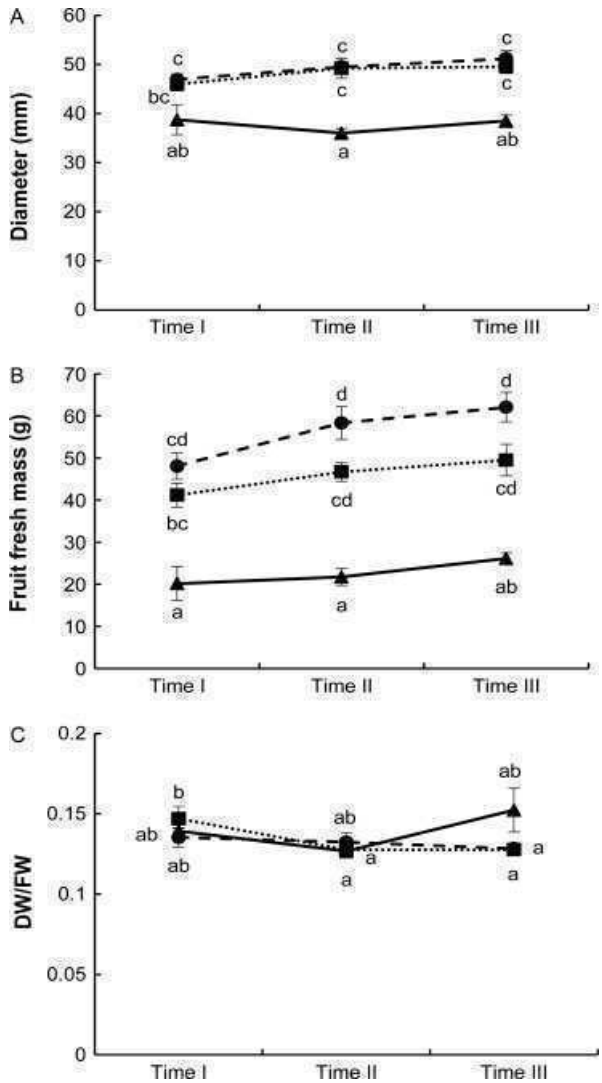


Figure 1. Changes in (A) diameter, (B) fresh mass and (C) DW/FW ratio of clementine fruits of high (– ▲ –), medium (· · · ■ · · ·) and low (– – ● – –) fruit load treatments during fruit ripening. Data are mean ± SE. Different letters indicate significant differences among treatments and dates ($\alpha = 0.05$).

Table 3. Changes in maturity index of clementine fruits of high, medium and low fruit load treatments during fruit ripening

Time	Treatments		
	High	Medium	Low
I	6.67 ± 0.67ab	9.41 ± 0.45b	9.58 ± 1.18b
II	6.46 ± 0.96a	7.43 ± 0.90ab	15.21 ± 0.90c
III	13.81 ± 1.97c	13.97 ± 1.95c	13.02 ± 0.79c

Data are mean ± SE. Different letters indicate significant differences among treatments ($\alpha = 0.05$).

III in any of the three treatments. While there was no difference in diameter between fruits of the medium and low (control) fruit load treatments, fruits of the high fruit load treatment were about 23% smaller at all three dates of measurement. The difference in fresh weight between fruits of the high fruit load treatment and those of the medium fruit load treatment (−50%) was less marked than that between fruits of the high fruit load treatment and those of the control (−64%). Although not significant, fresh weight was about 22% lower in fruits of the medium fruit load treatment than in those of the control.

Considering the maturity index (Table 3), fruits of the control had already reached maturity at time II, while fruits of the high and medium fruit load treatments required 1.5 months more to reach a maturity index of about 13.6. We can thus consider that the increase in fruit load clearly delayed fruit maturity. In the control the increase in maturity index from time I to time II was attributable to a concomitant increase in total sugar concentration (Fig. 2A) and decrease in total organic acid concentration (Fig. 2B). There was, however, no further significant change in total sugar concentration or total organic acid concentration from time II to time III, while the maturity index decreased slightly to reach 13. In the medium and high fruit load treatments the total sugar concentration (Fig. 2A) and maturity index (Table 3) increased from time II to time III. At time III, all three treatments had the same maturity index of about 13.6. We thus decided to compare treatments at that time.

The dry weight/fresh weight (DW/FW) ratio appeared to be unaffected by either treatment or time (Fig. 1C). We may therefore

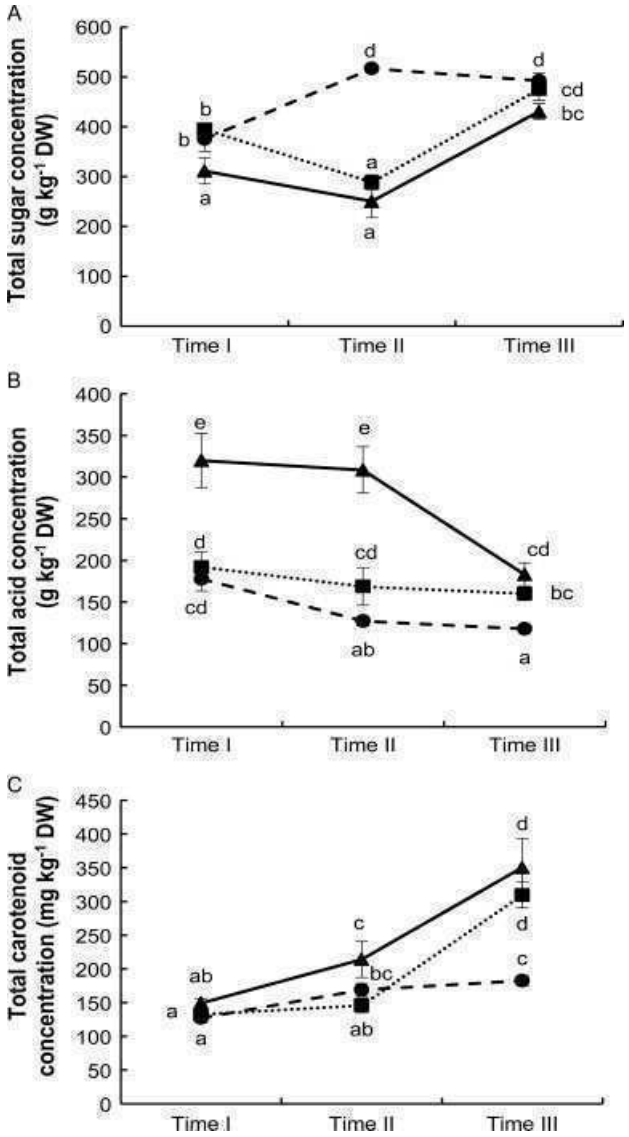


Figure 2. Changes in (A) sugars, (B) organic acids and (C) carotenoids in clementine fruits of high (– ▲ –), medium (· · · ■ · · · ·) and low (– – ● – –) fruit load treatments during fruit ripening. Data are mean ± SE. Different letters indicate significant differences among treatments and dates ($\alpha = 0.05$).

infer from this observation that all comparisons of concentrations made on a DW basis would also be valid if they were expressed on an FW basis.

Total carotenoids

The total carotenoid concentration increased from time I to time III in all treatments, with the exception of fruits of the control where a plateau was reached from time II, which is consistent with the above hypothesis, namely that fruits of the control had already reached maturity at time II (Fig. 2C). The total carotenoid concentration was much higher in fruits of the high and medium fruit load treatments (+106 and +82% respectively) than in those of the control at maturity. In contrast, there was no significant difference in total carotenoid concentration between the high and medium fruit load treatments at time III. Whereas the total carotenoid concentration was

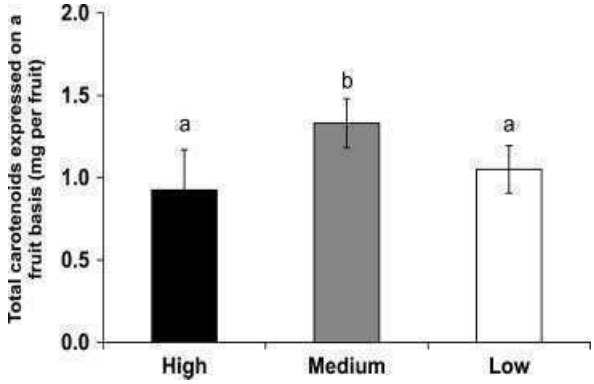


Figure 3. Total carotenoids in clementine fruits of high (■), medium (■) and low (□) fruit load treatments at time III. Data are mean ± SE. Different letters indicate significant differences among treatments ($\alpha = 0.05$).

highest in the high and medium fruit load treatments when expressed on a DW basis, it was highest in the medium fruit load treatment compared with the control (+26.7%) and the high fruit load treatment (+43.8%) when expressed on a per fruit basis (Fig. 3).

Differences in concentrations of sugars, organic acids and carotenoids at time III

The high fruit load treatment was characterised by substantially lower concentrations of sugars and higher concentrations of organic acids and carotenoids (Fig. 4). Values for the medium fruit load treatment were intermediate (Fig. 4). The total sugar concentration in the high fruit load treatment was 12.6 % lower than in the control as a consequence of decreases in fructose (−22.3%) and sucrose (−26.9%), whereas glucose was higher than in the control (+58.3%) (Fig. 4A). The total organic acid concentration was higher in the medium (+35.7%) and high (+55.4%) fruit load treatments than in the control (Fig. 4B). These differences were due to higher concentrations of succinic acid (+62.2 and +86.5% respectively). The medium and high fruit load treatments had higher concentrations of ascorbic acid and lower concentrations of malic acid than the control. The total carotenoid concentration was higher in the medium (+69.5%) and high (+93%) fruit load treatments than in the control (Fig. 4C). Nearly all carotenoids followed this pattern, the major contributor being β-cryptoxanthin, the most important carotenoid of clementine fruits (85.7%). Concentrations of phytoene, phytofluene, ζ-carotene, zeaxanthin, *cis*-β-cryptoxanthin, *cis*-antheraxanthin and β-carotene were 5.4–211% higher than in the control as a consequence of the higher fruit load.

Differences in percentages of different carotenoids, sugars and organic acids at time III

The composition of carotenoids, sugars and organic acids was modified by fruit load (Table 4). In the high fruit load treatment, upstream carotenoids of the biosynthetic pathway, such as phytoene (44.8%), phytofluene (59.9%) and ζ-carotene (18.7%), accumulated at the expense of β-carotene. Fructose, sucrose and malic acid were lower (−9.9, −16.6 and −62.9% respectively) and glucose, ascorbic acid and succinic acid higher (+81.2, +40.8 and +33.8% respectively) in the high fruit load treatment than in the control. In the medium fruit load treatment, carotenoids followed

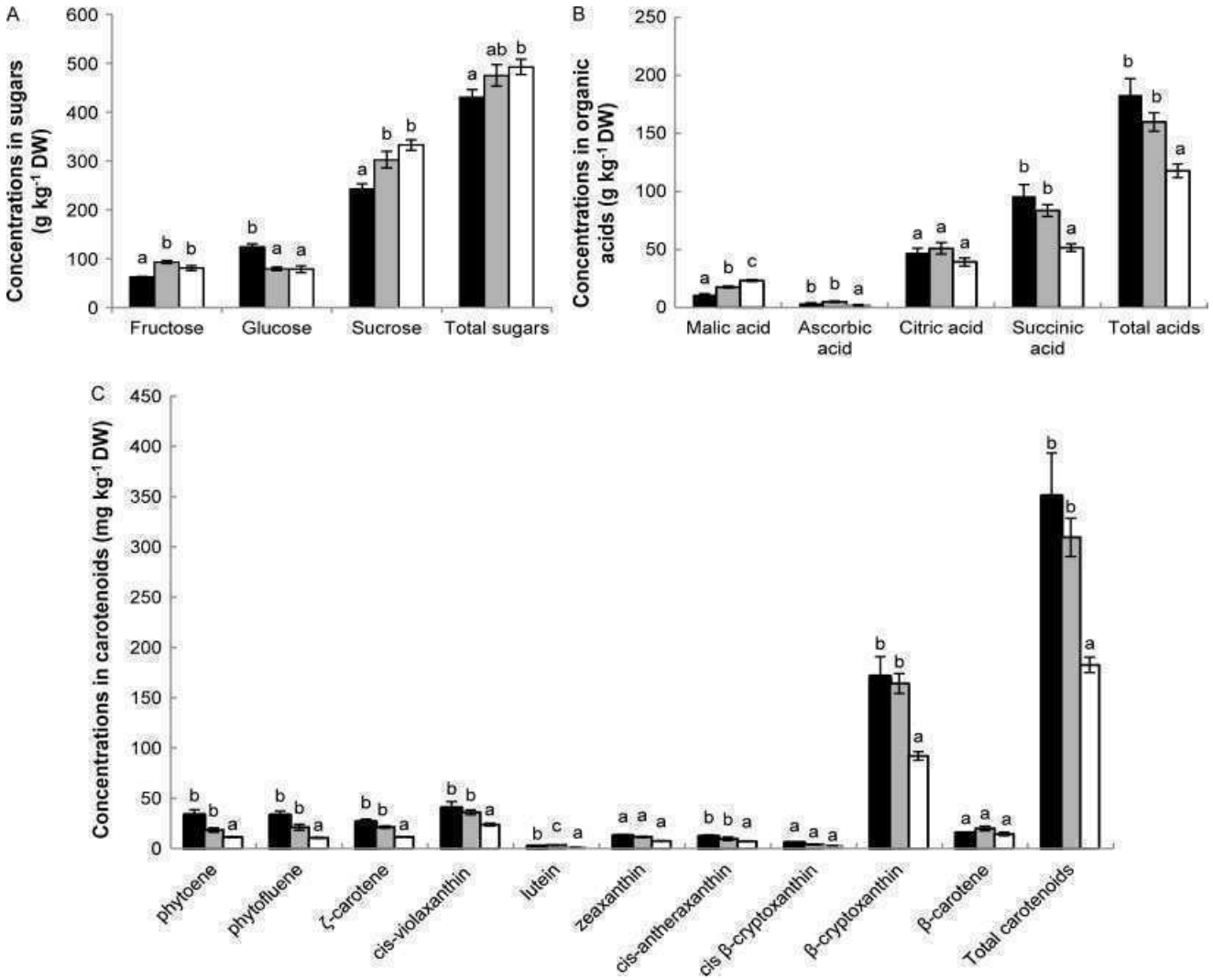


Figure 4. Concentrations of (A) sugars, (B) organic acids and (C) carotenoids in clementine fruits of high (■), medium (▒) and low (□) fruit load treatments at time III. Data are mean ± SE. Different letters for each compound indicate significant differences among treatments ($\alpha = 0.05$).

the same trend as in the control. However, β-cryptoxanthin and lutein were 4.9 and 84.2% higher respectively. Sucrose, glucose, malic acid, citric acid and succinic acid were intermediate, whereas fructose and ascorbic acid were substantially higher than in the control and the high fruit load treatment.

DISCUSSION

Fruit load strongly impacts quality criteria

Clearly, fruit load had a strong impact on criteria of fruit quality. Fruit size was substantially lower in the high fruit load treatment compared with the other two treatments (Fig. 1). Similarly, the concentration of sugars expressed on a DW basis was lower in the high fruit load treatment (Fig. 2A). These observations are consistent with observations made on grapevine, peach, tomato and mango.[18–20,27,28] Globally, organic acids followed a reverse trend, their concentration being lowest in the control (Fig. 2B), which fits with what we know about the dynamics of sugars and organic acids in fruits.[29] Taken together, our results confirm that high fruit load has a negative effect on the organoleptic quality criteria of fruits.

When considering carotenoids, the picture is completely different. Fruits of the control had the lowest concentration by far, suggesting that limiting carbon supply to the fruit can result in a strong improvement in its nutritional quality (Fig. 2C), at least when carotenoid concentration is expressed on a DW (or FW) basis. Indeed, the picture shifts again when expressing carotenoid concentration on a per fruit basis. Then, fruits of the intermediate treatment (medium fruit load) appear to be the best, with the highest amount of carotenoids per fruit (Fig. 3).

Our results do not support the hypothesis that carbohydrates directly determine carotenoid synthesis in fruits

Reports of a positive effect of sucrose supply on the synthesis of carotenoids in fruits can be found in the literature.[22,30] One may wonder why our own observations are not consistent with these (Fig. 2C). One possible explanation is that our observations were made on fruits that had all reached the same final stage of maturity, whereas the reported observations from the literature where made during the maturation process. In the reported observations made on citrus fruits, for instance, sucrose feeding was observed to promote colour break in an ethylene-dependent

Table 4. Changes in proportions (%) of carotenoids, sugars and organic acids in clementine fruits of high, medium and low fruit load treatments at time III

	Treatments		
Compound	High	Medium	Low
Carotenoids			
Phytoene	9.15 ± 0.63b	5.81 ± 0.31a	6.32 ± 0.30a
Phytofluene	9.24 ± 0.47b	6.67 ± 0.52a	5.78 ± 0.23a
ζ-Carotene	7.54 ± 0.36b	6.95 ± 0.32ab	6.35 ± 0.30a
cis-Violaxanthin[a]	11.31 ± 0.65a	11.65 ± 0.64a	13.03 ± 0.49a
Lutein	0.59 ± 0.08a	1.05 ± 0.10b	0.57 ± 0.18a
Zeaxanthin	3.49 ± 0.10a	3.76 ± 0.21a	4.18 ± 0.11a
cis-Antheraxanthin[a]	3.31 ± 0.21a	3.55 ± 0.25a	3.99 ± 0.15a
cis-β-Cryptoxanthin	1.49 ± 0.20a	1.29 ± 0.14a	1.56 ± 0.13a
β-Cryptoxanthin	49.40 ± 1.02a	52.88 ± 0.76b	50.40 ± 0.89a
β-Carotene	4.49 ± 0.25a	6.39 ± 0.46b	7.83 ± 0.14b
Sugars			
Fructose	14.79 ± 0.64a	19.74 ± 0.52c	16.41 ± 0.79b
Glucose	28.81 ± 0.44c	16.79 ± 0.48b	15.90 ± 1.30a
Sucrose	56.41 ± 0.59a	63.47 ± 0.98b	67.68 ± 1.14c
Organic acids[a]			
Malic acid	7.33 ± 0.67a	11.39 ± 0.90b	19.78 ± 0.78c
Oxalic acid	2.41 ± 0.60a	1.71 ± 0.53a	1.66 ± 0.27a
Ascorbic acid	2.21 ± 0.15b	3.07 ± 0.22c	1.57 ± 0.06a
Citric acid	29.69 ± 1.37a	31.65 ± 1.93a	33.38 ± 1.44a
Succinic acid	58.36 ± 1.80c	52.18 ± 1.67b	43.61 ± 1.49a

Data are mean ± SE. Different letters for each compound indicate significant differences among treatments ($\alpha = 0.05$).
[a] Clementine fruits contain 5,6-epoxycarotenoids (violaxanthin and antheraxanthin) and organic acids. To prevent isomerisation of 5,6-epoxycarotenoids to their corresponding 5,8-epoxy derivatives (luteoxanthin, auroxanthin and mutatoxanthin), $MgCO_3$ was added to neutralise acids liberated during extraction.

way,[22] which suggests that the positive effect of sucrose on carotenoid synthesis before maturity was an indirect one, i.e. mediated by the maturation process itself.

The hypothesis that carbohydrate availability determines carotenoid synthesis by influencing precursor availability has been repeatedly expressed in the literature (see e.g. Ref. 10) in spite of the fact that we know very little about the way the metabolite fluxes between primary and secondary metabolisms are orchestrated.[31] The strongest argument is that carotenoids derive from primary metabolites, more precisely 3-phosphoglycerate and pyruvate in the methylerythritol phosphate pathway,[32] and that they are costly to synthesise. There is, however, mounting evidence that availability of resources does not necessarily directly determine biogenesis of all types of secondary metabolite. Many secondary metabolites represent only a very slight proportion of the carbohydrates. Our own measurements show for instance that the order of magnitude of the concentration of total carotenoids in the pulp of ripe clementine fruits is in the 125–350 mg kg^{-1} DW range (Fig. 2C), while being in the 250–515 g kg^{-1} DW range for sugars (Fig. 2A). In other words, sugars are about 1000 times more abundant on a mass basis than carotenoids in the pulp of clementine fruits. In addition, in our trial the concentration of total carotenoids appears to be poorly correlated with the concentration of total sugars or sugars plus organic acids. If any, the correlation between sugars and carotenoids is a negative one, which clearly does not plead in favour of the idea that carbohydrates determine carotenoid synthesis by influencing precursor availability, at least in the range of concentrations considered within this study.

The ongoing characterisation of sugar signalling[33,34] has modified our simplistic view of the effect of carbohydrate availability. The emerging mechanistic view of physiologists is rather one of a modulating role of carbohydrates regarding biogenesis of secondary metabolites.[30,35,36] For instance, accumulation of soluble sugars in tomato leaves was observed to repress genes encoding enzymes of the carotenoid as well as the Rohmer pathway.[37] It would certainly be useful to investigate the effect of carbohydrate accumulation on the major genes of the biosynthetic pathway of carotenoids in fruits in the future.

Fruit load influences carotenoid composition

While total carotenoids increased as a consequence of a low leaf/fruit ratio, the proportion of upstream and downstream carotenoids increased and decreased respectively (Fig. 4C). In a previous study we had already observed that environmental conditions can influence carotenoid profiles of citrus fruits.[38] We found that mandarins and clementines from the Mediterranean area had markedly increased proportions of phytoene, phytofluene and β-carotene compared with fruits from subtropical and tropical growing areas. In tomato, Gautier *et al*.[39] found that increasing the temperature from 21 to 26 °C modified the carotenoid composition by decreasing carotenes but not lycopene. Taken together, these results suggest that environmental factors influence the relative proportions of carotenoids by modifying the flux into the carotenoid biosynthetic pathway and abscisic acid (ABA) production. Several mechanisms have been postulated by different authors as reviewed recently.[36] Phytoene biosynthesis is a rate-limiting step in carotenogenesis, and environmental variations seem to act on its expression. For example, stress has been observed to influence PSY (phytoene synthase) mRNA abundance in rice and maize and is positively correlated with increased carotenoid flux and ABA. Thus the decrease in the proportion of downstream carotenoids could be explained by a concomitant increase in 9-*cis*-epoxycarotenoid dioxygenase (NCED) expression and enhanced *cis*-violaxanthin degradation associated with ABA production. Other regulation mechanisms could be involved, such as negative feedback regulation by ABA accumulation or a shift in the limiting step from PSY to another enzyme of the carotenoid biosynthetic pathway, since both mechanisms would result in a modification of carotenoid proportions.

CONCLUSION

Our observations clearly underline that we do not really know how the metabolite fluxes between primary and secondary metabolisms are orchestrated. They do not support the common view that a high carbon status is favourable to the synthesis of carotenoids in fruits through its positive influence on precursor availability. The effect of carbon availability, if any, seems to a be a negative one, suggesting that sugars could repress genes encoding enzymes of the carotenoid or the Rohmer pathway, as has been observed in leaves so far, but not yet in fruits. Our observations clearly advocate for further studies to be conducted, especially about the way carbohydrates specifically regulate carotenoid biosynthesis but also degradation and storage in fruits. What is at stake here is our capacity to influence the nutritional quality of fruits while maintaining other quality criteria.

ACKNOWLEDGEMENTS

We thank E Carcouet, I Poggi and J Barbaggio for their assistance. This study was partly supported by the 2007-2013 Interreg IVA programme 'France–Italie Maritime' within the PYRGI project (B5H10000000006).

REFERENCES

1 Fraser PD and Bramley PM, The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res* **43**:228–265 (2004).
2 Krinsky NI and Johnson EJ, Carotenoid actions and their relation to health and disease. *Mol Aspects Med* **26**:459–516 (2005).
3 Combs GF, *The Vitamins: Fundamental Aspects in Nutrition and Health*. Academic Press, San Diego, CA (1995).
4 Bender DA, *Nutritional Biochemistry of the Vitamins*. Cambridge University Press, Cambridge (2003).
5 Bureau JL and Bushway RJ, HPLC determination of carotenoids in fruits and vegetables in the United States. *J Food Sci* **51**:128–130 (1986).
6 Abushita AA, Daood HG and Biacs PA, Change in carotenoids and antioxidant vitamins in tomato as a function of varietal and technological factors. *J Agric Food Chem* **48**:2075–2081 (2000).
7 Kidmose U, Knuthsen P, Edelenbos M, Justesen U and Hegelund E, Carotenoids and flavonoids in organically grown spinach (*Spinacia oleracea* L) genotypes after deep frozen storage. *Int. Conf. on the Nutrional Enhancement of Plant Foods*, pp. 918–923 (2000).
8 Sandmann G, Romer S and Fraser PD, Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab Eng* **8**:291–302 (2006).
9 Poiroux-Gonord F, Bidel LPR, Fanciullino AL, Gautier H, Lauri-Lopez F and Urban L, Health benefits of vitamins and secondary metabolites of fruits and vegetables and prospects to increase their concentrations by agronomic approaches. *J Agric Food Chem* **58**:12065–12082 (2010).
10 Cunningham FX, Regulation of carotenoid synthesis and accumulation in plants. *Pure Appl Chem* **74**:1409–1417 (2002).
11 Herms DA and Mattson WJ, The dilemma of plants – to grow or to defend. *Q Rev Biol* **67**:283–335 (1992).
12 Lorio PL, Growth differentiation balance – a basis for understanding southern pine-beetle tree interactions. *Forest Ecol Manag* **14**:259–273 (1986).
13 Luxmoore RJ, A source sink framework for coupling water, carbon, and nutrient dynamics of vegetation. *Tree Physiol* **9**:267–280 (1991).
14 Fanciullino AL, Dhuique-Mayer C, Luro F, Casanova J, Morillon R and Ollitrault P, Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. *J Agric Food Chem* **54**:4397–4406 (2006).
15 Roper TR and Williams LE, Net CO_2 assimilation and carbohydrate partitioning of grapevine leaves in response to trunk girdling and gibberellic-acid application. *Plant Physiol* **89**:1136–1140 (1989).
16 Schaper H and Chacko EK, Effect of irradiance, leaf age, chlorophyll content and branch-girdling on gas-exchange of cashew (*Anacardium occidentale* L.) leaves. *J Hort Sci* **68**:541–550 (1993).
17 Di Vaio C, Petito A and Buccheri M, Effect of girdling on gas exchanges and leaf mineral content in the 'Independence' nectarine. *J Plant Nutr* **24**:1047–1060 (2001).
18 Genard M, Lescourret F, Ben Mimoun M, Besset J and Bussi C, A simulation model of growth at the shoot-bearing fruit level. II. Test and effect of source and sink factors in the case of peach. *Eur J Agron* **9**:189–202 (1998).
19 Lechaudel M, Genard M, Lescourret F, Urban L and Jannoyer M, Modeling effects of weather and source–sink relationships on mango fruit growth. *Tree Physiol* **25**:583–597 (2005).
20 Lescourret F, Ben Mimoun M and Gènard M, A simulation model of growth at the shoot-bearing fruit level: I. Description and parameterization for peach. *Eur J Agron* **9**:173–188 (1998).
21 Lechaudel M, Jannoyer M and Genard M, Effects of the leaf : fruit ratio on growth and partitioning of water and dry matter in mango fruit. *Proc. Seventh Int. Mango Symp.*, pp. 429–433 (2004).
22 Iglesias DJ, Tadeo FR, Legaz F, Primo-Millo E and Talon M, *In vivo* sucrose stimulation of colour change in citrus fruit epicarps: interactions between nutritional and hormonal signals. *Physiol Plant* **112**:244–250 (2001).
23 Monteith JL, Evaporation and environment. *Symp Soc Exp Biol* **19**:205–234 (1965).
24 MS Ladaniya, *Citrus Fruit. Biology, Technology and Evaluation*. Academic Press, San Diego, CA (2007).
25 Albertini MV, Carcouet E, Pailly O, Gambotti C, Luro F and Berti L, Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. *J Agric Food Chem* **54**:8335–8339 (2006).
26 Fanciullino AL, Cercos M, Dhuique-Mayer C, Froelicher Y, Talon M, Ollitrault P, *et al*, Changes in carotenoid content and biosynthetic gene expression in juice sacs of four orange varieties (*Citrus sinensis*) differing in flesh fruit color. *J Agric Food Chem* **56**:3628–3638 (2008).
27 Genard M, Dauzat J, Franck N, Lescourret F, Moitrier N, Vaast P, *et al*, Carbon allocation in fruit trees: from theory to modelling. *Trees Struct Funct* **22**:269–282 (2008).
28 Lechaudel M, Genard M, Lescourret F, Urban L and Jannoyer M, Leaf-to-fruit ratio affects water and dry-matter content of mango fruit. *J Hort Sci Biotechnol* **77**:773–777 (2002).
29 Tadeo FR, Cercos M, Colmenero-Flores JM, Iglesias DJ, Naranjo MA, Rios G, *et al*, Molecular physiology of development and quality of citrus. *Adv Bot Res* **47**:147–223 (2008).
30 Telef N, Stammitti-Bert L, Mortain-Bertrand A, Maucourt M, Carde JP, Rolin D, *et al*, Sucrose deficiency delays lycopene accumulation in tomato fruit pericarp discs. *Plant Mol Biol* **62**:453–469 (2006).
31 Bouvier F, Rahier A and Camara B, Biogenesis, molecular regulation and function of plant isoprenoids. *Prog Lipid Res* **44**:357–429 (2005).
32 Rohmer M, Seemann M, Horbach S, BringerMeyer S and Sahm H, Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *J Am Chem Soc* **118**:2564–2566 (1996).
33 Gibson SI, Control of plant development and gene expression by sugar signaling. *Curr Opin Plant Biol* **8**:93–102 (2005).
34 Rolland F, Moore B and Sheen J, Sugar sensing and signaling in plants. *Plant Cell* **14**:185–205 (2002).
35 Fraser PD, Enfissi EMA, Halket JM, Truesdale MR, Yu DM, Gerrish C, *et al*, Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* **19**:4131–4132 (2007).
36 Cazzonelli CI and Pogson BJ, Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci* **15**:266–274 (2010).
37 Mortain-Bertrand A, Stammitti L, Telef N, Colardelle P, Brouquisse R, Rolin D, *et al*, Effects of exogenous glucose on carotenoid accumulation in tomato leaves. *Physiol Plant* **134**:246–256 (2008).
38 Dhuique-Mayer C, Fanciullino AL, Dubois C and Ollitrault P, Effect of genotype and environment on citrus juice carotenoid content. *J Agric Food Chem* **57**:9160–9168 (2009).
39 Gautier H, Diakou-Verdin V, Benard C, Reich M, Buret M, Bourgaud F, *et al*, How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance? *J Agric Food Chem* **56**:1241–1250 (2008).
40 Rodrigo MJ, Marcos JF and Zacarias L, Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *J Agric Food Chem* **52**:6724–6731 (2004).
41 Melendez-Martinez AJ, Britton G, Vicario IM and Heredia FJ, Identification of isolutein (lutein epoxide) as *cis*-antheraxanthin in orange juice. *J Agric Food Chem* **53**:9369–9373 (2005).

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.3

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Neurochemistry International 59 (2011) 729–738

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Different presynaptic nicotinic receptor subtypes modulate *in vivo* and *in vitro* the release of glycine in the rat hippocampus

Stefania Zappettini [a], Elisa Mura [b], Massimo Grilli [a], Stefania Preda [b], Alessia Salamone [a], Guendalina Olivero [a], Stefano Govoni [b], Mario Marchi [a,c,*]

[a] Section of Pharmacology and Toxicology, Department of Experimental Medicine, University of Genoa, Genoa, Italy
[b] Department of Drug Sciences, Centre of Excellence in Applied Biology, University of Pavia, Pavia, Italy
[c] Centre of Excellence for Biomedical Research, University of Genoa, Genoa, Italy

ARTICLE INFO

Article history:
Received 22 June 2011
Accepted 27 June 2011
Available online 5 July 2011

Keywords:
Synaptosomes
Glycine
Nicotinic receptor subtypes
Hippocampus
In vivo release
In vitro release

ABSTRACT

In the present study, using an *in vivo* approach (a microdialysis technique associated to HPLC with fluorimetric detection) and *in vitro* purified hippocampal synaptosomes in superfusion, we investigated the glycinergic transmission in the hippocampus, focusing on the nicotinic control of glycine (GLY) release. The acute administration of nicotine *in vivo* was able to evoke endogenous GLY release in the rat hippocampus. The specific nicotinic agonists PHA-543613 hydrochloride (PHA543613) selective for the α7 nicotinic receptor subtype administered *in vivo* also elicited GLY release in a similar extent, while the α4β2 agonist 5-IA85380 dihydrochloride (5IA85380) was less effective. Nicotine elicited GLY overflow also from hippocampal synaptosomes *in vitro*. This overflow was Ca^{2+}-dependent and inhibited by methyllycaconitine (MLA), but was not modified by dihydro-beta-erythroidine (DHβE, 1 μM). Choline(Ch)-evoked GLY overflow was Ca^{2+} dependent, unaltered in presence of DHβE and blocked by methyllycaconitine (MLA). Additionally, 5IA85380 elicited a GLY overflow, which in turn was Ca^{2+} dependent, was significantly inhibited by DHβE but was unaffected by MLA. The GLY overflow produced by these nicotinic agonists quantitatively resembles that evoked by 9 mM KCl. The effects of a high concentration of 5IA85380 (1 mM), in the presence of 2 μM DHβE, on the release of GLY was also studied comparatively to that on glutamate and aspartate release. The nicotinic agonist 5IA85380 tested at high concentration (1 mM) was able to produce a stimulatory effect of endogenous release of the three amino acids, even in the presence of 2 μM DHβE, indicating the existence of a DHβE resistant, α4β2 nAChR subtype with a functional role in the modulation of GLY, ASP, and GLU release. Our results show that in the rat hippocampus the release of GLY is, at least in part, of neuronal origin and is modulated by the activation of both α7 and α4β2 (low and high affinity) nAChR subtypes.

1. Introduction

It is well documented that glycine (GLY) is a primary inhibitory neurotransmitter in the brain stem and in the spinal cord (Aprison, 1990; Betz, 1992; Legendre, 2001). However, the presence of GLY is not limited to the above-mentioned CNS areas. Several lines of evidence show that [^{3}H]GLY is taken up through a presynaptic GLY transporter (GLYT2) into glycinergic nerve endings (Gomeza et al., 2003), and is subsequently released in several brain areas including the hippocampus (Russo et al., 1993; Raiteri et al., 2001; Luccini et al., 2008,2010; Romei et al., 2009), where it can exert a tonic inhibitory role controlling neuronal excitability (Mori et al., 2002; Semyanov et al., 2004; Wang and Xu, 2006; Zhang et al., 2008b; Keck and White, 2009). Glycine receptors (GLYRs) are widely expressed in hippocampal regions, further supporting a role for the glycinergic neurotransmission in this area. Nevertheless, to date, the physiological role for and conditions under which GLYRs are activated remain unknown. Interestingly, most of the GLY effects in the hippocampus, as well as in other brain areas, are reported to occur through the activation of extrasynaptic receptors, since GLY innervation in most brain areas is quantitatively modest, but in the cerebellum and auditory system, and the identification of GLY synapses in adult brain is difficult (Zafra et al., 1995a,b; Danglot et al., 2004; Zeilhofer et al., 2005).

Abbreviations: AUC, area under the curve; Ch, choline; DHβE, dihydro-β-erythroidine; GLY, glycine; GLYRs, glycine receptors; 5IA85380, 5-IA85380 dihydrochloride; MLA, methyllycaconitine; nAChR, nicotinic ACh receptor; PHA543613, PHA-543613 hydrochloride.

* Corresponding author at: Section of Pharmacology and Toxicology, Department of Experimental Medicine, University of Genoa, Genoa, Italy. Tel.: +39 010 3532657; fax: +39 010 3993360.
E-mail address: marchi@pharmatox.unige.it (M. Marchi).

0197-0186/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.neuint.2011.06.022

As far as other possible neuronal sources of GLY at the hippocampal level, it has to be recalled that GLY can be co-stored with GABA and can also be present in association with GLU in glutamatergic nerve terminals (Laube et al., 2002; Cubelos et al., 2005; Raiteri et al., 2005; Muller et al., 2007; Eichler et al., 2009; Legendre et al., 2009). Moreover, the possibility that GLY could be accumulated and possibly released from astrocytes has also to be taken into consideration (Yang et al., 2003; Zhang et al., 2008a,c).

Glycine may have a dual role in the CNS, acting as an inhibitory neurotransmitter when interacting with the strychnine-sensitive receptors, and playing a stimulatory role when co-activating excitatory NMDA receptors together with glutamate (Johnson and Ascher, 1987). A detailed knowledge of the GLY release and its regulation in the hippocampus could be therefore important particularly because GLY can determine the level of activation of NMDA receptors whose GLY-binding sites are not saturated in normal conditions (Berger et al., 1998). Moreover, due to its dual action, glycinergic transmission may modulate the balance between inhibitory and excitatory transmission and, thus, may constitute an effective system in the homeostatic regulation of hippocampal synaptic plasticity (Zhang et al., 2008a,c).

Therefore, it is important to determinate the mechanism controlling GLY release from GLY-containing nerve endings. Within this context presynaptic nicotinic acetylcholine receptors (nAChRs) may play a relevant role. Indeed, nicotine stimulates neurotransmitter release activating presynaptic nAChRs in different brain areas (Wonnacott, 1997; MacDermott et al., 1999; Vizi and Lendvai, 1999; Vizi et al., 2010). Studies to investigate the nicotinic modulation of glycinergic transmission were first performed in the spinal cord, since the cholinergic activation in this area account for nicotine-associated analgesia. Although the stimulatory effect of nicotine is confirmed, the information concerning the specific involvement of nAChRs subtypes in the modulation of GLY release is, so far, controversial. It has been reported that nicotine facilitates GLY release and glycinergic neurotransmission in both rat spinal cord neurons and in the cardiac vagal neurons via specific α4β2, but not throughout α7 nAChRs (Kiyosawa et al., 2001; Wang et al., 2003). However, another paper, by Bradaïa and Trouslard (2002) showed that both α4β2 and α7 nAChRs receptors seem to be involved in the nicotinic regulation of the release of GLY in the spinal cord (Bradaïa and Trouslard, 2002). α4β2, but not α7 nAChRs were also involved in the nicotinic modulation of synaptic activity in cultured embryonic rat spinal cord interneurons (Fucile et al., 2000). Glycine release from cultured cortical neurons was also increased by nicotine through a mechanism mediated by the opening of Ca^{2+} channels and not directly by the Ca^{2+} influx through the nAChR channel. Moreover, it has been found that nAChRs, different from the major central nervous system nAChR subtypes (i.e. α4β2 and α7), possibly α3β4, were highly expressed at the glycinergic inhibitory presynaptic terminals in the substantia gelatinosa of the adult spinal cord where they can play a role in the control of the inhibitory activity (Takeda et al., 2003). No data are available so far on the possible existence of nAChRs subtypes modulating GLY release at the hippocampal level.

In the present study, using an *in vivo* approach (microdialysis technique associated to HPLC with fluorimetric detection) and *in vitro* purified hippocampal synaptosomes in superfusion, we tried to fill the gap concerning the knowledge of glycinergic transmission in hippocampus, focusing on the nicotinic control of GLY release. Particularly, we investigated whether and to what extent some specific nicotinic agonists (Bencherif et al., 1998; Alkondon et al., 1999; Wishka et al., 2006) were able to evoke, *in vivo* and *in vitro*, endogenous GLY release in the rat hippocampus. In this brain area, the results indicate that: (a) the release of GLY is, at least in part, of neuronal origin, (b) the release of GLY is modulated by the activation of both α7 and α4β2 nAChR subtypes.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200–250 g, Harlan, Udine) were used for both *in vivo* experiments and as brain tissue source for *in vitro* experiments. Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (light 7 a.m.–7 p.m.). The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (Decreto Ministeriale number 124/2003-A). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

2.2. In vivo experiments

2.2.1. Microdialysis probe implantation

Rats were anesthetized with Equithesin 3 ml/kg (pentobarbital 9.7 g, chloral hydrate 42.5 g, $MgSO_4$ 21.3 g for 1L, 10% ethanol, 40% propylene glycol v/v) administered intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected, and cut with a sterile scalpel to expose the skull. A hole was drilled to allow the implantation of the probe into the brain parenchyma. The probe was implanted in the hippocampus (CA1/CA2 regions; AP −5.8 mm, ML ± 5.0 mm from bregma and DV −8.0 mm from dura) according to the Paxinos and Watson atlas (1986), and secured to the skull with one stainless steel screw and dental cement. All *in vivo* experiments were performed using microdialysis probes, made in our laboratory according to the original method described by Di Chiara (1990) (Emophan Bellco Artificial OR-internal diameter 200 μm, cutoff 40 KDa; Bellco, Mirandola, Italy), with a nominal active length of 5 mm. Finally, the skin was sutured, and the rats were allowed to recover from anesthesia for at least 24 h before the neurotransmitter release study.

2.2.2. Microdialysis samples collection

Microdialysis experiments were performed on conscious freely moving rats. On the day of the experiments (24 hr after the surgical procedure), the probe was perfused with artificial CSF containing 145 mM NaCl, 3.0 mM KCl, 1.26 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM Na_2HPO_4, buffered at pH 7.2–7.4 and filtered through a Millipore 0.2 μm pore membrane. In all experiments, the microdialysis membrane was allowed to stabilize for 1 h at the flow rate of 4 μl/min, without collecting samples. At the end of the stabilization period, three samples were collected to evaluate baseline release of GLY, and then the specific treatment started. All treatments were administered by manually switching syringes and tubing connections to allow drugs diluted in artificial CSF to flow through the probes. Tubing switches were performed taking care to maintain constant flow rates and collection volumes. Both basal and treatment samples were collected every 20 min in 100 μl Eppendorf tubes. The flow rate of 4 μl/min was maintained using a 1000 μl syringe (Hamilton) and a microinjection pump (CMA/100, CMA/Microdialysis AB). *In vitro* recovery of the probe for GLY was about 20%. Each rat was used for only one microdialysis session. At the end of each experiment animals were sacrificed by guillotine, rat brains were removed and the position of the microdialysis probe was verified by histological procedures, slicing the tissues by a cryostat microtome (LEICA CM 1510). Only data

from rats in which probe tracks were exactly located in the target area were used for statistical analysis.

2.3. In vitro experiments

2.3.1. Experiments of release

Rats were killed by decapitation and the hippocampus rapidly removed at 0–4 °C. Purified synaptosomes were prepared on Percoll® gradients (Sigma–Aldrich, St Louis, MO, USA) essentially according to Nakamura et al. (1993), with only minor modifications. Briefly, the tissue was homogenized in 6 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged (5 min, 1000 *g* at 4 °C) to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 *g* for 5 min at 4 °C. The layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in physiological HEPES-buffered medium having the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4 (Lu et al. 1998) synaptosomal protein content following purification was 10–15% of that in the supernatant stratified on the Percoll® gradient.

The synaptosomal suspension was layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37 °C (Raiteri and Raiteri, 2000; Superfusion System, Ugo Basile, Comerio, Varese, Italy). Synaptosomes were superfused at 1 ml/min with standard physiological medium as previously described. The system was first equilibrated during 36.5 min of superfusion; subsequently, four consecutive 90 s fractions of superfusate were collected. Synaptosomes were exposed to agonists for 90 s starting from the second fraction collected (t = 38 min), with antagonists being added 8 min before agonists. The evoked overflow was calculated by subtracting the corresponding basal release from each fraction and was expressed as pmol/mg of synaptosomal proteins. We have previously amply demonstrated that in our superfusion system the possible effects of drugs operated indirectly by other mediators in the monolayer of synaptosomes in superfusion are absolutely minimized (Raiteri and Raiteri, 2000).

2.4. Endogenous aminoacid determination

Endogenous GLY, GLU and ASP were measured by high performance liquid chromatography analysis following precolumn derivatization with *o*-phthalaldehyde and resolution through a C18-reverse phase chromatographic column (10 × 4.6 mm, 3 μm; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as internal standard. Buffers and gradient program were prepared and executed as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min.

2.5. Statistical analysis

2.5.1. In vivo experiments

Values were expressed either as amount of GLY measured in the dialysate (pmol/80 μL) or as area under the curve (AUC), evaluating the cumulative release over time. AUC was used as a measure of treatment exposure and was calculated, for each animal, using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA, USA), defining as baseline of the area the basal value (average concentration of three consecutive samples immediately preceding the drug dose).

D'Agostino–Pearson Omnibus Test (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA, USA) and Grubb's Test (GraphPad QuickCalcs, online calculator for scientists at http://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA) were used as preliminary tests in order to evaluate whether data were sampled from a Gaussian distribution and to detect outliers respectively. All outliers were excluded from the analysis. Data were then analyzed by analysis of variance (one- or two-way ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Data were considered significant for $P < 0.05$. The reported data are expressed as means ± SEM. The number of animals used for each experiment is reported in the legend to Fig. 1.

2.5.2. In vitro experiments

Multiple comparisons were performed with one- or two-way ANOVA followed by an appropriate *post hoc* test (Dunnett, Tukey–Kramer and Newman–Keuls). Data were considered significant for $P < 0.05$, at least. The EC_{50} and Hillslope have been calculated according to a four parameter logistic curve equation [y = min + max – min/1 + $(x/EC_{50})^{Hillslope}$] of Sigma Plot 8.0 (Jandel Scientific, San Rafael, CA, USA).

2.6. Chemicals

Percoll®, Choline Iodide, dimethyl sulfoxide, nicotine hydrogen tartrate salt (Sigma–Aldrich, St Louis, MO, USA); 5-Iodo-A-85380, RJR2429 dihydrochloride, PHA-543613 hydrochloride, dihydro-β-erythroidine hydrobromide, methyllycaconitine citrate, (Tocris Bioscience, Bristol, UK); all salts used for the preparation of aCSF (NaCl, KCl, $CaCl_2$, $MgCl_2$, Na_2HPO_4) and for Equithesin ($MgSO_4$) were purchased at Merck KGaA, Darmstadt, Germany; chloral hydrate, ethanol 96% and propylene glycol were used for the preparation of Equithesin and were obtained at VWR BDH Prolabo, Belgium.

3. Results

We first analyzed the effect of an acute administration of nicotine on hippocampal GLY release *in vivo*. The choice of the concentration of the cholinergic agonist was derived from literature data demonstrating that the administration, by microdialysis, of 50 mM nicotine was able to significantly increase the levels of GLY in hippocampal extracellular compartment (Toth et al., 1992; Toth, 1996). Under our experimental conditions a 40 min-long administration of 50 mM nicotine was able to greatly enhanced GLY release from basal values (42%). The time course of the endogenous GLY release evoked by nicotine *in vivo* is reported in the inset to Fig. 1. Basing on such time course, we analyzed the effect of nicotine exposure on the cumulative amount of GLY release over time, calculating the AUC. Then, we compared the average AUC after nicotine exposure to the average AUCs obtained after the separate perfusion of two selective nAChRs agonists, 5-IA85380 dihydrochloride (5IA85380), selective for α4β2 nAChRs, and PHA-543613 hydrochloride (PHA543613), selective for α7 subtypes. As shown in Fig. 1, nicotine (50 mM) and PHA543613 (1 mM) elicited almost the same amount of GLY release over time (average AUC nicotine = 259.6 ± 23.35 vs. average AUC PHA543613 = 205.1 ± 29.37) while 5IA85380 (1 mM) was much less effective (average AUC 5IA85380 = 90.96 ± 12.17; −65% compared to the AUC of GLY release elicited by nicotine).

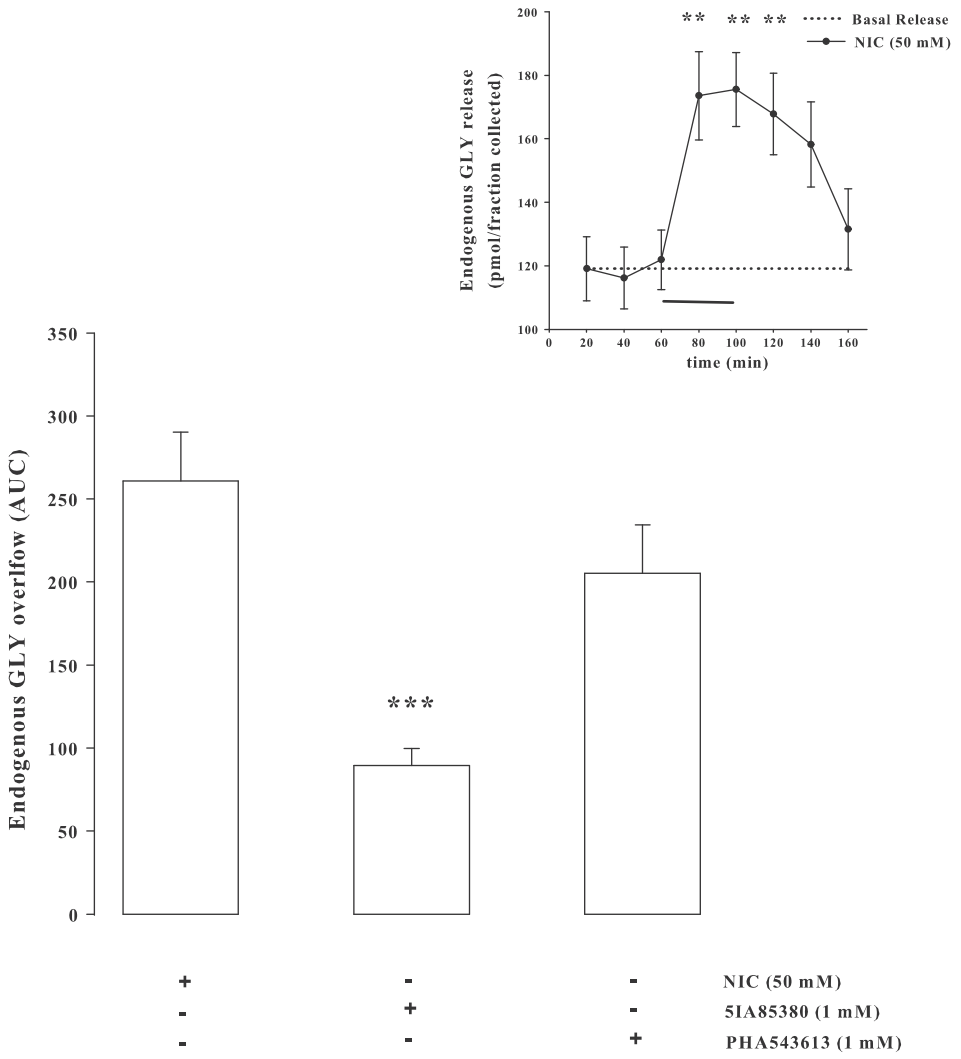


Fig. 1. Characterization of the *in vivo* nicotine-evoked endogenous GLY release. Effect of nicotine (NIC), 5IA85380 and PHA543613 on endogenous GLY overflow (AUC) from rat hippocampus. Data are mean ± SEM of 4–18 experiments. $^{***}P < 0.001$ versus nicotine-evoked GLY overflow. One way ANOVA followed by Tukey–Kramer *post hoc* test. Inset time course of GLY release in response to nicotine. Dotted line represents basal release while black line indicates permanent stimulation with nicotine. $^{**}P < 0.01$ versus basal release. Two way ANOVA followed by Bonferroni *post hoc* test. Values are from 18 experiments and represent mean ± SEM.

In order to better explore the type and localization of the nAChRs involved in the hippocampal nicotine-evoked GLY release, we performed *in vitro* experiments on purified isolated nerve endings. Fig. 2A illustrates the time course of the endogenous GLY release evoked *in vitro* by nicotine. The maximal effect is reached in fraction 41 (about 3 min after having stimulated the synaptosomes with nicotine) and then the release returns to the basal level. Nicotine in a concentration-dependent manner (0.01–0.1 mM) increased GLY overflow with an apparent EC_{50} value of 6.59 ± 4.84 μM (Hill coefficient: 0.99; Fig. 2B). The nicotine-evoked GLY overflow was almost totally Ca^{2+}-dependent and significantly inhibited (−70%) by methyllycaconitine (MLA) (10 nM) but not modified by dihydro-β-erythroidine (DHβE, 1 μM).

Table 1 shows the *in vitro* effects of different concentrations of KCl and four different nicotinic agonists on endogenous GLY release from rat purified hippocampal synaptosomes in superfusion. In this study, we used two α7 selective agonists, choline (Ch) and PHA543613, and the α4β2 selective compounds 5IA85380 and RJR2429 dihydrochloride. The GLY overflow elicited by Ch (1 mM) was significantly higher than that elicited by PHA543613 (100 μM) (211.57 ± 26.04 and 100.8 ± 26.92 respectively. The GLY overflow elicited by the selective α4β2 receptor agonists 5IA85380 (10 nM) was similar to that evoked by Ch and significantly higher than that elicited by RJR2429 dihydrochloride (3 μM) (216.28 ± 23.44 and 143.10 ± 19.34 respectively). The stimulatory effects of these four agonists have been compared to the GLY overflow evoked by depolarization with 9 and 15 mM KCl. In the presence of 9 and 15 mM KCl in the perfusion solution, the GLY overflows were 150.73 ± 31.60 and 447.34 ± 76.46, respectively. Therefore, the amount of endogenous GLY released by all the four nicotinic agonists was quantitatively very similar to that released by the lower concentration of KCl (9 mM).

Fig. 3A shows that the 5IA85380 (10 nM)-evoked GLY overflow was totally dependent upon external Ca^{2+}, almost totally blocked (−80%) by DHβE (1 μM) and unaffected by MLA (10 nM). In addition, the Ch(1 mM)-evoked overflow was totally dependent upon external Ca^{2+}, significantly antagonized (−77%) by MLA (10 nM) and unaffected by DHβE (1 μM), confirming the involvement of an α7 nAChR subtype (Fig. 3B). DHβE (1 μM) and MLA (10 nM) did not produce any significant effect on basal GLY release on their own.

Two distinct components of nicotinic agonist-mediated ^{86}Rb efflux were revealed in mouse brain synaptosomes (Marks et al., 1999). One that was relatively sensitive to inhibition by DHβE

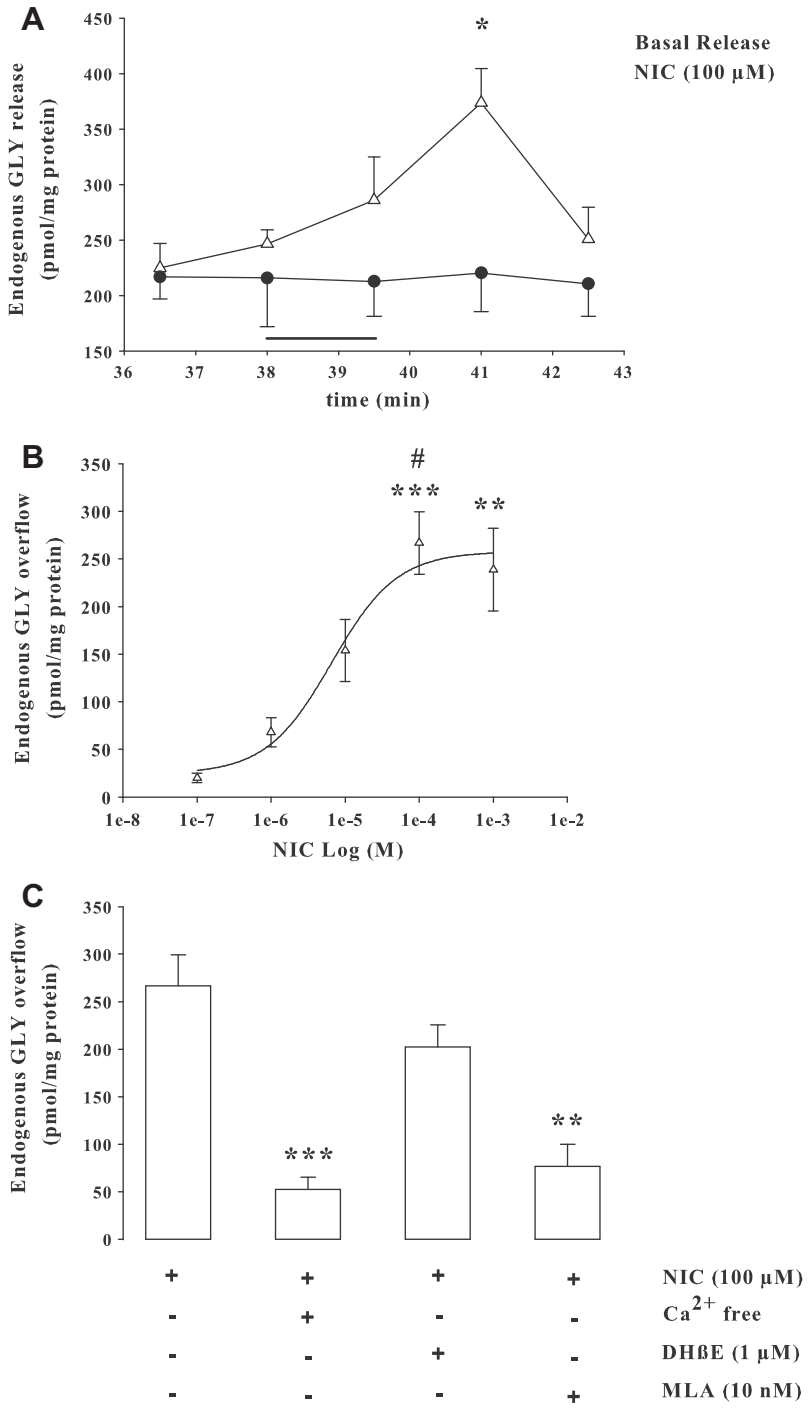


Fig. 2. (A) Time course of nicotine-evoked GLY release *in vitro*. Synaptosomes were depolarized with nicotine (NIC) for 90 s at $t = 38$ min of superfusion. Values represent mean ± SEM of at least eight replicate superfusion chambers per condition (basal or evoked release). $^{*}P < 0.05$, versus basal release. Two way ANOVA followed by Tukey–Kramer *post hoc* test. (B) Concentration-dependent effect of nicotine on endogenous GLY overflow from rat hippocampal synaptosomes. Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus nicotine 100 nM; $^{\#}P < 0.05$, versus nicotine 1 µM. One way ANOVA followed by Tukey–Kramer post hoc test. (C) Effect of Ca^{2+} free, DHβE and MLA on endogenous GLY overflow evoked by nicotine from rat hippocampal synaptosomes. Synaptosomes were depolarized with nicotine for 90 s at $t = 38$ min of superfusion. When appropriate antagonists were introduced 8 min before depolarization or Ca^{2+} was omitted 18 min before nicotine. Data are mean ± SEM of 3–6 experiments run in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus nicotine evoked GLY overflow, one way ANOVA followed by Dunnett post hoc test.

and one that was less sensitive to inhibition. In order to investigate on the presence of low affinity α4β2 nAChR subtypes modulating GLY release we extended our study and verified whether 5IA85380 at higher concentration (1 mM) in the presence of different concentrations of DHβE (2 µM–1 mM) was able to evoke GLY overflow. Moreover, we comparatively evaluated the 5IA85380 (1 mM) evoked overflow of GLU and ASP in the same experimental conditions. The results reported in Fig. 4 show that 5IA85380 at higher concentrations (1 mM) in the presence of 2 µM DHβE produced a stimulatory effect of endogenous overflow of all the three amino acids. The calculated overflow of GLY elicited by high concentration of 5IA85380 (1 mM) in presence of DHβE (2 µM) was very similar to that produced by 10 nM which was on the contrary completely blocked in presence of 2 µM DHβE (Fig. 3A). The

Table 1
Effects of selective nAChR subtype agonists on endogenous GLY overflow from rat hippocampal synaptosomes.

Drugs	Endogenous GLY overflow (pmol/mg protein)
α7 nAChR subtype agonists	
Ch (1 mM)	211.57 ± 26.04[a]
PHA543613 (100 μM)	100.8 ± 26.92[a],[b]
α4β2 nAChR subtype agonists	
5IA85380 (10 nM)	216.28 ± 23.44[a]
RJR2429 (3 μM)	143.10 ± 19.34[a],[c]
KCl (9 mM)	150.73 ± 31.60[a]
KCl (15 mM)	447.34 ± 76.46

Data are means ± SEM of three experiments run in triplicate. For experimental details see Section 2.

[a] $P < 0.01$ versus KCl 15 mM, one way ANOVA followed by Tukey–Kramer *post hoc* test.

[b] $P < 0.05$ versus Ch 1 mM, one way ANOVA followed by Tukey–Kramer *post hoc* test.

[c] $P < 0.05$ versus 5IA85380 10 nM, one way ANOVA followed by Tukey–Kramer *post hoc* test.

5IA85380 (1 mM) plus DHβE (2 μM) evoked GLU (119 ± 28 pmol/mg protein) and ASP (63 ± 13 pmol/mg protein) overflows were significantly lower than that of GLY (242 ± 35 pmol/mg protein) (Fig. 4). The antagonistic effect of DHβE on the overflow of GLY, GLU and ASP evoked by 5IA85380 (1 mM) becomes evident in presence of higher concentrations (10 μM–1 mM) and the maximal significant antagonistic effect was reached at the concentration of 1 mM. Of course we have to consider that both agonists and antagonists could be less specific at very high doses and therefore the possibility that 5IA85380 at this concentration activates also other presynaptic receptors, has to be considered.

4. Discussion

The present work demonstrates for the first time that nicotine can increase the *in vivo* release of endogenous GLY from rat hippocampus. We have previously shown (Fedele et al., 1998) a nicotine-evoked release of GLU and ASP, but we were unable to describe a nicotine-evoked release of GLY. In the present paper we used a different microdialysis approach, leading to the sampling of the CA1/CA2 area of the hippocampus, and we administered a higher nicotine concentration. In our experimental condition we clearly found a significant nicotine-evoked GLY release, we demonstrated that this stimulatory effect is probably due to the activation of two distinct nAChR subtypes since 5IA85380 and PHA543613, selective agonist of α4β2 and of α7 nAChRs respectively, also increase the *in vivo* GLY release. This is the first *in vivo* demonstration of the involvement of two different nAChRs subtypes on GLY release. However, the *in vivo* results do not permit the precise localization of such nAChRs which may reside either on the soma or at the level of the nerve terminal of glycinergic neurons, as well as on other non-glycinergic neurons (Laube et al., 2002; Cubelos et al., 2005; Raiteri et al., 2005; Muller et al., 2007; Eichler et al., 2009; Legendre et al., 2009). Additionally, since according to several authors, GLY is accumulated in the astrocytes the transmitter could be released also from these cells (Yang et al., 2003; Zhang et al., 2008a,c). In this regard, the existence of nAChRs on astrocytes has been reported (Oikawa et al., 2005; Xiu et al., 2005) and it has been also already demonstrated that α7 nAChRs modulate GLU release from hippocampal gliosomes (Patti et al., 2007) that are astrocyte sub-cellular particles isolated from the adult rat or mice brain (Nakamura et al., 1993. Stigliani et al., 2006).

As far as the precise localization of the nAChRs modulating GLY release our *in vitro* results demonstrate that, at least in part, the nicotine evoked GLY release comes from a neuronal source. Indeed, the selective α4β2 and α7 nAChRs agonists produced a significant Ca^{2+}-dependent enhancement of GLY release from purified isolated nerve endings, a preparation which almost totally excludes the presence of glial contamination (Stigliani et al., 2006), thus confirming the neuronal source of GLY release. Although, the glycinergic innervation in the rat hippocampus is quantitatively modest, the existence of selective glycinergic nerve endings has been demonstrated using the same subcellular preparation we used in this study (Russo et al., 1993; Raiteri et al., 2001; Luccini et al., 2008, 2010; Romei et al., 2009). Therefore, the possibility that different subtypes of nAChRs are present on these glycinergic terminals and elicit endogenous GLY release is very likely to occur.

The *in vivo* results suggest a prevalent role of the α7 nAChRs in the modulation of GLY release, while the data collected from *in vitro* experiments show that both receptor subtypes seem to be able to elicit GLY release with a similar potency. The discrepancy between the *in vivo* and *in vitro* experiments may have different causes. First of all, in hippocampus, *in vivo,* nAChRs are present on different neuronal populations. Hence, the levels of GLY measured *in vivo* are the final result of the interactions of hierarchically organized synapses ultimately controlling GLY release. These indirect mechanisms of control of neurotransmitter release are excluded in our *in vitro* model of synaptosomes in superfusion (Raiteri and Raiteri, 2000). Moreover, *in vivo* an amount of the total level of neurotransmitter measured in the dialysate may also derive from astrocytes, where a putative nicotinic control of GLY release has not well studied. In our microdialysis experiments we do not know the availability of the selective nicotinic agonists in the tissue after their perfusion through the probe. On the other hand, *in vivo* concentrations are higher than those used *in vitro* in order to guarantee the delivery to the tissue of sufficient amount of drugs. Finally, another difference between the two experimental models relies on the timing of exposure to experimental drugs (few seconds *in vitro* versus 40 min *in vivo*).

Another interesting finding of our work is the demonstration of the existence of a presynaptic α4β2, DHβE resistant, nAChRs which modulate the release of GLY indicating that high and low affinity α4β2 nAChRs are both involved in the modulation of the release of this aminoacid. The existence of DHβE resistant nAChRs may explain the results of Fig. 2, which show that the GLY overflow evoked by a high concentration of nicotine was partially inhibited by the α7 nAChRs antagonist MLA, but almost unaltered in presence of DHβE. The most likely explanation for the lack of effect of DHβE could be that, using very high concentration of nicotine, the evoked release of GLY was almost entirely due to the stimulation of an α4β2 DHβE resistant nAChRs. Interestingly, as shown in Fig. 4, also the release of GLU and ASP was elicited in a similar manner by a high concentration of 5IA85380 in presence of DHβE, demonstrating the existence of functional nAChR, DHβE resistant, also on GLU nerve endings. Therefore, our results demonstrate for the first time that DHβE resistant nAChRs play an important stimulatory role on the release of GLU, ASP and GLY. Moreover, at the hippocampal level the release of all these neurotransmitters seem to be elicited by nAChRs of the same subtypes.

Our *in vitro* data clearly demonstrate both the neuronal source of the GLY released and the presynaptic localization of the nAChRs. However, the direct identification of the precise neuronal terminals from which GLY is released is difficult to determine since it is possible that, besides the release from specific glycinergic neurones, GLY can be co-localized and co-released with other neurotransmitters such as GLU and GABA. In this regard, it has to be noted that double labeling confocal microscopy revealed the existence of the GLY transporter GLYT1, in a specific subpopulation of GLU nerve terminals in the hippocampus (Cubelos et al., 2005; Raiteri and Raiteri, 2000) confirming previous observations of a tight association between GLYT1 and glutamatergic neurotransmission

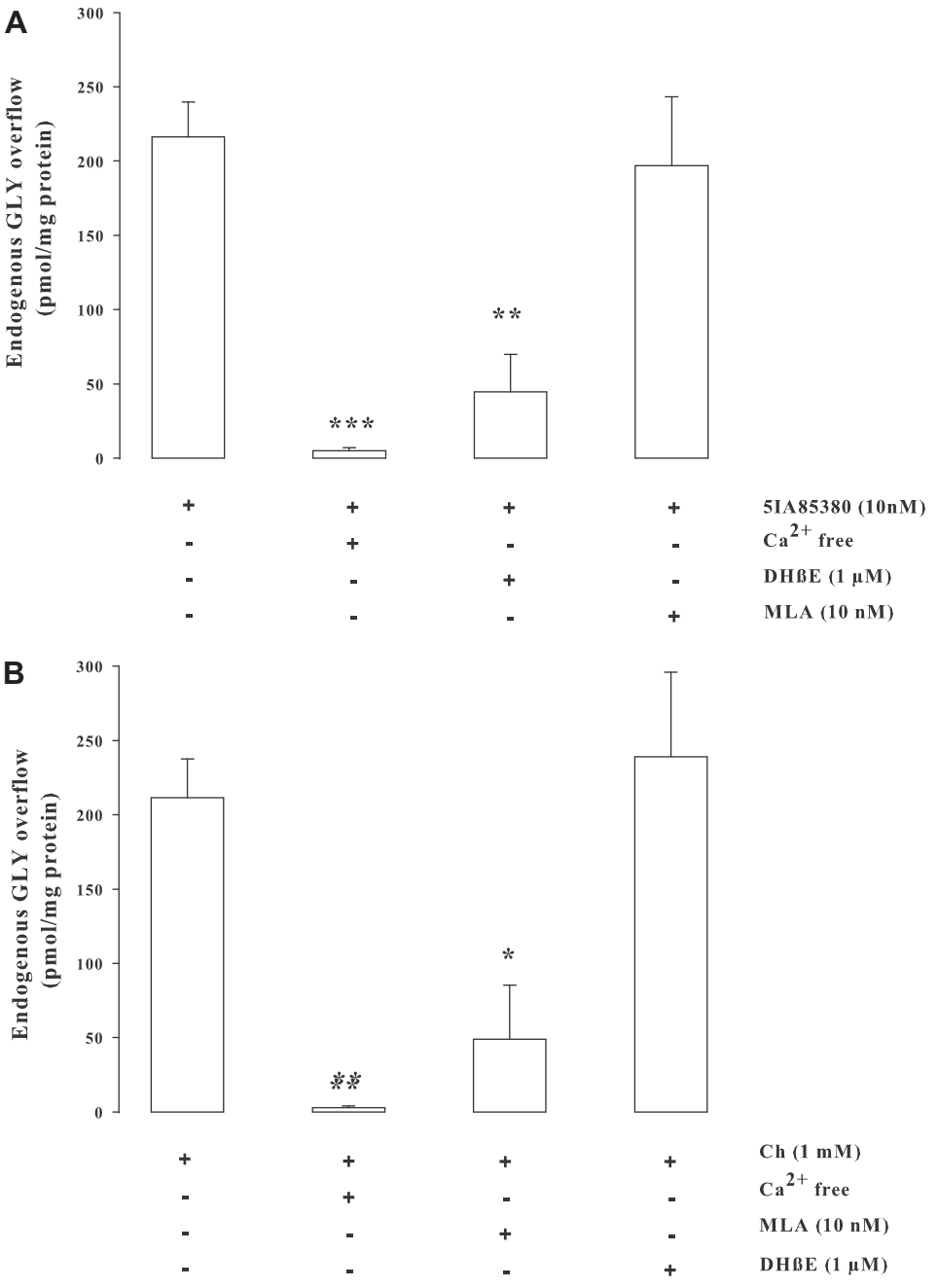


Fig. 3. (A) Effect of DHβE, MLA, and Ca^{2+} free on endogenous GLY overflow evoked by 5IA85380 from rat hippocampal synaptosomes. Synaptosomes were depolarized with 5IA85380 for 90 s at $t = 38$ min of superfusion. When appropriate antagonists were introduced 8 min before 5IA85380. Ca^{2+} was omitted 18 min before 5IA85380. Data are mean ± SEM of 3–6 experiments run in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus 5IA85380 evoked GLY overflow. One way ANOVA followed by Dunnett *post hoc* test. (B) Effect of MLA, DHβE, and Ca^{2+} free on endogenous GLY overflow evoked by Ch from rat hippocampal synaptosomes. Synaptosomes were depolarized with Ch for 90 s at $t = 38$ min of superfusion. When appropriate antagonists were introduced 8 min before Ch. Ca^{2+} was omitted 18 min before Ch. Data are mean ± SEM of 3–6 experiments run in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Ch evoked GLY overflow, one way ANOVA followed by Dunnett *post hoc* test.

at the mRNA level (Smith et al., 1992; Borowsky et al., 1993; Zafra et al., 1995a,b). Moreover, the co-localization of GLY and GABA in hippocampal interneurons, similar to spinal cord, brain stem, and cerebellum, suggests that this property is likely to be a general characteristic of inhibitory interneurons throughout the CNS.

The direct evidence for the nicotine-evoked GLY release from glutamatergic or GABAergic terminals has never been reported. However, it would be conceivable to hypothesize that, if GLY were co-released with these aminoacids, it should be possibly evoked by the activation of the same nAChRs subtypes present on GLU or GABA nerve endings. The demonstration that nAChRs of the same subtypes (α4β2, DHβE sensitive and resistant, and α7 nAChRs) stimulate the endogenous GLU, ASP and GLY release strongly support the possibility that, at least in part, the nicotine-evoked GLY release could be due to GLY co-stored in glutamate nerve endings. On the contrary to what expected, our results suggest that the source of the endogenous GLY release elicited by the low affinity, DHβE resistant, nAChRs is not the GLY which is possibly co-stored and co-released from the GABA neurons since, as previously reported, these nAChRs subtypes were not involved in the stimulation of endogenous GABA release (Zappettini et al., 2011). However, nicotine-evoked GLY and GABA release can be elicited

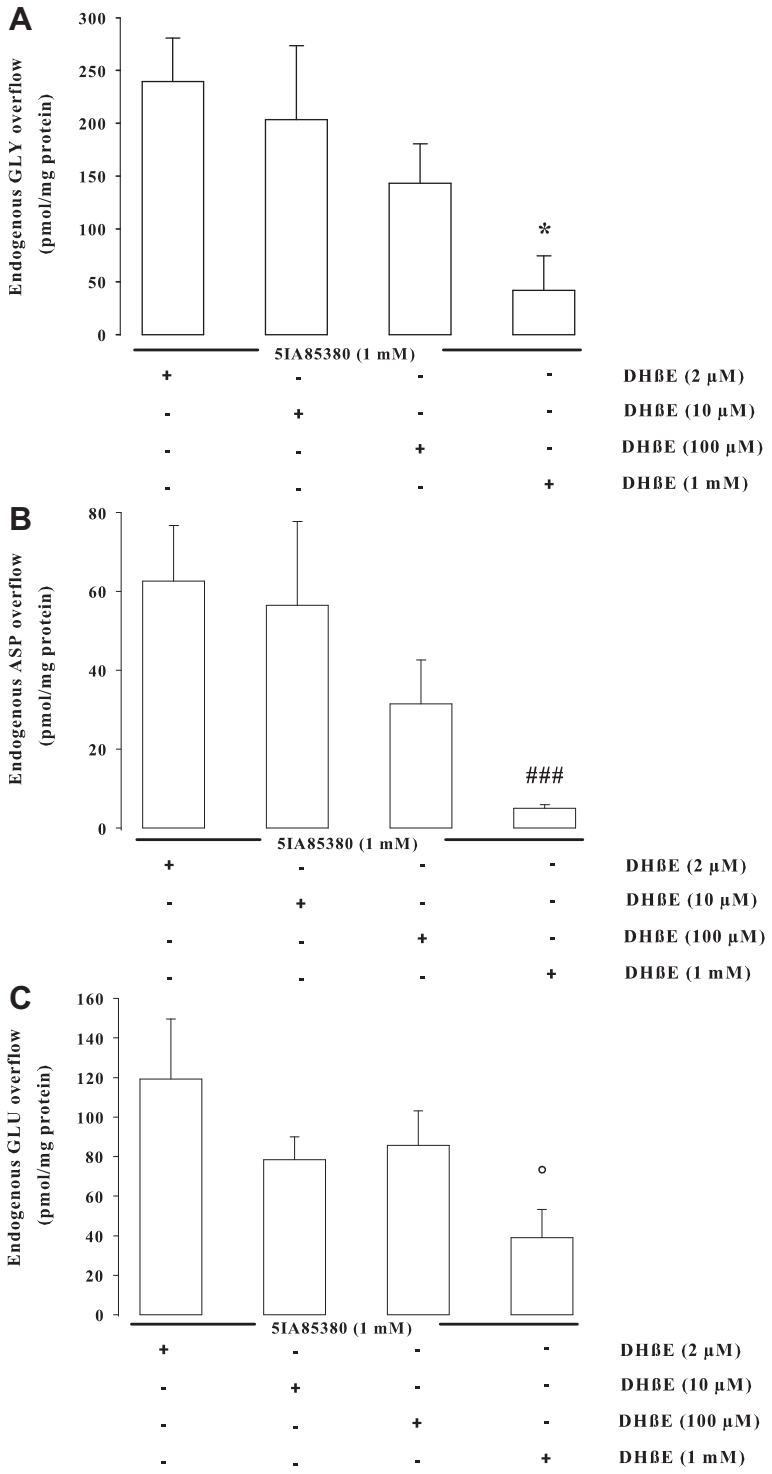


Fig. 4. Effect of 5IA85380 plus DHβE on endogenous overflow of GLY, (A) ASP (B) and GLU (C) from rat hippocampal synaptosomes. Synaptosomes were depolarized with 5IA85380 (1 mM) in presence of different concentrations of DHβE (2 μM–1 mM) for 90 s at $t = 38$ min of superfusion. Data are mean ± SEM of 3 experiments run in triplicate. $^{*}P < 0.05$ versus 5IA85380 (1 mM) plus DHβE (2 μM)-evoked GLY overflow; $^{###}P < 0.001$ versus (1 mM) plus DHβE(2 μM)-evoked ASP overflow. $P < 0.05$ versus (1 mM) plus DHβE(2 μM)-evoked GLU overflow. One way ANOVA followed by Dunnett *post hoc* test. The basal release of GLY, ASP and GLU in presence of DHβE (2 μM–1 mM) was 454 ± 10, 92 ± 12 and 213 ± 26, respectively.

also by α4β2, DHβE sensitive, and α7 nAChRs subtypes, which are present on GABA nerve endings (Zappettini et al., 2011) and modulate GLY release as we have shown in this paper. This view is compatible with a nicotinic-evoked release of GLY co-stored in GABAergic nerve terminals. Recent findings show that both α4β2 and α7 nAChR subtype modulate also *in vivo* the GABA release in rat hippocampus (Zappettini et al., 2011).

The idea that GLY can be released not only from pure glycinergic nerve endings but also from other neuronal sources is further supported by the analysis of the amount of GLY released comparatively to the release of GLU and GABA. In fact both the K^+-evoked and the nicotine-evoked GLY released from purified synaptosomes is 3–4 times higher in comparison to that of GLU and GABA (Zappettini et al., 2010, 2011), which would be in contrast with the very

scarce glycinergic innervation present in the hippocampus. The amount of glycine released following nicotine or KCl stimulation would therefore support, as suggested, the possibility that the major source of GLY released come from glutamatergic or GABAergic nerve endings.

In conclusion, we have demonstrated that hippocampal GLY release, possibly from glycinergic nerve endings, is stimulated presynaptically by three different nAChR subtypes ($\alpha4\beta2$, DHβE sensitive and resistant, and $\alpha7$ nAChRs). Moreover, the possibility that nAChRs present on GLU or GABA nerve terminals modulate GLY which is co-released with these neurotransmitters, is likely to occur.

The hypothesized nicotinic modulation of the co-release of GLY with GLU or GABA allows several speculations on putative role of this modulation in both various neuropsychiatric disease and in nicotine dependence, focusing the interest on the nicotinic regulation of glycinergic transmission as a target for drug investigation. It has to be considered that GLY is not only an agonist at the GLY receptors but also acts as essential co-agonist of GLU/ASP at the NMDA receptors. The NMDA receptor hypofunction (Lindsley et al., 2006; Yang and Svensson, 2008; Gaspar et al., 2009) and dysfunction of inhibitory neurotransmission (Lewis et al., 2005; Charych et al., 2009) have been implicated in the pathophysiology of severe psychiatric disorders such as schizophrenia. Interestingly, smoking occurs at higher rates than in the general population among individuals with schizophrenia, who may smoke more because nicotine helps them to cope with cognitive deficits that characterize these states (Kumari and Postma, 2005; Potter et al., 2006). The increase of GLY concentration at NMDA receptor level was found to be beneficial in the therapy of schizophrenic patients. A selective stimulatory effect by nicotine agonists of the co-release of GLY and GLU/ASP should therefore produce an improvement of the hypofunction of the NMDA receptors and could be useful in this pathology.

Acknowledgements

Mario Marchi would like to express deep gratitude to the colleague and friend Maurizio Raiteri for having, long time ago, introduced him to the exciting world of "synaptosomes in superfusion" and all times inspired and encouraged his research. We wish to thank Maura Agate for editorial assistance and Dr. Silvia E. Smith (University of Utah School of Medicine) for reviewing the manuscript. This work was supported by the Italian Ministero Università Ricerca to Prof. Mario Marchi (prot. N 20072BTSR2_002) and to Prof. Govoni (MIUR 2009), by Compagnia di San Paolo; AROMA (ALCOTRA 2007–2013), European project PYRGI.

References

Alkondon, M., Pereira, E.F.R., Eisenberg, H.M., Albuquerque, E.X., 1999. Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. J. Neurosci. 19, 2693–2705.

Aprison, M.K., 1990. The discovery of the neurotransmitter role of glycine. In: Ottersen, O.P., Storm Mathisen, J. (Eds.), Glycine Transm.. Wiley, New York, pp. 1–24.

Bencherif, M., Schmitt, J.D., Bhatti, B.S., Crooks, P., Caldwell, W.S., Lovette, M.E., Fowler, K., Reeves, L., Lippiello, P.M., 1998. The heterocyclic substituted pyridine derivative (±)-2-(-3-pyridinyl)-1-Azabicyclo[2.2.2]octane (RJR-2429): a selective ligand at nicotinic acetylcholine receptors. J. Pharmacol. Exp. Ther. 284, 886–894.

Berger, A.J., Dieudonné, S., Ascher, P., 1998. Glycine uptake governs glycine site occupancy at NMDA receptors of excitatory synapses. J. Neurophysiol. 80, 3336–3340.

Betz, H., 1992. Structure and function of inhibitory glycine receptors. Q. Rev. Biophys. 25, 381–394.

Borowsky, B., Mezey, E., Hoffman, B.J., 1993. Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron 10, 851–863.

Bradaïa, A., Trouslard, J., 2002. Nicotinic receptors regulate the release of glycine onto lamina X neurons of the rat spinal cord. Neuropharmacology 43, 1044–1054.

Charych, E.I., Liu, F., Moss, S.J., Brandon, N.J., 2009. $GABA_A$ receptors and their associated proteins: implications in the etiology and treatment of schizophrenia and related disorders. Neuropharmacology 57, 481–495.

Cubelos, N., Gimenéz, C., Zafra, F., 2005. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb. Cortex 15, 448–459.

Danglot, L., Rostaing, P., Triller, A., Bessis, A., 2004. Morphologically identified glycinergic synapses in the hippocampus. Mol. Cell. Neurosci. 27, 394–403.

Di Chiara, G., 1990. *In-vivo* brain dialysis of neurotransmitters. Trends Pharmacol. Sci. 11, 116–121.

Eichler, S.A., Forstera, B., Smolinsky, B., Juttner, R., Lehmann, T.N., Fahling, M., Schwarz, G., Legendre, P., Meier, J.C., 2009. Splice-specific roles of glycine receptor alpha3 in the hippocampus. Eur. J. Neurosci. 30, 1077–1091.

Fedele, E., Varnier, G., Ansaldo, M.A., Raiteri, M., 1998. Nicotine administration stimulates the *in vivo* *N*-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. Br. J. Pharmacol. 125, 1042–1048.

Fucile, S., De Saint Jan, D., de Carvalho, L.P., Bregestovski, P., 2000. Fast potentiation of glycine receptor channels of intracellular calcium in neurons and transfected cells. Neuron 28, 571–583.

Gaspar, P.A., Bustamante, M.L., Silva, H., Aboitiz, F., 2009. Molecular mechanisms underlying glutamatergic dysfunction in schizophrenia: therapeutic implications. J. Neurochem. 111, 891–900.

Gomeza, J., Ohno, K., Hülsmann, S., Armsen, W., Eulenburg, V., Richter, D.W., Laube, B., Betz, H., 2003. Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron 40, 797–806.

Johnson, J.W., Ascher, P., 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325, 529–531.

Keck, T., White, J.A., 2009. Glycinergic inhibition in the hippocampus. Rev. Neurosci. 20, 13–22.

Kiyosawa, A., Katsurabayashi, S., Akaike, N., Pang, Z.P., Akaike, N., 2001. Nicotine facilitates glycine release in the rat spinal dorsal horn. J. Physiol. 536, 101–110.

Kumari, V., Postma, P., 2005. Nicotine use in schizophrenia: the self medication hypotheses. Neurosci. Biobehav. Rev. 29, 1021–1034.

Laube, B., Maksay, G., Schemm, R., Betz, H., 2002. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? Trends Pharmacol. Sci. 23, 519–527.

Legendre, P., 2001. The glycinergic inhibitory synapse. Cell. Mol. Life Sci. 58, 760–793.

Legendre, P., Forstera, B., Juttner, R., Meier, J.C., 2009. Glycine receptors caught between genome and proteome—functional implications of RNA editing and splicing. Front. Mol. Neurosci. 2, 23.

Lewis, D.A., Hashimoto, T., Volk, D.W., 2005. Cortical inhibitory neurons and schizophrenia. Nat. Rev. Neurosci. 6, 312–324.

Lindsley, C.W., Shipe, W.D., Wolkenberg, S.E., Theberge, C.R., Williams Jr., D.L., Sur, C., Kinney, G.G., 2006. Progress towards validating the NMDA receptor hypofunction hypothesis of schizophrenia. Curr. Top. Med. Chem. 6, 771–785.

Lu, Y., Grady, S., Marks, M.J., Picciotto, M., Changeux, J.-P., Collins, A.C., 1998. Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. J. Pharmacol. Exp. Ther. 287, 648–657.

Luccini, E., Romei, C., Raiteri, L., 2008. Glycinergic nerve endings in hippocampus and spinal cord release glycine by different mechanisms in response to identical depolarizing stimuli. J. Neurochem. 105, 2179–2189.

Luccini, E., Romei, C., Di Prisco, S., Raiteri, M., Raiteri, L., 2010. Ionic dysregulations typical of ischemia provoke release of glycine and GABA by multiple mechanisms. J. Neurochem. 114, 1074–1084.

MacDermott, A.B., Role, L.W., Siegelbaum, S.A., 1999. Presynaptic ionotropic receptors and the control of transmitter release. Annu. Rev. Neurosci. 22, 443–485.

Marks, M.J., Whiteaker, P., Calcaterra, J., Stitzel, J.A., Bullock, A.E., Grady, S.R., Picciotto, M.R., Changeux, J.P., Collins, A.C., 1999. Two pharmacologically distinct components of nicotinic receptor-mediated rubidium efflux in mouse brain require the beta2 subunit. J. Pharmacol. Exp. Ther. 289, 1090–1103.

Mori, M., Gähwiler, B.H., Gerber, U., 2002. Beta-alanine and taurine as endogenous agonists at glycine receptors in rat hippocampus in vitro. J. Physiol. 539, 191–200.

Muller, E., Bakkar, W., Legendre, P., Bergeron, R., Martina, M., 2007. Site and mechanism of glycine release at glutamatergic synapse in mouse hippocampus. Society for Neuroscience, San Diego, CA, Program No. 358.12/J20.

Nakamura, Y., Iga, K., Shibata, T., Shudo, M., Kataoka, K., 1993. Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. Glia 9, 48–56.

Oikawa, H., Nakamichi, N., Kambe, Y., Ogura, M., Yoneda, Y., 2005. An increase in intracellular free calcium ions by nicotinic acetylcholine receptors in a single cultured rat cortical astrocyte. J. Neurosci. Res. 79, 535–544.

Patti, L., Raiteri, L., Grilli, M., Zappettini, S., Bonanno, G., Marchi, M., 2007. Evidence that alpha-7 nicotinic receptor modulates glutamate release from mouse neocortical gliosomes. Neurochem. Int. 51, 1–7.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic coordinates, second ed. Academic Press, New York.

Potter, A.S., Newhouse, P.A., Bucci, D.J., 2006. Central nicotinic cholinergic systems: a role in the cognitive dysfunction in attention-deficit/hyperactivity disorder? Behav. Brain. Res. 175, 201–211.

Raiteri, L., Raiteri, M., 2000. Synaptosomes still viable after 25 years of superfusion. Neurochem. Res. 25, 1265–1274.

Raiteri, L., Raiteri, M., Bonanno, G., 2001. Glycine is taken up through $GLYT_1$ and $GLYT_2$ transporters into mouse spinal cord axon terminals and causes vesicular release of its proposed cotransmitter GABA. J. Neurochem. 76, 1823–1832.

Raiteri, L., Zappettini, S., Stigliani, S., Paluzzi, S., Raiteri, M., Bonanno, G., 2005. Glutamate release induced by activation of glycine and GABA transporters in spinal cord is enhanced in a mouse model of amyotrophic lateral sclerosis. Neurotoxicology 26, 883–892.

Romei, C., Luccini, E., Raiteri, M., Raiteri, L., 2009. $GABA_B$ presynaptic receptors modulate glycine exocytosis from mouse spinal cord and hippocampus glycinergic nerve endings. Pharmacol. Res. 59, 154–159.

Russo, C., Marchi, M., Andrioli, G.C., Cavazzani, P., Raiteri, M., 1993. Enhancement of glycine release from human brain cortex synaptosomes by acetylcholine acting at M4 muscarinic receptors. J. Pharmacol. Exp. Ther. 266, 142–146.

Semyanov, A., Walker, M.C., Kullmann, D.M., Silver, R.A., 2004. Tonically active GABAA receptors: modulating gain and maintaining the tone. Trends Neurosci. 27, 262–269.

Smith, K.E., Borden, L.A., Hartig, P.R., Branchek, T., Weinshank, R.L., 1992. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 8, 927–935.

Stigliani, S., Zappettini, S., Raiteri, L., Passalacqua, M., Melloni, E., Venturi, C., Tacchetti, C., Diaspro, A., Usai, C., Bonanno, G., 2006. Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. J. Neurochem. 96, 656–668.

Takeda, D., Nakatsuka, T., Papke, R., Gu, J.G., 2003. Modulation of inhibitory synaptic activity by a non-alpha4beta2, non-alpha7 subtype of nicotinic receptors in the substantia gelatinosa of adult rat spinal cord. Pain 101, 13–23.

Toth, E., 1996. Effect of nicotine on the level of extracellular amino acids in the hippocampus of rat. Neurochem. Res. 21, 903–907.

Toth, E., Sershen, H., Hashim, A., Vizi, E.S., 1992. Effect of nicotine on extracellular levels of neurotransmitters assessed by microdialysis in various brain regions: role of glutamic acid. Neurochem. Res. 17, 265–271.

Vizi, E.S., Lendvai, B., 1999. Modulatory role of presynaptic nicotinic receptors in synaptic and non-synaptic chemical communication in the central nervous system. Brain Res. Rev. 30, 219–235.

Vizi, E.S., Fekete, A., Karoly, R., Mike, A., 2010. Non-synaptic receptors and transporters involved in brain functions and targets of drug treatment. Br. J. Pharmacol. 160, 785–809.

Wang, J., Wang, X., Irnaten, M., Venkatesan, P., Evans, C., Baxi, S., Mendelowitz, D., 2003. Endogenous acetylcholine and nicotine activation enhances GABAergic and glycinergic inputs to cardiac vagal neurons. J. Neurophysiol. 89, 2473–2481.

Wang, W., Xu, T.L., 2006. Chloride homeostasis differentially affects GABA(A) receptor- and glycine receptor-mediated effects on spontaneous circuit activity in hippocampal cell culture. Neurosci. Lett. 406, 11–16.

Wishka, D.G., Walker, D.P., Yates, K.M., Reitz, S.C., Jia, S., Myers, J.K., Olson, K.L., Jacobsen, E.J., Wolfe, M.L., Groppi, V.E., Hanchar, A.J., Thornburgh, B.A., Cortes-Burgos, L.A., Wong, E.H., Staton, B.A., Raub, T.J., Higdon, N.R., Wall, T.M., Hurst, R.S., Walters, R.R., Hoffmann, W.E., Hajos, M., Franklin, S., Carey, G., Gold, L.H., Cook, K.K., Sands, S.B., Zhao, S.X., Soglia, J.R., Kalgutkar, A.S., Arneric, S.P., Rogers, B.N., 2006. Discovery of N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide, an agonist of the alpha7 nicotinic acetylcholine receptor, for the potential treatment of cognitive deficits in schizophrenia: synthesis and structure–activity relationship. J. Med. Chem. 49, 4425–4436.

Wonnacott, S., 1997. Presynaptic nicotinic ACh receptors. Trends Neurosci. 20, 92–98.

Xiu, J., Nordberg, A., Zhang, J.T., Guan, Z.Z., 2005. Expression of nicotinic receptors on primary cultures of rat astrocytes and up-regulation of the alpha7, alpha4 and beta2 subunits in response to nanomolar concentrations of the beta-amyloid peptide(1–42). Neurochem. Int. 47, 281–290.

Yang, C.R., Svensson, K.A., 2008. Allosteric modulation of NMDA receptor via elevation of brain glycine and D-serine: the therapeutic potentials for schizophrenia. Pharmacol. Ther. 120, 317–332.

Yang, Y., Ge, W., Chen, Y., Zhang, Z., Shen, W., Wu, C., Poo, M., Duan, S., 2003. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc. Natl. Acad. Sci. USA 100, 15194–15199.

Zafra, F., Aragón, C., Olivares, L., Danbolt, N.C., Giménez, C., Storm-Mathisen, J., 1995a. Glycine transporters are differentially expressed among CNS cells. J. Neurosci. 15, 3952–3969.

Zafra, F., Gomeza, J., Olivares, L., Aragón, C., Giménez, C., 1995b. Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. Eur. J. Neurosci. 7, 1342–1352.

Zappettini, S., Grilli, M., Salamone, A., Fedele, E., Marchi, M., 2010. Presynaptic nicotinic receptors evoke endogenous glutamate and aspartate release from hippocampal synaptosomes by way of distinct coupling mechanisms. Br. J. Pharmacol. 161, 1161–1171.

Zappettini, S., Grilli, M., Lagomarsino, F., Fedele, E., Marchi, M., 2011. Presynaptic nicotinic $\alpha7$ and non-$\alpha7$ receptors stimulate endogenous GABA release from rat hippocampal synaptosomes through two distinct mechanisms of action. PLoS One 6, e16911.

Zeilhofer, H.U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bösl, M.R., Fritschy, J.M., 2005. Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. J. Comp. Neurol. 482, 123–141.

Zhang, L.H., Gong, N., Fei, D., Xu, L., Xu, T.L., 2008a. Glycine uptake regulates hippocampal network activity via glycine receptor-mediated tonic inhibition. Neuropsychopharmacology 33, 701–711.

Zhang, X.B., Sun, G.C., Liu, L.Y., Yu, F., Xu, T.L., 2008b. Alpha2 subunit specificity of cyclothiazide inhibition on glycine receptors. Mol. Pharmacol. 73, 1195–1202.

Zhang, Z., Gong, N., Wang, W., Xu, L., Xu, T.L., 2008c. Bell-shaped D-serine actions on hippocampal long-term depression and spatial memory retrieval. Cereb. Cortex 18, 2391–2401.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.4

OPEN ACCESS Freely available online

PLoS one

Dual Effect of Beta-Amyloid on α7 and α4β2 Nicotinic Receptors Controlling the Release of Glutamate, Aspartate and GABA in Rat Hippocampus

Elisa Mura[1☯], Stefania Zappettini[2☯], Stefania Preda[1], Fabrizio Biundo[1], Cristina Lanni[1], Massimo Grilli[2], Anna Cavallero[2], Guendalina Olivero[2], Alessia Salamone[2], Stefano Govoni[1]*, Mario Marchi[2,3]

1 Department of Drug Sciences, Centre of Excellence in Applied Biology, University of Pavia, Pavia, Italy, **2** Section of Pharmacology and Toxicology, Department of Experimental Medicine, University of Genoa, Genoa, Italy, **3** Centre of Excellence for Biomedical Research, University of Genoa, Genoa, Italy

Abstract

Background: We previously showed that beta-amyloid (Aβ), a peptide considered as relevant to Alzheimer's Disease, is able to act as a neuromodulator affecting neurotransmitter release in absence of evident sign of neurotoxicity in two different rat brain areas. In this paper we focused on the hippocampus, a brain area which is sensitive to Alzheimer's Disease pathology, evaluating the effect of Aβ (at different concentrations) on the neurotransmitter release stimulated by the activation of pre-synaptic cholinergic nicotinic receptors (nAChRs, α4β2 and α7 subtypes). Particularly, we focused on some neurotransmitters that are usually involved in learning and memory: glutamate, aspartate and GABA.

Methodology/Findings: We used a dual approach: *in vivo* experiments (microdialysis technique on freely moving rats) in parallel to *in vitro* experiments (isolated nerve endings derived from rat hippocampus). Both *in vivo* and *in vitro* the administration of nicotine stimulated an overflow of aspartate, glutamate and GABA. This effect was greatly inhibited by the highest concentrations of Aβ considered (10 μM *in vivo* and 100 nM *in vitro*). *In vivo* administration of 100 nM Aβ (the lowest concentration considered) potentiated the GABA overflow evoked by nicotine. All these effects were specific for Aβ and for nicotinic secretory stimuli. The *in vitro* administration of either choline or 5-Iodo-A-85380 dihydrochloride (α7 and α4β2 nAChRs selective agonists, respectively) elicited the hippocampal release of aspartate, glutamate, and GABA. High Aβ concentrations (100 nM) inhibited the overflow of all three neurotransmitters evoked by both choline and 5-Iodo-A-85380 dihydrochloride. On the contrary, low Aβ concentrations (1 nM and 100 pM) selectively acted on α7 subtypes potentiating the choline-induced release of both aspartate and glutamate, but not the one of GABA.

Conclusions/Significance: The results reinforce the concept that Aβ has relevant neuromodulatory effects, which may span from facilitation to inhibition of stimulated release depending upon the concentration used.

Citation: Mura E, Zappettini S, Preda S, Biundo F, Lanni C, et al. (2012) Dual Effect of Beta-Amyloid on α7 and α4β2 Nicotinic Receptors Controlling the Release of Glutamate, Aspartate and GABA in Rat Hippocampus. PLoS ONE 7(1): e29661. doi:10.1371/journal.pone.0029661

Editor: Maria A. Deli, Biological Research Center of the Hungarian Academy of Sciences, Hungary

Received July 21, 2011; **Accepted** December 1, 2011; **Published** January 11, 2012

Funding: This work was supported by the Italian Ministero Università Ricerca to Prof. S. Govoni (prot. N° 2007HJCCSF and MIUR 2009) and to Prof. M. Marchi (prot. N° 20072BTSR2_002), by Compagnia di San Paolo; AROMA ([ALCOTRA] Alpi Latine Cooperazione Transfrontaliera 2007–2013), European project PYRGI. Dr. E. Mura is an awardee of the Alzheimer's Association (NIRG-11-205183). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: govonis@unipv.it

☯ These authors contributed equally to this work.

Introduction

In 1984 Glenner and Wong sequenced a small peptide isolated from the brains of Alzheimer's disease (AD) patients. This peptide, known as beta-amyloid (Aβ), was subsequently recognized as the main pathogenetic marker of AD [1]. The increased levels of the peptide in extracellular sites lead to subsequent events that include the aggregation and deposition of Aβ, the hyperphosphorylation of tau protein, the occurrence of neurotoxic phenomena and consequent neuronal death and, finally, dementia [2,3].

Although the neurotoxic role of Aβ is unchallenged, the scientific community has emphasized the existence of physiological roles for the peptide (as reviewed in [4]). The idea is that Aβ may be important in normal brain functioning, but when it exceeds certain concentrations the peptide may become neurotoxic. Both different aggregates and isoforms of Aβ may have different biological actions in a continuum from physiology to pathology determining and participating to the subsequent stages of the disease [4]. In this regard, we previously showed that Aβ acts as a neuromodulator, affecting neurotransmitter release in the absence of evident signs of neurotoxicity [5–7]. This role may be at borderline between physiology and pathology. In a physiological context, the neuromodulatory role of Aβ would be important for the proper balance among neurotransmitter systems. On the contrary, in pathological conditions the Aβ-mediated synaptic modulation might be related to Aβ-driven functional alterations of neurotransmission in addition and before the well known neurodegenerative events. The dysregulation of neurotransmission

may in turn produce early cognitive and non-cognitive disturbances based on the neurotransmitter systems and the brain area involved.

For all these reasons, we started to systematically explore the effect of the peptide on different brain areas and neurotransmitter systems both *in vivo* and *in vitro*. Our previous studies showed that Aβ inhibits the cholinergic control of both dopamine and γ-aminobutyric acid (GABA) release in the nucleus accumbens and caudate putamen [5–7]. In these brain areas, Aβ affects neurotransmitter release acting downstream muscarinic receptors, particularly on protein kinase C [5].

The purpose of the present study is to evaluate whether nicotinic acetylcholine receptors (nAChRs) may also be involved in the neuromodulatory action of Aβ. In this regard, several findings suggest that Aβ interacts with high affinity with nAChRs. The peptide can bind to α7 and with 100–5000 times lower affinity to α4β2 nAChRs [8]. Interestingly, the Aβ-nAChRs interaction may serve non-neurotoxic roles (as the control of synaptic plasticity and neuronal homeostasis), as well as contributing to AD etiology [9]. There are conflicting data concerning the type of effect exerted by Aβ on nAChRs, with some authors showing the activation of the receptor, whereas others report an antagonist action [10]. These differences may be related either to the experimental model investigated or to the Aβ species and concentrations administered [11].

An intriguing brain area for the purpose of this study is the hippocampus, a neuroanatomical structure that is implicated in learning and memory and that is involved in AD from the early stages of the disease [12]. Cholinergic projections to the hippocampus mainly derive from the medial septum-diagonal band via the fimbria fornix [13,14]. Acetylcholine released from these projections acts on both muscarinic and nicotinic targets modulating neurotransmission [15,16]. Interestingly, we recently demonstrated that the activation of both α7 and α4β2 nAChRs subtypes promotes the hippocampal release of inhibitory and excitatory neurotransmitters such as GABA, glutamate (Glu), and aspartate (Asp) [17,18] This cholinergic control of GABAergic and glutamatergic systems may have important effects concerning the intra-hippocampal circuits modulating synaptic plasticity, a process relevant to memory trace formation [19]. Moreover, a putative modulatory effect of Aβ on excitatory and inhibitory transmitters may be relevant in the derangements of synaptic activity preceding and accompanying neurodegenerative processes associated with Aβ deposition in the course of the disease.

We used only Aβ1–40 peptide in our experiments, for two main reasons. First, physiologically the 40-amino-acid long peptide is the most abundant form [20–22]. Second, Aβ1–42 has been reported to aggregate faster than Aβ1–40 [23] and thus it is considered as the most neurotoxic species [24]. With the aim of exploring new effects of Aβ other than the neurotoxic ones, we chose to avoid this potentially confounding element.

For all these reasons, using both *in vivo* (microdialysis) and *in vitro* (synaptosomes in superfusion) techniques, we studied whether Aβ1–40 (pM-μM) may affect the nicotine (Nic)-evoked release of GABA, Glu and Asp in hippocampus. In particular, we evaluated whether the neuromodulatory action of Aβ may be exerted on both α7 and α4β2 nAChRs subtypes.

The results presented here show the Aβ capability to regulate the nicotinic control of aspartate, glutamate and GABA release in absence of evident signs of neurotoxicity, and in a way that depends upon the concentration used and the nicotinic receptor subtype involved.

Results

In vivo results

We first performed immunohistochemical analysis in order to test whether the administration of Aβ1–40 through the dialysis probe allowed the delivery of the peptide to the tissue. Fig. 1 shows the presence of the peptide for all the concentrations tested (100 nM–10 μM) within the hippocampus. As expected, there was a visible positive correlation between the concentration administered and the signal of Aβ immunoreactivity in the tissue.

We characterized the Aβ peptide conformation by using Western Blot procedure, starting from the stock solution (100 μM Aβ1–40 solution). With SDS-PAGE, all Aβ1–40 preparations analyzed resolved to immunoreactive species consistent with Aβ monomer (Fig. S1).

We then analyzed the effect of an acute administration of Nic on the release of GABA, Glu, and Asp in hippocampus *in vivo*.

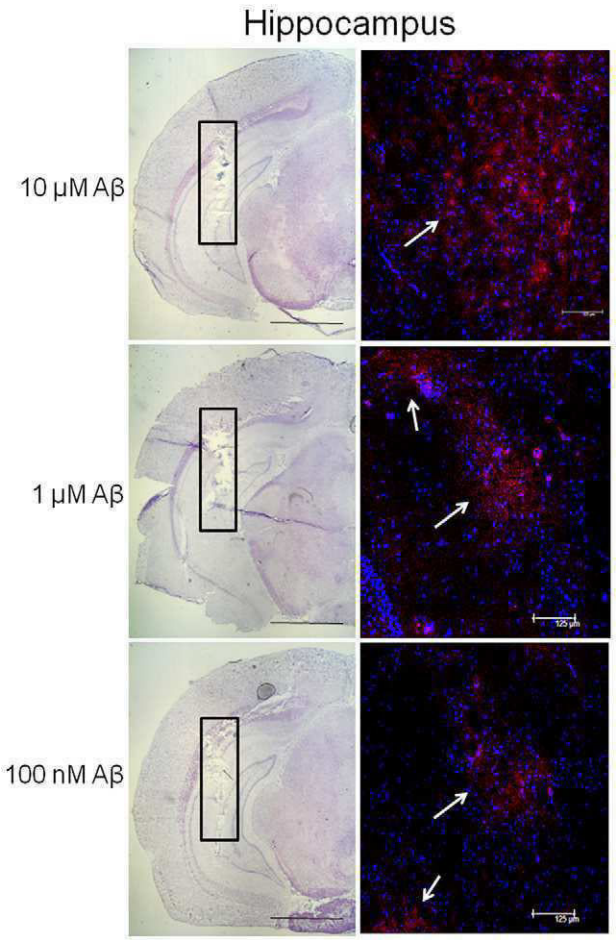


Figure 1. Immunohistochemical analysis showing beta-amyloid (Aβ) content in hippocampal tissue after its administration at different concentrations. Coronal section indicating the location of the probe (hippocampus, black rectangle) counterstained with Mayer Hematoxylin and relative fluorescence micrographs of the area within the black rectangle showing the presence of human Aβ protein. Aβ immunoreactivity (red-PE staining, white arrows) immediately after perfusion of 10 μM, 1 μM and 100 nM Aβ1–40. Nuclear DNA was counterstained with Hoechst 33342 (blue staining). Scale bars for Mayer hematoxylin sections: 200 μm. Scale bars for fluorescent micrographs: 125 μm.
doi:10.1371/journal.pone.0029661.g001

Previously published data have shown that the administration by microdialysis of 20 mM Nic is not enough to evoke a GABA overflow in hippocampus [25]. In similar experimental conditions Toth [26] showed that 50 mM Nic is able to significantly increase the levels of Glu and Asp in hippocampal extracellular compartment. Despite Toth [26] was able to obtain a substantial release using lower concentrations, in our *in vivo* experiments the preliminary concentration curve performed (not shown) indicated 50 mM Nic as the best working condition. Hence, we evaluated 50 mM Nic. It should be stressed that microdialysis application of drugs can be considered as a point source in the brain, having a sphere of action with decreasing drug concentration. The effective concentrations used in the *in vivo* experiments cannot be specified; hence the concentration in the probe was given. Therefore the current drug concentration could be supraphysiological [27]. Future experiments comparing other administration methods may help to provide insight on this aspect.

In our experimental conditions a 40 minutes-long administration of 50 mM Nic was able to greatly enhanced GABA release from basal values. The effect of Nic peaked after 20 minutes of perfusion (1321%, Fig. 2A inset) and persisted for additional 20 minutes after the end of the treatment (+742% compared to basal values, Fig. 2A inset). Moreover, a 40 minutes-long treatment with 50 mM Nic was also effective in stimulating the release of excitatory amino acids, thus supporting the previously published data. Concerning Glu, the peak effect of Nic was observed at the end of the treatment (61%), then the release returned to the basal level (Fig. 2B inset). The time course of the endogenous Asp release evoked by Nic (50 mM) *in vivo* is reported in the inset to Fig. 2C. The effect of Nic on Asp release reached the top at the end of the perfusion with Nic (190%).

Based on the previously described time courses, we analyzed the effect of Nic exposure on the cumulative amount of neurotransmitter released over time, calculating the area under the curve (AUC). Then, we compared the average AUC after 50 mM Nic exposure to the average AUCs obtained after the separate administration of Nic in co-perfusion with Aβ1–40. Particularly, we created a dose-response curve evaluating different concentrations of the peptide (100 nM–10 μM). The Nic-evoked GABA overflow was inhibited by 10 μM Aβ1–40 (59%) and potentiated by 100 nM Aβ1–40 (35%) (Fig. 2A). Concerning 1 μM Aβ1–40 (the middle concentration evaluated *in vivo*), there was a trend to exert an inhibitory effect which, however, did not reach the statistical significance (Fig. 2A). As far as excitatory neurotransmitters, 10 μM Aβ1–40 was able to inhibit the Nic-induced overflow of both Glu (57%, Fig. 2B) and Asp (38%, Fig. 2C). Also 1 μM Aβ1–40 impaired the Nic-induced overflow of both Glu (70%) and Asp (61%), whereas 100 nM Aβ1–40 was ineffective (Fig. 2B and 2C). The reverse peptide Aβ40–1 (tested at 10 μM) did not modify the Nic-induced release of GABA (Fig. 2A), Glu (Fig. 2B) and Asp (Fig. 2C). None of the concentrations of Aβ1–40 tested *in vivo* (100 nM, 1 μM and 10 μM) affected the basal level of GABA, Glu and Asp in the hippocampus (Fig. S2).

In order to evaluate whether the effects of Aβ1–40 were selective for the nicotinic control of neurotransmitter release, we chose veratridine (Ver) as another secretory stimulus. Based on previous microdialysis publications [28,29], we evaluated two different concentrations for this depolarizing stimulus. As shown in Fig. 3A–3C insets, 100 μM Ver elicited the release of the three neurotransmitters studied although to a different extent (GABA = 1253%; Glu = 170%; Asp = 309%). The lower concentration of Ver (50 μM) was able to elicit the release of GABA (615%) but not the one of Glu and Asp. For these reasons we used 100 μM Ver in the following experiments. We then evaluated the effect of Aβ1–40 at 100 nM and 10 μM (the lowest and highest concentration that had been considered *in vivo*, respectively) on the release of neurotransmitters evoked by 100 μM Ver, comparing the specific AUC. Both of these two concentrations of Aβ1–40 did not affect the Ver-induced overflow of GABA, Glu, and Asp (Fig. 3A, Fig. 3B and Fig. 3C, respectively).

In vitro results

Likewise, Nic was able to evoke an overflow of GABA, Glu, and Asp from hippocampal nerve endings *in vitro* experiments. Figures 4A, 4B, and 4C respectively show the time course of the endogenous GABA, Glu, and Asp release enhanced by 100 μM Nic. Concerning GABA release, the peak effect (60%) of the nicotinic agonist was observed at the end of the 90 s-long treatment. In the case of both Asp and Glu the maximal effect of Nic (60% and 50% respectively) was reached in fraction 41 (about 3 minutes after having stimulated the synaptosomes with the cholinergic agonist), after which the release returns to the basal level.

Figures 4A, 4B, and 4C also show that the presence of Aβ1–40 in the medium of perfusion did not modify basal release of all three selected neurotransmitters.

We then evaluated the effects of different concentrations of Aβ1–40 (from 100 pM up to 100 nM) on the Nic-evoked overflow of GABA, Glu, and Asp. Fig. 5 shows that the highest concentration of Aβ1–40 tested *in vitro* (100 nM) greatly inhibited the Nic-induced overflow of GABA (70%), Glu (85%), and Asp (70%), whereas all the other concentrations (10 nM, 1 nM, 100 pM) were ineffective. Therefore, *in vitro* the Nic-induced GABA overflow was not potentiated by low concentrations of the peptide, as observed *in vivo*. The inhibitory effect was specific for Aβ1–40 since the reverse peptide (Aβ40–1) at the same concentration (100 nM) did not modify the Nic-stimulated release of all three neurotransmitters (Fig. 5).

Parallel to *in vivo* experiments, we also evaluated the effect of Aβ1–40 on the transmitter release stimulated by Ver *in vitro*. 10 μM Ver stimulated an overflow of GABA that was 836.81 ± 124.66 pmol mg^{-1} protein (Fig. 6A). In regard to excitatory neurotransmitters, the Ver-induced overflow of Glu and Asp was 1039.28 ± 174.84 and 179.26 ± 24.49 pmol mg^{-1} protein, respectively (Fig. 6B). Similarly to the *in vivo* results, also *in vitro* Aβ1–40 (tested at 100 pM and 100 nM, thus at the lowest and highest concentration that had been evaluated on synaptosomes) did not modify the release of GABA, Glu, and Asp that was stimulated by a depolarizing stimulus such as Ver (Fig. 6). Moreover, we also evaluated the effects of Aβ1–40 on another depolarizing secretory stimulus such as potassium (K^+). 12 mM K^+ evoked an overflow of GABA, Glu and Asp that was 694.00 ± 144.00, 827.00 ± 158.00, 102.00 ± 15.00, respectively. Figure 6 shows that 100 nM Aβ1–40 did not modify the K^+-induced release of GABA, Glu and Asp.

Our *in vitro* method (*i.e.,* synaptosomes in superfusion), permits us to unequivocally identify the receptor targeted by specific drugs and anatomically localize it onto a precise synaptosomal population, and to pharmacologically characterize it [30]. Moreover, the possibility that the evoked release of Glu, Asp and GABA may influence the release of other measured neurotransmitters can be almost totally excluded in our experimental set up. Indeed, as previously described, synaptosomes are plated as very thin layers on microporous filters and up-down superfused with physiological solutions (see [30] and references therein). Under these experimental conditions, the transmitters released are removed by the superfusion fluid before they can accumulate and activate presynaptic auto- and heteroreceptors, as

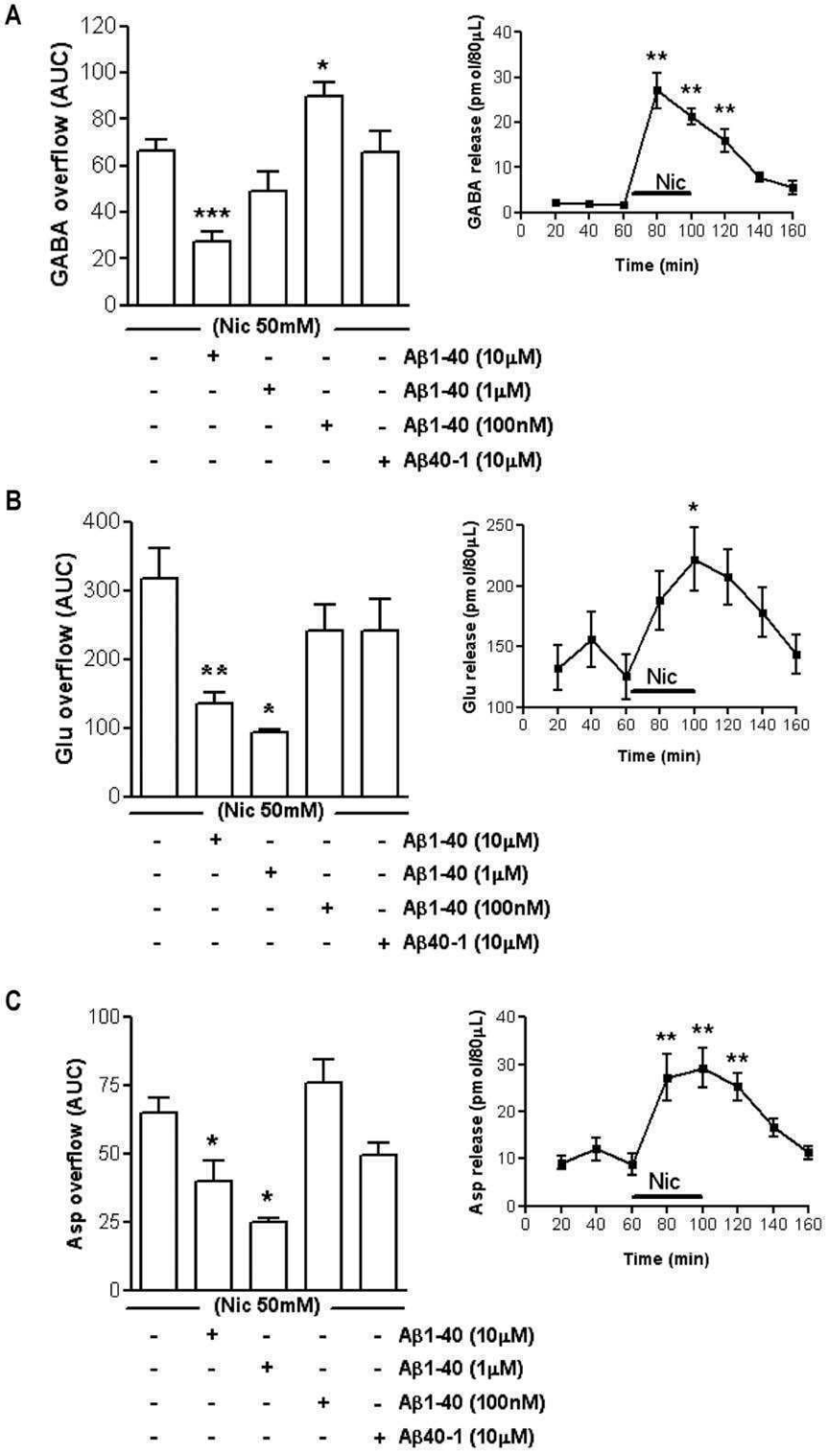


Figure 2. *In vivo* effect of different concentrations of beta-amyloid on the nicotine-induced overflow of hippocampal neurotransmitters. Effect of beta-amyloid (Aβ)1–40 (100 nM–10 µM) on 50 mM nicotine (Nic)-induced overflow of GABA (**A**), glutamate (Glu, **B**) and aspartate (Asp, **C**). *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. Nic (One-way ANOVA followed by Dunnett's Multiple Comparison Test). Data are expressed as mean ± SEM of 4–15 individual rats for each experimental group. Insets show the time course of 50 mM Nic-induced release of GABA (**A inset**), Glu (**B inset**) and Asp (**C inset**). *$p<0.05$, **$p<0.01$ vs. basal release (One-way ANOVA followed by Bonferroni *post hoc* test). Data are expressed as mean ± SEM of 13–15 individual rats.
doi:10.1371/journal.pone.0029661.g002

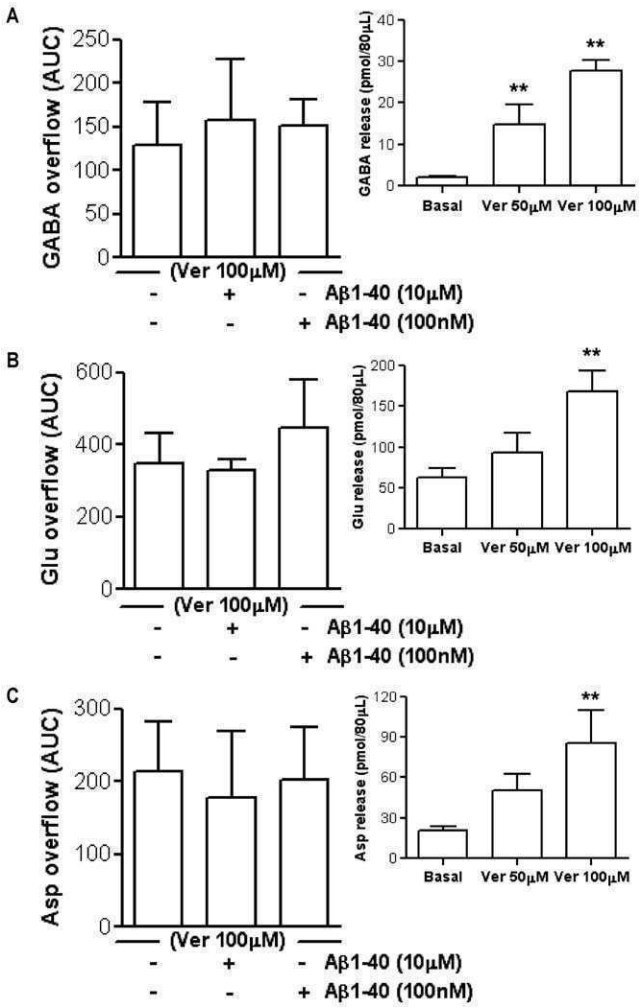


Figure 3. Lack of effect of beta-amyloid on the veratridine-induced neurotransmitter release in hippocampus *in vivo*. Effect of beta-amyloid (Aβ)1–40 (100 nM and 10 μM) on 100 μM veratridine (Ver)-induced overflow of GABA (**A**), glutamate (Glu, **B**) and aspartate (Asp, **C**). (One-way ANOVA followed by Dunnett's Multiple Comparison Test). Data are expressed as mean ± SEM of 4 individual rats for each experimental group. Insets show the effect of two different concentrations of Ver (50 μM and 100 μM) on the release of GABA (**A inset**), Glu (**B inset**) and Asp (**C inset**). **$p<0.01$ vs. Basal (One-way ANOVA followed by Dunnett's Multiple Comparison Test). Data are expressed as mean ± SEM of 4–9 individual rats for each experimental group.
doi:10.1371/journal.pone.0029661.g003

well as reuptake carriers, thus excluding the possibility of indirect effects. Using this technique, we recently demonstrated that the hippocampal release of GABA, Glu, and Asp is stimulated by the activation of both α7 and α4β2 nAChRs at presynaptic level [17,18]. Therefore, using the same experimental conditions, we evaluated whether the effects of Aβ1–40 on the Nic-evoked overflow of GABA, Glu, and Asp may be mediated by either α7 or α4β2 nAChRs or both. In order to do that, we first confirmed the presence and functional effect of both α7 and α4β2 nAChRs on glutamatergic and GABAergic hippocampal nerve endings by using specific agonists. We chose choline (Ch) and 5-Iodo-A-85380 dihydrochloride (5IA85380) as selective agonists for α7 and α4β2, respectively, and we administered them at the same concentration used in our previous studies [17,18]. In regard to GABA, 1 mM Ch evoked an overflow of 41.69±3.46 pmol mg^{-1} protein that was the same than that elicited by 10 nM 5IA85380 (40.41±3.98 pmol mg^{-1} protein (Figures 7A and 7C). As far as the excitatory neurotransmitters, both 1 mM Ch and 10 nM 5IA85380 evoked an overflow of Glu (61.07±5.22 and 76.91±8.86 pmol mg^{-1} protein respectively) and of Asp (45.52±3.16 and 56.19±4.28 pmol mg^{-1} protein respectively) (Figures 7B and 7D).

There was a dual effect of Aβ1–40 on the release of neurotransmitters evoked by Ch, the selective agonist for α7 nAChR subtype. 100 nM Aβ1–40 (the highest concentration considered *in vitro*) greatly inhibited the Ch-induced overflow of GABA (45%), Glu (75%) and Asp (70%) (Figures 7A and 7B). On the contrary, low concentrations of Aβ1–40 (100 pM and 1 nM) greatly enhanced (100% and 55%, respectively) the Ch-induced Glu release (Fig. 7B). At 100 pM (the lowest concentration considered *in vitro*) Aβ1–40 also potentiated (55%) the Asp release evoked by 1 mM Ch (Fig. 7B). The potentiating effect of low concentrations of Aβ1–40 was not observed in the case of GABA release (Fig. 7A).

As far as the α4β2-selective agonist, 100 nM Aβ1–40 (the highest concentration considered *in vitro*) greatly inhibited the 5IA85380-induced overflow of all three neurotransmitters (GABA = 60%; Glu = 70%; Asp = 85%) (Figures 7C and 7D). All the other concentrations were ineffective.

Discussion

In the present work we found for the first time that Aβ1–40 affects *in vivo* and *in vitro* the nicotinic-control of the hippocampal release of GABA, Glu, and Asp, in absence of gross signs of neurodegeneration.

Some strategies let us to avoid neurotoxic effects of the peptide. First, the study was focused on Aβ1–40 since it is the most abundant form that is present in physiological state. In fact, the concentration of secreted Aβ1–42 is about 10% that of Aβ1–40 [31]. Moreover, Aβ1–40 and Aβ1–42 have different profiles of aggregation [32]. Aβ1–40 has been reported to aggregate more slowly than Aβ1–42; therefore, the latter is considered as the most neurotoxic species [24]. On the other hand, in our previous *in vivo* experiments in nucleus accumbens, at variance with Aβ1–40, Aβ1–42 was ineffective since it was retained inside the dialysis probe and did not reach the brain tissue, as shown by immunohistochemical analysis [5]. We subsequently administered a freshly prepared solution of Aβ1–40 in order to minimize its aggregation. Finally, we used a dialysis membrane with a cutoff size of 40 KDa to allow the passage through the dialysis fiber of soluble Aβ monomers or small molecular weight oligomers and avoid high molecular weight oligomers (the neurotoxic species as shown by [33]). In our experimental conditions, the peptide did not aggregate (Fig. S1), therefore it did not give origin to neurotoxic oligomeric species.

Following the administration of different concentrations of Aβ1–40 (100 nM–10 μM), immunohistochemical analysis shows that the peptide diffuses through the dialysis membrane to the hippocampal tissue where it is found in proximity of the dialysis probe (Fig. 1). No apoptotic-related phenomena were observed within the area of amyloid diffusion as shown by Hoechst staining. However, we cannot exclude the presence, even at this early time, after Aβ treatment, of more subtle signs of toxicity such as synaptic degeneration and neurite retraction.

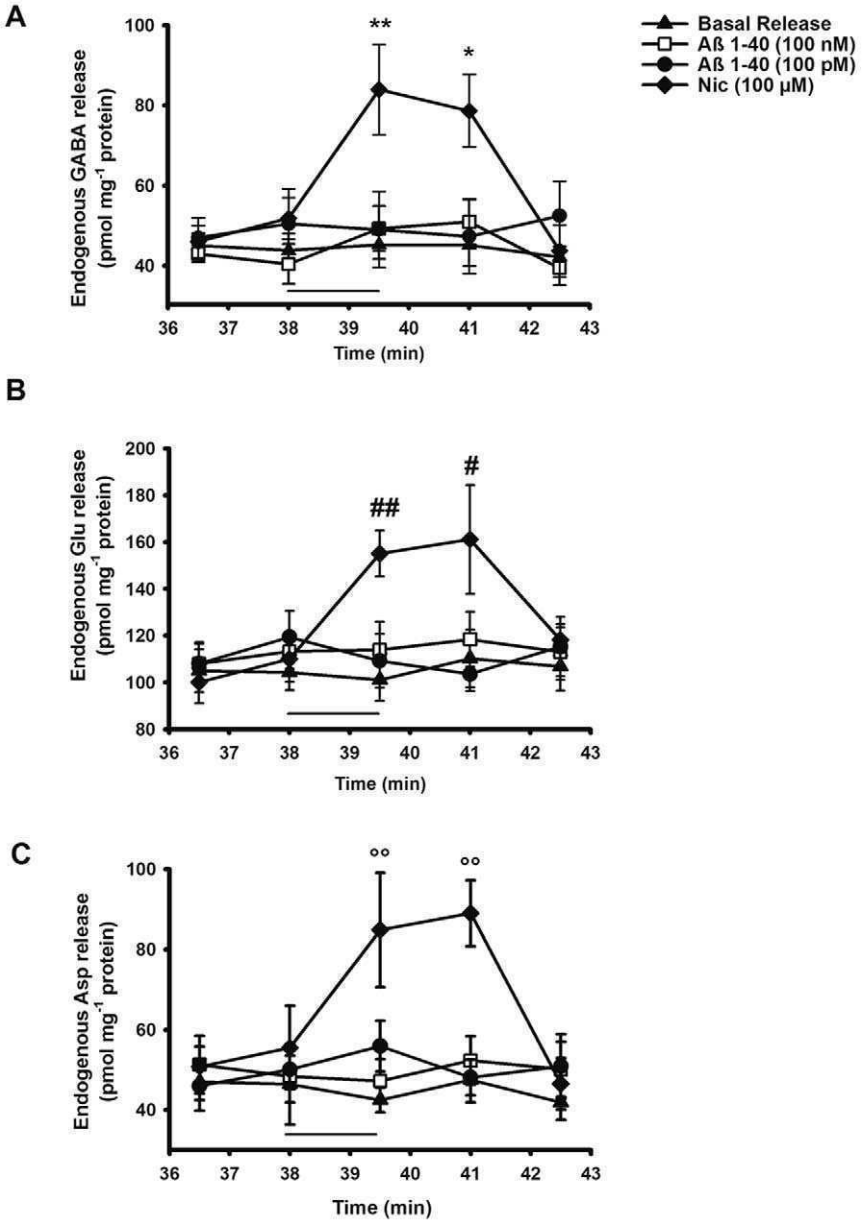


Figure 4. Time course of nicotine and beta-amyloid evoked GABA (A), glutamate (B) and aspartate (C) release *in vitro*. Synaptosomes were depolarized with nicotine (Nic) or beta-amyloid (Aβ)1–40 for 90 s at $t = 38$ min of superfusion (black bar). Values represent mean ± SEM of at least eight replicate superfusion chambers per condition (basal or evoked release). *$p<0.05$, **$p<0.01$ vs. GABA basal release; $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. glutamate (Glu) basal release; $^{\circ\circ}p<0.01$ vs. aspartate (Asp) basal release. Two way ANOVA followed by Bonferroni *post hoc* test.
doi:10.1371/journal.pone.0029661.g004

In vivo and *in vitro* inhibition of Glu and Asp release by Aβ1–40 and dual effects on GABA release

We observed both *in vivo* and *in vitro* that high concentrations of Aβ1–40 (10 µM and 100 nM, the two highest concentrations respectively used *in vivo* and *in vitro*) greatly inhibit the Nic-induced release of GABA, Glu, and Asp in the hippocampus (Figures 2 and 5). The observed inhibitory effect is consistent with that previously described in the nucleus accumbens and striatum at similar concentrations when studying dopamine and GABA release [5–7]. The inhibitory effect on Glu and Asp but not on GABA release was observed also using lower *in vivo* concentrations (1 µM) of administered Aβ (Fig. 2). Surprisingly enough, *in vivo* 100 nM Aβ1–40 (the lowest concentration considered) was able to potentiate the GABA overflow evoked by 50 mM Nic (Fig. 2). To our knowledge, this is the first demonstration of the Aβ1–40 capability to modulate both positively and negatively the nicotinic cholinergic control of GABA release *in vivo*. The observed dual effect of the peptide is in line with the hypothesis that Aβ may have different biological effects increasing the concentrations, possibly in a continuum from physiology to pathology [4]. Interestingly, 100 nM Aβ1–40 potentiates the Nic-induced GABA release (function), 1 µM Aβ1–40 is ineffective (loss of function) and 10 µM Aβ1–40 has an inhibitory effect (gain of new function). The dual effect of Aβ on Nic-induced GABA release was not appreciated *in vitro*.

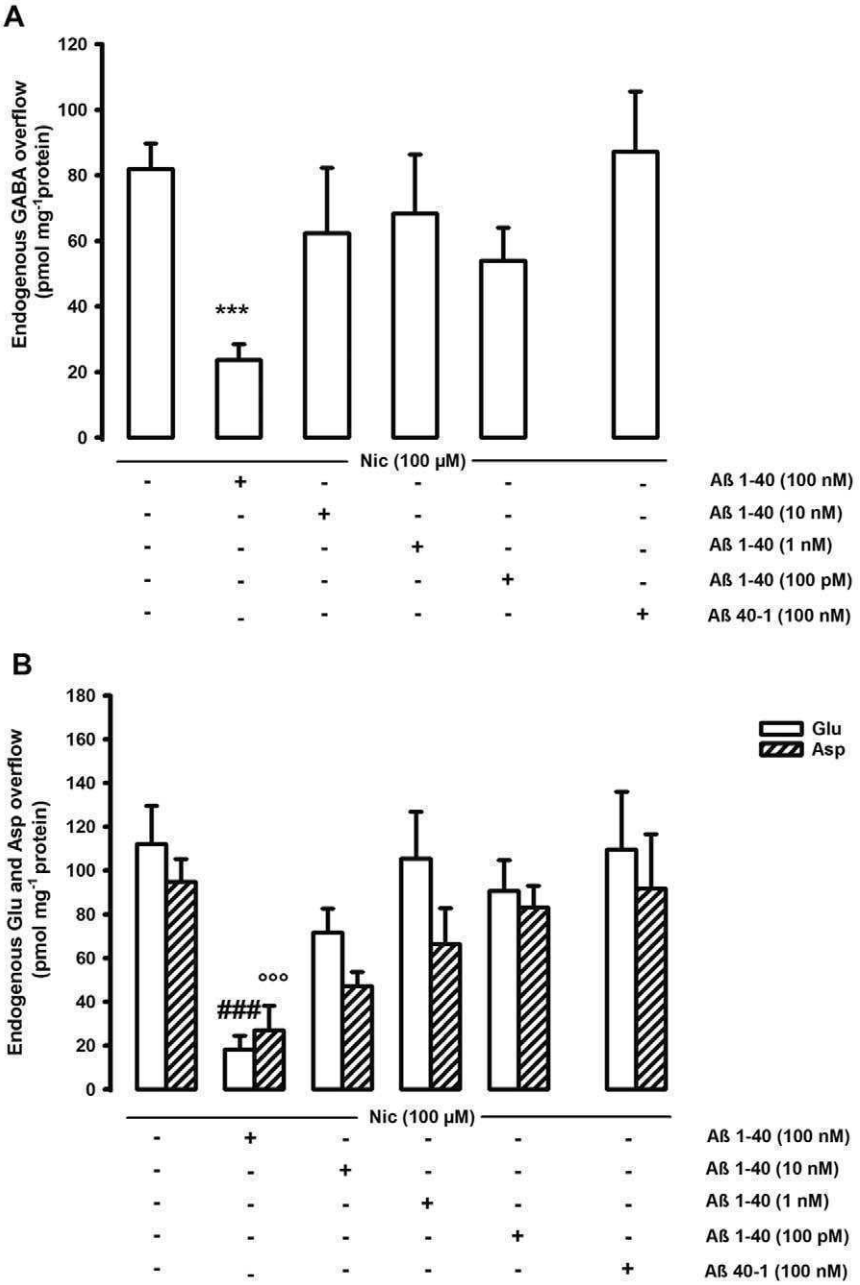


Figure 5. *In vitro* effect of beta-amyloid on the nicotine-induced overflow of hippocampal neurotransmitters. Effect of different concentrations of beta-amyloid (Aβ)1–40 on the nicotine (Nic)-induced overflow of endogenous GABA (**A**), glutamate and aspartate (Glu and Asp respectively, **B**) from rat hippocampal synaptosomes. Synaptosomes were depolarized with Nic for 90 s at $t = 38$ min of superfusion. When appropriate Aβ was introduced 8 min before Nic. Data are mean ± SEM of 5–8 experiments run in triplicate. ***$p<0.001$ vs. Nic-evoked GABA overflow; ###$p<0.001$ vs. Nic-evoked Glu overflow; °°°$p<0.001$ vs. Nic-evoked Asp overflow. One way ANOVA followed by Dunnett *post hoc* test.
doi:10.1371/journal.pone.0029661.g005

It is difficult to compare *in vivo* and *in vitro* results for many reasons. First, *in vivo* concentrations are higher than those used *in vitro* in order to guarantee the delivery to the tissue of sufficient amount of drugs. Despite the fact that immunohistochemical micrographs (Fig. 1) show that there is a positive correlation among the Aβ concentrations administered and the amount of immunostaining in the hippocampal tissue, we do not know the exact amount of Aβ that reaches the tissue during a microdialysis experiment and its accumulation/disposal with time. Another difference between the two experimental models is the timing of exposure to experimental drugs (few seconds *in vitro* versus 40 minutes *in vivo*). Moreover, the observation of an *in vivo* potentiating effect of low concentrations of Aβ on the Nic-induced GABA release may be explained by indirect mechanisms of control of neurotransmitter release present *in vivo*. In fact, the levels of both excitatory and inhibitory neurotransmitters measured *in vivo* are the final result of the interactions of hierarchically organized synapses ultimately controlling Glu, Asp, and GABA release. In this regard, the *in vivo* GABA increase mediated by low concentrations of Aβ could be due to an indirect modulatory role

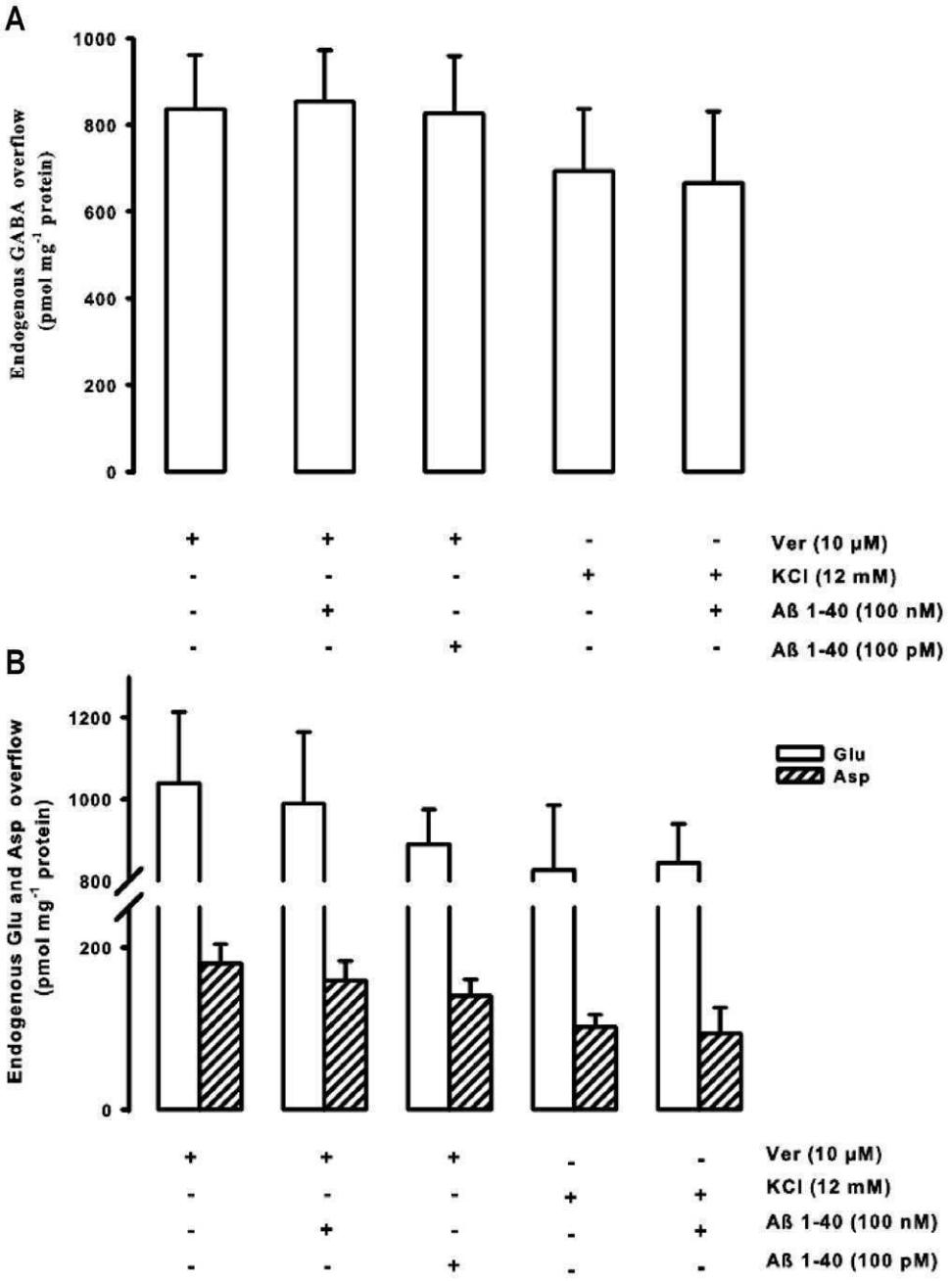


Figure 6. Lack of effect of beta-amyloid on both potassium- and veratridine-induced neurotransmitter release in hippocampus *in vitro*. Effect of different concentrations of beta-amyloid (Aβ)1–40 on both potassium (K^+)- and veratridine (Ver)-evoked overflow of endogenous GABA (**A**), glutamate and aspartate (Glu and Asp respectively, **B**) from rat hippocampal synaptosomes. Synaptosomes were depolarized either with Ver or with K^+ for 90 s at $t = 38$ min of superfusion. When appropriate Aβ was introduced 8 min before Ver or K^+. Data are mean ± SEM of 3–6 experiments run in triplicate.
doi:10.1371/journal.pone.0029661.g006

of Glu and/or Asp; indeed, we have demonstrated that low Aβ concentrations potentiate the release of Glu and Asp from synaptosomes, which might in turn stimulate GABA release through the activation of glutamatergic receptors on GABAergic neurons [34]. On the contrary, indirect control mechanisms of neurotransmitter release are excluded in our *in vitro* model of synaptosomes in superfusion. In fact, *in vitro* data obtained on perfused synaptosomes are due to the direct effects of the added drugs, which have to act upon receptors or modulatory sites located on the same synaptosome from which occurs the release of the transmitter [30].

Effect of Aβ on Glu, Asp and GABA release elicited by depolarizing stimuli

In vivo and *in vitro* both low and high concentrations of the peptide did not modify the overflow of Glu, GABA, and Asp that was elicited by a depolarizing stimulus such as Ver (Fig. 3 and 6). This observation is consistent with our previous *in vivo* and *in vitro* data demonstrating that neurotransmitter release enhanced by another depolarizing stimulus, such as K^+, was not affected by Aβ in two different brain areas [5,6], even if it should be mentioned that Lee and Wang [35] and Kar and colleagues [36] (the latter

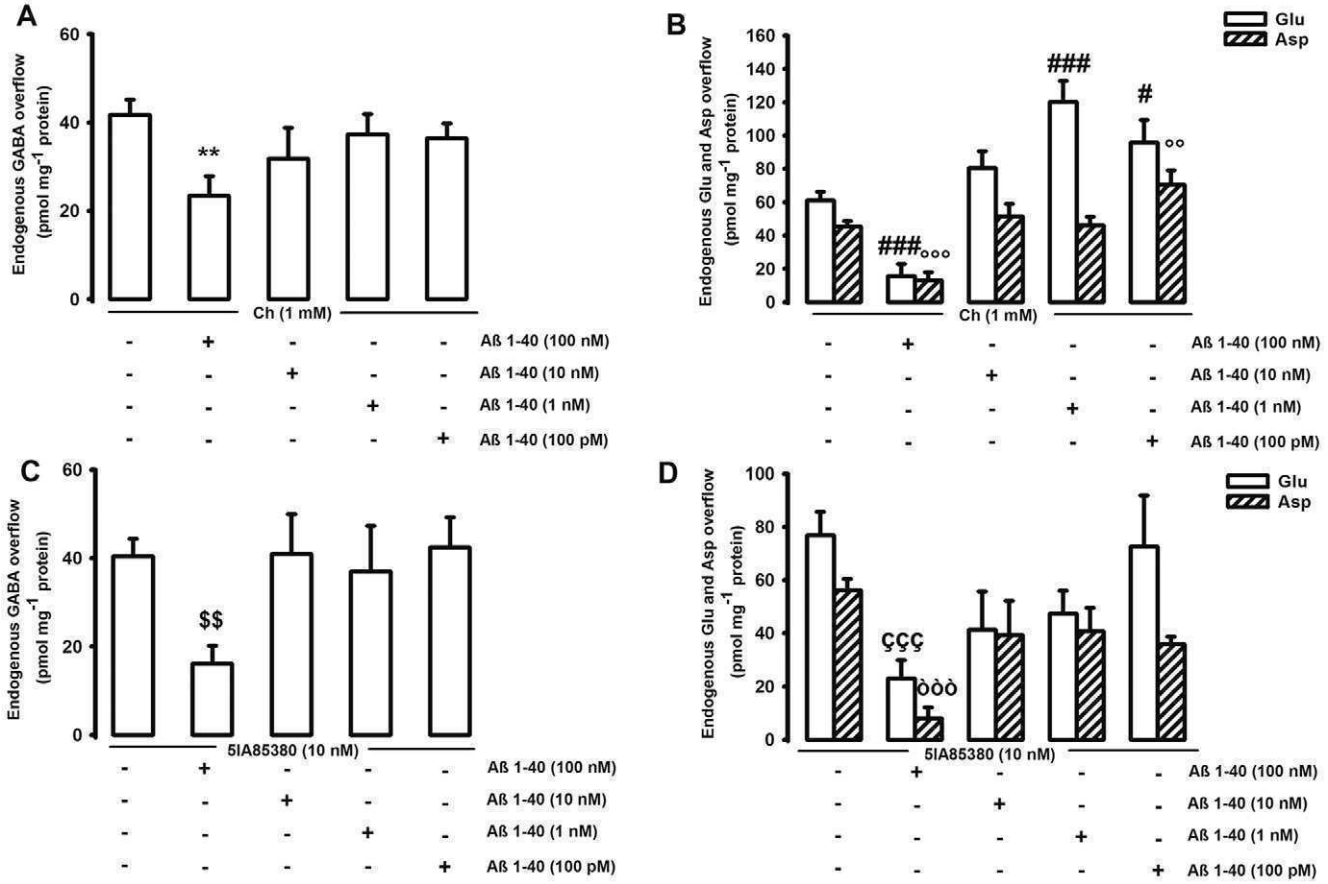


Figure 7. *In vitro* effect of beta-amyloid on hippocampal neurotransmitter release elicited by specific nicotinic agonists. Effect of different concentrations of beta-amyloid (Aβ)1–40 on choline (Ch)-evoked overflow of endogenous GABA (**A**), glutamate and aspartate (Glu and Asp respectively, **B**) and on the 5IA85380-evoked overflow of endogenous GABA (**C**), Glu and Asp (**D**) from rat hippocampal synaptosomes. Synaptosomes were depolarized either with Ch or with 5IA85380 for 90 s at $t = 38$ min of superfusion. When appropriate Aβ was introduced 8 min before the specific nicotinic agonist. Data are mean ± SEM of 3–6 experiments run in triplicate. **p<0.01 vs. Ch-evoked GABA overflow; #p<0.05, ###p<0.001 vs. Ch-evoked Glu overflow; °°p<0.01, °°°p<0.001 vs. Ch-evoked Asp overflow. $$p<0.01 vs. 5IA85380-evoked GABA overflow; çççp<0.001 vs. 5IA85380-evoked Glu overflow; ôôôp<0.001 vs. 5IA85380-evoked Asp overflow. One way ANOVA followed by Dunnett *post hoc* test.
doi:10.1371/journal.pone.0029661.g007

using hippocampal slices) found an inhibitory action of low Aβ concentrations. Ver is a lipid-soluble neurotoxin that is an activator of Na^+ channels. It targets the neurotoxin receptor site 2 and preferentially binds to activated Na^+ channels causing persistent activation [37]. This persistent Na^+ induces bursts of action potentials and sustains membrane depolarization, which is subsequently correlated with an opening of voltage-gated Ca^{2+} channels [38]. The final result is the enhancement of neurotransmitter release [39]. In our experimental setting, the lack of effect of Aβ on Ver stimulus suggests that Aβ1–40 may affect the Nic-triggered neurotransmitter release directly binding to nAChRs or acting to substrates that are specific for nAChRs-induced intracellular signaling. The action is specific for Aβ1–40 sequence since the reverse peptide was ineffective (Fig. 2 and 5).

In vitro pharmacological dissection of the effect of Aβ on Glu, Asp and GABA release elicited by specific nicotinic agonists

Another possible explanation for the differences of Aβ effect on GABA release observed *in vivo* (dual effect) and *in vitro* (inhibition only) may depend on the differential contribution to the described effect of α7 and α4β2 nAChRs. This aspect was approached *in vitro* by using specific nicotinic agonists. We previously demonstrated that hippocampal glutamatergic and GABAergic nerve endings show both α7 and α4β2 nAChRs that are capable to control neurotransmitter release [17,18]. The reported results show that the stimulation of both α7 and α4β2 nAChRs elicits the release of Asp, Glu, and GABA, thus indicating that both receptors are positively linked to the release of the studied neurotransmitters, even if it is possible that they reside on different nerve endings populations and that they operate through distinct cellular mechanisms [40]. At high concentrations (100 nM), Aβ is always inhibitory on the release stimulated by both Ch (α7 selective agonist) and 5IA85380 (selective for α4β2 nAChRs) (Fig. 7). On the contrary, low concentrations of Aβ1–40 (1 nM and 100 pM) selectively act on α7 subtypes potentiating the Ch-induced release of both Asp and Glu (Fig. 7B), but not the one elicited by 5IA85380. Therefore, we were able to observe dual effects of Aβ *in vitro* when using specific nAChRs agonists. However, somewhat surprisingly, the stimulatory activity of low Aβ concentrations was associated with Glu and Asp and not with GABA release, as expected. Interestingly, some papers show that the physiological range of concentrations of the peptide is from pM to low nM [41,42]. Hence, the relevance of our data to AD is that high, likely not physiological, concentrations of Aβ greatly impair cholinergic

responses mediated by two different type of nAChRs. On the contrary, physiological concentrations of the peptide may potentiate the positive nicotinic control of neurotransmitter release, specifically acting on α7 subtypes. This last observation is consistent with some data showing the capability of picomolar Aβ to activate α7 receptors currents [43]. This view contrasts with the fact that we did not observe an impact of Aβ on basal neurotransmitter release from synaptosomes. On the other hand, the possibility of a direct activation on α7 receptors by the peptide is consistent with the demonstration that a low picomolar concentration of Aβ enhances hippocampal long term potentiation (LTP) and memory with a mechanism dependent upon activation of α7 nAChRs [44]. Interestingly, Puzzo and collaborators also showed that, physiologically, the presence of the peptide is required for the modulation of LTP and memory formation in a mechanism dependent on α7 nAChRs [45].

Our data show that low concentrations of Aβ (100 pM and 1 nM) seem to selectively modulate α7-depending functions whereas 100 nM Aβ interacts with both receptors subtypes negatively affecting their function. In this regard, Wang and collaborators [8] showed that the peptide can bind with picomolar affinity to α7 subtypes and with 100–5000 times lower affinity to α4β2 nAChRs, suggesting that, in our experimental conditions, a possible mechanism of action of Aβ concerns the physical interaction to nAChRs. Particularly, it seems that Aβ binds nAChRs near the nicotine binding site [46]. However, it has also been hypothesized that Aβ influences the function of α7 subtype by altering the packing of lipids within the plasma membrane, instead of directly binding to the receptors [47]. Since some authors have shown the capability of neurons to internalize Aβ [48], it cannot be excluded that the peptide may enter synaptosomes and act on cytosolic substrates downstream nAChRs.

Our results are at partial variance with those published by Mehta and colleagues [49] showing that in the hippocampus low concentrations of Aβ1–42 increase pre-synaptic Ca^{++} through the action on α4β2 nAChRs, whereas in cortex the involved receptors are mainly the α7 subtypes. The two experimental models, however, are too different to allow a direct comparison. Mehta and colleagues used knockout mice (C57Bl/6J) to demonstrate the selective involvement of nAChRs and measured intrasynaptosomal calcium fluxes, while we measured the release of transmitters altered by the treatment with Aβ1–40 using Wistar rat synaptosomes. As already discussed, in our case we also observed important differences between *in vivo* (in a "wired" system) and *in vitro* experiments. Moreover, it is possible that in knockout mice adaptive phenomena take place with time. In spite of so many differences, it is noteworthy that both our data and those by Mehta and collaborators agree on the possibility that low concentrations of Aβ peptides may stimulate synaptosomal activity through the interaction with nAChRs. Both sets of data show that Aβ peptides may act differently on α4β2 and α7 nAChRs depending on the adopted experimental settings. Interestingly, when using hyppocampal synaptosomes derived from non-transgenic rats the same group of Mehta found that picomolar Aβ directly activates presynaptic α7 nAChRs to increase nerve terminal Ca^{2+} [50] in line with our results.

In regard to the functional effect of the peptide, the dual action of low and high Aβ concentrations on α7-mediated neurotransmitter release may be explained by different ways. First, a desentization-related mechanism, as concentrations of Aβ1–40 in the range 10 pM–1 nM have been reported to activate α7 nAChRs, whereas an higher concentration (100 nM) induces desentization of the receptor [43]. Moreover, increasing the concentrations Aβ may change its targets. In fact, at low concentrations Aβ can selectively activate α7 nAChRs. However, at high concentrations the peptide may act simultaneously on different targets, possibly downstream nAChRs, leading to the oxidative modification of synaptosomal proteins [51] and perhaps to an inhibitory action on neurotransmitter release.

The lack of potentiating effect of low Aβ concentrations on *in vitro* GABA release elicited by Ch further supports the idea that the *in vivo* observed potentiation is due to indirect mechanisms involving a neuronal circuit. On the other hand, it is difficult to explain why low Aβ concentrations potentiate the α7-stimulated release of Glu and Asp, but not the one of GABA. One possibility is that the synaptosomal release machinery of Glu, Asp, and GABA recruited by α7 stimulation differ, as suggested in the case of the sensitivity of glutamatergic and GABAergic release to botulin toxins [52]. Alternatively, it is either possible that variants of α7 receptors [53] may control the release of Glu, Asp, and GABA, or that the differential pacing in the desensitization mechanism of the nAChRs residing on Glu, Asp, and GABA terminals may affect the action of Aβ.

Conclusions

Altogether, the results reinforce the concepts that Aβ has relevant neuromodulatory effects, which may span from facilitation to inhibition of stimulated release depending upon the concentration. Of particular interest is the observation that, at least *in vitro*, the nicotinic cholinergic control of two excitatory neurotransmitters, Asp and Glu, was particularly sensitive to the effect of low Aβ concentrations in synaptosomes derived from the hippocampus. Moreover, the involved receptor seems to be the α7 one, which other lines of evidence suggest to be a target of Aβ. These actions may be relevant to the early stages of AD which, rather than being characterized by neurodegeneration, may be associated with synaptic dysfunctions affecting more than a transmitter system, albeit with peculiar sensitivity of mechanisms associated with nicotinic cholinergic transmission. It may be hypothesized that the early derangement of Aβ production may lead to trespass the threshold beyond which Aβ loses the ability to co-promote Asp and Glu release, which may be linked to an efficient memory trace formation, and subsequently gains the ability to directly inhibit the ability of cholinergic stimuli to promote Glu and Asp release. This may further impair the signal to noise ratio of Glu transmission associated with synaptic trace formation. The neurotoxicity may finally eventually become the leading event. Moreover, parallel to this impairment of cholinergic control of Glu and Asp release, other effects may be present in other brain areas involving other neurotransmitters, thus providing the basis for a multitransmitter deficit in the disease. This is turn may be responsible for the various psychiatric symptoms that characterize subsets of patients, in addition to the more easily recognized memory deficits. These observations may help to redirect the pharmacological approaches toward multiple neurotransmitter targets in the early stages of the disease.

Materials and Methods

Chemicals

5-Iodo-A-85380 dihydrochloride (3-[(2S)-2-Azetidinylmethoxy]-5-iodopyridine dihydrochloride) was purchased from Tocris Bioscience (Bristol, UK); nicotine hydrogen tartrate salt, veratridine, Aβ1–40 and Aβ40–1, Percoll®, Choline Iodide, dimethyl sulfoxide were obtained from Sigma Aldrich (Milan, Italy); all salts used for the preparation of artificial cerebrospinal fluid (aCSF) (NaCl, KCl, $CaCl_2$, $MgCl_2$, Na_2HPO_4) and for Equithesin ($MgSO_4$) were purchased at Merck KGaA, Darmstadt, Germany;

chloral hydrate, ethanol 96% and propylene glycol were used for the preparation of Equithesin and were obtained at VWR BDH Prolabo, Belgium.

Preparation of Aβ1–40 solutions

In the case of both *in vivo* and *in vitro* experiments, synthetic human Aβ1–40 (Sigma Aldrich, Milan, Italy) was dissolved in aCSF at a concentration of 100 μM (stock solution). Then, this solution was filtered through a Millipore 0.2 μm pore membrane and stocked in small aliquots at −80°C. Working solutions were freshly prepared by diluting an aliquot of Aβ1–40 stock solution at the final concentrations (10 μM, 1 μM, or 100 nM Aβ1–40 for *in vivo* experiments, 100 nM, 10 nM, 1 nM, or 100 pM for *in vitro* analysis).

Animals

Young male Wistar rats (275–300 g; Harlan, Udine Italy), housed in standard conditions (temperature 23±1°C; humidity 50%) with 12:12 light/dark cycles, water and food *ad libitum*, were used either for microdialysis experiments or as brain tissue source for the *in vitro* experiments. The use of animals for the preparation of synaptosomes was approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (Decreto Ministeriale number 124/2003-A). The *in vivo* protocol was approved by Ethical Committee of Pavia's University (registered as 2/2008) according to international regulations for the care and treatment of laboratory animals, to the Italian Act (DL n 116, GU, suppl 40, 18 February, 1992) and to EEC Council Directive (86/609, OJ L 358, 1, 12 December, 1987). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

In vivo experiments

Microdialysis probe implantation. Rats were anesthetized with Equithesin 3 ml/kg (pentobarbital 9.7 g, chloral hydrate 42.5 g, $MgSO_4$ 21.3 g for 1 L, 10% ethanol, 40% propylene glycole v/v) administered intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected, and cut with a sterile scalpel to expose the skull. A hole was drilled to allow the implantation of the probe into the brain parenchyma. The probe was implanted in the hippocampus (CA1/CA2 regions; AP −5.8 mm, ML ±5.0 mm from bregma and DV–8.0 mm from dura, according to [54]) and secured to the skull with one stainless steel screw and dental cement. All experiments were performed using microdialysis probes, made in our laboratory according to the original method described by Di Chiara and colleagues [55] (Emophan Bellco Artificial OR-internal diameter 200 μm, cutoff 40 KDa; Bellco, Mirandola, Italy), with a nominal active length of 5 mm.

Finally, the skin was sutured, and the rats were allowed to recover from anesthesia.

Microdialysis samples collection. Microdialysis experiments were performed on conscious freely moving rats. On the day of the experiments (24 hours after the surgical procedure), the probe was perfused with aCSF containing 145 mM NaCl, 3.0 mM KCl, 1.26 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM Na_2HPO_4, buffered at pH 7.2–7.4 and filtered through a Millipore 0.2 μm pore membrane. In all experiments, the microdialysis membrane was allowed to stabilize for 1 hour at the flow rate of 4 μl/min, without collecting samples. At the end of the stabilization period, three samples were collected to evaluate baseline release of GABA, Asp, and Glu; then, the specific treatment started. In the case of Aβ administration, a pre-treatment of 20 min with the peptide preceded its co-perfusion either with Nic or with Ver. Anyway, the perfusion of the peptide did not last more than 100 min. All treatments were administered by manually switching syringes and tubing connections to allow drugs diluted in aCSF to flow through the probes. Tubing switches were performed taking care to maintain constant flow rates and collection volumes. Both basal and treatment samples were collected every 20 min in 100 μl Eppendorf tubes at a flow rate of 4 μl/min, using a 1000 μl syringe (Hamilton) and a micro-injection pump (CMA/100, CMA/Microdialysis AB). Levels of GABA, Glu and Asp in the dialysate were measured by high performance liquid chromatography (HPLC) with fluorometric detection (see **Chromatography**). *In vitro* recovery of the dialysis probe was 13.88±0.25 for Asp, 15.21±0.42 for Glu and 7.91±0.25 for GABA. Each rat was used for only one microdialysis session. At the end of each experiment, animals were sacrificed by guillotine, rat brains were removed, and the position of the microdialysis probe was verified by histological procedures, slicing the tissues by a cryostat microtome (LEICA CM 1510). Only data from rats in which probe tracks were exactly located in the target area were used for statistical analysis.

In vitro experiments

Experiments of release. Rats were killed by decapitation and the hippocampus was rapidly removed at 0–4°C. Purified synaptosomes were prepared on Percoll® gradients (Sigma-Aldrich, St Louis, MO, USA) according to the original method described by Dunkley et al., [56], with only minor modifications. Briefly, the tissue was homogenized in 6 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged (5 min, 1000 g at 4°C) to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33500 g for 5 min at 4°C. The layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in physiological HEPES-buffered medium having the following composition (mM): NaCl 128, KCl 2.4, CaCl2 3.2, KH2PO4 1.2, MgSO4 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4 [57]. Synaptosomal protein content following purification was 10–15% of that in the supernatant stratified on the Percoll® gradient.

The synaptosomal suspension was layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37°C [30] (Superfusion System, Ugo Basile, Comerio, Varese, Italy). Synaptosomes were superfused at 1 ml/min with standard physiological medium as previously described. The system was first equilibrated during 36.5 min of superfusion; subsequently, four consecutive 90 s fractions of superfusate were collected. Synaptosomes were exposed to agonists for 90 s starting from the second fraction collected (t = 38 min), with antagonists being added 8 min before agonists. We have previously amply demonstrated that in our superfusion system the possible effects of drugs operated indirectly by other mediators in the monolayer of synaptosomes in superfusion are absolutely minimized [30].

Chromatography

In both dialysates and fractions collected from synaptosomes in superfusion levels of endogenous GABA, Glu, and Asp were measured by HPLC analysis following precolumn derivatization

with o-phthalaldehyde and resolution through a C18-reverse phase chromatographic column (10×4.6 mm, 3 μm; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as internal standard. Buffers and gradient program were prepared and executed as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min.

Immunohistochemical analysis

Immunohistochemical analysis was performed to verify the presence of Aβ in the perfused tissue and to confirm (according to HOECHST 33342 staining) the absence of neurotoxic-induced apoptotic phenomenon. Brain tissue samples were frozen and stored at −80°C. For immunodetection of infused Aβ peptide, 10 μm coronal sections (obtained on a cryostat Leica CM 1510) were incubated with a primary monoclonal antibody recognizing Aβ protein (clone 4G8; Chemicon International). Sections were then incubated with a mouse anti-IgG antibody RPE conjugated (Dako). After the fluorescent labeling procedures, sections were finally counterstained for DNA with HOECHST 33342 and mounted in a drop of Mowiol (Calbiochem, Inalco SpA, Milan, Italy). Fluorescent micrographs were acquired with a Leica TCS SP5 II confocal microscope. After acquisition of fluorescent micrographs, the slides were demounted and then the same sections were slightly counterstained with Mayer hematoxylin, dehydrated and mounted in DPX for microanatomical analysis. The images were acquired with a BX51 Olympus microscope.

Western blotting procedure

Samples were subjected to SDS-PAGE (15%) and then transferred onto PVDF membrane (DuPont NEN, Boston MA). The membrane was blocked for 1 hour with 5% non fat dry milk in Tris-buffered saline containing 1% Tween 20 (TBST). Membranes were immunoblotted with the antibody 6E10, recognizing residues 1–17 of Aβ (Chemicon-Prodotti Gianni, Milano, Italy). The detection was carried out by incubation with horseradish peroxidase conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburgh, MD U.S.A.) for 1 h. The blots were then washed extensively and Aβ visualized using an enhanced chemiluminescent methods (Pierce, Rockford, IL, USA). Molecular mass was estimated by molecular weight markers (Invitrogen).

Statistical analysis

***In vivo* experiments.** Values were expressed either as amount of neurotransmitter measured in the dialysate (pmol/80 μL) or as area under the curve (AUC), evaluating the cumulative release over time. AUC was used as a measure of treatment exposure and was calculated for each animal using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA, USA). The basal value (average concentration of three consecutive samples immediately preceding the drug dose) was used as baseline to calibrate the calculation.

D'Agostino-Pearson Omnibus Test (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA, USA) and Grubb's Test (GraphPad QuickCalcs, online calculator for scientists at http://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA) were used as preliminary tests in order to evaluate whether data were sampled from a Gaussian distribution and to detect outliers respectively. All outliers were excluded from the analysis. Data were analyzed by analysis of variance (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Data were considered significant for $p<0.05$. The reported data are expressed as means ± S.E.M. The number of animals used for each experiment is reported in the legend to Figures 2 and 3.

***In vitro* experiments.** The evoked overflow was calculated by subtracting the corresponding basal release from each fraction. All data are expressed as $pmol \cdot mg^{-1}$ protein and represent mean ± SEM of the number of experiments reported in the figure legends. Multiple comparisons were performed with one-or two way ANOVA followed by an appropriate post hoc test (Dunnett or Bonferroni). Data were considered significant for $p<0.05$ at least, using KyPlot 2.0 beta 15.

Supporting Information

Figure S1 Characterization of beta-amyloid (Aβ) conformation by using Western Blot procedure. SDS-PAGE showing immunoreactive species consistent with Aβ monomer in all the preparations analyzed: the stock solution (100 μM Aβ1–40) freshly prepared, the stock solution (100 μM Aβ1–40) maintained for 100 min at room temperature and the most concentrated working solution evaluated *in vivo* (10 μM Aβ1–40) maintained for 100 min (maximum length of Aβ1–40 perfusion during microdialysis experiments) at room temperature.
(TIF)

Figure S2 Lack of effect of beta-amyloid on the basal neurotransmitter release in hippocampus *in vivo*. Effect of beta-amyloid (Aβ)1–40 (100 nM–10 μM) on the basal release of GABA (**A**), glutamate (Glu, **B**) and aspartate (Asp, **C**). One-way ANOVA. Data are expressed as mean ± SEM of 4–9 individual rats for each experimental group.
(TIF)

Acknowledgments

We thank Centro Grandi Strumenti of University of Pavia for acquisition of fluorescent micrographs with the Leica TCS SP5 II confocal microscope.

Author Contributions

Conceived and designed the experiments: EM SG SP SZ MG MM. Performed the experiments: EM SP CL FB SZ GO AS AC. Analyzed the data: EM SP SG CL SZ MG MM. Contributed reagents/materials/analysis tools: SG MM. Wrote the paper: EM SP SG SZ MG MM.

References

1. Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120: 885–90.
2. Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. Science 256: 184–5.
3. Hardy J (2006) Alzheimer's disease: the amyloid cascade hypothesis: an update and reappraisal. J Alzheimers Dis 9: 151–3.
4. Mura E, Lanni C, Preda S, Pistoia F, Sarà M, et al. (2010a) Beta-amyloid: a disease target or a synaptic regulator affecting age-related neurotransmitter changes? Curr Pharm Des 16: 672–83.

5. Preda S, Govoni S, Lanni C, Racchi M, Mura E, et al. (2008) Acute beta-amyloid administration disrupts the cholinergic control of dopamine release in the nucleus accumbens. Neuropsychopharmacology 33: 1062–70.
6. Mura E, Preda S, Govoni S, Lanni C, Trabace L, et al. (2010b) Specific neuromodulatory actions of amyloid-beta on dopamine release in rat nucleus accumbens and caudate putamen. J Alzheimers Dis 19: 1041–53.
7. Grilli M, Lagomarsino F, Zappettini S, Preda S, Mura E, et al. (2010) Specific inhibitory effect of amyloid-beta on presynaptic muscarinic receptor subtypes modulating neurotransmitter release in the rat nucleus accumbens. Neuroscience 167: 482–9.
8. Wang HY, Lee DH, Davis CB, Shank RP (2000) Amyloid peptide Abeta(1–42) binds selectively and with picomolar affinity to alpha7 nicotinic acetylcholine receptors. J Neurochem 75: 1155–61.
9. Buckingham SD, Jones AK, Brown LA, Sattelle DB (2009) Nicotinic acetylcholine receptor signalling: roles in Alzheimer's disease and amyloid neuroprotection. Pharmacol Rev 61: 39–61.
10. Jürgensen S, Ferreira ST (2010) Nicotinic receptors, amyloid-beta, and synaptic failure in Alzheimer's Disease. J Mol Neurosci 40: 221–229.
11. Parri RH, Dineley TK (2010) Nicotinic acetylcholine receptor interaction with beta-amyloid: molecular, cellular, and physiological consequences. Curr Alzheimer Res 7: 27–39.
12. Thal DR, Griffin WS, Braak H (2008) Parenchymal and vascular Abeta-deposition and its effects on the degeneration of neurons and cognition in Alzheimer's disease. J Cell Mol Med 12: 1848–62.
13. Alonso JR, Amaral DG (1995) Cholinergic innervation of the primate hippocampal formation. Distribution of choline acetyltransferase immunoreactivity in the Macaca fascicularis and Macaca mulatta monkeys. J Comp Neurol 355: 135–70.
14. Yoshida K, Oka H (1995) Topographical projections from the medial septum-diagonal band complex to the hippocampus: a retrograde tracing study with multiple fluorescent dyes in rats. Neurosci Res 21: 199–209.
15. Hefft S, Hulo S, Bertrand D, Muller D (1999) Synaptic transmission at nicotinic acetylcholine receptors in rat hippocampal organotypic cultures and slices. J Physiol 515: 769–76.
16. Descarries L, Gisiger V, Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 53: 603–25.
17. Zappettini S, Grilli M, Salamone A, Fedele E, Marchi M (2010) Pre-synaptic nicotinic receptors evoke endogenous glutamate and aspartate release from hippocampal synaptosomes by way of distinct coupling mechanisms. Br J Pharmacol 161: 1161–71.
18. Zappettini S, Grilli M, Lagomarsino F, Cavallero A, Fedele E, et al. (2011) Presynaptic nicotinic α7 and non-α7 receptors stimulate endogenous GABA release from rat hippocampal synaptosomes through two mechanisms of action. PLoS One 6: e16911.
19. Ji D, Lape R, Dani JA (2001) Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. Neuron 31: 131–41.
20. Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, et al. (2003) APP processing and synaptic function. Neuron 37: 925–37.
21. Terai K, Iwai A, Kawabata S, Tasaki Y, Watanabe T, et al. (2001) beta-amyloid deposits in transgenic mice expressing human beta-amyloid precursor protein have the same characteristics as those in Alzheimer's disease. Neuroscience 104: 299–310.
22. Walsh DM, Selkoe DJ (2007) A beta oligomers - a decade of discovery. J Neurochem 101: 1172–84.
23. Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB, et al. (2003) Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. Proc Natl Acad Sci U S A 100: 330–5.
24. Verdile G, Fuller S, Atwood CS, Laws SM, Gandy SE, et al. (2004) The role of beta amyloid in Alzheimer's disease: still a cause of everything or the only one who got caught? Pharmacol Res 50: 397–409.
25. Fedele E, Varnier G, Ansaldo MA, Raiteri M (1998) Nicotine administration stimulates the in vivo N-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. Br J Pharmacol 125: 1042–8.
26. Toth E (1996) Effect of nicotine on the level of extracellular amino acids in the hippocampus of rat. Neurochem Res 21: 903–7.
27. Benowitz NL, Porchet H, Jacob PI (1990) Pharmacokinetics, metabolism, and pharmacodynamics of nicotine. In: Wonnacott S, Russell MAH, Stolerman IP, eds. Nicotine Psychopharmacology: Molecular, Cellular, and Behavioural Aspects. Oxford, England: Oxford University Press. pp 112–157.
28. Battaglia G, Monn JA, Schoepp DD (1997) In vivo inhibition of veratridine-evoked release of striatal excitatory amino acids by the group II metabotropic glutamate receptor agonist LY354740 in rats. Neurosci Lett 229: 161–4.
29. Ahmad S, Fowler LJ, Whitton PS (2004) Effects of acute and chronic lamotrigine treatment on basal and stimulated extracellular amino acids in the hippocampus of freely moving rats. Brain Res 1029: 41–7.
30. Raiteri L, Raiteri M (2000) Synaptosomes still viable after 25 years of superfusion. Neurochem Res 25: 1265–1274.
31. Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., et al. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264: 1336–40.
32. Kirkitadze MD, Kowalska A (2005) Molecular mechanisms initiating amyloid β-fibril formation in Alzheimer's disease. Acta Biochim Pol 52: 417–423.
33. Sabella S, Quaglia M, Lanni C, Racchi M, Govoni S, et al. (2004) Capillary electrophoresis studies on the aggregation process of beta-amyloid 1–42 and 1–40 peptides. Electrophoresis 25: 3186–94.
34. Wu M, Hajszan T, Leranth C, Alreja M (2003) Nicotine recruits a local glutamatergic circuit to excite septohippocampal GABAergic neurons. European Journal of Neuroscience 18: 1155–1168.
35. Lee DH, Wang HY (2003) Differential physiologic responses of alpha7 nicotinic acetylcholine receptors to beta-amyloid1–40 and beta-amyloid1–42. J Neurobiol 55: 25–30.
36. Kar S, Seto D, Gaudreau P, Quirion R (1996) Beta-amyloid-related peptides inhibit potassium-evoked acetylcholine release from rat hippocampal slices. J Neurosci 16: 1034–40.
37. Wang SY, Wang GK (2003) Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. Cell Signal 15: 151–9.
38. Fekete A, Franklin L, Ikemoto T, Rózsa B, Lendvai B, et al. (2009) Mechanism of the persistent sodium current activator veratridine-evoked Ca elevation: implication for epilepsy. J Neurochem 111: 745–56.
39. Luccini E, Romei C, Di Prisco S, Raiteri M, Raiteri L (2010) Ionic dysregulations typical of ischemia provoke release of glycine and GABA by multiple mechanisms. J Neurochem 114: 1074–84.
40. Dickinson JA, Kew JN, Wonnacott S (2008) Presynaptic alpha 7- and beta 2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. Mol Pharmacol 74: 348–59.
41. Ida N, Hartmann T, Pantel J, Schröder J, Zerfass R, et al. (1996) Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. J Biol Chem 271: 22908–14.
42. Brody DL, Magnoni S, Schwetye KE, Spinner ML, Esparza TJ, et al. (2008) Amyloid-beta dynamics correlate with neurological status in the injured human brain. Science 321: 1221–4.
43. Dineley KT, Bell KA, Bui D, Sweatt JD (2002) Beta -Amyloid peptide activates alpha 7 nicotinic acetylcholine receptors expressed in Xenopus oocytes. J Biol Chem 277: 25056–61.
44. Puzzo D, Privitera L, Leznik E, Fà M, Staniszewski A, et al. (2008) Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci 28: 14537–45.
45. Puzzo D, Privitera L, Fa' M, Staniszewski A, Hashimoto G, et al. (2011) Endogenous amyloid-β is necessary for hippocampal synaptic plasticity and memory. Ann Neurol 69: 819–30.
46. Magdesian MH, Nery AA, Martins AH, Juliano MA, Juliano L, et al. (2005) Peptide blockers of the inhibition of neuronal nicotinic acetylcholine receptors by amyloid beta. J Biol Chem 280: 31085–90.
47. Small DH, Maksel D, Kerr ML, Ng J, Hou X, et al. (2007) The β-amyloid protein of Alzheimer's disease binds to membrane lipids but does not bind to the α7 nicotinic acetylcholine receptor. Journal of Neurochemistry 101: 1527–1538.
48. Saavedra L, Mohamed A, Ma V, Kar S, de Chaves EP (2007) Internalization of beta-amyloid peptide by primary neurons in the absence of apolipoprotein E. J Biol Chem 282: 35722–32.
49. Mehta TK, Dougherty JJ, Wu J, Choi CH, Khan GM, et al. (2009) Defining pre-synaptic nicotinic receptors regulated by beta amyloid in mouse cortex and hippocampus with receptor null mutants. J Neurochem 109: 1452–8.
50. Dougherty JJ, Wu J, Nichols RA (2003) Beta-amyloid regulation of presynaptic nicotinic receptors in rat hippocampus and neocortex. J Neurosci 23: 6740–7.
51. Boyd-Kimball D, Castegna A, Sultana R, Poon HF, Petroze R, et al. (2005) Proteomic identification of proteins oxidized by Abeta(1–42) in synaptosomes: implications for Alzheimer's disease. Brain Res 1044: 206–15.
52. Verderio C, Grumelli C, Raiteri L, Coco S, Paluzzi S, et al. (2007) Traffic of botulinum toxins A and E in excitatory and inhibitory neurons. Traffic 8: 142–53.
53. Stitzel JA, Blanchette JM, Collins AC (1998) Sensitivity to the seizure-inducing effects of nicotine is associated with strain-specific variants of the alpha 5 and alpha 7 nicotinic receptor subunit genes. J Pharmacol Exp Ther 284: 1104–11.
54. Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic coordinates. Second Edition, New York: Academic Press.
55. Di Chiara G (1990) In-vivo brain dialysis of neurotransmitters. Trends Pharmacol Sci 11: 116–121.
56. Dunkley PR, Jarvie PE, Heath JW, Kidd GJ, Rostas JA (1986) A rapid method for isolation of synaptosomes on Percoll gradients. Brain Res 372: 115–29.
57. Lu Y, Grady S, Marks MJ, Picciotto M, Changeux J-P, et al. (1998) Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. J Pharmacol Exp Ther 287: 648–657.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.5

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Neurochemistry International xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes

Mario Marchi [a,b,c,*], Stefania Zappettini [a], Guendalina Olivero [a], Anna Pittaluga [a,b], Massimo Grilli [a]

[a] Section of Pharmacology and Toxicology, Department of Experimental Medicine, University of Genoa, Genoa, Italy
[b] Center of Excellence for Biomedical Research, University of Genoa, Italy
[c] National Institute of Neuroscience, Genoa, Italy

ARTICLE INFO

Article history:
Received 13 January 2012
Received in revised form 22 February 2012
Accepted 27 February 2012
Available online xxxx

Keywords:
Nicotinic receptors
Chronic nicotine treatment
Neurotransmitter release
Glutamate
Aspartate
GABA
Isolated nerve endings

ABSTRACT

The effect of chronic nicotine treatment on the release of endogenous glutamate (GLU), aspartate (ASP) and GABA evoked in vitro by KCl, 4-aminopyridine (4AP) and nicotinic agonists in synaptosomes of rat hippocampus was investigated. Rats were chronically administered with nicotine bitartrate or saline vehicle each for 14 days using osmotic mini-pumps. Hippocampal synaptosomes were stimulated with KCl, 4AP, nicotine or with choline (Ch) and 5-iodo-A-85380 dihydrochloride (5IA85380). The GLU and ASP overflow evoked by Ch, nicotine, KCl and 4AP were increased in treated animals while the nicotine-evoked GABA overflow was reduced and that evoked by Ch, KCl and 4AP was unaffected. The 5IA85380-evoked overflow of the three aminoacids (AAs) was always reduced. The increase of ASP and GLU overflow evoked by KCl, 4AP or Ch was blocked by DL-threo-β-benzyloxyaspartic acid (DL-TBOA), a carrier transporter inhibitor, and by inhibitors of the Na^+/Ca^{2+} exchangers 2-[[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester (SN-6) and 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R7943). In conclusion long-term nicotine treatment may selectively increase GLU and ASP overflow elicited by KCl, 4AP and Ch through the activation of a carrier-mediated release mechanism and completely abolished the stimulatory effects of α4β2 nAChRs which modulate the release of all the three AA.

1. Introduction

Accumulating evidences indicate that changes in the function and activities of several important components of glutamate (GLU) neurotransmission play a fundamental role in mediating the rewarding action of nicotine (Markou, 2008 and references therein; Kalivas, 2009). Moreover nicotinic-glutamate interaction may have important implications in cognitive functions and may be critical to the long-term effects of several drugs of abuse including nicotine.

It is well established that chronic nicotine administration affects NMDA receptor binding and receptor subunits composition (Delibas et al., 2005; Levin et al., 2005; Lee et al., 2007; Wang et al., 2007; Rezvani et al., 2008; Kenny et al., 2009), induces changes of alfa-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA) and of group I metabotropic receptors (Kane et al., 2005) and alters the functional responses of NMDA and AMPA in different brain areas including hippocampus (Risso et al., 2004; Yamazaki et al., 2006; Vieyra-Reyes et al., 2008). However most of these papers do not provide insights into the mechanisms through which nicotine produced these effects although the release of GLU might obviously play a crucial role in generating them. At this regard it is well known that acute nicotine treatment increase both the *in vivo* (Fedele et al., 1996; Lallemand et al., 2006) and in vitro release of GLU in different brain areas (Gray et al., 1996; Gioanni et al., 1999; López et al., 2001) however, so far, very little information are available on the effect of chronic administration of nicotine on this parameter (Lallemand et al., 2006).

Evidence of a releasing effect due to the activation of different subtypes of nAChRs in the CNS has been already provided (Toth et al., 1992, 1993; Toth, 1996; Wonnacott, 1997; Vizi and Lendvai, 1999; Jones and Wonnacott, 2004; Rousseau et al., 2005). It has

Abbreviations: 4AP, 4-aminopyridine; 5IA85380, 5-iodo-A-85380 dihydrochloride; AAs, aminoacids; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolone propionate; ASP, aspartate; Ch, choline; DHβE, dihydro-β-erythroidine; DL-TBOA, DL-threo-β-benzyloxyaspartic acid; GLU, glutamate; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate; nAChR, nicotinic acetylcholine receptor; SN-6, 2-[[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester.

* Corresponding author at: Sezione di Farmacologia e Tossicologia, Dipartimento di Medicina Sperimentale, Università di Genova, Viale Cembrano 4, 16148 Genova, Italy.
E-mail address: marchi@pharmatox.unige.it (M. Marchi).

0197-0186/$ - see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.neuint.2012.02.032

been also reported that in the rat prefrontal cortex as well as in the hippocampus the nicotine modulation of GLU release occurs through the activation of both α7 and α4β2 nicotinic acetylcholine receptors (nAChRs) (Dickinson et al., 2007, 2008; Parikh et al., 2008; Zappetttini et al., 2010). Very recently it has also been shown that in the rat hippocampus nicotine modulates also the release of ASP and GABA in a similar manner (Zappettini et al., 2010, 2011).

To extend the knowledge on the effect of chronic nicotine treatment on the glutamatergic neurotransmission in the rat hippocampus we investigated on the effects of this treatment on the in vitro release of endogenous GLU and aspartate (ASP) evoked by KCl and 4-aminopyridine (4AP). Moreover we have also investigated on the possible functional changes of the in vitro responses of the presynaptic α7 and α4β2 nAChR subtypes modulating endogenous GLU and ASP release from synaptosomes. This because after chronic nicotine treatment the nAChR subtypes may be not equally responsive to nicotine activation and therefore might elicit a release of a different amount of GLU which, in turn might influence the glutamatergic receptors function. In addition we have comparatively extended our study to the release of endogenous GABA evoked by the same stimuli as above described to study whether and in what extent some functional features were changed in nicotine treated rats compared to those from animals treated with saline. The results indicate that chronic exposure to nicotine may a) selectively increase the GLU and ASP overflow elicited by different depolarizing stimuli (KCl or 4AP) and by the α7 nAChRs through the activation of a carrier-mediated release mechanism and b) completely abolished the stimulatory effects of α4β2 nAChRs which modulate the release of all the three AAs.

2. Materials and methods

2.1. Animals and brain tissue preparation

Adult male Sprague–Dawley rats (200–250 g) were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (light 7 a.m–7 p.m.). Food and water were freely available. Animals were chronically administered (−) nicotine bitartrate or saline vehicle each at pH 7.4, for 14 days via chronic infusion using osmotic mini-pumps. The osmotic minipumps used deliver (-)nicotine or vehicle with a pumping rate of 5 μL/h for 14 days (model 2ML2; Alza Co., Palo Alto, CA, USA); osmotic mini-pumps were pre-filled with either vehicle or (-)nicotine bitartrate to deliver 0.125 mg/kg/h (free base; Mugnaini et al., 2006). At the end of the treatment with osmotic mini-pumps, the brain nicotine concentration was approximately 1.5 μM and dropped to almost zero after 1-week withdrawal (Mugnaini et al., 2006). These concentrations are compatible with those found during the day in the venous plasma nicotine concentration of the typical human heavy smokers (Benowitz, 1988; for a review see Matta et al., 2007). The animals were killed by decapitation and the hippocampus rapidly removed at 0–4 °C. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (Decreto Ministeriale n. 124/2003-A). All efforts were made to minimize animal suffering and to use a minimum number of animals necessary to produce reliable results.

2.2. Release experiments

Purified synaptosomes were prepared from a vast population of neuronal cells on Percoll gradients (Sigma–Aldrich, St. Louis, MO, USA) essentially according to Nakamura et al., 1993) with only minor modifications. Briefly, the tissue was homogenized in 6 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass–Teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged (5 min, 1000*g* at 4 °C) to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500*g* for 5 min at 4 °C.

The layer between 10% and 20% Percoll (synaptosomal fraction) was collected, washed by centrifugation and resuspended in physiological HEPES buffered medium having the following composition (mM): 128 NaCl, 2.4 KCl, 3.2 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 HEPES, pH 7.5, 10 glucose, pH 7.2–7.4. Synaptosomal protein content following purification was 10–15% of that in the supernatant stratified on the Percoll gradient. The synaptosomal suspension was layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37 °C (Raiteri and Raiteri, 2000; Superfusion System, Ugo Basile, Comerio, Varese, Italy). Synaptosomes were superfused at 1 ml/min with standard physiological medium as previously described (Grilli et al., 2009). The system was first equilibrated during 36.5 min of superfusion; subsequently, four consecutive 90 s fractions of superfusate were collected. Synaptosomes were exposed to agonists for 90 s after the first fraction had been collected (t = 38 min), while antagonists were added 8 min before. Appropriate controls were always run in parallel. The evoked overflow was calculated by subtracting the corresponding basal release from each fraction collected, and was expressed as pmol/mg of synaptosomal proteins. In some experiments, synaptosomes were depolarized with KCl (15 mM) for 90 s in the absence of any drug. In this case, the overflow was calculated by subtracting the corresponding basal release from the outflow induced by KCl. We have previously demonstrated that in our superfusion system, the neurotransmitter released is immediately removed and cannot be taken up by synaptosomes (for a review, see Raiteri and Raiteri, 2000).

2.3. Endogenous amino acid determination

Endogenous amino acid content was measured by HPLC analysis following pre-column derivatization with *o*-phthalaldehyde and resolution through a C18 reversed-phase chromatographic column (10 × 4.6 mm; 3 μm; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as internal standard. Buffers and gradient program were prepared and executed as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min.

2.4. Monitoring cytoplasmic Ca^{2+} concentration

Cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored in purified synaptosomes using the fluorescent dye FURA PE 3-AM (Grynkiewicz et al., 1985). Synaptosomes were incubated for 40 min at 37 °C in the dark, while gently shaking, in a medium containing 20 mM of $CaCl_2$ and 5 mM FURA PE 3-AM (and 1% DMSO). Control synaptosomes containing 1% DMSO, but no FURA PE 3-AM, were prepared to measure auto-fluorescence. Synaptosomal suspension was washed to remove extra-particle FURA PE 3-AM. Pellets were resuspended in ice-cold medium, divided into 200 μl aliquots (each containing 200 μg proteins) and stored on ice until use. Measures were obtained within 2 h. To estimate the apparent ($[Ca^{2+}]_i$),

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

a 200 µl aliquot of synaptosomes was diluted into 1.8 ml of physiological medium, containing 3.2 mM $CaCl_2$, and incubated at 37 °C for 5 min. Fluorescence was recorded for at least 1 min before addition of 15 mM KCl or 10 nM 5IA85380 or 1 mM Ch. Measurements were made at 37 °C in a thermostated cuvette under continuous stirring using a RF-5301PC dual wavelength spectrofluorometer (Shimadzu, Japan) and by alternating the excitation wavelength of 340 and 380 nm.

Fluorescent emission was monitored at 510 nm. Calibration of the fluorescent signals was performed at the end of each experiments by adding 10 mM ionomycin in the presence of 3.2 mM Ca^{2+}, to obtain Fmax, followed by 10 mM EGTA (adjusted to pH 8 with 3 mM Tris), to obtain Fmin (Grynkiewicz et al., 1985). Intrasynaptosomal FURA PE 3-AM was determined for each synaptosomal preparation by adding 40 mM Mn^{2+} to quench the extracellular fluorescence; this Mn^{2+}-quenched fluorescence comprised 7–10% of the total fluorescence at two wavelengths and was stable for the duration of the experiments. After correcting for the extracellular dye, $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al., (1985), using a KD of 250 nM for the Ca^{2+}/FURA PE 3-AM complex.

2.5. Statistical analysis

All data are expressed as pmol/mg protein and represent mean ± SEM of the number of experiments reported in the figure legends. Multiple comparisons were performed with two-way ANOVA followed by an appropriate (Bonferroni and Newman Keuls) *post hoc* test. Differences were considered significant for $p < 0.05$, at least.

2.6. Chemicals

Percoll, *o*-phthalaldehyde, homoserine, choline chloride, EGTA, FURA PE 3-AM, 4AP were obtained from (Sigma–Aldrich, St. Louis, MO, USA); 5-Iodo-A-85380 dihydrochloride (5IA85380), DL-TBOA, DHβE, SN-6, KB-R7943 mesylate were obtained from (Tocris Bioscience, Bristol, UK).

3. Results

Fig. 1 shows that the GLU and ASP overflow elicited both by 15 mM KCl and by 30 µM 4AP from hippocampal nerve endings were potentiated in the treated animals compared to the saline treated rats while the GABA overflow did not show any significant change.

To further analyze some characteristics of this very important increase of amino acid overflow in the treated animals we investigated the effects of the excitatory aminoacid transporter inhibitor DL-TBOA and of two inhibitors of Na^+/Ca^{2+} exchanger, SN-6 and 2-[2-[4-(4-nitrobenzyloxy)phenyl] ethyl] isothiourea mesylate (KB-R7943) on the endogenous AA overflow. The KCl (15 mM)-evoked increase of GLU overflow was strongly reduced by DL-TBOA (10 µM), SN-6 (30 µM) and KB-R7943 (1 µM) (Fig. 2A), while the ASP overflow was completely blocked (Fig. 2B). Both GLU and ASP overflow evoked by KCl (15 mM) were significantly dependent on external Ca^{2+} both in synaptosomes treated with nicotine or saline (Fig. 2).

Interestingly also the overflow of endogenous GLU and ASP evoked by nicotine (100 µM) was strongly and significantly increased in hippocampal synaptosomes of chronically treated animals compared to controls. Conversely the nicotine (100 µM)-evoked GABA overflow was significantly reduced (Fig. 3).

In order to clarify this point we have studied the effects of two different agonists, Ch and 5IA85380 known to act selectively on the α7 and α4β2 nAChR subtypes respectively (Mukhin et al., 2000; Uteshev et al., 2003; Dickinson et al., 2008; Zappettini et al., 2010). The Ch (1 mM)-evoked GLU and ASP overflow in the treated animals was strongly potentiated compared to controls while the Ch (1 mM)-evoked GABA overflow was unaffected (Fig. 4). The 5IA85380 (10 nM)-evoked overflow of all the three aminoacids was significantly reduced compared to controls in an almost similar manner (Fig. 4).

Quite interestingly in our experimental conditions also the increase of the Ch-evoked GLU and ASP overflow in treated animals, mediated by the α7 nAChRs, was due to a carrier-mediated, Ca^{2+}-dependent mechanism since was totally blocked in presence of DL-TBOA(10 µM) (Fig. 5A and B) and also blocked by the inhibitors Na^+/Ca^{2+} exchanger SN-6 (30 µM) (Fig. 5A and B) similarly to what occurred when synaptosomes were depolarized with KCl. Either the GLU and ASP overflow in the animals chronically treated with saline or nicotine was significantly dependent on external Ca^{2+} (Fig. 4A and B). When Ch was used as nicotinic agonist, KB-R7943 was not tested due to the possible interaction of this compound with nAChRs as previously reported (Pintado et al., 2000).

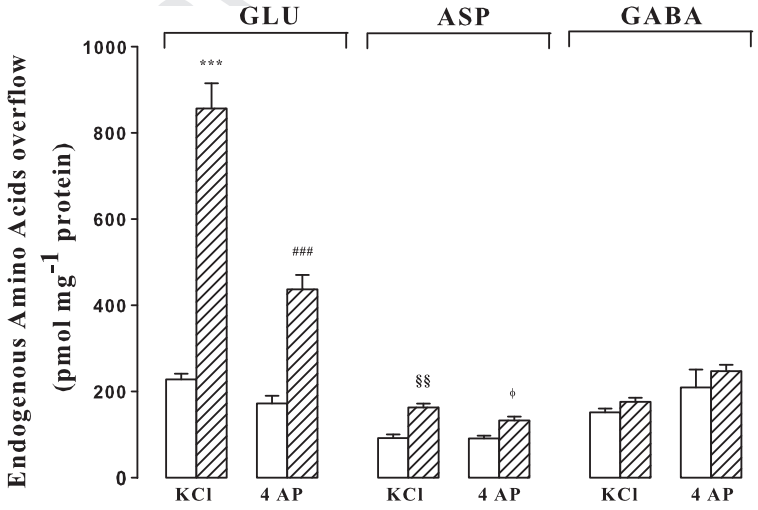


Fig. 1. Effect of KCl (15 mM) and 4AP (30 µM) on endogenous GLU, ASP and GABA overflow from hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were depolarized with KCl (15 mM) and 4AP (30 µM) for 90 s at $t = 38$ min of superfusion. Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{***}p < 0.001$ versus KCl-evoked GLU overflow in vehicle-treated rats; $^{\#\#\#}p < 0.001$ versus 4AP-evoked GLU overflow in vehicle-treated rats; $^{\S\S}p < 0.01$ versus KCl-evoked ASP overflow in vehicle-treated rats; $^{\phi}p < 0.05$ versus 4AP-evoked ASP overflow in vehicle-treated rats. The two-way ANOVA followed by Bonferroni *post hoc* test was used in this figure to Fig. 6.

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

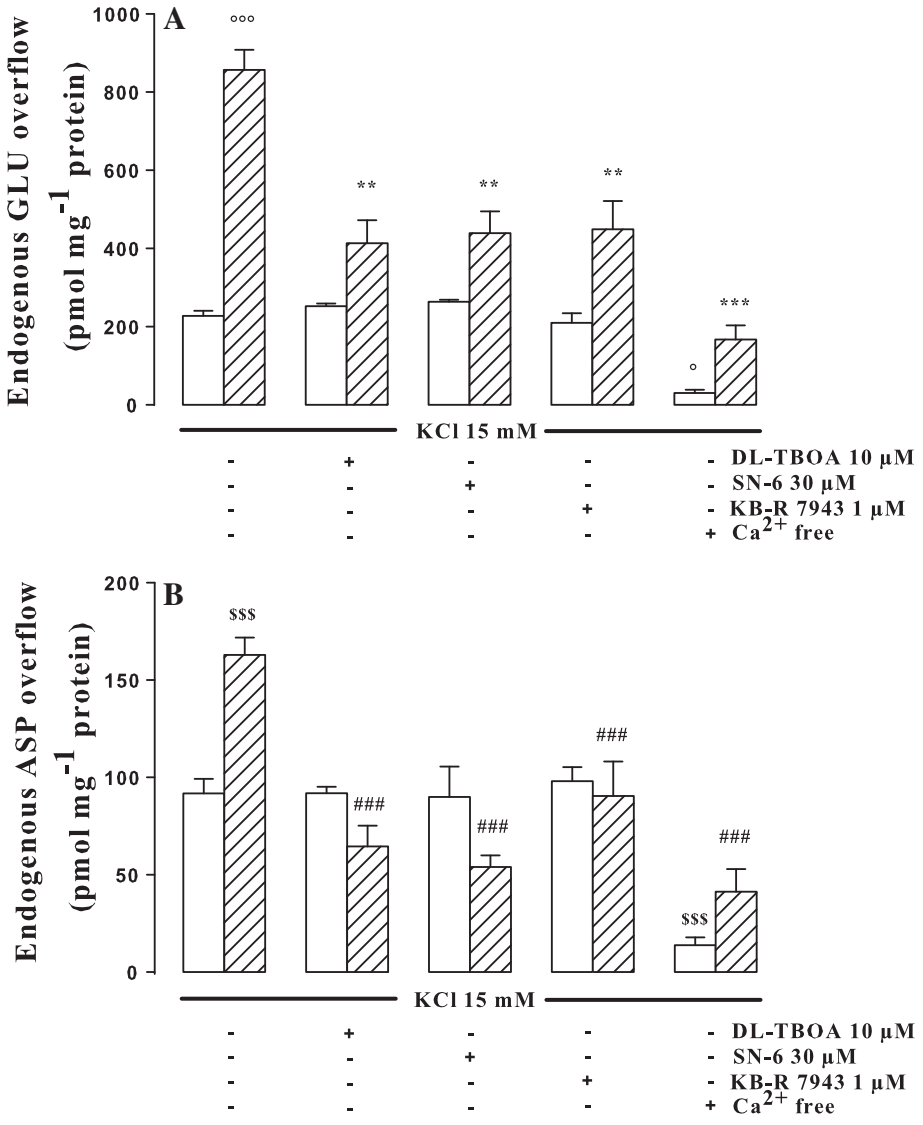


Fig. 2. Effect of DL-TBOA (10 µM), SN-6 (30 µM), KB-R7943 (1 µM) and Ca^{2+}-free, on endogenous GLU (panel A) and ASP (panel B) overflow evoked by 15 mM KCl from hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were depolarized with KCl for 90 s at $t = 38$ min of superfusion. When appropriate antagonists were introduced 8 min before depolarization and Ca^{2+} was omitted 18 min before KCl., (A) Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{°°°}p < 0.001$; $^{°}p < 0.05$ versus KCl -evoked GLU overflow in vehicle-treated rats; $^{***}p < 0.001$; $^{**}p < 0.01$ versus KCl -evoked GLU overflow in nicotine-treated rats., (B) Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{\$\$\$}p < 0.001$ versus KCl -evoked ASP overflow in vehicle-treated rats; $^{\#\#\#}p < 0.001$ versus KCl -evoked ASP overflow in nicotine-treated rats.

As far as the concentrations of the nicotinic drugs used in our experiment it has to be noted that we have previously shown that, at the concentrations used, their efficacy in the stimulation of AA release was relatively mild being comparable to the stimulatory effects of a low concentration (9 mM) of KCl (Zappettini et al., 2010).

The existence of a low affinity nAChRs, dihydro-β-erythroidine (DHβE) insensitive, modulating rubidium efflux has been demonstrated to be present in rat hippocampus (Marks et al., 1999). We have recently demonstrated that low affinity α4β2 nAChRs are present on GLU nerve terminals since 5IA85380 at higher concentration (1 mM) in presence of 2 µM DHβE was able to evoke the overflow of GLU and ASP from rat hippocampal synaptosomes (Zappettini et al., 2010). The results reported in Fig. 6 confirm that 5IA85380 at higher concentrations (1 mM) in presence of 2 µM DHβE produced a stimulatory effect of endogenous overflow of GLU and ASP but not of GABA. This nicotinic stimulatory effect was almost completely abolished in the nicotine treated rats.

Fig. 7 shows that KCl (15 mM), 5IA85380 (10 nM) and Ch (1 mM) increased significantly the apparent $[Ca^{2+}]_i$ in synaptosomes of vehicle treated animals. In the treated rats the effect of 5IA85380 was almost completely abolished while the increase in the $[Ca^{2+}]_i$ in synaptosomes in the response to KCl or Ch stimulation was quantitatively comparable to that found in the vehicle treated animals.

4. Discussion

One of the aims of the present study was to determine the effect of prolonged nicotine exposure on the overflow of endogenous GLU, ASP and GABA evoked in vitro by KCl or 4AP in isolated nerve endings of rat hippocampus. Previous findings have reported that the KCl-evoked overflow of several neurotransmitters (ACh, NA, DA) did not change in animals chronically treated with nicotine (Risso et al., 2004; Grilli et al., 2005, 2009). Here we show, on the contrary, that both the KCl and the 4AP-evoked overflow of GLU and ASP, but not that of GABA, were strongly increased in synaptosomes from chronically treated animals. Surprisingly this selective

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

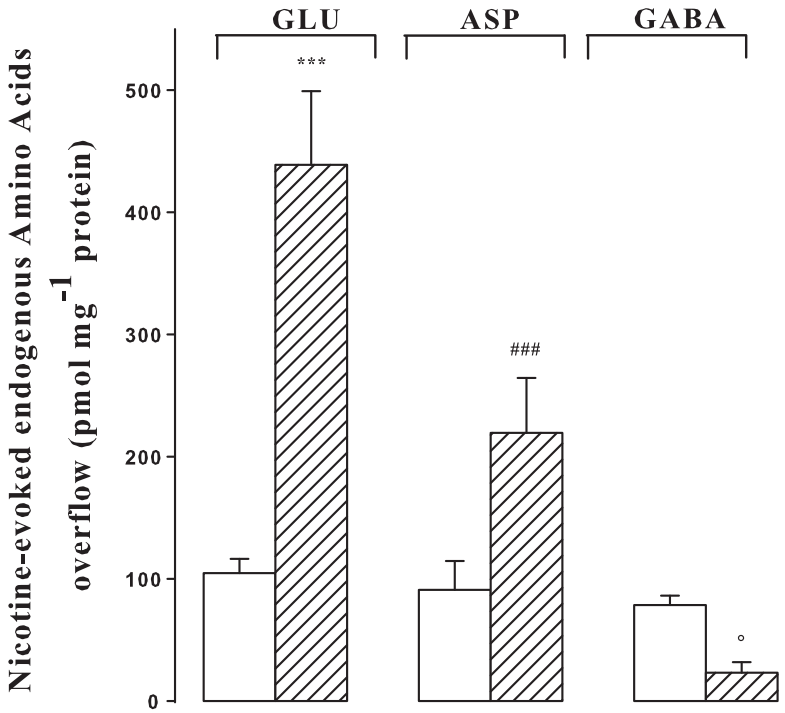


Fig. 3. Effect of nicotine-evoked endogenous GLU, ASP and GABA overflow from hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were stimulated with nicotine (100 μM) for 90 s at $t = 38$ min of superfusion, Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{***}p < 0.001$ versus nicotine-evoked GLU overflow in vehicle-treated rats; $^{\#\#\#}p < 0.001$ versus nicotine-evoked ASP overflow in vehicle-treated rats; $^{\circ}p < 0.05$ versus nicotine-evoked GABA overflow in vehicle-treated rats.

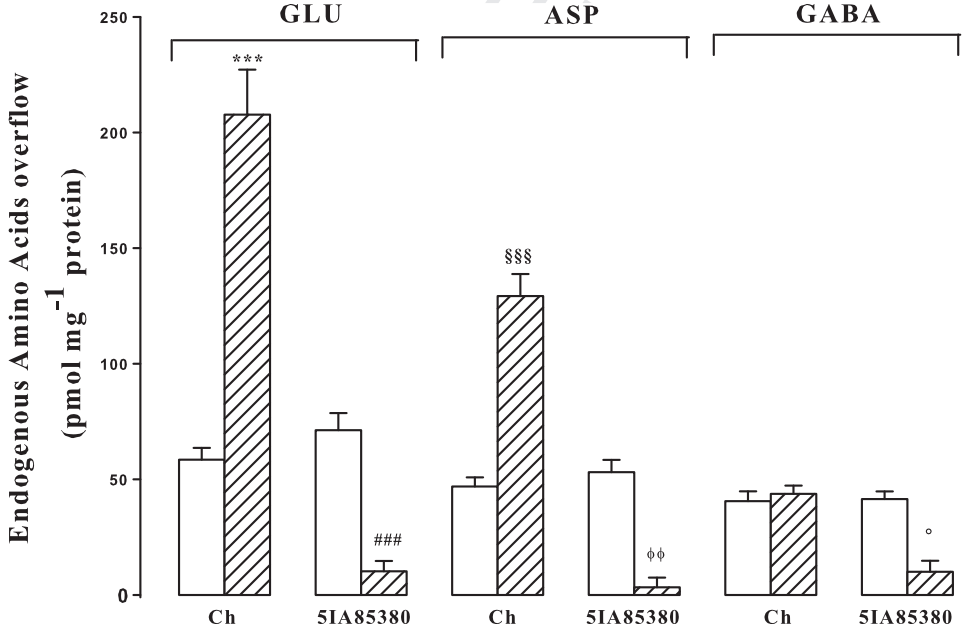


Fig. 4. Effect of Ch (1 mM) and 5IA85380 (10 nM) on endogenous GLU, ASP and GABA overflow from hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were stimulated with Ch (1 μM) and 5IA85380 (10 nM) for 90 s at $t = 38$ min of superfusion. Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{***}p < 0.001$ versus Ch-evoked GLU overflow in vehicle-treated rats; $^{\#\#\#}p < 0.001$ versus 5IA85380-evoked GLU overflow in vehicle-treated rats; $^{§§§}p < 0.001$ versus Ch-evoked ASP overflow in vehicle-treated rats; $^{\phi\phi}p < 0.01$ versus 5IA85380-evoked ASP overflow in vehicle-treated rats; $^{\circ}p < 0.05$ versus 5IA85380-evoked GABA overflow in vehicle-treated rats.

potentiating effect, which occurred also for the Ch- evoked release but does not appear to be exclusively dependent on the acute activation of nAChRs, was not due to an increase of the normal exocytotic process but to the activation of a carrier-mediated release. The possibility that the release of different neurotransmitters could occur not only by exocytosis but also through the reversal of the function of the carriers is well established (Levi and Raiteri, 1993 and references therein). However this carrier-mediated release was Ca^{2+}-dependent, a finding quite unexpected since the carrier-mediated release of neurotransmitters is usually a Na^{+}-dependent, Ca^{2+}-independent mechanism. Previous results have shown that the Ch-evoked GLU and ASP release from hippocampal synap-

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

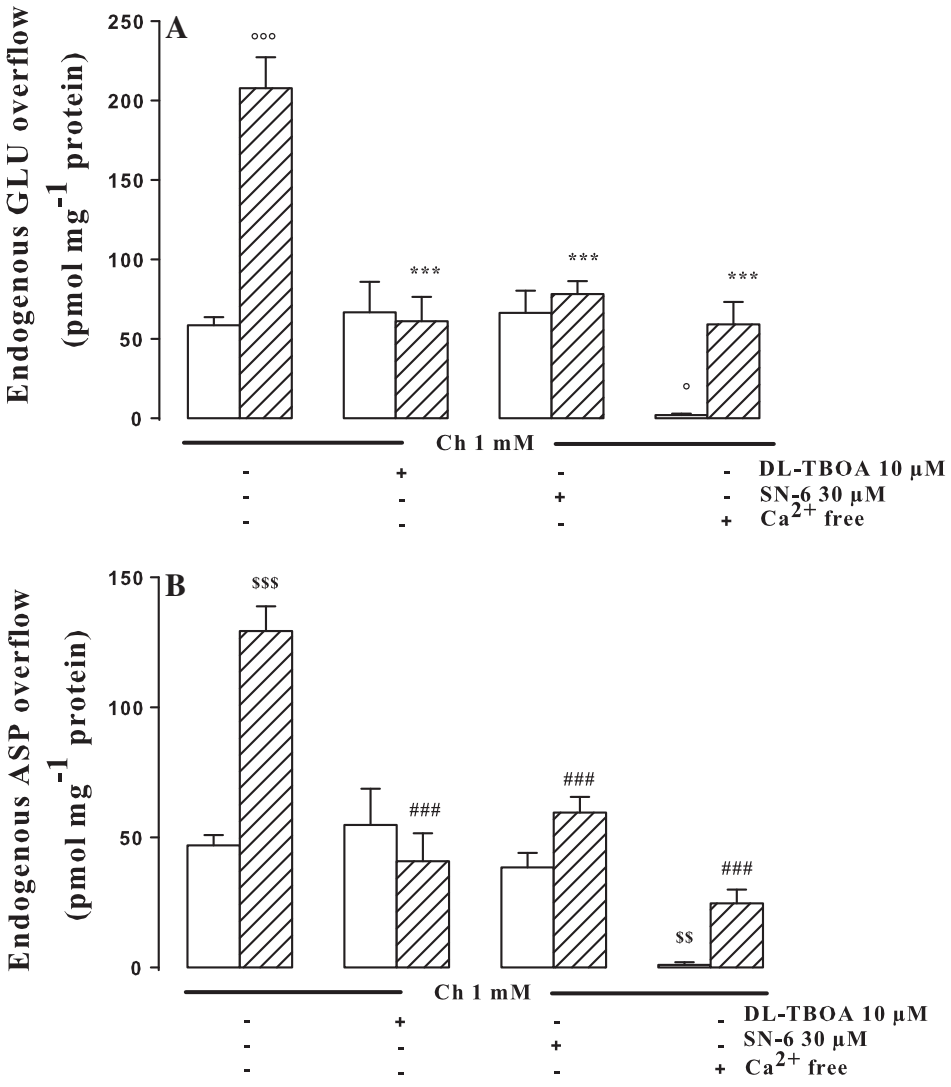


Fig. 5. Effect of DL-TBOA (10 µM), SN-6 (30 µM) and Ca^{2+}-free, on endogenous GLU (panel A) and ASP (panel B) o overflow evoked by Ch from hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were stimulated with Ch (1 mM) for 90 s at t = 38 min of superfusion. When appropriate antagonists were introduced 8 min before depolarization and Ca^{2+} was omitted 18 min before Ch., (A) Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{°°°}p < 0.001$, $^{°}p < 0.05$ versus Ch-evoked GLU overflow in vehicle-treated rats; $^{***}p < 0.001$ versus Ch-evoked GLU overflow in nicotine-treated rats., (B) Data are mean ± SEM of 3–6 experiments for each concentration run in triplicate. $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$ versus Ch-evoked ASP overflow in vehicle-treated rats; $^{\#\#\#}p < 0.001$ versus Ch-evoked ASP overflow in nicotine-treated rats.

tosomes was Ca^{2+}-dependent and largely sensitive to dantrolene, xestospongin C and thapsigargin, but was not altered in the presence of TTX or Cd^{2+}, indicating that it was not due to classical membrane depolarization. It seems therefore that Ch can increase Ca^{2+} influx directly through the α7 nAChR channel that generates Ca^{2+}-induced calcium release from endoplasmic reticulum stores, which ultimately leads to GLU and ASP exocytosis. It is worth noting that in pre-fontal cortex synaptosomes and hippocampal mossy fiber terminals, activation of α7 nAChRs by nicotine has been shown to enhance [^{3}H]D-ASP and endogenous GLU release with a similar mechanism (Dickinson et al., 2008; Bancila et al., 2009).

Our results therefore show that in the synaptosomes of the treated animals the selective increase of the overflow of GLU and ASP following either the KCl depolarization or the α7 nAChRs stimulation by Ch is due to the activation of a carrier-mediated mechanism. Interestingly this event is dependent from the Ca^{2+} concentration in the terminal regardless if this is a consequence of Ca^{2+} entry after a depolarizing stimuli or entry through the receptor channel and also dependent from the activity of the Na^+/Ca^{2+} exchangers. In other words we could speculate that the KCl depolarization or the α7 nAChRs activation would first trigger the influx of Ca^{2+} into GLU terminals (i), the possible increase in the function and/or in the number of the Na^+/Ca^{2+} exchangers might accelerate Ca^{2+} efflux with a consequent elevation of Na^+ influx (ii), the increased concentration of intra-terminal Na^+ would than trigger the carrier-mediated GLU and ASP overflow (iii). In agreement with hypothesis it has been recently reported that in primary cultured neuronal cells from mouse cerebral cortex chronic nicotine treatment induced an increase of the protein levels of the Na^+/Ca^{2+} exchanger isoforms NCX1 and NCX2 (Katsura et al., 2002; Kimura et al., 2009).

Another interesting result of our study is that in the nerve endings from animals chronically treated with nicotine, the in vitro administration of nicotine triggered different responses according to the AA studied. Indeed, while the nicotine-evoked GABA overflow was significantly inhibited, the nicotine-evoked overflow of GLU and ASP was strongly potentiated. This different effect of the

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

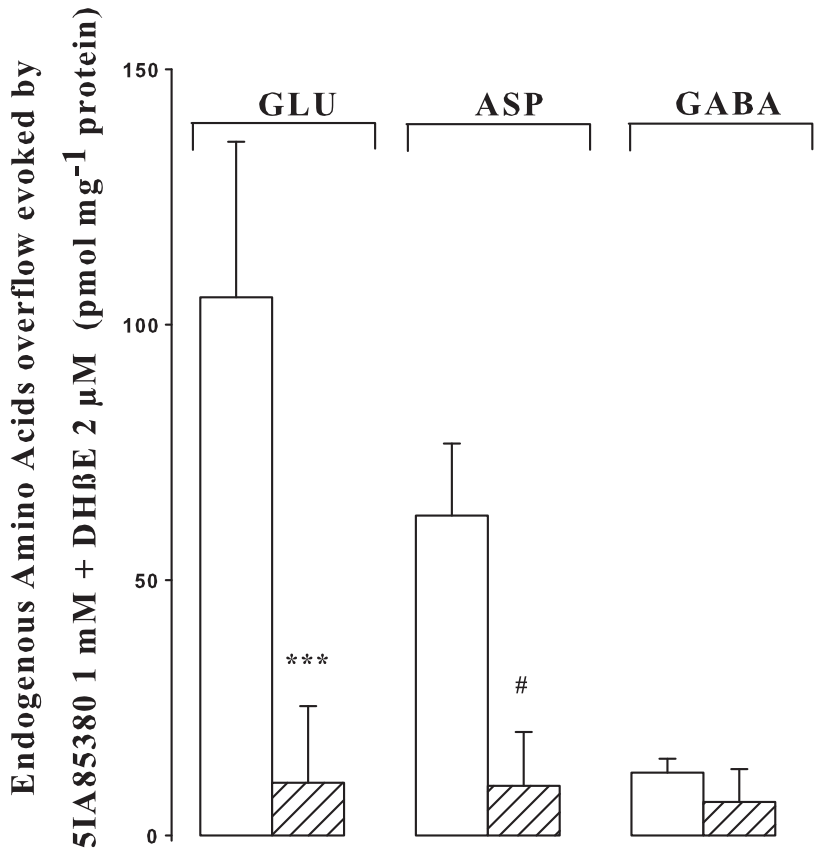


Fig. 6. Effect of 5IA85380 (1 mM) plus DHβE (2 μM) on endogenous release of GLU, ASP and GABA from rat hippocampal synaptosomes prepared from vehicle (white) or nicotine (hatched bar) treated rats. Synaptosomes were stimulated with 5IA85380 (1 mM) in presence of DHβE (2 μM) for 90 s at $t = 38$ min of superfusion. Data are mean ± SEM of 3–6 experiments run in triplicate. $^{***}p < 0.001$ versus DHβE (2 μM)-evoked GLU release in vehicle-treated rats; $^{\#}p < 0.01$ versus DHβE (2 μM)-evoked ASP release in vehicle-treated rats.

nicotine stimulation is apparently difficult to explain since GLU, ASP and GABA release in rat hippocampal synaptosomes are elicited by functionally similar α7 and α4β2 nAChR subtypes (Zappettini et al., 2010,2011). Our results show that in the nerve endings from animals chronically treated with nicotine there was a selective increase of the stimulating response of Ch on α7 nAChRs, which modulate GLU and ASP overflow. No changes were found in the functional responses of Ch on α7 nAChRs eliciting GABA overflow. Conversely the α4β2 nAChRs, which modulate the overflow of GLU, ASP and GABA, when stimulated in vitro by 5IA85380, were almost completely ineffective. Interestingly, also the α4β2 low affinity, DHβE insensitive, nAChRs were completely ineffective in accordance with recent data reporting that exposure to nicotine caused a desensitization of both high- and low-sensitivity subtypes stimulating $^{86}Rb^{+}$ efflux from mice cortical and thalamic nerve endings (Marks et al., 2010). Therefore the increase of the stimulatory effect of nicotine on the release of GLU and ASP in the treated animals seems due to a prevalence of the stimulation of the α7 over the inhibition of α4β2 nAChRs while, on the contrary, the inhibitory effects on GABA release could be explained as due to the prevalence of the inhibitory effect of nicotine on α4β2 nAChRs.

In conclusion our results show for the first time that long-term treatment with nicotine selectively alters the mechanism of GLU and ASP release. Indeed, in the treated animals the selective increase of GLU and ASP overflow occurs through a new and specific activation of a carrier-mediated release mechanism. Secondly the α7 and the α4β2 nAChRs, which modulate presynaptically AA release, respond in a different manner after chronic exposure to nicotine. The α4β2 nAChRs are desensitized and are ineffective when stimulated in vitro. The lack of the stimulatory effect of the Ca^{2+} concentration in synaptosomes found in presence of the selective α4β2 nAChRs agonist 5IA85380 in the treated animals confirms this finding. Conversely the functional response of the α7 nAChRs do not seem to be changed compared to controls. Indeed, the Ch-evoked release of GLU and ASP evaluated in presence of DL-TBOA and SN-6 was very similar to that found in the control animals. Moreover the activation of the α7 nAChRs in the treated animals elicits an increase of GLU and ASP overflow through a carrier-mediated mechanism similar to that elicited by KCl.

Chronically administered nicotine can also increase the functional response of NMDA and AMPA receptors (Risso et al., 2004; Grilli et al., 2009). These findings and our present results confirm the possibility, although speculative, that the chronic treatment with nicotine could promote modification of the synaptic function at hippocampal level. The combination of the nAChR-mediated presynaptic facilitation of GLU and ASP overflow, which may occur after chronic nicotine treatment, with a coincident increase of the postsynaptic depolarization of the neurons could produce the kind of coincidence that is necessary for the synaptic plasticity and the LTP induction to occur. Whether this effect may actually happen in the hippocampus and play a role in the rewarding actions of nicotine could be matter of future investigation. Interestingly, a facilitation of LTP in the amygdala and dentate gyrus after exposure to nicotine was found to be dependent on the activation of NMDA receptors and has been prevented by blocking both α7 (Welsby et al., 2006; Huang et al., 2008) and also by α4β2 nAChRs (Huang et al., 2008). Moreover it has been also recently reported that the modulation of synaptic transmission by dendritic α7-nAChRs pres-

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

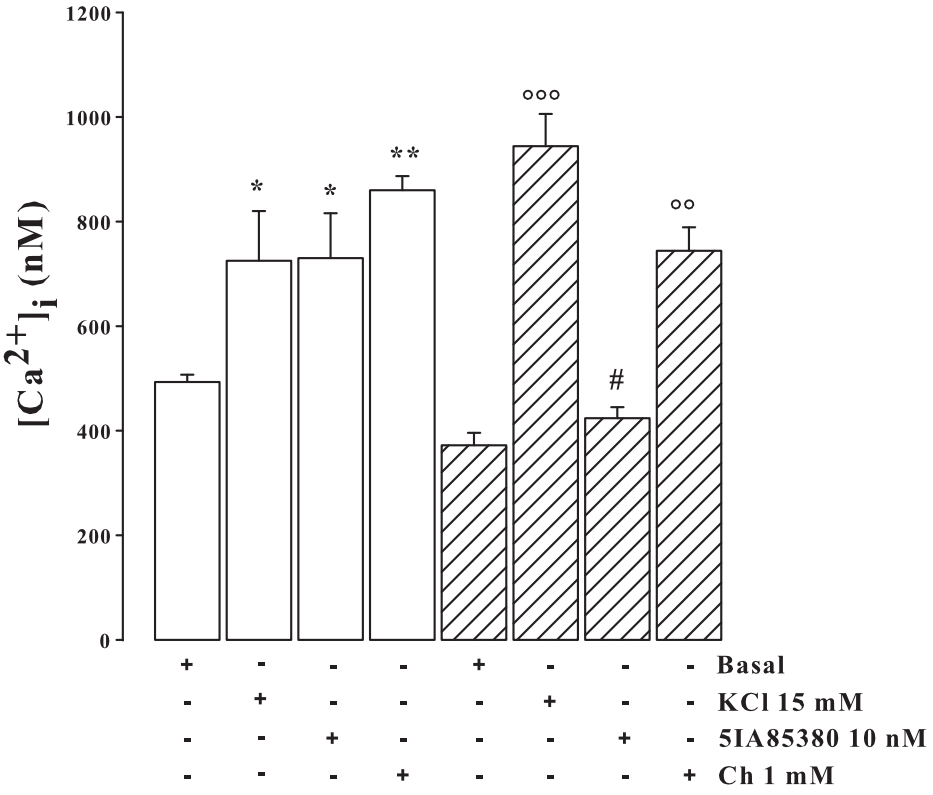


Fig. 7. Effect of 15 mM KCl, Ch (1 mM) and 5IA85380 (10 nM) on the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ induced in rat hippocampal synaptosomes. Synaptosomes were loaded with FURA PE-3 AM, resuspended in standard HEPES-buffered medium and incubated for 40 min before fluorometric measurements. Basal Ca^{2+} levels were measured for 2 min before addition of Ch, 5IA85380 or KCl. Data are expressed as means ± SEM of three experiments run in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ versus basal $[Ca^{2+}]_i$ in control animals; $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ versus basal $[Ca^{2+}]_i$ in treated animals; $^{\#}p < 0.05$, versus 5IA85380 in control animals (Two-way ANOVA followed by Newman Keuls Multiple Comparison Test).

ent on GABAergic interneurons and the LTP boosted by these receptors may play a determining role in hippocampal functions (Rozsa et al., 2008).

According to the information available in the literature there are several neurotransmitter involved in the process of nicotine dependence and tolerance (Proctor et al., 2011; Wooters et al., 2011) and in accordance there are several different research approaches to identify new drugs which have potential for development as pharmacotherapies for tobacco smoking cessation. Our findings demonstrating that long-term nicotine treatment may selectively increase GLU and ASP overflow through the activation of a carrier-mediated release might suggest that medications that target GLU transporters and/or Na^+/Ca^{2+} exchangers might be a new treatment for nicotine dependence.

Acknowledgements

This work was supported by from the Italian Ministero Università Ricerca to Prof. Mario Marchi (prot. No. 20072BTSR2_002 and No. 2009R7WCZS_003), by Compagnia di San Paolo by University of Genoa 'Progetto Ricerca Ateneo' and by project AROMA (ALCOTRA 2007-2013) by the European Community Project PYRGI, project No. B51H10000000006 and by the project 7FP-KBBE BAMMBO. We wish to thank Maura Agate for editorial assistance and Dr. Silvia E. Smith, Ph.D. (University of Utah) for reviewing the manuscript.

References

Bancila, V., Cordeiro, J.M., Bloc, A., Dunant, Y., 2009. Nicotine-induced and depolarisation-induced glutamate release from hippocampus mossy fibre synaptosomes: two distinct mechanisms. J. Neurochem. 110, 570–580.

Benowitz, N.L., 1988. Drug therapy. Pharmacologic aspects of cigarette smoking and nicotine addition. N. Engl. J. Med. 319, 1318–1330.

Delibas, N., Doguc, D.K., Sutcu, R., Eroglu, E., Gökalp, O., 2005. Effect of nicotine on hippocampal nicotinic acetylcholine alpha7 receptor and NMDA receptor subunits 2A and 2B expression in young and old rats. Int. J. Neurosci. 115, 1151–1163.

Dickinson, J.A., Hanrott, K.E., Mok, M.H., Kew, J.N., Wonnacott, S., 2007. Differential coupling of alpha7 and non-alpha7 nicotinic acetylcholine receptors to calcium-induced calcium release and voltage-operated calcium channels in PC12 cells. J. Neurochem. 100, 1089–1096.

Dickinson, J.A., Kew, J.N., Wonnacott, S., 2008. Presynaptic alpha 7- and beta 2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. Mol. Pharmacol. 74, 348–359.

Fedele, E., Jin, Y., Varnier, G., Raiteri, M., 1996. In vivo microdialysis study of a specific inhibitor of soluble guanylyl cyclase on the glutamate receptor/nitric oxide/cyclic GMP pathway. Br. J. Pharmacol. 119, 590–594.

Gioanni, Y., Rougeot, C., Clarke, P.B., Lepousé, C., Thierry, A.M., Vidal, C., 1999. Nicotinic receptors in the rat prefrontal cortex: increase in glutamate release and facilitation of mediodorsal thalamo-cortical transmission. Eur. J. Neurosci. 11, 18–30.

Gray, R., Rajan, A.S., Radcliffe, K.A., Yakehiro, M., Dani, J.A., 1996. Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature 383, 713–716.

Grilli, M., Parodi, M., Raiteri, M., Marchi, M., 2005. Chronic nicotine differentially affects the function of nicotinic receptor subtypes regulating neurotransmitter release. J. Neurochem. 93, 1353–1360.

Grilli, M., Pittaluga, A., Merlo-Pich, E., Marchi, M., 2009. NMDA-mediated modulation of dopamine release is modified in rat prefrontal cortex and nucleus accumbens after chronic nicotine treatment. J. Neurochem. 108, 408–416.

Grynkiewicz, G., Poenie, M., Tsien, R., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Huang, Y.Y., Kandel, E.R., Levine, A., 2008. Chronic nicotine exposure induces a long-lasting and pathway-specific facilitation of LTP in the amygdala. Learn. Mem. 15, 603–610.

Jones, I.W., Wonnacott, S., 2004. Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. J Neurosci. 15, 11244–11252.

Kalivas, P.W., 2009. The glutamate homeostasis hypothesis of addiction. Nature Rev. Neurosci. 10, 561–572.

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

Kane, J.K., Hwang, Y., Konu, O., Loughlin, S.E., Leslie, F.M., Li, M.D., 2005. Regulation of Homer and group I metabotropic glutamate receptors by nicotine. Eur. J. Neurosci. 21, 1145–1154.

Katsura, M., Mohri, Y., Shuto, K., Hai-Du, Y., Amano, T., Tsujimura, A., Sasa, M., Ohkuma, S., 2002. Up-regulation of L-type voltage-dependent calcium channels after long term exposure to nicotine in cerebral cortical neurons. J. Biol. Chem. 277, 7979–7988.

Kenny, P.J., Chartoff, E., Roberto, M., Carlezon Jr., W.A., Markou, A., 2009. NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-administration: role of the ventral tegmental area and central nucleus of the amygdala. Neuropsychopharmacology 34, 266–281.

Kimura, J., Ono, T., Sakamoto, K., Ito, E., Watanabe, S., Maeda, S., Shikama, Y., Yatabe, M.S., Matsuoka, I., 2009. Na^+–Ca^{2+} exchanger expression and its modulation. Biol. Pharm. Bull. 32, 325–331.

Lallemand, F., Ward, R.J., Dravolina, O., De Witte, P., 2006. Nicotine-induced changes of glutamate and arginine in naive and chronically alcoholized rats: an in vivo microdialysis study. Brain Res. 1111, 48–60.

Lee, K.J., Kim, D.H., Choi, S.H., Shin, Y.C., Park, S.H., Moon, B.H., Kang, S.W., Cho, E., Choi, S.H., Chun, B.G., Lee, M.S., Shin, K.H., 2007. Effects of repeated nicotine treatment on the changes in glutamate receptor subunits levels in mesocorticolimbic dopamine areas. Korean J. Physiol. Pharmacol. 11, 139–144.

Levi, G., Raiteri, M., 1993. Carrier-mediated release of neurotransmitters. Trends in Neurosciences 16, 415–419.

Levin, E.D., Tizabi, Y., Rezvani, A.H., Caldwell, D.P., Petro, A., Getachew, B., 2005. Chronic nicotine and dizocilpine effects on regionally specific nicotinic and NMDA glutamate receptor binding. Brain Res. 1041, 132–142.

López, E., Arce, C., Vicente, S., Oset-Gasque, M.J., González, M.P., 2001. Nicotinic receptors mediate the release of amino acid neurotransmitters in cultured cortical neurons. Cereb. Cortex 11, 158–163.

Markou, A., 2008. Neurobiology of nicotine dependence. Phil. Trans. R. Soc. B 363, 3159–3168.

Marks, M.J., Whiteaker, P., Calcaterra, J., Stitzel, J.A., Bullock, A.E., Grady, S.R., Picciotto, M.R., Changeux, J.P., Collins, A.C., 1999. Two pharmacologically distinct components of nicotinic receptor-mediated rubidium efflux in mouse brain require the beta2 subunit. J. Pharmacol. Exp. Ther. 289, 1090–1103.

Marks, M.J., Meinerz, N.M., Brown, R.W., Collins, A.C., 2010. 86Rb+ efflux mediated by alpha4beta2∗-nicotinic acetylcholine receptors with high and low-sensitivity to stimulation by acetylcholine display similar agonist-induced desensitization. Biochem. Pharmacol. 80, 1238–1251.

Matta, S.G., Balfour, D.J., Benowitz, N.L., Boyd, R.T., Buccafusco, J.J., Caggiula, A.R., Craig, C.R., Collins, A.C., Damaj, M.I., Donny, E.C., Gardiner, P.S., Grady, S.R., Heberlein, U., Leonard, S.S., Levin, E.D., Lukas, R.J., Markou, A., Marks, M.J., McCallum, S.E., Parameswaran, N., Perkins, K.A., Picciotto, M.R., Quik, M., Rose, J.E., Rothenfluh, A., Schafer, W.R., Stolerman, I.P., Tyndale, R.F., Wehner, J.M., Zirger, J.M., 2007. Guidelines on nicotine dose selection for in vivo research. Psychopharmacology (Berl.) 190, 269–319.

Mugnaini, M., Garzotti, M., Sartori, I., Pilla, M., Repeto, P., Heidbreder, C.A., Tessari, M., 2006. Selective down-regulation of [(125)I]Y0-alpha-conotoxin MII binding in rat mesostriatal dopamine pathway following continuous infusion of nicotine. Neuroscience 137, 565–572.

Mukhin, A.G., Gundisch, D., Horti, A.G., Koren, A.O., Tamagnan, G., Kimes, A.S., Chambers, J., Vaupel, D.B., King, S.L., Picciotto, M.R., Innis, R.B., London, E.D., 2000. 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand for nicotinic acetylcholine receptors. Mol. Pharmacol. 57, 642–649.

Nakamura, Y., Iga, K., Shibata, T., Shudo, M., Kataoka, K., 1993. Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. Glia 9, 48–56.

Parikh, V., Man, K., Decker, M.W., Sarter, M., 2008. Glutamatergic contributions to nicotinic acetylcholine receptor agonist-evoked cholinergic transients in the prefrontal cortex. J. Neurosci. 28, 3769–3780.

Pintado, A.J., Herrero, C.J., Garcia, A.G., Montiel, C., 2000. The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. Br. J. Pharmacol. 130, 1893–1902.

Proctor, W.R., Dobelis, P., Moritz, A.T., Wu, P.H., 2011. Chronic nicotine treatment differentially modifies acute nicotine and alcohol actions on GABA(A) and glutamate receptors in hippocampal brain slices. Br. J. Pharmacol. 162, 1351–1363.

Raiteri, L., Raiteri, M., 2000. Synaptosomes still viable after 25 years of superfusion. Neurochem. Res. 25, 1265–1274.

Rezvani, A.H., Tizabi, Y., Getachew, B., Hauser, S.R., Caldwell, D.P., Hunter, C., Levin, E.D., 2008. Chronic nicotine and dizocilpine effects on nicotinic and NMDA glutamatergic receptor regulation: interactions with clozapine actions and attentional performance in rats. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 1030–1040.

Risso, F., Parodi, M., Grilli, M., Molfino, F., Raiteri, M., Marchi, M., 2004. Chronic nicotine causes functional upregulation of ionotropic glutamate receptors mediating hippocampal noradrenaline and striatal dopamine release. Neurochem. Int. 44, 293–301.

Rousseau, S.J., Jones, I.W., Pullar, I.A., Wonnacott, S., 2005. Presynaptic alpha7 and non-alpha7 nicotinic acetylcholine receptors modulate [^{3}H]D-aspartate release from rat frontal cortex in vitro. Neuropharmacology 49, 59–72.

Rozsa, B., Katona, G., Kaszas, A., Szipocs, R., Vizi, E.S., 2008. Dendritic nicotinic receptors modulate backpropagating action potentials and long-term plasticity of interneurons. Eur. J. Neurosci. 27, 364–377.

Toth, E., Sershen, H., Hashim, A., Vizi, E.S., Lajtha, A., 1992. Effect of nicotine on extracellular levels of neurotransmitters assessed by mycrodialysis in various brain regions: role of glutamic acid. Neurochem. Res. 17, 265–271.

Toth, E., Vizi, E.S., Lajtha, A., 1993. Effect of nicotine on levels of extracellular amino acids in regions of the rat brain in vivo. Neuropharmacology 32, 827–832.

Toth, E., 1996. Effect of nicotine on the level of extracellular amino acids in the hippocampus of rat. Neurochem. Res. 21, 903–907.

Uteshev, V.V., Meyer, E.M., Papke, R.L., 2003. Regulation of neuronal function by choline and 4OH-GTS-21 through alpha 7 nicotinic receptors. J. Neurophysiol. 89, 1797–1806.

Vieyra-Reyes, P., Picciotto, M.R., Mineur, Y.S., 2008. Voluntary oral nicotine intake in mice down-regulates GluR2 but does not modulate depression-like behaviors. Neurosci. Lett. 434, 18–22.

Vizi, E.S., Lendvai, B., 1999. Modulatory role of presynaptic nicotinic receptors in synaptic and non-synaptic chemical communication in the central nervous system. Brain Res. Rev. 30, 219–235.

Wang, F., Chen, H., Steketee, J.D., Sharp, B.M., 2007. Upregulation of ionotropic glutamate receptor subunits within specific mesocorticolimbic regions during chronic nicotine self-administration. Neuropsychopharmacology 32, 103–109.

Welsby, P., Rowan, M., Anwyl, R., 2006. Nicotinic receptor-mediated enhancement of long-term potentiation involves activation of metabotropic glutamate receptors and ryanodine-sensitive calcium stores in the dentate gyrus. Eur. J. Neurosci. 24, 3109–3118.

Wonnacott, S., 1997. Presynaptic nicotinic ACh receptors. Trends Neurosci. 20, 92–98.

Wooters, T.E., Smith, A.M., Pivavarchyk, M., Siripurapu, K.B., McIntosh, J.M., Zhang, Z., Crooks, P.A., Bardo, M.T., Dwoskin, L.P., 2011. BPiDI: a novel selective ?6?2∗ nicotinic receptor antagonist and preclinical candidate treatment for nicotine abuse. Br. J. Pharmacol. 163, 346–357.

Yamazaki, Y., Jia, Y., Wong, J.K., Sumikawa, K., 2006. Chronic nicotine-induced switch in Src-family kinase signaling for long-term potentiation induction in hippocampal CA1 pyramidal cells. Eur. J. Neurosci. 24, 3271–3284.

Zappettini, S., Grilli, M., Salamone, A., Fedele, E., Marchi, M., 2010. Pre-synaptic nicotinic receptors evoke endogenous glutamate and aspartate release from hippocampal synaptosomes by way of distinct coupling mechanisms. Br. J. Pharmacol. 161, 1161–1171.

Zappettini, S., Grilli, M., Lagomarsino, F., Cavallero, A., Fedele, E., Marchi, M., 2011. Presynaptic nicotinic α7 and non-α7 receptors stimulate endogenous GABA release from rat hippocampal synaptosomes through two mechanisms of action. PLoS One 6, e16911.

Please cite this article in press as: Marchi, M., et al. Chronic nicotine exposure selectively activates a carrier-mediated release of endogenous glutamate and aspartate from rat hippocampal synaptosomes. Neurochem. Int. (2012), doi:10.1016/j.neuint.2012.02.032

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.6

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA

REGIONE LIGURIA

REGIONE TOSCANA

Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale

Programme cofinancé par le Fonds Européen de Développement Régional

The Myrtle (*Myrtus communis* L.) Case: from a Wild Shrub to a New Fruit Crop

M. Mulas[a]
Department of Economics and Tree Systems
University of Sassari
Via De Nicola 9, 07100 Sassari
Italy

Keywords: Mediterranean maquis, aromatic berries, liqueur industry, phenological description, molecular markers

Abstract

Myrtle is an aromatic shrub spontaneously growing in the Mediterranean region. The edible fruits of this plant are widely used to produce a typical liqueur by hydroalcoholic infusion. The myrtle liqueur industry was completely based on the supply of the fruit harvest from wild plants with a consequent risk of selective pressure on the spontaneous species, low quality standardization and difficulties to assure every year an increasing quantity of raw material. In order to avoid the erosion of natural genetic resources a domestication process of the species was carried out starting in 1995. A mass selection of about 130 mother plants was the first step of a research completely performed on the Sardinia island, where the myrtle is a part of the Mediterranean maquis. Accessions were described for the main plant characters and morphology following a special *descriptor list* and only the pure clonal lines obtained by agamic propagation (softwood cuttings) were planted in a repository located in the experimental farm of the University of Sassari in Oristano (Central Western Sardinia). Yield quantity, plant vigor, rooting ability and resistance to nursery management were the main characters evaluated in the first stage of selection. The field observation of candidate selections produced a list of about 40 cultivars completely described and newly tested for fruit production and quality in different localities. Cultivars were compared also for fruit chemical composition and quality of the corresponding liqueurs, as well as for tolerance to the main transplant pathogens and phytoplasms. In vitro propagation of the better cultivars was the strategy adopted to provide nurseries of a great number of plants of few cultivars in the space of 4-5 years. The result was the plantation of about 200 ha that today may mitigate the effects of the harvest of wild fruits with a better quality standardization and yield security, with respect to seasonal meteoric events.

INTRODUCTION

Myrtle (*Myrtus communis* L.) is a typical shrub growing in the Mediterranean maquis of Sardinia island (Mulas et al., 2000a). Berries and leaf biomass showed aromatic properties and are widely harvested by local populations and processed to produce typical liqueurs by hydroalcoholic infusions. This activity until 30 years ago was exclusively craftmade and small quantities of biomass were required. Today is an interesting growing industry, with more than 30 factories of different size, and a production around 3.5 millions of liters. The harvest of berries and leaves is partially improved for organization and efficiency but the risk of an ecological selective pressure on the species is always relevant. Moreover, because of the high variability induced by genotypes and ecological conditions, the harvest of the raw materials from spontaneous plants is not able to provide good quality standards to the industry (Mulas et al., 2000b, c).

Since 1995 myrtle cultivation was proposed like a possible complement of harvest from spontaneous plants by means of a specific program of species domestication and

[a] mmulas@uniss.it

cultivar selection from wild genetic resources (Mulas et al., 1998; Mulas and Cani, 1999; Mulas, 2001, 2004).

In this note the main results of some studies are shortly presented with some consideration regarding the diffusion of the new fruit crop on about 200 ha.

MATERIALS AND METHODS

The research program for myrtle domestication started in 1995 with some studies on the land aimed to improve the knowledge of the ecology of the species *Myrtus communis*. The general objective was to optimize the management of the natural populations of the species (Mulas et al., 2000a, c; Perinu et al., 2002) and to have a picture of its phenotypic variability (Mulas and Deidda, 1998; Mulas and Cani, 1999; Mulas et al., 1996, 1998a, 2002). Other useful information for species domestication were obtained on flower biology (Mulas and Fadda, 2004), phenology (Mulas and Perinu, 2004), agamic propagation ability, technological characteristics of fruits (Melis and Mulas, 2004; Fadda and Mulas, 2010) and of leaf biomass (Melis et al., 2004; Mulas and Melis, 2008, 2011).

A repository of all selected cultivars of myrtle has been made at Fenosu in the Central Western Sardinia. Here the accessions are studied for morphological and technological characters while further information on their adaptability to cultivation have been obtained by other experimental plots located in different areas of the region.

In this note the main characteristics of some particularly valuable cultivars are shortly presented as well as some considerations on further needs of investigation to complete the domestication of the species.

RESULTS AND DISCUSSION

The selection from spontaneous flora of myrtle cultivars adapted to cultivation started with the study of mother plants in their natural environment (Mulas et al., 1999a, b). Agamic propagation of these pre-selections was performed by mean of softwood treatment with IBA powder at 1% and subsequent rooting during three weeks on a perlite frame and under mist irrigation (Mulas and Cani, 1996; Mulas et al., 1998b). Myrtle showed a good rooting ability under this treatment and it was consequently possible the nursery growth and planting in a repository of more than 60 selections (Fig. 1). Twenty of these have been widely described and are available to nurseries and growers (Mulas et al., 2002b, c, d, e). Many observations have been made on the pomological characters (Fig. 2) and on the yield performance of the candidate cultivars (Fig. 3).

In the first myrtle experimental orchards different cultivars were managed without any plant shaping and pruning, in order to observe the natural shape of every selection. Moreover, after three to five years, depending on the vigor of some selections and the tendency to a bush shape of others, the need to give to the plant a defined shape was absolutely strong. The choice was to shape the plant with a central axis in order to improve the secondary branch distribution and to facilitate both the mechanical and hand harvest (Fig. 4). The hand harvest and the mechanical harvest with vineyard harvester showed good results in order to have a good yield of safe fruits for the industry (Paschino et al., 2005). The best application of both harvest systems, however, is possible with cultivars having natural upright habits or pruned to obtain well defined a central axis canopy (Fig. 5). This shrub shape permitted a concentration of yield in the optimal spatial range of the harvester (Mulas, 2004, 2005). The canopy management may start in nursery with appropriate pruning of the young plants.

One of the most serious problems detected in the over 100 ha planted in the last years is the diffusion of visible symptoms of phytoplasm infections (Fig. 6) that may determine the loss of the yield in myrtle plant (Garau et al., 2005). A large variability in the cultivar sensitivity to phytoplasm was found (Fig. 7) and, consequently, a list of recommended cultivar for myrtle cultivation was indicated on the basis of yield, plant vigor, fruit pigmentation, and low sensitivity to phytoplasm symptoms (Table 1).

ACKNOWLEDGEMENTS

The Author is grateful to the Special Grant PYRGI for the financial support to the research.

Literature Cited

Fadda, A. and Mulas, M. 2010. Chemical profile changes during myrtle (*Myrtus communis* L.) fruit development and ripening. Scientia Hortic. 125:477-485.

Garau, R., Prota, V.A., Paltrinieri, S., Tolu, G., Sechi, A. and Bertaccini, A. 2005. Problemi fitopatologici della coltivazione del mirto. Proc. Third Congress on Myrtle. Sassari, Italy 23 September. p.51-58.

Melis, R.A.M. and Mulas, M. 2004. Valutazione della composizione di foglie di cultivar di mirto (*Myrtus communis* L.) per la produzione di liquori. Italus Hortus 11(4):344-346.

Melis, R.A.M., Parpinello, L., Biscaro, S., Perinu, B., Cauli, E. and Mulas, M. 2004. Qualità di bacche di mirto (*Myrtus communis* L.) provenienti da piante coltivate e produzione di liquori. Italus Hortus 11(4):340-343.

Mulas, M. 2001. Nuove acquisizioni e ricerche per la coltivazione del mirto (*Myrtus communis* L.). Rivista di Frutticoltura 63(10):55-58.

Mulas, M. 2004. Problematiche legate alla coltivazione del mirto. Italus Hortus 11(4):308-312.

Mulas, M. and Cani, M.R. 1996. Variability of rooting ability of softwood cuttings in myrtle germplasm. Proc. Intl. Symposium on "Breeding Research on Medicinal and aromatic Plants". Quedlinburg, Germany 30 June-4 July. p.191-194.

Mulas, M. and Cani, M.R. 1999a. Germplasm evaluation of spontaneous myrtle (*Myrtus communis* L.) for cultivar selection and crop development. J. Herbs, Spices & Medicinal Plants 6(3):31-49.

Mulas, M. and Deidda, P. 1998. Domestication of woody plants from Mediterranean maquis to promote new crops for mountain lands. Acta Hort. 457:295-301.

Mulas, M. and Fadda, A. 2004. First observation on biology and organ morphology of myrtle (*Myrtus communis* L.) flower. Agricultura Mediterranea 134:223-235.

Mulas, M. and Melis, R.A.M. 2008. Influence of growing area, year, season, and cultivar on the composition of myrtle leaves and infusions. HortScience 43(2):549-553.

Mulas, M. and Melis, R.A.M. 2011. Essential oil composition of myrtle (*Myrtus communis*) leaves. J. Herbs, Spices & Medicinal Plants 17(1):21-34.

Mulas, M. and Perinu, B. 2004. Osservazioni fenologiche su quattro cultivar di mirto (*Myrtus communis* L.) in due ambienti di coltivazione. Italus Hortus 11(4):347-350.

Mulas, M., Cani, M.R., Brigaglia, N. and Deidda, P. 1998b. Selezione varietale da popolazioni spontanee per la coltivazione di mirto e corbezzolo in Sardegna. Frutticoltura 60(3):45-50.

Mulas, M., Cani, M.R., Brigaglia, N. and Deidda, P. 1999b. Study of myrtle (*Myrtus communis* L.) genetic resources to promote extensive crop as integration of spontaneous harvests. Acta Hort. 502:85-88.

Mulas, M., Cani, M.R. and Deidda, P. 1996. First observation of myrtle (*Myrtus communis* L.) germplasm for characters related to intensive plant cultivation. Proc. Intl. Symposium on "Breeding Research on Medicinal and Aromatic Plants". Quedlinburg, Germany 30 June-4 July. p.29-32.

Mulas, M., Cani, M.R. and Brigaglia, N. 1998a. Characters useful to cultivation in spontaneous populations of *Myrtus communis* L. Acta Hort. 457:271-278.

Mulas, M., Francesconi, A.H.D. and Perinu, B. 2002a. Myrtle (*Myrtus communis* L.) as a new aromatic crop: cultivar selection. J. Herbs, Spices & Medicinal Plants 9(2/3):127-131.

Mulas, M., Francesconi, A.H.D., Perinu, B. and Fadda, A. 2002b. 'Barbara' and 'Daniela': two cultivars for myrtle berries production. Acta Hort. 576:169-175.

Mulas, M., Francesconi, A.H.D., Perinu, B., Peana, I., Nieddu, M.A. and Fadda, A. 2002c. 'Giovanna', 'Grazia' e 'Ilaria': tre nuove cultivar per la coltivazione del mirto. Rivista di Frutticoltura 64(1):49-51.
Mulas, M., Perinu, B. and Francesconi, A.H.D. 2000b. Rigenerazione della biomassa e tecnica selvicolturale del mirto (*Myrtus communis* L.). Monti e Boschi 51(1):43-50.
Mulas, M., Spano, D., Biscaro, S. and Parpinello, L. 2000c. Parametri di qualità dei frutti di mirto (*Myrtus communis* L.) destinati all'industria dei liquori. Industrie delle Bevande 29(169):494-498.
Mulas, M., Perinu, B. and Francesconi, A.H.D. 2000d. Biomass yield and forest management of myrtle (*Myrtus communis* L.) in the Mediterranean maquis. Proc. II Congress on "Applications and perspectives of the forestry research in Italy". Bologna, Italy 20-22 October 1999. p.61-64.
Mulas, M., Perinu, B., Francesconi, A.H.D., Nieddu, M.A., Fadda, A. and Peana, I. 2002e. Primi risultati sperimentali per la coltivazione del mirto (*Myrtus communis* L.): la selezione varietale. L'Informatore Agrario 4:55-59.
Paschino, F., Gambella, F. and Pinna, G. 2005. La meccanizzazione della raccolta del mirto. Proc. Third Congress on Myrtle. Sassari, Italy 23 September. p.43-50.
Perinu, B., Mulas, M., Fadda, A., Cauli, E. 2002a. Mediterranean maquis management and myrtle (*Myrtus communis* L.) biomass yield. Actes du Congrès Intl. "Environnement et Identité en Méditerranée". Corte, France 3-5 July. p.327-334.

Tables

Table 1. Myrtle cultivar bearing pigmented fruits and recommended for cultivation on the basis of plant vigor, shape, yield and phytoplasm sensitivity*.

Cultivar	Vigor	Shape	Yield	Phytoplasm sensitivity
Barbara	medium-low	medium	high	1
Carla	medium	medium-upright	high	0
Erika	medium-high	upright	medium-high	1
Giovanna	high	upright	high	1
Giuseppina	medium-high	medium-upright	medium-high	0
Maria Antonietta	medium-high	upright	high	0
Maria Rita	medium	medium-compact	medium-high	1
Nadia	medium-high	medium-upright	medium-high	1
Rosella	medium	upright	medium	1
Tonina	medium-high	medium-upright	high	1

*: 0 = no symptom evidence; 1 = light symptoms occurrence; 2 = medium symptoms; 3 = severe symptoms.

Figures

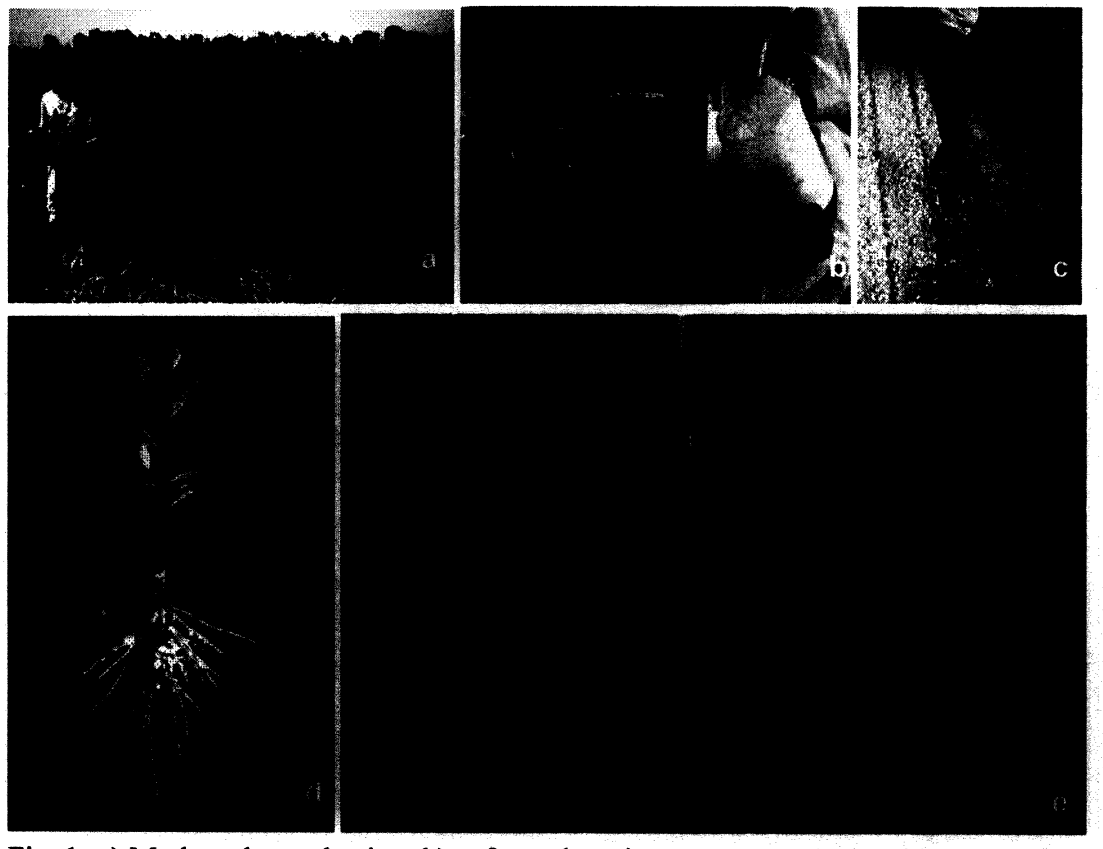


Fig. 1. a) Mother plant selection; b) softwood cutting treatment; c) agamic propagation of accessions; d) good rooting of myrtle softwood cutting; e) nursery growth of plants.

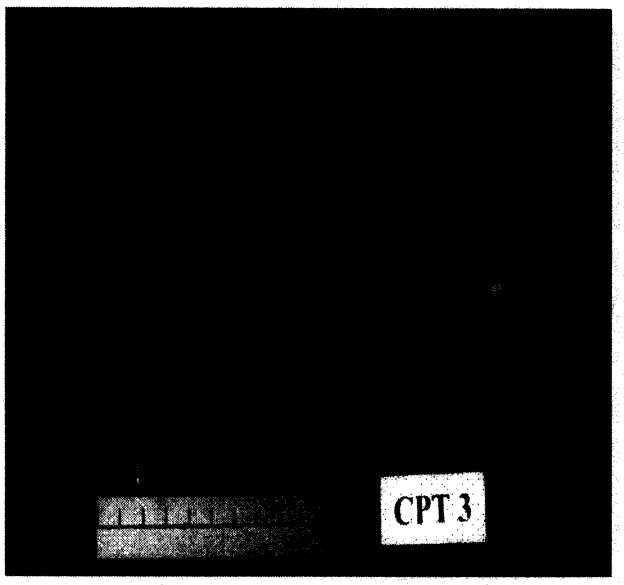


Fig. 2. Pomological plate of the cultivar 'Maria Rita', one of the most cultivated and appreciated for the early ripening fruit, high aromatic and anthocyan content.

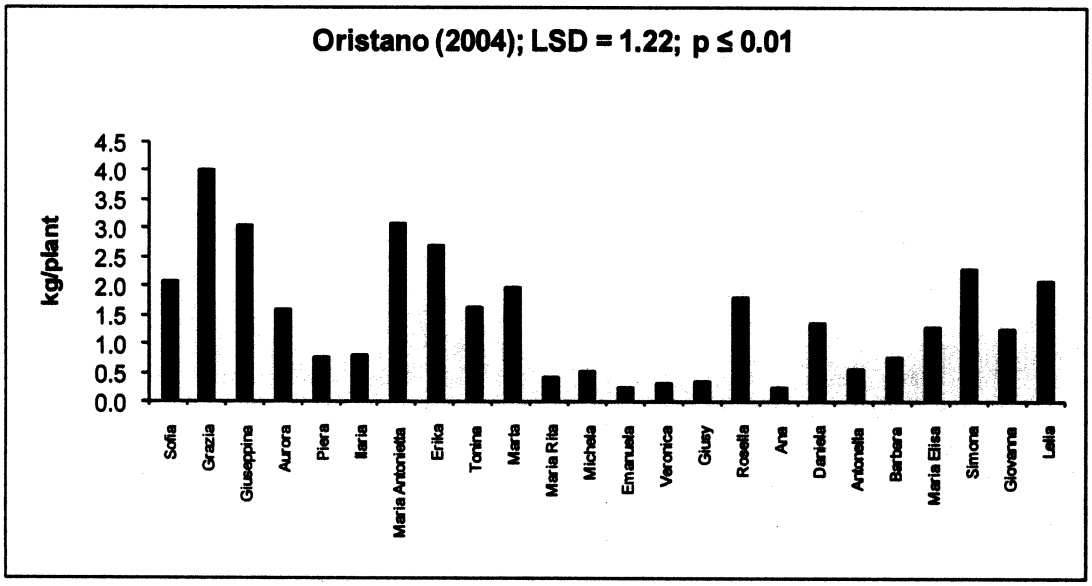


Fig. 3. Yield per plant in the experimental plot of Oristano in 2004.

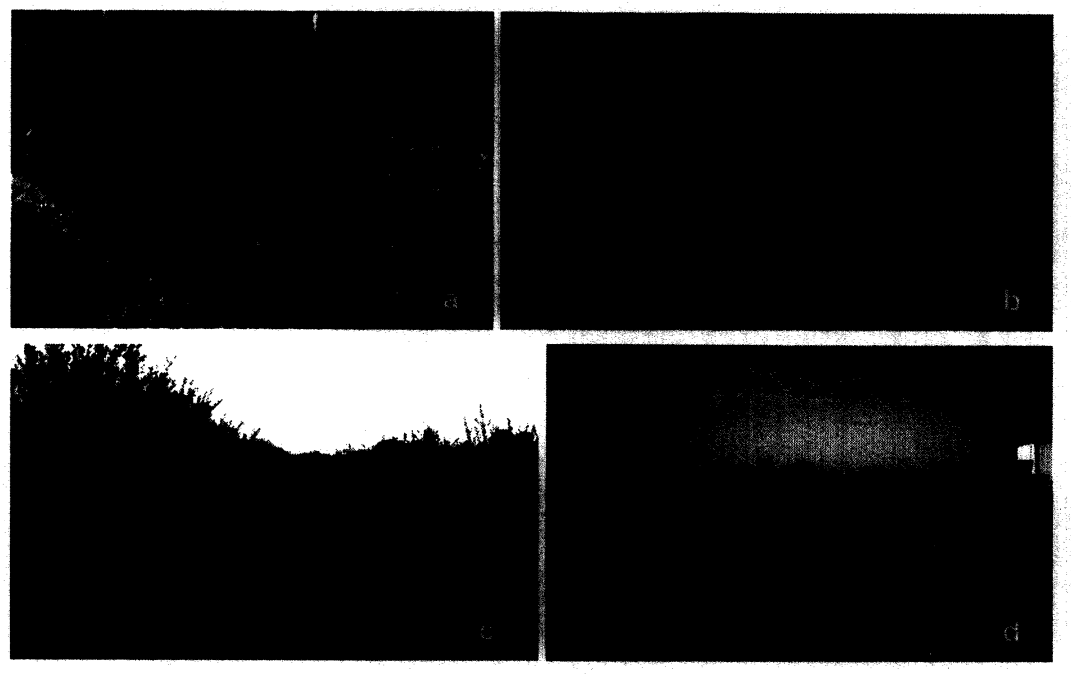


Fig. 4. a) myrtle orchard one year after planting; b) myrtle orchard two years after planting; c) myrtle orchard five years after planting; d) myrtle orchard after shape pruning at 6th year after planting.

Fig. 5. Myrtle harvest with shaking comb (left) or vineyard harvester (right).

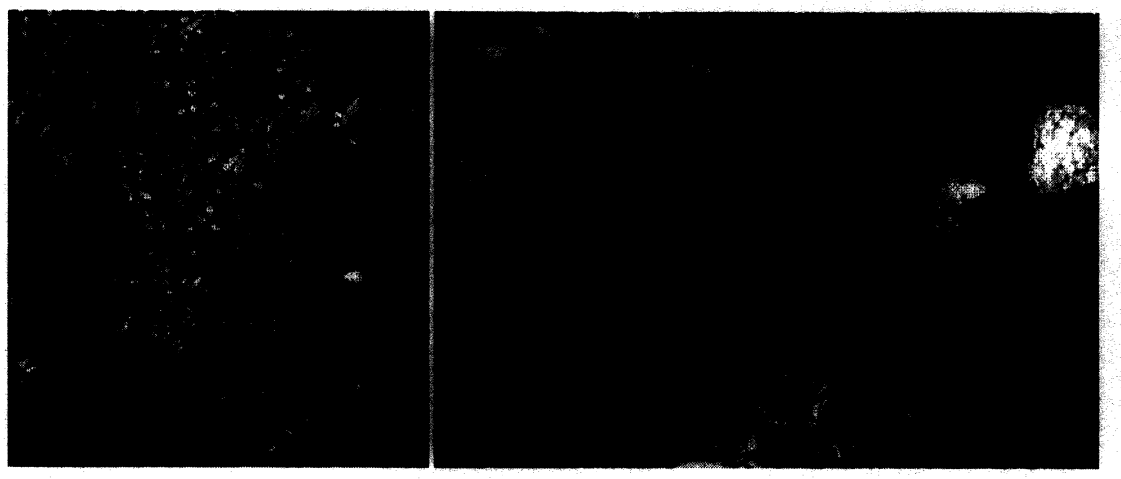


Fig. 6. Phytoplasm infection symptoms on 'Daniela' cultivar: mychrophylls, dwarf plants and geotropism inversion of shoots.

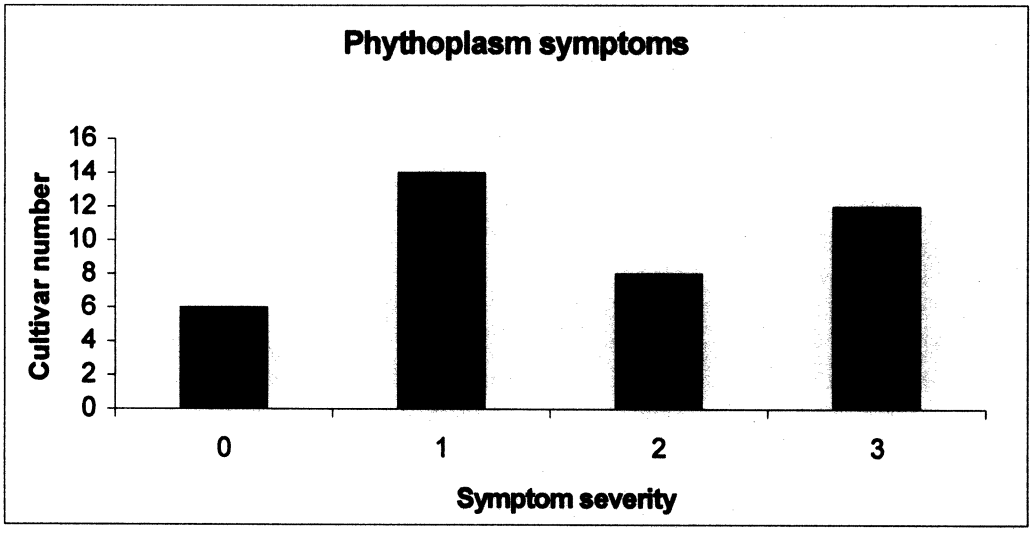


Fig. 7. Distribution of 40 myrtle cultivar in classes of visual sensitivity to phytoplasm after evaluation of severity score of observed symptoms (0 = absence; 1 = light symptoms; 2 = medium severity symptoms; 3 = severe symptoms).

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.7

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Neuropharmacology 63 (2012) 916–926

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

In vitro exposure to nicotine induces endocytosis of presynaptic AMPA receptors modulating dopamine release in rat nucleus accumbens nerve terminals

Massimo Grilli [a,1], Maria Summa [a,1], Alessia Salamone [a], Guendalina Olivero [a], Stefania Zappettini [a], Silvia Di Prisco [a], Marco Feligioni [d], Cesare Usai [b], Anna Pittaluga [a,c,1], Mario Marchi [a,c,*,1]

[a] Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy
[b] Institute of Biophysics, National Research Council, via De Marini 6, 16149 Genova, Italy
[c] Center of Excellence for Biomedical Research, University of Genoa, Italy
[d] Pharmacology of Synaptic Plasticity Unit, European Brain Research Institute, 00143 Rome, Italy

ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form
19 June 2012
Accepted 24 June 2012

Keywords:
Nicotinic receptors
AMPA receptors
Nicotine treatment
Neurotransmitter release
Isolated nerve endings
Nucleus accumbens

ABSTRACT

Here we provide functional and immunocytochemical evidence supporting the presence on Nucleus Accumbens (NAc) dopaminergic terminals of cyclothiazide-sensitive, alfa-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA) receptors, which activation causes Ca^{2+}-dependent [^{3}H]dopamine ([^{3}H]DA) exocytosis. These AMPA receptors cross-talk with co-localized nicotinic receptors (nAChRs), as suggested by the finding that *in vitro* short-term pre-exposure of synaptosomes to 30 μM nicotine caused a significant reduction of both the 30 μM nicotine and the 100 μM AMPA-evoked [^{3}H]DA overflow. Entrapping pep2-SVKI, a peptide known to compete for the binding of GluA2 subunit to scaffolding proteins involved in AMPA receptor endocytosis, in NAC synaptosomes prevented the nicotine-induced reduction of AMPA-mediated [^{3}H]DA exocytosis, while pep2-SVKE, used as negative control, was inefficacious. Immunocytochemical studies showed that a significant percentage of NAc terminals were dopaminergic and that most of these terminals also posses GluA2 receptor subunits. Western blot analysis of GluA2 immunoreactivity showed that presynaptic GluA2 proteins in NAc terminals were reduced in nicotine-pretreated synaptosomes when compared to the control. The nACh-AMPA receptor −receptor interaction was not limited to dopaminergic terminals since nicotine pre-exposure also affected the presynaptic AMPA receptors controlling hippocampal noradrenaline release, but not the presynaptic AMPA receptors controlling GABA and acetylcholine release. These observations could be relevant to the comprehension of the molecular mechanisms at the basis of nicotine rewarding.

1. Introduction

Accumulating evidence indicates that nicotine-induced glutamatergic synaptic plasticity may have important implications in mediating the rewarding actions of nicotine and might be associated with the initiation of nicotine-seeking behavior (Liechti and Markou, 2008; Reissner and Kalivas, 2010; D'Souza and Markou, 2011). It is well established that chronic nicotine administration affects N-methyl-D-aspartic acid (NMDA) receptor subunits composition (Delibas et al., 2005; Levin et al., 2005; Lee et al., 2007; Wang et al., 2007; Rezvani et al., 2008; Kenny et al., 2009), that induces changes to alfa-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA) and group I metabotropic receptors (Kane et al., 2005), and that it alters the functional responses of NMDA and AMPA in different brain areas including the hippocampus (Risso et al., 2004a; Yamazaki et al., 2006; Vieyra-Reyes et al., 2008; Lin et al., 2010). Inasmuch, electrophysiological results showed that also acute application of nicotine might induce changes in the glutamatergic functions and that such a cholinergic–glutamatergic functional cross-talk could involve ionotropic glutamate receptors at the presynaptic level. Indeed, the *in vitro* application of nicotine increased the AMPA-mediated excitatory postsynaptic current amplitude in the VTA

Abbreviations: ACh, acetylcholine; AMPA, alfa-amino-3-hydroxy-5-methyl-4-isoxazolone propionate; DA, dopamine; DAT, dopamine transporter; ECL, enhanced chemiluminescence; NAc, nucleus accumbens; nAChRs, nicotinic acetylcholine receptors; NMDA, N-methyl-D-aspartic acid; NSSP, non-synaptic synaptosomal protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Post, postsynaptic component of the synaptic active zone; Pre, presynaptic component of the synaptic active zone; (NSSP), non-synaptic components; Stx-1A, anti-syntaxin-1A; Syn, synaptosomes; t–TBS, Tris-buffered saline–Tween.

* Corresponding author. Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy. Tel.: +39 010 3532657; fax: +39 010 3993360.
E-mail address: marchi@pharmatox.unige.it (M. Marchi).
[1] Equally contributed.

0028-3908/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2012.06.049

dopaminergic neurons through a presynaptic/preterminal mechanism (Mansvelder and McGehee, 2000). Interestingly, previous data obtained from VTA slices indicated that nicotine, by activating presynaptic nAChRs, enhanced glutamatergic transmission and modulated LTP induction with a short onset time (less than 10 min; Mansvelder and McGehee, 2000; Mansvelder et al., 2002). The possibility that different receptors, which coexist on the same nerve endings, may functionally interact and modulate neurotransmitter release has been amply demonstrated (Rodrigues et al., 2006; Marchi and Grilli, 2010; Prezeau et al., 2010; Albizu et al., 2011). In particular, the functional cross-talk involving both presynaptic NMDA and metabotropic GLU receptors with nAChRs coexisting on the same nerve endings have been already investigated (Risso et al., 2004b; Parodi et al., 2006), while no information are available so far on the coexistence and the functional cross-talk involving presynaptic nicotine and AMPA receptors on NAc dopaminergic terminals.

To provide new evidence in the understanding of nicotine-induced adaptation of glutamatergic synaptic activity in the NAc, we have investigated, with functional and immunocytochemical analysis, the possible cross-talk between presynaptic nAChRs and AMPA receptors coexisting on isolated nerve endings. Our results demonstrate the presence of cyclothiazide-sensitive, AMPA receptors, which are co-localized with nAChRs on NAc dopaminergic terminals. Our data also show that nicotine causes a rapid decrease in the number of surface located coexisting neuronal AMPA receptors. This effect is selective for AMPA receptors present on DA and NA nerve endings, but could not be observed when investigating the release regulating presynaptic AMPA receptors present on GABAergic and cholinergic nerve endings. A better understanding of the functional interaction involving nicotine and ionotropic glutamate receptors could aid in the identification of new potential therapeutic targets for tobacco smoking cessation.

2. Material and methods

2.1. Animals and brain tissue preparation

Adult male rats (Sprague–Dawley, 200–250 g) were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light-dark schedule (light 7.00 a.m.–7.00 p.m.). Food and water were freely available. The animals were killed by decapitation and the brain was rapidly removed at 0–4 °C. Fresh tissue was dissected according to Paxinos and Watson Atlas (1986; sections between Bregma 0.7–2.2 mm for NAc). The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (protocol number 124/2003-A). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

2.2. Release experiments from synaptosomes

Crude synaptosomes from the nucleus accumbens (NAc) and the hippocampus of adult rats were homogenized in 40 volumes of 0.32 M sucrose, buffered to pH 7.4 with phosphate (final concentration 0.01 M, Raiteri et al., 1974). The homogenate was centrifuged at 1000 *g* for 5 min, to remove nuclei and cellular debris, and crude synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in physiological medium having the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4.

In release experiments, NAc synaptosomes were incubated for 20 min at 37 °C with [^{3}H]dopamine ([^{3}H]DA; final concentration 0.03 μM) in the presence of 6-nitroquipazine (final concentration 0.1 μM) to avoid false labeling of serotonergic terminals and of desipramine (final concentration 0.1 μM) to avoid false labeling of noradrenergic terminals, [^{3}H]acetylcholine ([^{3}H]ACh; final concentration 0.08 μM). In a set of experiments, hippocampal synaptosomes were incubated with [^{3}H]GABA (final concentration 0.02 μM) or [^{3}H]noradrenaline ([^{3}H]NA; final concentration 0.03 μM) in the presence of 6-nitroquipazine (to avoid false labeling of serotonergic terminals).

Identical portions of the synaptosomal suspension were then layered on microporous filters at the bottom of parallel superfusion chambers thermostated at 37 °C (Raiteri et al., 1974). Synaptosomes were superfused at 0.5 ml/min with standard physiological medium as previously described. Starting from $t = 36$ min to $t = 48$ min of superfusion four consecutive 3-min fractions (b1–b4) were collected. Synaptosomes were exposed to agonists or to depolarizing agent (4-aminopyridine) at $t = 39$ min, till the end of superfusion, while antagonists were present from 8 min before agonists. When indicated, at $t = 29$ min of superfusion, synaptosomes were exposed for 10 min to nicotine (30 μM) in the absence or in the presence of nicotine agonists. When indicated, rat hippocampus was homogenized in buffered sucrose containing 20 mM of the peptides under study in order to entrap these agents into subsequently isolated synaptosomes (see Pittaluga et al., 2006). Based on estimates made by entrapping of [^{3}H]sucrose, the intrasynaptosomal concentration of the compounds is about 5% of the original concentration in the homogenization medium (Raiteri et al., 2000). Samples collected and superfused synaptosomes were then counted for radioactivity (fractional efflux) and agonist-induced effect was expressed as "% induced overflow". And were evaluated by subtracting the neurotransmitter content released in the four fractions collected under basal condition (no drug added) from that released in presence of the stimulus.

2.3. Sub-synaptic fractionation of nerve terminals

Purified synaptosomes were prepared on Percoll gradients (Sigma–Aldrich, St Louis, MO) essentially according to Dunkley et al. (1986), with minor modifications. Briefly, the tissue was homogenized in 6 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged at 1000 × *g* for 5 min, at 4 °C, to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min at 4 °C. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected, washed by centrifugation and resuspended in physiological HEPES-buffered medium having the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4. Protein content was quantified by applying Bradford (1976) method.

Synaptosomes were washed by centrifugation and sub-synaptic fractions were prepared as described in literature (Phillips et al., 2001; Rebola et al., 2003; Feligioni et al., 2006). Briefly, nucleus accumbens synaptosomes (Syn, total synaptosomal fraction) were resuspended in 300 μl of buffered sucrose (0.32 M sucrose; Tris, 0.01 M; $CaCl_2$ 0.1 mM), diluted 1:10 in ice-cold 0.1 mM $CaCl_2$ and mixed with an equal volume of 2× Triton X-100 buffer (2% Triton X-100, 40 mM Tris, pH 6.0; 4 °C). Protease inhibitors were used in all purification steps. Following a 30-min incubation at 4 °C, the insoluble material (synaptic junctions) was pelleted (40,000 *g*, 30 min, 4 °C) and the supernatant (non-synaptic synaptosomal protein; NSSP) was decanted, diluted with 6 vol of acetone at −20 °C and proteins precipitated by centrifugation (18,000 *g*; 30 min; −18 °C). The synaptic junction pellet was resuspended in 10 vol of 1× Triton X-100 buffer (1% Triton X-100, 20 mM Tris, pH 8.0; 4 °C), incubated for 30 min at 4 °C and then centrifuged (40,000 *g*, 30 min, 4 °C). The pellet contained the insoluble postsynaptic density (Post) and the supernatant contained the presynaptic active zone (Pre). The protein in the supernatant (presynaptic fraction) was acetone precipitated and collected as above. In a set of experiments aimed at investigating the effect of nicotine pretreatment on the sub-synaptic distribution of GluA2 subunits, synaptosomes were firstly incubated in physiological medium at 37 °C in a rotary water bath for 10 min in absence or in presence of nicotine (30 μM) and then sub-synaptic fractions prepared as previously described.

2.4. Reversible biotinylation measuring endocytosis of GluA2 subunit

Synaptosomes were prepared and purified as described above from the brain of 5 pulled rats NAcs. Synaptosomes proteins were counted by applying Bradford (1976) method and then resuspended in HEPES-buffered medium at 4 °C. Two aliquots of synaptosomes [500 μg proteins each, namely the synaptosomal fraction (Syn) and the streptavidin pull-down synaptosomal lysate (L), respectively, were collected and stored at 4 °C. GluA2 endocytosis measurement was evaluated by performing reversible biotinylation as described in literature (Jayanthi et al., 2004; Johnson et al., 2005) with minimal modifications. Briefly, purified synaptosomes were divided in 4 aliquots (500 μg proteins each) and labeled with 2 mg/ml of sulfo–NHS–SS–biotin for 1 h at 4 °C. Synaptosomes were then washed twice with ice-cold 100 mM glycine in PBS/Ca–Mg to quench biotin. One aliquot was used to measure total biotinylated GluA2, whereas another one was used for detecting the minimum of biotinylation. In the latter case, synaptosomes were stripped with the non-permeable reducing agent Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The other 2 aliquots were incubated for 10 min at 37 °C under mild shacking after which one aliquot was treated for 10 min with nicotine (T, nicotine f.c. 30 μM) while the other one was kept as control (C). The reaction was stopped with cold washing buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6). Biotinylated surface proteins were stripped by using 50 mM TCEP in washing buffer for 1 h at 4 °C. Synaptosomes were then washed twice in ice-cold washing buffer and following were lysate in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate). Streptavidin magnetic beads (20 μl

each sample over-night at 4 °C) were added to biotinylated synaptosomes (nicotine treated, T, and nicotine untreated, C, synaptosomes) to pull-down biotinylated proteins as well as to non-biotinylated synaptosomes to evaluate streptavidin pull-down specificity (B). After extensive washes with washing buffer, 1× SDS-PAGE buffer were added and samples were boiled for 5 min at 95 °C before electrophoresis gel loading.

2.5. Immunoblotting

Proteins were loaded on 10% Sodium Dodecyl Sulphate-PAGE gel and then transferred onto PVDF membranes. Non-specific binding sites were blocked 1 h at room temperature with Tris-buffered saline–Tween (t–TBS; 0.02 M Tris, 0.150 M NaCl, and 0.05% Tween 20) containing 5% non-fat dried milk and probed for protein of interest with the following primary antibodies: rabbit anti-GluA2 (1:500), mouse anti-syntaxin-1A (Stx-1A; 1:10000), mouse anti-synaptophysin (1:10000), rabbit anti-PSD95 (1:1000). After washes, membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody (1:2000), and immunoblots were visualized with an ECL (enhanced chemiluminescence) Plus Western blotting detection system to detect protein bands. In a set of experiments aimed at investigating the GluA2 redistribution in the subsynaptic fractions, the presynaptic components the synaptic active zone (Pre), the postsynaptic components the synaptic active zone Post and the non-synaptic (NSSP) components isolated from control and from nicotine pre-treated synaptosomes were blotted as previously described. In this case, the level of GluA2 subunit immunoreactivity was monitored in the total synaptosomes (Syn) and in the Pre, in the Post and in the NSSP fractions of control and nicotine pretreated NAc; β-actin immunoreactivity [quantified by using mouse anti-β-actin (1:5000)] was used as internal control. In the experiments carried out to investigate GluA2 endocytosis with the reversible biotinylation approach, the biotinylated surface proteins from control synaptosomes (lane C), from nicotine-treated synaptosomes (lane T), from the streptavidin pull-down synaptosomal lysate (lane L), from the beads control (lane B) and from the synaptosomal fraction (lane Syn) were blotted. GluA2 subunit immunoreactivity was monitored in all the fractions.

2.6. Immunocytochemical analysis in rats nucleus accumbens nerve terminals

Crude synaptosomes from NAc were prepared as previously described. For immunocytochemical analysis 50 μg of synaptosomal proteins were placed onto cover-slips which were previously coated with poly-L-lysine, were fixed with 2% paraformaldehyde for 15 min and were washed with phosphate-buffered saline (PBS). After extensive washes with 0.5% bovine serum albumin (BSA) PBS, synaptosomes were first incubated with the following primary antibodies diluted in PBS containing 3% albumin: the rabbit anti-GluA2 receptor (1:2000) and the anti-dopamine transporter (DAT; 1:2000) for 1 h at room temperature and then permeabilized with 0.05% Triton X-100 PBS for 5 min and exposed to the mouse anti-syntaxin-1A (Stx-1A; 1:10000). Synaptosomes were then washed with 0.5% BSA PBS and incubated for 30 min at room temperature with AlexaFluor-647 (red) donkey anti-rabbit IgG antibodies, Cy3 (yellow) goat anti-rat IgG antibodies, and AlexaFluor-488 (green)-labeled donkey anti-mouse IgG antibodies (1:500 for all), as appropriate. Fluorescence image acquisition was performed with a multi-channel Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543 and 633 nm excitation lines. Images (512_ 512_ 8 bit) were taken through a planapochromatic oil immersion objective 63×/NA1.4. Light collection configuration was optimized according to the combination of chosen fluorochromes and sequential channel acquisition was performed to avoid cross-talk phenomena. Leica LasAF software package was used for acquisition, storage and visualization. Each coverslip was analyzed by acquisition of at least three different fields.

2.7. Statistical analysis

Multiple comparisons were performed with one- or two-way ANOVA followed by the Bonferroni *post hoc* test. Data were considered significant for $p < 0.05$, at least. The IC_{50} and Hillslope have been calculated according to a four parameter logistic curve using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA, USA). GluA2 and β-actin bands from sub-fractionated samples as well as GluA2 bands from *Reversible biotinylation experiments* were quantified by using the ImageJ 1.46b software (Wayne Rasband, NIH, MD, USA). Protein bands used were within linear range of standard curves that were obtained by loading increasing amounts of samples and were normalized for β-actin level in the same membrane.

The quantitative estimation of co-localized proteins in immunocytochemical studies was performed calculating the co-localization coefficients from the red- and green two colour-channel scatter plots (Manders et al., 1993). Co-localization coefficients express the fraction of co-localizing molecular species in each component of a dual colour image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition (Gonzalez and Wintz, 1987). If two molecular species are co-localized, the overlay of their spatial distributions has a correlation value higher than what would be expected by chance alone. Costes et al. (2004) developed an automated procedure to evaluate correlation between first and the second colour channel with a significance level >95%. The same procedure automatically determines an intensity threshold for each colour channel based on a linear least-square fit of the two colours intensities in the image's 2D correlation cytofluorogram. Costes' et al. approach was employed using macro routines integrated as plug-ins (WCIF Co-localization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.46b software (Wayne Rasband, NIH, MD, USA).

2.8. Materials

1-[7,8-^{3}H]noradrenaline (5–15 Ci(185–555 GBq)/mmol), [^{3}H]GABA (70–100Ci(2.59–3.70TBq)/mmol), [^{3}H]choline (60–90Ci (2220–3330 GBq)/mmol) and [7,8-^{3}H]dopamine (>70 Ci/mmol (2.59 TBq/mmol) were purchased from Perkin Elmer SpA. Nicotine hydrogen tartrate salt, 4-aminopyridine (4AP), Choline, Mecamylamine (Sigma–Aldrich, St Louis, MO, USA); 5-Iodo-A-85380, Cytisine, (S)alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate [AMPA], cyclothiazide, pep2-SVKI, pep2-SVKE, were from Tocris (Tocris Bioscience, Bristol, UK). Anti-GluA2 receptor monoclonal rabbit monoclonal immunoaffinity purified IgG, anti-syntaxin-1A monoclonal mouse IgG and Cy3 goat anti-rat IgG antibodies were obtained from Gene Tex (Irvine, CA, USA). Anti-synaptophysin monoclonal mouse IgG was purchased from Synaptic System (Goettingen, Germany); rabbit anti-PSD95 polyclonal immunoaffinity IgG was from Affinity BioReagents (Golden, CO, USA). Rat anti-DAT antibody was obtained from Novus Biologicals (Littleton, CO, USA); Sulfo–NHS–SS–biotin, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and Streptavidin Magnetic Beads were purchased from Pierce Thermo Scientific (Rockford, IL, USA), β-actin monoclonal mouse IgG1, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Western blotting detection system was purchased from GeHealthcare (Italy). AlexaFluor-647 or AlexaFluor-488 antibodies are purchased from Molecular Probes (Alfagene, OR, USA).

3. Results

An important characteristic of our approach studying release from isolated synaptosomes in superfusion is that any change of one neurotransmitter outflow can be attributed exclusively to an action on the nerve terminal releasing that transmitter. Synaptosomes can be selectively labeled by adding the appropriate [^{3}H] neurotransmitter which is taken up by the synaptosomal population which possesses the specific carriers for that neurotransmitter. This permits the unequivocal localization of a given receptor protein (i.e. nACh or AMPA receptors as in this case) involved in the modulation of neurotransmitter release from a define subpopulation of the nerve terminal releasing the neurotransmitters under investigation.

Exposure of rat nerve terminals isolated from rat NAc to 100 μM AMPA caused a marked release of preloaded [^{3}H]DA, which was totally prevented by the concomitant addition of 10 μM NBQX or when omitting Ca^{2+} ions in the superfusion medium (Fig. 1). The release of [^{3}H]DA evoked by 100 μM AMPA was significantly potentiated (+59%) when 10 μM cyclothiazide, inactive on its own, was added concomitantly to the agonist (Fig. 1).

Fig. 2 shows that 30 μM nicotine and 100 μM AMPA caused a marked release of prelabeled [^{3}H]DA that quantitatively corresponds to that caused by 10 μM 4AP. *In vitro* pre-exposure of synaptosomes for 10 min to 30 μM nicotine caused a marked reduction (−83%) of the nicotine-induced [^{3}H]DA overflow, suggesting that nicotine receptors could undergo agonist-induced receptor desensitization (Fig. 2A). Furthermore, pretreatment of NAc synaptosomes with nicotine also caused a significant reduction of the 100 μM AMPA-evoked overflow of dopamine (−59%), but failed to modify the 4AP-induced dopamine overflow (Fig. 2A,). The lack of effect on 4AP-induced dopamine overflow indicates that changes in the exocytotic machinery do not account for nicotine-induced modifications of nicotine or AMPA receptor-induced DA overflow. Fig. 2A also shows that nicotine-induced reduction of 100 μM AMPA-evoked [^{3}H]DA overflow was impeded when mecamylamine (20 μM) was added concomitantly to nicotine. At the concentration applied mecamylamine failed to affect on its own the spontaneous release of [^{3}H]DA, nor it modified the 100 μM AMPA-evoked [^{3}H]DA overflow (not shown). To further explore the

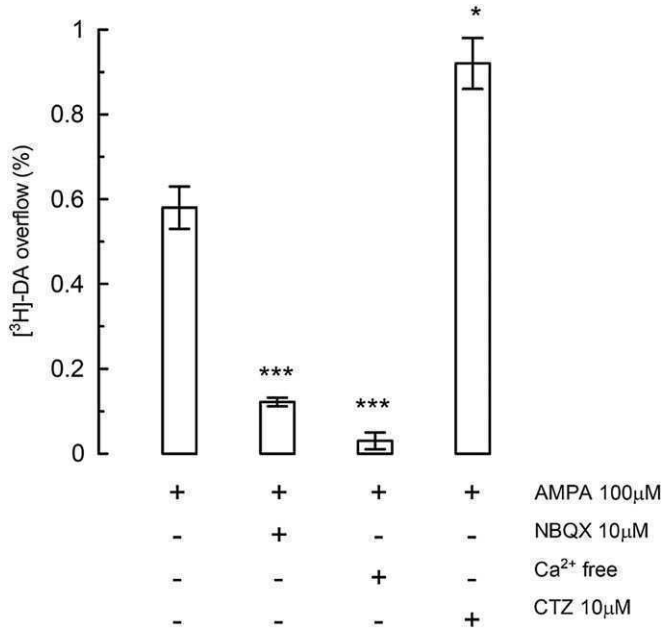


Fig. 1. Effect of AMPA on the spontaneous release of [^{3}H]dopamine from rat nucleus accumbens synaptosomes: Ca^{2+}-dependency, cyclothiazide-sensitivity and antagonism by NBQX. Synaptosomes were prepared as previously described, preloaded with the radioactive tracer and exposed in superfusion to 100 μM AMPA. Antagonist was added 8 min before agonist; when studying the calcium dependency of the AMPA-induced release of tritium, at $t = 20$ min of superfusion, standard medium was replaced with a solution where Ca^{2+} was omitted and 0.5 mM EGTA was added. Results are expressed as induced overflow. Data are means ± SEM of *five* experiments run in triplicate (three superfusion chambers for each experimental condition). $^{*}p < 0.05^{***}$, $p < 0.001$ vs. 100 μM AMPA.

interaction between nAChRs and AMPA receptors regarding the [^{3}H]-DA release we performed some experiments to evaluate the acute effects on [^{3}H]-DA release by the simultaneous activation of these two receptors. As reported in Fig. 2A the amount of ^{3}H-DA elicited by the simultaneous application of nicotine and AMPA, correspond to the sum of the stimulation by each single agonist. This suggests that the significant reduction of the 100 μM AMPA-evoked overflow of dopamine does not occur when nicotine is co-applied with AMPA but specifically takes place after prolonged nicotine exposure of NAc synaptosomes.

The selective α4β2 agonists 5IA-85380 (10 nM) and cytisine (100 μM) produced similar inhibitory effects while choline used as specific agonist for α7 nAChR failed to produce an inhibitory effect (Fig. 2B). Notably, the nicotine-induced change of AMPA-evoked overflow of [^{3}H]DA occurred in a concentration-dependent fashion, the nicotine-induce effects on AMPA receptor function being maximally evident when synaptosomes were preexposed to 30 μM nicotine (apparent IC_{50} value 0.913 ± 3.21 μM; Hillslope 0.57 ± 0.14; Fig. 2C).

Experiments were then carried out to investigate whether nicotine-induced modification of AMPA-evoked [^{3}H]DA release could be ascribed to changes in AMPA receptor constitutive trafficking. To this aim, the effect of pep2-SVKI, a peptide known to compete for the binding of GluA2 subunit to scaffolding proteins involved in AMPA receptor endocytosis (namely the glutamate receptor-interacting protein, GRIP, the AMPA receptor binding protein, ABP and the protein interacting with C kinase 1 PICK1; Dong et al., 1997; Xia et al., 1999; Braithwaite et al., 2000; Daw et al., 2000; Malinow and Malenka, 2002), was studied on the inhibitory effect of nicotine pretreatment on AMPA receptors. To avoid prolonged incubation, the peptides were entrapped into nerve terminals during synaptosomal preparation (Raiteri et al., 2000).

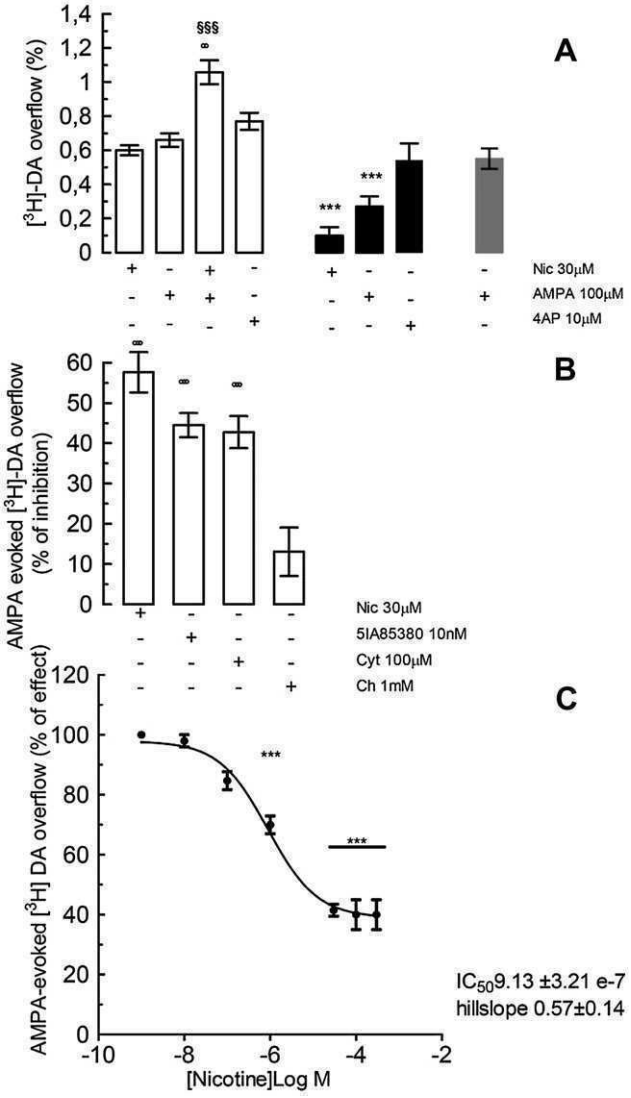


Fig. 2. Effect of nicotine pretreatment on the release of [^{3}H]dopamine evoked by nicotine, AMPA and 4-aminopyridine from rat nucleus accumbens synaptosomes: antagonism by mecamylamine, subtypes selectivity and concentration effect relationship. (A). Empty bar: control synaptosomes, black bar: nicotine pretreated synaptosomes, grey bar: synaptosomes pretreated with 20 μM mecamylamine together with nicotine 10 min before AMPA. Control (no drug pretreatment) and nicotine pretreated synaptosomes were exposed to 30 μM nicotine, 100 μM AMPA and 100 μM 4-aminopyridine. Results are expressed as induced overflow. Data are means ± SEM of *six* experiments run in triplicate. $^{§§§}p < 0.001$ vs. 30 μM nicotine, $^{○○}p < 0.01$ vs. 100 μM AMPA, $^{***}p < 0.001$ vs. control, respectively. (B) Synaptosomes were exposed to different nicotinic agonists and then stimulated with 100 μM AMPA. Results are expressed as percent of inhibition. Data are means ± SEM of five experiments run in triplicate. $^{○○○}p < 0.001$ vs. nicotine induced inhibition. (C) Synaptosomes were pretreated with increasing concentration (0.01–100 μM) of nicotine and then exposed to 100 μM AMPA. Results are expressed as percent of AMPA-induced releasing effect from control (no drug pretreated) synaptosomes. Data are means ± SEM of *x* experiments run in triplicate. $^{***}p < 0.001$ vs. control. The IC_{50} and the Hillslope were calculated according to a four parameter logistic curve using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA).

Aspecific interactions were excluded by analyzing in parallel experiments the effects of the inactive peptide pep2-SVKE, used as negative control (Collingridge and Isaac, 2003; Henley, 2003). Fig. 3A shows that AMPA-induced [^{3}H]DA overflow from nicotine pretreated NAc dopaminergic terminals was significantly increased (+81%) in synaptosomes which entrapped pep2-SVKI but not pep2-SVKE. On the contrary, the drastic reduction of nicotine-evoked overflow of [^{3}H]DA due to nicotine pretreatment was unchanged

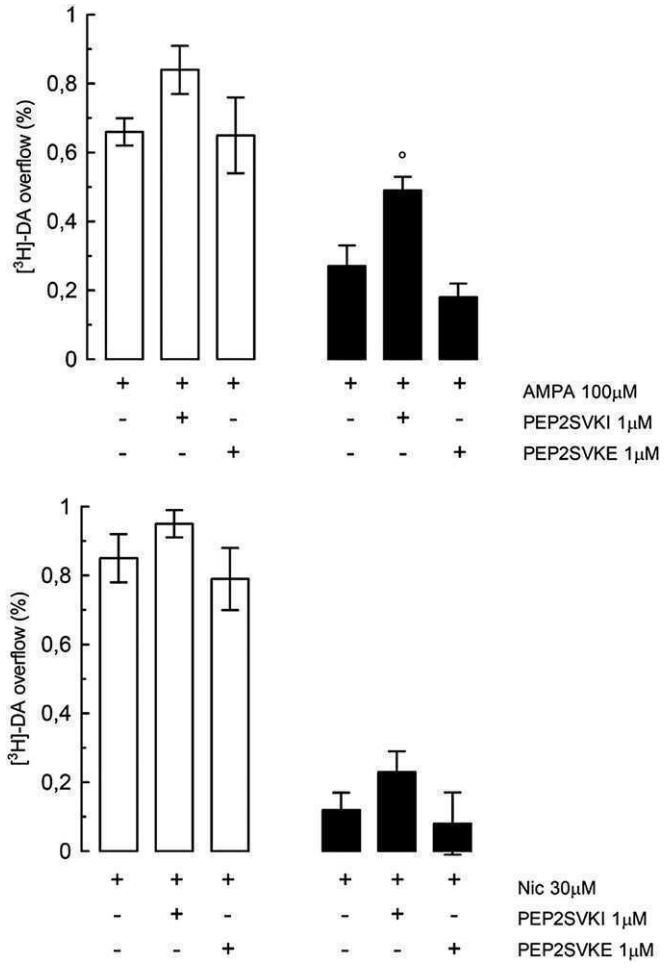


Fig. 3. Effects of peptides that interfere with AMPA receptor internalization on the AMPA and the nicotine-evoked release of [^{3}H]dopamine from control and nicotine pretreated rat nucleus accumbens synaptosomes. White bar: [^{3}H]DA release evoked by 100 μM AMPA (panel A) or by 30 μM nicotine (panel B) from control (no entrapped peptide) or peptide (pep2-SVKI or pep2-SVKE, as indicated) entrapped NAc synaptosomes. Black bar: [^{3}H]DA release evoked by 100 μM AMPA (panel A) or by 30 μM nicotine (panel B) from control (no entrapped peptide) or peptide (pep2-SVKI or pep2-SVKE, as indicated) entrapped NAc synaptosomes preexposed to 30 μM nicotine. Results are expressed as induced overflow. Data are means ± SEM of *six* experiments run in triplicate. $^{*}p < 0.05$ vs. 100 μM AMPA in nicotine-treated animals.

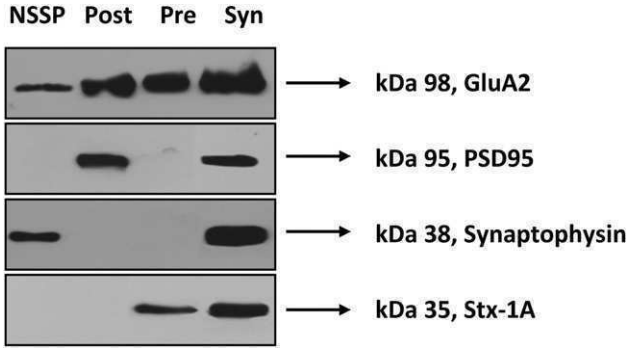


Fig. 4. GluA2 subunit receptor protein expression in rat nucleus accumbens synaptosomes. Western blotting of GluA2 proteins in the NSSP fraction, in the Post, in the Pre and in the whole initial synaptosomal fraction from which fractionation was performed (Syn), here used as loading control. Anti-PSD95, anti-synaptophysin and anti-Stx-1A were used as markers for ultrasynaptic fraction to determine the purity of the preparation that is more than 90%. Protein weights are expressed in kDa. The blots are representative of six analyses.

in pep2-SVKI or pep2SKE-enriched NAc synaptosomes (Fig. 4B). Entrapped peptides did not affect on their own the spontaneous release of [^{3}H]DA (not shown).

In order to confirm the presence of GluA2 receptor subunit at the sub-synaptic level in NAc synaptosomal membranes, we performed some experiments using a specific purification technique (Phillips et al., 2001; Pinheiro et al., 2003; Rebola et al., 2003; Feligioni et al., 2006; Summa et al., 2011) that allows the simultaneous analysis of the presynaptic component of the synaptic active zone (Pre), of the postsynaptic component of synaptosomal membranes (Post), and of the non-synaptic component (NSSP) of synaptosomal membranes. GluA2 immunoreactivity was also analyzed in synaptosomal membranes (Syn) as internal control. Western blot analysis unveiled a significant GluA2 immunoreactivity in all the three presynaptic components the synaptic active zone (Pre), the postsynaptic components of synaptosomal membranes (Post) and the non-synaptic components (NSSP) of synaptosomal membranes synaptosomal subfractions as well as in the Syn (Fig. 4). In parallel analysis the separation of the Pre and of the Post subfractions from other presynaptic proteins not located in the active zone, the NSSP proteins were validated. As shown in Fig. 4, PSD95 was found to be predominant in the postsynaptic fraction, syntaxin-1A (Stx-1A) in the presynaptic component, while synaptophysin in the NSSP fraction. In order to determine whether changes in AMPA-induced releasing effect caused by the transient preexposure of NAc synaptosomes to 30 μM nicotine could be paralleled by changes in GluA2 subunits in synaptosomal membranes, the level of GluA2 subunit immunoreactivity was monitored in the total synaptosomes (Syn), as well as in the Pre, Post, and in the NSSP fractions of control and nicotine pretreated NAc synaptosomes (Fig. 5A). As shown in Fig. 5A, the loss of function of AMPA receptors in nicotine pretreated synaptosomes was accompanied by a decreased insertion of GluA2 subunit proteins in the presynaptic component of synaptic plasma membranes nicotine-treated synaptosomes when compared to control, while the level of the GluA2 immunoreactivity was not significantly modified in the NSSP and in Post fractions (Fig. 5B).

GluA2 receptor subunit endocytosis induced by exposure of NAc synaptosomes to 30 μM nicotine can also be analyzed by studying the fraction of surface receptors that are internalized. For this purpose we applied the reversible biotinylation approach (Jayanthi et al., 2004; Johnson et al., 2005) and quantified .the internalized biotinylated GluA2 subunits. A significant increase (+41.41%) of internalized presynaptic GluA2 biotin tagged (Fig. 5C, lane T) was observed following exposure of synaptosomes to nicotine treatment when compared to control (Fig. 5C, lane C). Total streptavidin pull-down synaptosomal lysate (Fig. 5C, lane L) was added as input to detect synaptosomal GluA2. TCEP biotin stripping (0% biotin) and 100% biotinylation efficacy were also tested (not shown).

Because the fractionation procedure used above does not allow the purification of only the dopaminergic synaptic fraction from the entire nerve terminal population, we undertook a complementary immuno-cytochemistry study aimed at identifying the presence of GluA2 receptor subunits at dopaminergic nerve endings, identified as terminals labeled with antibody against the dopamine transporter (DAT). In these experiments, the presynaptic location of GluA2 subunits proteins was confirmed by analyzing the co-localization of this protein in the Stx-1A-immunoreactive elements. Our data indicate that 75 ± 6% Stx-1A -immunoreactive particles were also DAT immunoreactive in the total populations of NAc nerve terminals, while 71 ± 6% of DAT positive terminals were also labeled with anti-Stx-1A antibody (see for a representative image Fig. 6, panels a–c). Inasmuch, they also allow to conclude that only 48 ± 9% of the Stx-1A positive particles were GluA2-immunoreactive elements and, reciprocally, that 73 ± 4% of the

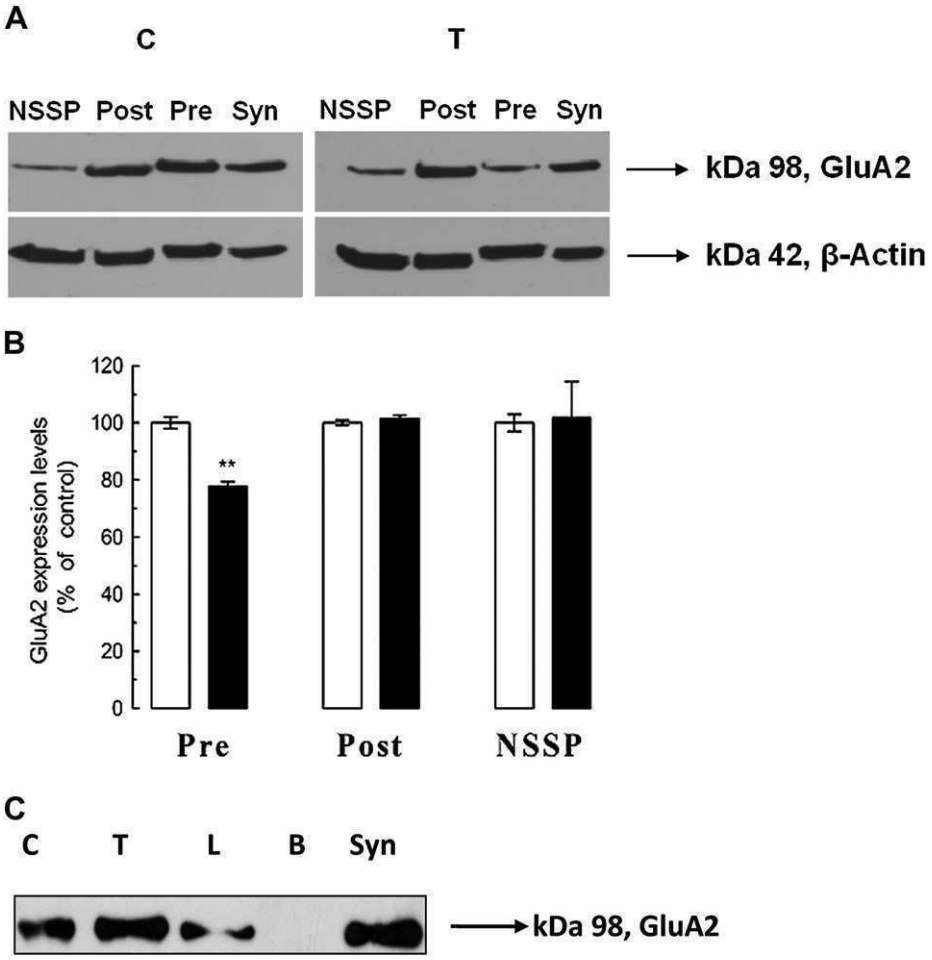


Fig. 5. Effects of nicotine pretreatment on GluA2 subunit distribution in synaptosomal subfractions isolated from nucleus accumbens nerve terminals. Panel A: Western blotting of GluA2 and of β-actin proteins in the NSSP, the Post, the Prefractions and in the Syn protein, here used as loading control from nicotine pretreated and control NAc synaptosomes. Protein weights are expressed in kDa. The blots are representative of analysis from four different synaptosomal preparations. Panel B: Nicotine pretreatment alters the synaptic distribution of GluA2 subunits. Quantitative analysis of the synaptic distributions of GluA2 subunits in control (empty bar) and nicotine pretreated (black bar) rat NAc synaptosomes. GluA2 immunoreactivity was expressed as percentage of β-actin immunoreactivity. Panel C: Nicotine treatment induces an endocytosis of presynaptic GluA2 subunits. Nicotinic stimulated synaptosomes in reversible biotinylation experiment. Western blotting of internalized biotinylated protein collected by streptavidin pull-down. GluA2 immunoreactivity in control (lane C), treated (lane T), total synaptosomes (L) and streptavidin pull-down control (lane B). Changes in NAc synaptic GluA2 level in nicotine pretreated NAc synaptosomes are calculated as percentage of control synaptosomes (expressed as 100% ± SEM) and are indicated as mean ± SEM of separate immunoblots from four different preparations. $^{**}p < 0.01$ vs. control.

GluA2 positive particles were also Stx-1A-immunoreactive elements (see for a representative image Fig. 6, panels g–i). Finally, among the DAT-immunopositive terminals, 45 ± 9% of the particles were positive for GluA2 immunoreactivity and reciprocally 74 ± 5% of the GluA2-immunoreactive elements were immunolabeled with anti-DAT antibodies (see for a representative image Fig. 6, panels d–f). Altogether, these observations led us to conclude that a significant percentage of nerve terminals isolated from the NAc of adult rats, identified as Stx-1A positive elements, were dopaminergic. Among these terminals, a significant percentage of terminals also possess GluA2 receptor subunits (Fig. 6, panel j).

To further study whether the internalization of AMPA receptors which modulate [^{3}H]-DA release is a common feature of other presynaptic AMPA receptors we investigated whether the preincubation with nicotine may influence also the function of AMPA receptors which modulate [^{3}H]-ACh, [^{3}H]-GABA and [^{3}H]-NA (Desce et al., 1991; Pittaluga and Raiteri, 1992; Malva et al., 1994; Pittaluga et al., 1997). It is well known that nAChRs are present on nerve terminals and elicit exocytotic release of various neurotransmitters including NA, DA, ACh and GABA in a Ca^{2+} dependent manner (Wonnacott et al., 1989; Levin, 1992; Wessler et al., 1992; Guo et al., 1998; Li et al., 1998; Zappettini et al., 2011; Wilkie et al., 1996; Sershen et al., 1997). The finding that the nicotine-induced release of [^{3}H]-NA the release was tetrodotoxin sensitive (Sershen et al., 1997) has been explained by local depolarization and subsequent generation of action potentials further supporting the presence of these receptors on the nerve endings. Evidence also exist supporting the presynaptic localization of AMPA receptors whose activation elicits the Ca^{2+}-dependent, vesicular-like release of NA (Pittaluga and Raiteri, 1992; Malva et al., 1994; Br; Pittaluga et al., 1997, 2006), of DA (Desce et al., 1991; Malva et al., 1994; Pittaluga et al., 2006,), of GABA (Pittaluga et al., 1997) and ACh (Pittaluga et al., 1997, 2006). Well in line with these functional observations, the existence of presynaptic AMPA receptors is also supported by results from immunocytochemical (Martin et al., 1998; Fujiyama et al., 2004; Carr et al., 2010) and autoradiographic (Tarazi et al., 1998) investigations.

Fig. 7 (panel A) shows that exposure of NAc synaptosomes preloaded with [^{3}H]GABA to nicotine (30 μM) caused a significant overflow of the radioactive tracer. A comparable releasing effect could be observed when GABAergic NAc terminals were exposed

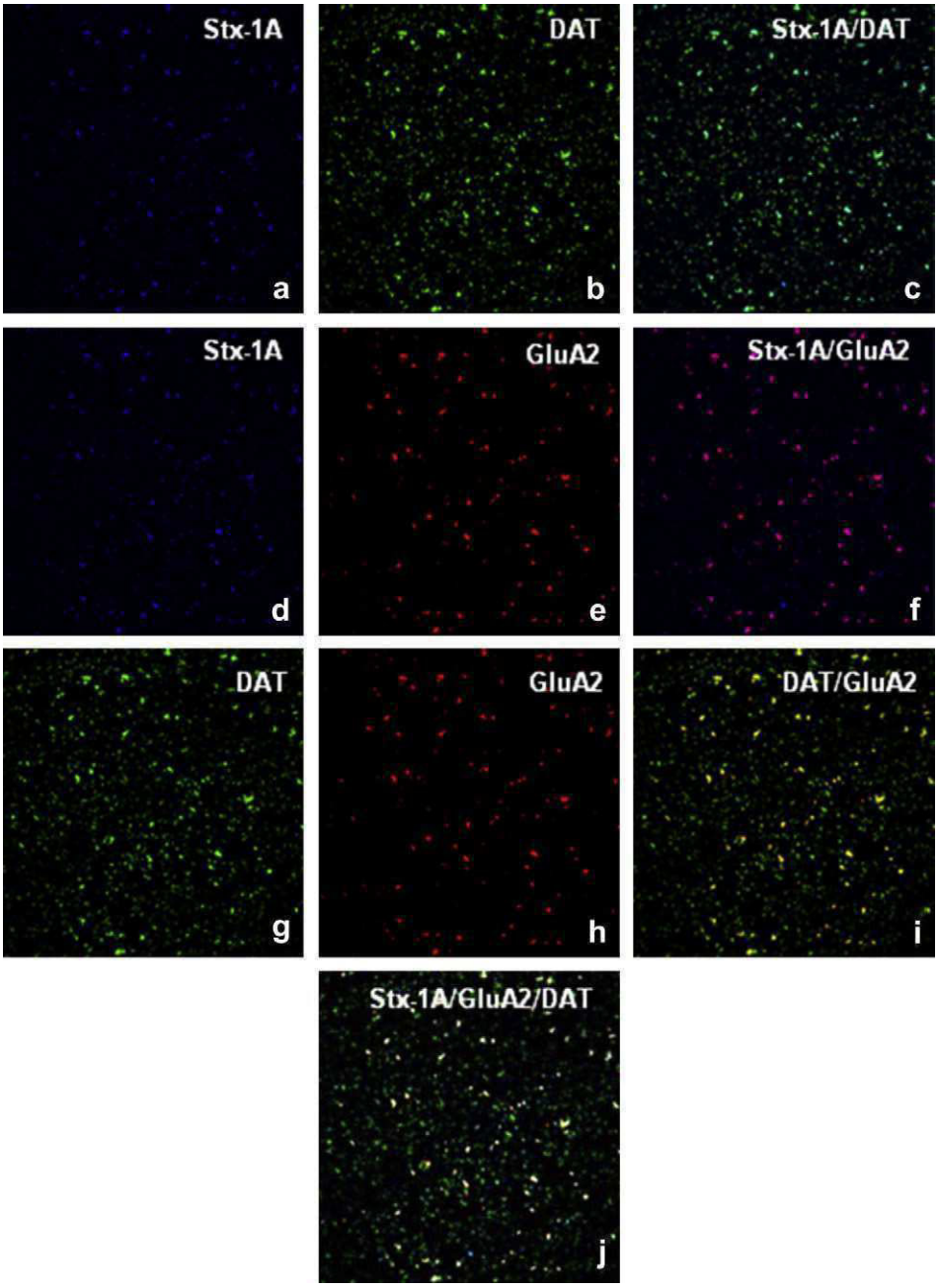


Fig. 6. GluA2 receptor subunit and dopamine transporter protein are co-localized on rat nucleus accumbens dopaminergic terminals. Representative double-labeling images of anti-dopamine transporter (DAT, specific marker of dopaminergic nerve terminals) and of anti-GluA2 with anti-syntaxin-1 (Stx-1A, marker of all nerve terminals, panel c and panel i, respectively) and of anti-GluA2 with anti-DAT (panel f). Representative triple image of anti-GluA2 with anti-DAT and with anti-Stx-1A. The image is representative of *n* different images taken on different days.

to 100 μM AMPA. Pre-exposure of NAc synaptosomes to 30 μM nicotine caused a dramatic decrease (−99%) of the stimulatory effect of nicotine receptors but failed to cause significant changes to the overflow of [^{3}H]GABA elicited by 100 μM AMPA. Similar results were observed when studying the overflow of [^{3}H]ACh from NAc synaptosomes. Exposure of NAc terminals preloaded with [^{3}H]Ch to 30 μM nicotine or to 100 μM AMPA caused a marked overflow of [^{3}H]ACh; synaptosomes pre-exposure to 30 μM nicotine drastically reduced (−73%) the nicotine but not the AMPA-induced stimulatory effect (Fig. 7, panel B). Finally, both 30 μM nicotine and 100 μM AMPA released [^{3}H]NA from hippocampal nerve endings. However, differently from GABAergic and cholinergic terminals, nicotine pretreatment caused a significant reduction of both agonist-induced stimulating effects (−99%, −57%, respectively) (Fig. 7, panel C).

4. Discussion

Several lines of evidence in laboratory animals and preclinical reports demonstrate that glutamatergic system is critically involved in nicotine dependence. Moreover, it is well known that different glutamatergic receptors participate in the mediation of several aspects of nicotine-mediated effects (Liechti and Markou, 2008; Reissner and Kalivas, 2010; D'Souza and Markou, 2011; Timofeeva and Levin, 2011). Therefore, as far as the glutamate–nicotine interaction is concerned, one potentially

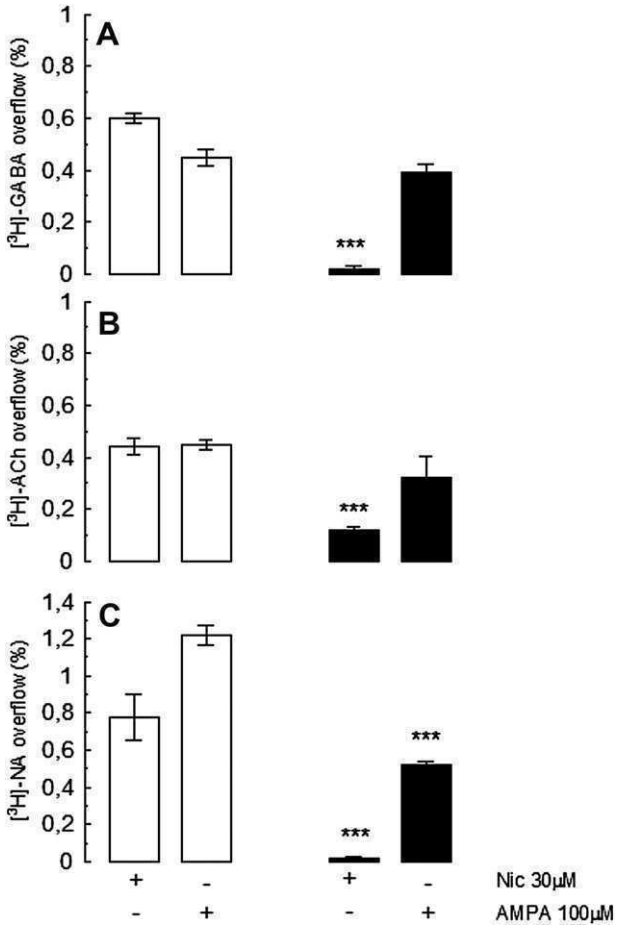


Fig. 7. Effects of nicotine pretreatment on the nicotine and the AMPA-induced release of different neurotransmitters from distinct synaptosomal preparations. Synaptosomes isolated from the NAc and the hippocampus of adult rats were preloaded with [^{3}H]GABA, [^{3}H]Ch (NAc) and [^{3}H]NA (hippocampus), respectively and the release of preloaded radioactive tracers caused by exposing synaptosomes to 30 μM nicotine or to 100 μM AMPA was quantified. Empty bar: agonist-evoked release from control synaptosomes. Black bar: agonist-evoked release from 30 μM nicotine pretreated synaptosomes. Results are expressed as induced overflow. Data are means ± SEM of five experiments run in triplicate. $^{***}p < 0.001$ vs. respective control.

relevant hypothesis is that nicotine and glutamate receptors be functionally coupled and control dopamine outputs in basal ganglia.

If this were the case, we previously demonstrated that, at the presynaptic level, nAChRs functionally interact with other coexisting glutamatergic (NMDA and metabotropic) receptors through different mechanisms (Marchi and Grilli, 2010 and references therein). Here we report on the existence, in the rat NAc dopaminergic nerve terminals, of a population of cyclothiazide-sensitive, GluA2-containing AMPA-preferring receptor subtypes (Pittaluga et al., 1997; Ghersi et al., 2003), whose activation elicits the release of the catecholamine.

The presence of these receptors is strongly strengthened by the data from immuno-cytochemistry studies indicating that a significant percentage of nerve terminals isolated from the NAc of adult rats, identified as Stx-1A positive elements, were dopaminergic. Among these terminals, a significant percentage of nerve endings also posses GluA2 receptor subunits (Fig. 6, panel j).

The second important finding is that these presynaptic AMPA heteroreceptors functionally interact with coexisting nAChRs. In fact, pretreatment of NAc synaptosomes with nicotine caused a significant reduction of the AMPA-evoked overflow of [^{3}H]DA but failed to modify the 4AP-induced [^{3}H]DA overflow, suggesting that changes in the exocytotic machinery do not account for nicotine-induced modifications of nicotine or AMPA receptor-induced [^{3}H]DA overflow (Fig. 2A). Furthermore, this event was impeded by mecamylamine, further supporting the hypothesis that nicotine and presynaptic AMPA receptors co-localize on NAc dopaminergic terminals and that activation of nicotine receptor represents the triggering event accounting for the loss of function of AMPA receptors. To investigate the molecular cascade of events, which may justify the decrease of the functional response of AMPA receptors here described, we first considered that presynaptic cyclothiazide-sensitive AMPA-preferring presynaptic receptors located on NAc could undergo constitutive trafficking in synaptosomal membranes. Similarly to what observed postsynaptically, in recent years evidence have been provided showing that scaffolding proteins involved in the constitutive trafficking of AMPA receptors also locate presynaptically, where they co-localize with GluR2 subunit and Syntaxin-1A proteins (Haglerød et al., 2009). Inasmuch, interfering with GluA2-GRIP/PICK1 interaction at the presynaptic level was reported to cause quantitative changes to AMPA-induced releasing effect, an event that was related to modification in the number of AMPA receptors actively inserted in synaptosomal membranes (Pittaluga et al., 2006; Summa et al., 2011). If this is the case, the possibility that nicotine pretreatment could decrease the functional response of AMPA receptors by enhancing their internalization could be envisaged. To this aim, we analyzed the effects of pep2-SVKI, a peptide known to compete for the binding of GluA2 subunit to scaffolding proteins involved in AMPA receptor internalization. Interestingly the decrease of the AMPA-induced [^{3}H]DA overflow in nicotine pretreated NAc dopaminergic terminals was significantly counteracted in synaptosomes containing entrapped pep2-SVKI when compared to controls, while pep2-SVKE, the inactive peptide, was ineffective. On the contrary, the drastic reduction of nicotine-evoked overflow of [^{3}H]DA due to nicotine pretreatment was unchanged in pep2-SVKI or pep2SKE-entrapped NAc synaptosomes. Therefore, the finding that the nicotine-induced decrease of the function of AMPA receptors on dopaminergic NAc terminals could be, at least partially restored by impeding GluA2/GRIP/ABP/PICK1 interaction led us to speculate that the nicotine effect was due to the increased rate of internalization of cyclothiazide-sensitive presynaptic AMPA-preferring receptors, which are characterized by the presence of the GluA2 subunit. Accordingly, the loss of function of AMPA receptors in nicotine pre-treated synaptosomes was accompanied by a reduced presence of GluA2 subunit proteins in the presynaptic component of synaptic plasma membranes in the nicotine-treated nerve endings, but not in the control. Additionally, the level of the GluA2 immunoreactivity was not significantly modified in the NSSP and in Post fractions (Fig. 6 B). As far as the relatively small reduction in the presynaptic expression of GluA2 (Fig. 6B) it has to be considered that these experiments have been performed using the whole population of synaptosomes and the data are not referred only to the DA nerve endings and therefore the value could be even underestimated.

One may therefore propose that nicotine pretreatment could favour the internalization of GluA2-containing, cyclothiazide-sensitive AMPA heteroreceptors located on NAc terminals. This event, however, seems to occur in a restricted population of nerve terminals, since AMPA-induced releasing effect was halved, but not abolished, by nicotine. The reasons for the limited efficacy of nicotine in reducing AMPA-induced releasing effects could be manifold. For instance, it's possible that NAc dopaminergic terminals possess distinct AMPA receptor subpopulations, each having different subunit assembly and sensitivity to nicotine. Considering that GluA2-containing receptors are scarcely permeable to Ca^{2+}

ions and have low channel conductance, their forced internalization should slightly affect the AMPA-induced dopamine exocytosis. Thus, also exists the possibility that new GluA2-lacking AMPA receptors could be actively inserted in plasma membranes, then compensating for the loss of function due to GluA2 subunit internalization. To note, such a compensatory event was already reported to occur in NAc synapses after withdrawal from cocaine self administration (Wolf, 2010). Finally, it's plausible that only a limited number of dopaminergic terminals possess co-expressed nicotine and AMPA receptors should be taken in consideration. Whatever the explanation, the events here described could be relevant to the comprehension of the molecular mechanisms at the basis of tabagism.

The trafficking of neurotransmitter receptors is a crucial mechanism for the modulation of synaptic transmission and the cellular processes that govern this trafficking and the regulation of membrane proteins are highly complex. However as far as the mechanisms that initiate the endocytosis there are several convincing evidences that calcium regulates all form of receptor endocytosis even at the level of nerve terminals and calmodulin is the calcium sensor involved (see Wu et al., 2009 and references therein).

Interestingly, the nicotine-induced changes to release regulating, presynaptic AMPA heteroreceptors was not limited to dopaminergic terminals in the NAc, but also could be observed when studying the AMPA-evoked release of noradrenaline from hippocampal nerve terminals. Nicotine-induced adaptation of AMPA receptors however does not represent a general molecular event. Indeed, the pre-exposure of NAc synaptosomes to 30 μM nicotine failed to cause significant changes of the AMPA-evoked overflow of [^{3}H]GABA and [^{3}H]ACh from these terminals (Fig. 7). Whether the decrease of AMPA responses could be partially explained by an allosteric modulation or by desensitization of nAChRs due to long-lasting exposure to nicotine is difficult to foresee (Lendvai and Vizi, 2008). Indeed in cholinergic and GABAergic nerve terminals the nAChRs which modulate neurotransmitter release are desensitized while the function of AMPA receptor did not change. However, the possibility that the desensitization of nAChRs may play a role in the decrease of AMPA responses cannot be excluded (Lin et al., 2010). Due to the very heterogeneous presence of nAChRs on these nerve endings (Wilkie et al., 1996; Sershen et al., 1997; Wonnacott, 1997; Kulak et al., 2001; Zoli et al., 2002; Collins et al., 2009; McClure-Begley et al., 2009; Grady et al., 2010; Wu and Lukas, 2011; Quik et al., 2011), it is very difficult to correlate specific nAChR subtypes with the internalization of AMPA receptors. Of course, the possibility that nAChR and AMPA receptors might not coexist on GABAergic and cholinergic nerve endings has to be also considered. In any case, the involvement of α7 nAChR subtype can be excluded on the basis of the lack of effect of the specific α7 agonist choline due to the absence of these nAChR subtypes on DA and NA terminal, as reported above. It has to be noted that DA and NA are stored and released from large-dense core vesicles while GABA and ACh from small synaptic vesicles; it is therefore likely that the functional coupling between presynaptic nACh and AMPA receptors plays a specific role in the regulation of neurotransmitter release from the large-dense core vesicles and not from other types of vesicles.

It has been demonstrated that neither acute nor chronic exposure to nicotine has any effect on the clearance of the surface nAChRs from the plasma membranes or they degradation in the lysosome (Darsow et al., 2005). This finding strongly suggests that nicotine-dependent regulation of nAChR surface expression, in particular that of the α4β2 nAChRs, does not occur via changes in endocytotic trafficking or lysosomal degradation rate of the surface nAChRs (Darsow et al., 2005) as previously suggested by other authors (Peng et al., 1994). Our results show that pre-exposure of synaptosomes to 30 μM nicotine also caused a marked reduction of the nicotine-induced overflow of the four neurotransmitter studied, suggesting that these nAChRs could undergo an agonist-induced receptor desensitization. Moreover, at the presynaptic level the desensitization of nAChRs modulating ACh, GABA and DA release following acute or chronic exposure to nicotine has been already reported (Lapchak et al., 1989; Grilli et al., 2005).

The most likely explanation for the selective endocytosis of AMPA receptors which modulate DA and NA release is that the selective population of cyclothiazide-sensitive AMPA receptors endowed with GluA2 subunits, which are selectively present on these terminals, is susceptible to the internalization triggered by the nicotinic stimuli, while those receptors located on cholinergic and GABAergic terminals are not. Notably, the study of the pharmacological profile unveiled that the AMPA receptors controlling acetylcholine and GABA release were cyclothiazide-insensitive and did not undergo a GRIP/ABP/PICK1-dependent constitutive trafficking (Pittaluga et al., 1997, 2006).

In conclusion, the present results demonstrate with functional evidence and immunocytochemical analysis, the presence of cyclothiazide-sensitive AMPA receptors that are co-localized with nAChRs on NAc dopaminergic terminals. Our results also show that nicotine, through a mechanism never described before, causes a rapid decrease in the number of surface located coexisting neuronal AMPA receptors. This effect is selective for AMPA receptors present on DA and NA nerve endings and does not occur to AMPA receptors present on GABAergic and cholinergic nerve endings, and seems to be related to the internalization of the specific GluA2 subunits.

As previously (see for a review Marchi and Grilli, 2010) and currently shown, we should consider that nicotine may not only stimulate neurotransmitter release but that it also exerts, at the presynaptic level through the interaction with other coexisting receptors, a functional modulatory role. As far as the nicotinic modulation of glutamate receptor function, we have already demonstrated that, at the presynaptic level, the activation of nAChRs positively modulates both NMDA and glutamate metabotropic receptors coexisting on the same nerve endings (Risso et al., 2004b; Parodi et al., 2006). The modulation of ionotropic AMPA receptors is an important mechanism of the control of excitatory synaptic efficacy and plasticity (Kessel and Malinow, 2009). Here we report that the AMPA receptor function can be dynamically negatively regulated in neurons in response to activation of nAChRs present on the same nerve endings. This dynamic control by cholinergic nicotinic system of both NMDA and AMPA receptors may therefore be crucial for many forms of synaptic plasticity including LTP and LTD (Song and Huganir, 2002).

Acknowledgements

This work was supported by grants from Italian MIUR (Ministero dell'Istruzi'one, dell'Universita' e della Ricerca, Projects n. 2009P7WHNR_003 AP; Projects n. 2009R7WCZS_003 MM) from Compagnia di San Paolo, Turin, Italy to MM, from University of Genoa 'Progetto Ricerca Ateneo' to MM and AP, and from ALCOTRA (Alpi Latine Cooperazione Transfrontaliera – Project n. 231 «Developpement commercial des potentialite' s du genre salvia: nouvelles productions); from the European Community (Project Strategia d'Impresa in Settori di Nicchia per l'Economia Agro-Industriale del Mediterraneo, Pyrgi, project n° B51H10000000006 and project Progetto 7FP-KBBE BAMMBO). We wish to thank Maura Agate for careful editorial assistance and Dr. Silvia E. Smith, University of Utah, for reviewing the manuscript.

References

Albizu, L., Holloway, T., González-Maeso, J., Sealfon, S.C., 2011. Functional crosstalk and heteromerization of serotonin 5-HT2A and dopamine D2 receptors. Neuropharmacology 61, 770–777.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Braithwaite, S.P., Meyer, G., Henley, J.M., 2000. Interactions between AMPA receptors and intracellular proteins. Neuropharmacology 39, 919–930.

Carr, K.D., Chau, L.S., Cabeza de Vaca, S., Gustafson, K., Stouffer, M., Tukey, D.S., Restituito, S., Ziff, E.B., 2010. AMPA receptro subunit GluR1 downstream of D1 dopamine receptor stimulation in nucleus accumbens shell mediates increased drug reward magnitude in food-restricted rats. Neuroscience 165, 1074–1092.

Collingridge, G.L., Isaac, J.T., 2003. Functional roles of protein interactions with AMPA and kainate receptors. Neurosci. Res. 4, 3–15.

Collins, A.C., Salminen, O., Marks, M.J., Whiteaker, P., Grady, S.R., 2009. The road to discovery of neuronal nicotinic cholinergic receptor subtypes. Handb. Exp. Pharmacol. 192, 85–112.

Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G., Lockett, S., 2004. Automatic and quantitative measurement of protein–protein colocalization in live cells. Biophys. J. 86, 3993–4003.

Darsow, T., Booker, T.K., Piña-Crespo, J.C., Heinemann, S.F., 2005. Exocytic trafficking is required for nicotine-induced up-regulation of alpha 4 beta 2 nicotinic acetylcholine receptors. J. Biol. Chem. 280, 18311–18320.

Daw, M.I., Chittajallu, R., Bortolotto, Z.A., Dev, K.K., Duprat, F., Henley, J.M., Collingridge, G.L., Isaac, J.T.R., 2000. PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. Neuron 28, 873–886.

Delibas, N., Doguc, D.K., Sutcu, R., Eroglu, E., Gökalp, O., 2005. Effect of nicotine on hippocampal nicotinic acetylcholine alpha7 receptor and NMDA receptor subunits 2A and 2B expression in young and old rats. Int. J. Neurosci. 115, 1151–1163.

Desce, J.M., Godeheu, G., Galli, T., Artaud, F., Chéramy, A., Glowinski, J., 1991. Presynaptic facilitation of dopamine release through D,L-alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors on synaptosomes from the rat striatum. J. Pharmacol. Exp. Ther. 259, 692–698.

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F., Huganir, R.L., 1997. GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. Nature 386, 279–284.

D'Souza, M.S., Markou, A., 2011. Neuronal mechanisms underlying development of nicotine dependence: implications for novel smoking-cessation treatments. Addict. Sci. Clin. Pract. 6, 4–16.

Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kiddn, G.J.E., Rostas, J.A.P., 1986. A rapid method for isolation of synaptosomes on Percoll gradient. Brain Res. 372, 115–129.

Feligioni, M., Holman, D., Haglerød, C., Davanger, S., Henley, J.M., 2006. Ultrastructural localisation and differential agonist-induced regulation of AMPA and kainate receptors present at the presynaptic active zone and postsynaptic density. J. Neurochem. 99, 549–560.

Fujiyama, F., Kuramoto, E., Okamoto, K., Hioki, H., Furuta, T., Zhou, L., Nomura, S., Kaneko, T., 2004. Presynaptic localization of an AMPA-type glutamate receptor in corticostriatal and thalamostriatal axon terminals. Eur. J. Neurosci. 20, 3322–3330.

Ghersi, C., Bonfanti, A., Manzari, B., Feligioni, M., Raiteri, M., Pittaluga, A., 2003. Pharmacological heterogeneity of release-regulating presynaptic AMPA/kainate receptors in the rat brain: study with receptor antagonists. Neurochem. Int. 42, 283–292.

Gonzalez, R.C., Wintz, P., 1987. Digital Image Processing, second, ed. Addison-Wesley, Reading, MA, USA.

Grady, S.R., Salminen, O., McIntosh, J.M., Marks, M.J., Collins, A.C., 2010. Mouse striatal dopamine nerve terminals express alpha4alpha5beta2 and two stoichiometric forms of alpha4beta2*-nicotinic acetylcholine receptors. J. Mol. Neurosci. 40, 91–95.

Grilli, M., Parodi, M., Raiteri, M., Marchi, M., 2005. Chronic nicotine differentially affects the function of nicotinic receptor subtypes regulating neurotransmitter release. J. Neurochem. 93, 1353–1360.

Guo, J.Z., Tredway, T.L., Chiappinelli, V.A., 1998. Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. J. Neurosci. 18, 1963–1969.

Haglerød, C., Kapic, A., Boulland, J.L., Hussain, S., Holen, T., Skare, O., Laake, P., Ottersen, O.P., Haug, F.M., Davanger, S., 2009. Protein interacting with C kinase 1 (PICK1) and GluR2 are associated with presynaptic plasma membrane and vesicles in hippocampal excitatory synapses. Neuroscience 158, 242–252.

Henley, J.M., 2003. Proteins interactions implicated in AMPA receptor trafficking: a clear destination and an improving route map. Neurosci. Res. 4, 243–254.

Jayanthi, L.D., Samuvel, D.J., Ramamoorthy, S., 2004. Regulated internalization and phosphorylation of the native norepinephrine transporter in response to phorbol esters. Evidence for localization in lipid rafts and lipid raft-mediated internalization. J. Biol. Chem. 30, 19315–19326.

Johnson, L.A., Furman, C.A., Zhang, M., Guptaroy, B., Gnegy, M.E., 2005. Rapid delivery of the dopamine transporter to the plasmalemmal membrane upon amphetamine stimulation. Neuropharmacology 49, 750–758.

Kane, J.K., Hwang, Y., Konu, O., Loughlin, S.E., Leslie, F.M., Li, M.D., 2005. Regulation of Homer and group I metabotropic glutamate receptors by nicotine. Eur. J. Neurosci. 21, 1145–1154.

Kenny, P.J., Chartoff, E., Roberto, M., Carlezon Jr., W.A., Markou, A., 2009. NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-administration: role of the ventral tegmental area and central nucleus of the amygdala. Neuropsychopharmacology 34, 266–281.

Kessel, H.W., Malinow, R., 2009. Synaptic AMPA receptor plasticity and behaviour. Neuron 12, 340–350.

Kulak, J.M., McIntosh, J.M., Yoshikami, D., Olivera, B.M., 2001. Nicotine-evoked transmitter release from synaptosomes: functional association of specific presynaptic acetylcholine receptors and voltage-gated calcium channels. J. Neurochem. 77, 1581–1589.

Lapchak, P.A., Araujo, D.M., Quirion, R., Collier, B., 1989. Effect of chronic nicotine treatment on nicotinic autoreceptor function and N-[^{3}H]methylcarbamylcholine binding sites in the rat brain. J. Neurochem. 52, 483–491.

Lee, K.J., Kim, D.H., Choi, S.H., Shin, Y.C., Park, S.H., Moon, B.H., Kang, S.W., Cho, E., Choi, S.H., Chun, B.G., Lee, M.S., Shin, K.H., 2007. Effects of repeated nicotine treatment on the changes in glutamate receptor subunits levels in mesocorticolimbic dopamine areas. Kor. J. Physiol. Pharmacol. 11, 139–144.

Lendvai, B., Vizi, S., 2008. Nonsynaptic chemical transmission trough nicotinic acetylcholine receptors. Physiol. Rev. 88, 333–349.

Levin, E.D., 1992. Nicotinic systems and cognitive function. Psychopharmacology 108, 417–431.

Levin, E.D., Tizabi, Y., Rezvani, A.H., Caldwell, D.P., Petro, A., Getachew, B., 2005. Chronic nicotine and dizocilpine effects on regionally specific nicotinic and NMDA glutamate receptor binding. Brain Res. 1041, 132–142.

Liechti, M.E., Markou, A., 2008. Role of the glutamatergic system in nicotine dependence: implications for the discovery and development of new pharmacological smoking cessation therapies. CNS Drug 22, 705–724.

Li, X., Rainnie, D.G., McCarley, R.W., Greene, R.W., 1998. Presynaptic nicotinic receptors facilitate monoaminergic transmission. J. Neurosci. 18, 1904–1912.

Lin, H., Vicini, S., Hsu, F.C., Doshi, S., Takano, H., Coulter, D.A., Lynch, D.R., 2010. Axonal α7 nicotinic ACh receptors modulate presynaptic NMDA receptor expression and structural plasticity of glutamatergic presynaptic boutons. Proc. Natl. Acad. Sci. U.S.A 107, 16661–16666.

Malinow, R., Malenka, R.C., 2002. AMPA receptor trafficking and synaptic plasticity. Annu. Rev. Neurosci. 25, 103–126.

Malva, J.O., Carvalho, A.P., Carvalho, C.M., 1994. Modulation of dopamine and noradrenaline release and of intracellular Ca^{2+} concentration by presynaptic glutamate receptors in hippocampus. Br. J. Pharmacol. 113, 1439–1447.

Manders, E.M.M., Verbeek, F.J., Aten, J.A., 1993. Measurement of co-localization of objects in dual-color confocal images. J. Microsc. 169, 375–382.

Mansvelder, H.D., McGehee, D.S., 2000. Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron 27, 349–357.

Mansvelder, H.D., Keath, J.R., McGehee, D.S., 2002. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33, 905–919.

Marchi, M., Grilli, M., 2010. Presynaptic nicotinic receptors modulating neurotransmitter release in the central nervous system: functional interactions with other coexisting receptors. Prog. Neurobiol. 92, 105–111.

Martin, L.J., Furuta, A., Blackstone, C.D., 1998. AMPA receptor protein in developping rat brain: glutamate receptor-1 expression and localization change at regional, cellular and subcellular levels with maturation. Neuroscience 83, 917–928.

McClure-Begley, T.D., King, N.M., Collins, A.C., Stitzel, J.A., Wehner, J.M., Butt, C.M., 2009. Acetylcholine-stimulated [^{3}H]GABA release from mouse brain synaptosomes is modulated by alpha4beta2 and alpha4alpha5beta2 nicotinic receptor subtypes. Mol. Pharmacol. 75, 918–926.

Parodi, M., Patti, L., Grilli, M., Raiteri, M., Marchi, M., 2006. Nicotine has a permissive role on the activation of metabotropic glutamate 5 receptors coexisting with nicotinic receptors on rat hippocampal noradrenergic nerve terminals. Neurochem. Int. 48, 138–143.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, second ed. Academic Press, New York.

Peng, X., Gerzanich, V., Anand, R., Whiting, P.J., Lindstrom, J., 1994. Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. Mol. Pharmacol. 46, 523–530.

Phillips, G.R., Huang, J.K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, W.S., Arndt, K., Frank, M., Gordon, R.E., Gawinowicz, M.A., Zhao, Y., Colman, D.R., 2001. The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. Neuron 32, 63–77.

Pinheiro, P.S., Rodrigues, R.J., Silva, A.P., Cunha, R.A., Oliveira, C.R., Malva, J.O., 2003. Solubilization and immunological identification of presynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the rat hippocampus. Neurosci. Lett. 336, 97–100.

Pittaluga, A., Raiteri, M., 1992. N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Location on axon terminals and pharmacological characterization. J. Pharmacol. Exp. Ther. 260, 232–237.

Pittaluga, A., Bonfanti, A., Raiteri, M., 1997. Differential desensitization of ionotropic non-NMDA receptors having distinct neuronal location and function. Naunyn Schmiedeberg's Arch. Pharmacol. 356, 29–38.

Pittaluga, A., Feligioni, M., Longordo, F., Luccini, E., Raiteri, M., 2006. Trafficking of presynaptic AMPA receptors mediating neurotransmitter release: neuronal selectivity and relationships with sensitivity to cyclothiazide. Neuropharmacology 50, 286–296.

Prezeau, L., Rives, M.L., Comps-Agrar, L., Maurel, D., Kniazeff, J., Pin, J.P., 2010. Functional crosstalk between GPCRs: with or without oligomerization. Curr. Opin. Pharmacol. 10, 6–13.

Quik, M., Perez, X.A., Grady, S.R., 2011. Role of α6 nicotinic receptors in CNS dopaminergic function: relevance to addiction and neurological disorders. Biochem. Pharmacol. 82, 873–882.
Raiteri, M., Angelini, F., Levi, G., 1974. A simple apparatus for studying the release of neurotransmitters from synaptosomes. Eur. J. Pharmacol. 25, 411–414.
Raiteri, M., Sala, R., Fassio, A., Rossetto, O., Bonanno, G., 2000. Entrapping of impermeant probes of different size into nonpermeabilized synaptosomes as a method to study presynaptic mechanisms. J. Neurochem. 74, 423–431.
Rebola, N., Pinheiro, P.C., Oliveira, C.R., Malva, J., Cunha, R.A., 2003. Subcellular localization of adenosine A(1) receptors in nerve terminals and synapses of the rat hippocampus. Brain Res. 987, 49–58.
Reissner, K.J., Kalivas, P.W., 2010. Using glutamate homeostasis as a target for treating addictive disorders. Behav. Pharmacol. 21, 514–522.
Rezvani, A.H., Tizabi, Y., Getachew, B., Hauser, S.R., Caldwell, D.P., Hunter, C., Levin, E.D., 2008. Chronic nicotine and dizocilpine effects on nicotinic and NMDA glutamatergic receptor regulation: interactions with clozapine actions and attentional performance in rats. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 1030–1040.
Risso, F., Parodi, M., Grilli, M., Molfino, F., Raiteri, M., Marchi, M., 2004a. Chronic nicotine causesfunctional upregulation of ionotropic glutamate receptors mediating hippocampal noradrenaline and striatal dopamine release. Neurochem. Int. 44, 293–301.
Risso, F., Grilli, M., Parodi, M., Bado, M., Raiteri, M., Marchi, M., 2004b. Nicotine exerts a permissive role on NMDA receptor function in hippocampal noradrenergic terminals. Neuropharmacology 47, 65–71.
Rodrigues, R.J., Almeida, T., de Mendonça, A., Cunha, R.A., 2006. Interaction between P2X and nicotinic acetylcholine receptors in glutamate nerve terminals of the rat hippocampus. J. Mol. Neurosci. 30, 173–176.
Sershen, H., Balla, A., Lajtha, A., Vizi, E.S., 1997. Characterization of nicotinic receptors involved in the release of noradrenaline from the hippocampus. Neuroscience 77, 121–130.
Song, I., Huganir, R.L., 2002. Regulation of AMPA receptors during synaptic plasticity. Trends Neurosci. 25, 578–588.
Summa, M., Di Prisco, S., Grilli, M., Marchi, M., Pittaluga, A., 2011. Hippocampal AMPA autoreceptors positively coupled to NMDA autoreceptors traffic in a constitutive manner and undergo adaptative changes following enriched environment training. Neuropharmacology 61, 1282–1290.
Tarazi, F.I., Campbell, A., Yeghiyan, S.K., Baldessarini, R.J., 1998. Localization of ionotropic glutamate erceptors in caudate-putamnn and nucleus accumnbens septi of rat brain: comparison of NMDA, AMPA and kainate receptors. Synapse 30, 227–235.
Timofeeva, O.A., Levin, E.D., 2011. Glutamate and nicotinic receptor interactions in working memory: importance for the cognitive impairment of schizophrenia. Neuroscience 195, 21–36.
Vieyra-Reyes, P., Picciotto, M.R., Mineur, Y.S., 2008. Voluntary oral nicotine intake in mice down-regulates GluR2 but does not modulate depression-like behaviors. Neurosci. Lett. 434, 18–22.
Wang, F., Chen, H., Steketee, J.D., Sharp, B.M., 2007. Upregulation of ionotropic glutamate receptor subunits within specific mesocorticolimbic regions during chronic nicotine self-administration. Neuropsychopharmacology 32, 103–109.
Wessler, I., Apel, C., Garmsen, M., Klein, A., 1992. Effects of nicotine receptor agonists on acetylcholine release from the isolated motor nerve, small intestine and trachea of rats and guinea-pigs. Clin. Investig. 70, 182–189.
Wilkie, G.I., Hutson, P., Sullivan, J.P., Wonnacott, S., 1996. Pharmacological characterization of a nicotinic autoreceptor in rat hippocampal synaptosomes. Neurochem. Res. 21, 1141–1148.
Wolf, M.E., 2010. Regulation of AMPA receptor trafficking in the nucleus accumbens by dopamine and cocaine. Neurotox. Res. 18, 393–409.
Wonnacott, S., Irons, J., Rapier, C., Thorne, B., Lunt, G.G., 1989. Presynaptic modulation of transmitter release by nicotinic receptors. Prog. Brain Res. 79, 157–163.
Wonnacott, S., 1997. Presynaptic nicotinic ACh receptors. Trends Neurosci. 20, 92–98.
Wu, J., Lukas, R.J., 2011. Naturally-expressed nicotinic acetylcholine receptor subtypes. Biochem. Pharmacol. 82, 800–807.
Wu, X., McNeil, B.D., Xu, J., Fan, J., Xue, L., Melicoff, E., Adachi, R., Bai, L., Wu, L., 2009. Ca^{2+} and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. Nat. Neurosci. 12, 1003–1011.
Xia, J., Zhang, X., Staudinger, J., Huganir, R.L., 1999. Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22, 179–187.
Yamazaki, Y., Jia, Y., Niu, R., Sumikawa, K., 2006. Nicotine exposure in vivo induces long-lasting enhancement of NMDA receptor-mediated currents in the hippocampus. Eur. J. Neurosci. 23, 1819–1828.
Zappettini, S., Grilli, M., Lagomarsino, F., Cavallero, A., Fedele, E., Marchi, M., 2011. Presynaptic nicotinic alpha7 and non-alpha7 receptors stimulate endogenous GABA release from rat hippocampal synaptosomes through two mechanisms of action. PLoS One 6, e16911.
Zoli, M., Moretti, M., Zanardi, A., McIntosh, J.M., Clementi, F., Gotti, C., 2002. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J. Neurosci. 22, 8785–8789.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.8

frontiers in PHARMACOLOGY

Front Pharmacol. 2012; 3: 146. PMCID: PMC3406330
Published online 2012 July 27. doi: 10.3389/fphar.2012.00146

Beta Amyloid Differently Modulate Nicotinic and Muscarinic Receptor Subtypes which Stimulate *in vitro* and *in vivo* the Release of Glycine in the Rat Hippocampus

Stefania Zappettini,[1] Massimo Grilli,[1] Guendalina Olivero,[1] Elisa Mura,[2] Stefania Preda,[2] Stefano Govoni,[2] Alessia Salamone,[1] and Mario Marchi[1,3,*]

Author information ► Article notes ► Copyright and License information ►

Abstract

Go to:

Using both *in vitro* (hippocampal synaptosomes in superfusion) and *in vivo* (microdialysis) approaches we investigated whether and to what extent β amyloid peptide 1–40 (Aβ 1–40) interferes with the cholinergic modulation of the release of glycine (GLY) in the rat hippocampus. The nicotine-evoked overflow of endogenous GLY in hippocampal synaptosomes in superfusion was significantly inhibited by Aβ 1–40 (10 nM) while increasing the concentration to 100 nM the inhibitory effect did not further increase. Both the Choline (Ch; α7 agonist; 1 mM) and the 5-Iodo-A-85380 dihydrochloride (5IA85380, α4β2 agonist; 10 nM)-evoked GLY overflow were inhibited by Aβ 1–40 at 100 nM but not at 10 nM concentrations. The KCl evoked [^{3}H]GLY and [^{3}H]Acetylcholine (ACh) overflow were strongly inhibited in presence of oxotremorine; however this inhibitory muscarinic effect was not affected by Aβ 1–40. The effects of Aβ 1–40 on the administration of nicotine, veratridine, 5IA85380, and PHA543613 hydrochloride (PHA543613; a selective agonist of α7 subtypes) on hippocampal endogenous GLY release *in vivo* were also studied. Aβ 1–40 significantly reduced (at 10 μM but not at 1 μM) the nicotine-evoked *in vivo* release of GLY. Aβ 1–40 (at 10 μM but not at 1 μM) significantly inhibited the PHA543613 (1 mM)-elicited GLY overflow while was ineffective on the GLY overflow evoked by 5IA85380 (1 mM). Aβ 40–1 (10 μM) did not produce any inhibitory effect on nicotine-evoked GLY overflow both in the *in vitro* and *in vivo* experiments. Our results indicate that (a) the cholinergic modulation of the release of GLY occurs by the activation of both α7 and α4β2 nicotinic ACh receptors (nAChRs) as well as by the activation of inhibitory muscarinic ACh receptors (mAChRs) and (b) Aβ 1–40 can modulate cholinergic evoked GLY release exclusively through the interaction with α7 and the α4β2 nAChR nicotinic receptors but not through mAChR subtypes.

Keywords: β amyloid, glycine release, nicotinic receptors, muscarinic receptors, microdialysis

Introduction

Go to:

Nicotinic and muscarinic receptors are widely expressed in the brain and implicated in the pathophysiology of many neurological conditions, including Alzheimer's disease (AD), where typical symptoms include the loss of cognitive function and dementia. The presence of extracellular neuritic plaques composed of β amyloid (Aβ) peptide is a characteristic feature of AD, however, although neurotoxicity is a prominent feature of Aβ, recent data emphasize the existence of synaptic functional roles of this peptide which can modulate the release of several neurotransmitters (see Mura et al., 2012 and references therein). Accordingly, Aβ isoforms and oligomers of increasing molecular dimensions may have different biological actions in a continuum from physiology to pathology, determining loss and gains of function along the course of the disease (Mura et al., 2010a). Indeed, it has been shown that non-neurotoxic Aβ 1–40 concentrations were able to modulate (predominantly, but not exclusively, to inhibit) the release of several neurotransmitters (dopamine, γ aminobutyric acid, aspartate, glutamate) elicited by the stimulation of cholinergic muscarinic and nicotinic receptor [muscarinic ACh receptors (mAChR); nicotinic ACh receptors (nAChR)] subtypes in different brain areas; (Preda et al., 2008; Puzzo et al., 2008; Grilli et al., 2009; Jürgensen and Ferreira, 2010;Mura et al., 2010a,b, 2012; Ondrejcak et al., 2010). The hippocampus, an area which is particularly vulnerable and early target of Alzheimer's disease and in which the cholinergic pathways are critical for modulation of attention and memory (Parri et al., 2011), Aβ regulates the nicotine-evoked release of both excitatory (glutamate and aspartate) and inhibitory (γ aminobutyric acid) aminoacids (Mura et al., 2012).

Increasing evidence demonstrate that glycine (GLY) is an important aminoacid at hippocampal level which may have a dual role acting as an inhibitory neurotransmitter, when interacting with the strychnine-sensitive receptors, and playing a stimulatory role, when co-activating excitatory *N*-Methyl-D-aspartic acid receptors together with glutamate (Johnson and Ascher, 1987; Luccini et al., 2008; Romei et al., 2009, 2011; Zappettini et al., 2011). No data are available so far on the possible effects of Aβ on the cholinergic receptors which modulate GLY release at the hippocampal level.

In the present study using both *in vitro* (hippocampal synaptosomes in superfusion) and *in vivo* (microdialysis) approaches we investigated whether and to what extent Aβ interferes with the cholinergic modulation of the release of GLY in the rat hippocampus.

The results indicate that (a) the cholinergic modulation of the release of GLY occurs by the activation of both α7 and α4β2 (nAChRs) as previously reported (Zappettini et al., 2011) as well as by the activation of inhibitory mAChRs; (b) the nicotinic modulation of GLY release by α4β2, and α7 nAChRs is inhibited *in vitro* in presence of a nanomolar concentration of Aβ 1–40 which is on the contrary ineffective on the inhibitory mAChR receptor subtypes.

Materials and Methods

Go to:

Animals

Adult male Wistar rats (200–250 g, Harlan, Udine) were used for both *in vivo* experiments and as brain tissue source for *in vitro* experiments. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (light 7 a.m. to 7 p.m.). The *in vitro* experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (Decreto Ministeriale number 124/2003-A). The *in vivo* protocol was approved by Ethical Committee of Pavia's University (registered as 2/2008) according to international regulations for the care and treatment of laboratory animals, to the Italian Act (DL n 116, GU, suppl 40, 18 February, 1992) and to EEC Council Directive (86/609, OJ L 358, 1, 12 December, 1987). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

In vitro experiments

Experiments of release Rats were killed by decapitation and the hippocampus rapidly removed at 0–4°C. Purified synaptosomes were prepared on Percoll® gradients (Sigma-Aldrich, St Louis, MO, USA) essentially according to (Nakamura et al., 1993), with only minor modifications. Briefly, the tissue was homogenized in six volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged (5 min, 1000 × *g* at 4°C) to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10, and 20% v/v in Tris-buffered sucrose) and

centrifuged at 33,500 × *g* for 5 min at 4°C. The layer between 10 and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation, and resuspended in physiological HEPES-buffered medium having the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4 (Lu et al., 1998). Synaptosomal protein content following purification was 10–15% of that in the supernatant stratified on the Percoll® gradient.

The synaptosomal suspension was layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri and Raiteri, 2000; Superfusion System, Ugo Basile, Comerio, Varese, Italy). Synaptosomes were superfused at 1 ml/min with standard physiological medium as previously described. The system was first equilibrated during 36.5 min of superfusion; subsequently, four consecutive 90 s fractions of superfusate were collected and the endogenous GLY content was measured by high performance liquid chromatography as below described. Synaptosomes were exposed to agonists for 90 s starting from the second fraction collected (t = 38 min), with antagonists being added 8 min before agonists. The evoked overflow was calculated by subtracting the corresponding basal release from each fraction and was expressed as pmol/mg of synaptosomal proteins. We previously demonstrated that in our superfusion system the indirect drug effects exerted by other mediators in the monolayer of synaptosomes in superfusion are absolutely minimized (Raiteri and Raiteri, 2000).

When studying the release of [^{3}H]GLY or [^{3}H]Acetylcholine (ACh) hippocampal synaptosomes were incubated for 20 min at 37°C with [^{3}H]GLY (final concentration 0.1 μM) in the presence of the selective GLY transporter 1 transporter blocker *N*-[(3R)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine hydrochloride (final concentration 0.3 μM) or with [^{3}H]Choline (Ch, final concentration 0.08 μM). The K^+-induced overflow from synaptosomes was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2). The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux).

Endogenous GLY determination

Endogenous GLY was measured by high performance liquid chromatography analysis following precolumn derivatization with *o*-phthalaldehyde and resolution through a C18-reverse phase chromatographic column (10 mm × 4.6 mm, 3 μm; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as internal standard. Buffers and gradient program were prepared and executed as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min.

In vivo experiments

Microdialysis probe implantation Rats were anesthetized with Equithesin 3 ml/kg (pentobarbital 9.7 g, chloral hydrate 42.5 g, $MgSO_4$ 21.3 g for 1 l, 10% ethanol, 40% propylene glycol v/v) administered intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected, and cut with a sterile scalpel to expose the skull. A hole was drilled to allow the implantation of the probe into the brain parenchyma. The probe was implanted in the hippocampus (CA1/CA2 regions; AP −5.8 mm, ML ± 5.0 mm from bregma, and DV −8.0 mm from dura) according to the Paxinos and Watson (1986) atlas, and secured to the skull with one stainless steel screw and dental cement. All *in vivo* experiments were performed using microdialysis probes, made in our laboratory according to the original method described by Di Chiara (1990; Emophan Bellco Artificial OR-internal diameter 200 μm, cutoff 40 kDa; Bellco, Mirandola, Modena, Italy), with a nominal active length of 5 mm. Finally, the skin was sutured, and the rats were allowed to recover from anesthesia for at least 24 h before the neurotransmitter release study.

Microdialysis samples collection Microdialysis experiments were performed on conscious freely moving rats. On the day of the experiments (24 h after the surgical procedure), the probe was perfused with artificial CSF containing 145 mM NaCl, 3.0 mM KCl, 1.26 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM Na_2HPO_4, buffered at pH 7.2–7.4, and filtered through a Millipore 0.2 μm pore membrane. In all experiments, the microdialysis membrane was allowed to stabilize for 1 h at the flow rate of 4 μl/min, without collecting samples. At the end of the stabilization period, three samples were collected to evaluate baseline release of GLY and then the specific treatment started. All treatments were administered by manually switching syringes and tubing connections to allow drugs diluted in artificial CSF to flow through the probes. Tubing switches were performed taking care to maintain constant flow rates and collection volumes. Both basal and treatment samples were collected every 20 min in 100 μl Eppendorf tubes. The flow rate of 4 μl/min was maintained using a 1000-μl syringe (Hamilton) and a microinjection pump (CMA/100, CMA/Microdialysis AB). *In vitro* recovery of the probe for GLY was about 20%. Each rat was used for only one microdialysis session. At the end of each experiment animals were sacrificed by guillotine, rat brains were removed and the position of the microdialysis probe was verified by histological procedures, slicing the tissues by a cryostat microtome (LEICA CM 1510). Only data from rats in which probe tracks were exactly located in the target area were used for statistical analysis.

Immunohistochemical analysis Immunohistochemical analysis was performed to verify the presence of Aβ in the perfused tissue and to confirm (according to HOECHST 33342 staining) the absence of neurotoxic-induced apoptotic phenomenon. Brain tissue samples were frozen and stored at −80°C. For immunodetection of infused Aβ peptide, 10 μm coronal sections (obtained on a cryostat Leica CM 1510) were incubated with a primary monoclonal antibody recognizing Aβ protein (clone 4G8; Chemicon International). Sections were then incubated with a mouse anti-IgG antibody RPE conjugated (Dako). After the fluorescent labeling procedures, sections were finally counterstained for DNA with HOECHST 33342 and mounted in a drop of Mowiol (Calbiochem, Inalco SpA, Milan, Italy). Fluorescent micrographs were acquired with a Leica TCS SP5 II confocal microscope. After acquisition of fluorescent micrographs, the slides were demounted and then the same sections were slightly counterstained with Mayer hematoxylin, dehydrated, and mounted in DPX for microanatomical analysis. The images were acquired with a BX51 Olympus microscope.

Statistical analysis

In vitro experiments Multiple comparisons were performed with one-way ANOVA followed by an appropriate *post hoc* test (Dunnett and Bonferroni). Data were considered significant for $p < 0.05$, at least.

In vivo experiments Values were expressed either as amount of GLY measured in the dialyzate (pmol/80 μl) or as area under the curve (AUC), evaluating the cumulative release over time. AUC was used as a measure of treatment exposure and was calculated, for each animal, using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA, USA), defining as baseline of the area the basal value (average concentration of three consecutive samples immediately preceding the drug dose).

D'Agostino–Pearson Omnibus Test (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA, USA) and Grubb's Test (GraphPad QuickCalcs, online calculator for scientists at http://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA) were used as preliminary tests in order to evaluate whether data were sampled from a Gaussian distribution and to detect outliers respectively. All outliers were excluded from the analysis. Data were then analyzed by analysis of variance (one- or two-way ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Data were considered significant for $p < 0.05$. The reported data are expressed as means ± SEM. The number of animals used for each experiment is reported in the legend to figures.

Preparation of beta amyloid solutions

In the case of both *in vivo* and *in vitro* experiments, synthetic human Aβ 1–40 (Sigma-Aldrich, Milan, Italy) was dissolved in aCSF at a concentration of 100 mM (stock solution). Then, this solution was filtered through a Millipore 0.2 μm pore membrane and stocked in small aliquots. Working solutions were freshly prepared by diluting an aliquot of Aβ 1–40 stock solution at the final concentrations (10 mM, 1 mM, or 100 nM Aβ 1–40 for *in vivo* experiments, 100 nM, 10 nM, 1 nM, or 100 pM for *in vitro* analysis).

Chemicals

Beta amyloid (1–40; 40–1), percoll®, choline, himbacine, dimethyl sulfoxide, veratridine, nicotine hydrogen tartrate salt (Sigma-Aldrich, St Louis, MO, USA); NFPS (ALX 5407), 5IA85380, PHA543613, AQRA741 (Tocris Bioscience, Bristol, UK); all salts used for the preparation of aCSF (NaCl, KCl, $CaCl_2$, $MgCl_2$, Na_2HPO_4) and for Equithesin ($MgSO_4$) were purchased at Merck KGaA, Darmstadt, Germany; chloral hydrate, ethanol 96%, and propylene glycol were used for the preparation of Equithesin and were obtained at VWR BDH Prolabo, Belgium;[^{3}H]Choline (specific activity: 60–90 Ci/mmol) and [^{3}H]Glycine (specific activity: 15 Ci/mmol) were purchased from Perkin Elmer SpA.

Results

Go to:

It is known that Aβ peptides structure and aggregation properties depend on several factors. It is therefore important when using Aβ peptides to specify both the concentration of the soluble Aβ but also the methods used to prepare Aβ solution and to verify the presence of Aβ aggregates (see Parri et al., 2011). This facilitates the interpretation of the results when reporting on Aβ effects and, which is even more important, would facilitate the comparison of data obtained in different laboratories. Hence, in our previous paper we characterized the Aβ peptide conformation we administered *in vivo* by Western Blot procedure (Mura et al., 2012) showing that we administered, at least predominantly, Aβ monomers. We cannot completely exclude by the adopted methods that small amounts of Aβ oligomers are present and may participate to produce the observed effects. In regard to the *in vitro* Aβ preparations, since we did not observe aggregation at the concentrations and the timing (up to 40 min) analyzed *in vivo*, we also do not expect to observe aggregation at the lower concentrations and shorter times used *in vitro* in light of the fact that aggregation is a concentration and time-dependent process.

Figure 1A shows that the nicotine-evoked overflow of endogenous GLY was significantly inhibited by Aβ 1–40 already at a concentration of 10 nM while increasing the concentration to 100 nM the inhibitory effect did not further increase. The 1-nM Aβ 1–40 concentration was ineffective. The reverse peptide Aβ 40–1 was ineffective even at high (100 nM) concentration. Aβ 1–40 (100 nM) did not affect the veratridine (10 nM)-evoked GLY overflow.

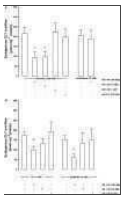


Figure 1

(A) Shows the concentration dependence effects of Aβ on nicotine- and veratridine-evoked endogenous GLY overflows from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments for each concentration **...**

Since it has been shown that two different nAChR subtypes, α7 and α4β2, modulate GLY release (Zappettini et al., 2011), the possibility that Aβ may differentially inhibit the nicotinic control of GLY release has been investigated. In order to verify this point we have studied the effects of two different agonists, Ch and 5-IA85380 hydrochloride (5IA85380), known to act selectively on the α7 and α4β2 nAChR subtypes respectively (Mukhin et al., 2000; Uteshev et al., 2003; Dickinson et al., 2007; Zappettini et al., 2010). Ch (1 mM) and the 5IA85380 (10 nM)-evoked a similar overflow of GLY confirming the involvement of the two receptor subtypes. Both the Ch (1 mM) and the 5IA85380 (10 nM)-evoked GLY overflow were significantly inhibited by Aβ 1–40 at 100 nM but not at 10–1 nM concentrations compared to controls (Figure 1B). Aβ 1–40 (100 nM) did not modify the basal release of endogenous GLY (data not shown).

It has been demonstrated that also different mAChR subtypes are involved in the modulation of both ACh and GLY release from brain hippocampal synaptosomes (Raiteri et al., 1984; Russo et al., 1993). We investigated whether Aβ was able to affect the muscarinic control of the release of these two transmitters as it was able to disrupt the nicotinic control of GLY release. Figure 2A shows that the KCl evoked [^{3}H]GLY overflow was strongly inhibited in presence of oxotremorine. This inhibitory effect was totally antagonized by himbacine but was not affected by Aβ 1–40 (100 nM). In presence of oxotremorine also the KCl evoked release of [^{3}H]ACh from hippocampal nerve endings was significantly inhibited (Figure 2B). This result was unexpected but demonstrates quite interestingly that the cholinergic muscarinic modulation of GLY release could be different according to the different brain areas or the different species studied since previous findings using human cortical nerve endings have shown a potentiating effect of oxotremorine on GLY release (Russo et al., 1993). Interestingly also the inhibitory effect of oxotremorine on [^{3}H]ACh release was antagonized, as expected, by the specific M2 mAChR antagonist AQRA741 but was not affected by Aβ 1–40 (100 nM; Figure 2B).

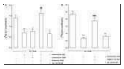


Figure 2

(A) Shows the effects of Aβ on muscarinic receptors controlling KCl evoked [^{3}H]GLY overflow from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments run in triplicate. *$p < 0.05$; **...**

Based on the *in vitro* data we then analyzed the effects of Aβ 1–40 on the administration of nicotine, veratridine, 5IA85380, and PHA543613 hydrochloride (PHA543613, a selective agonist of α7 subtypes) on hippocampal GLY release *in vivo*. In order to test whether the administration of Aβ 1–40 through the dialysis probe allowed the delivery of the peptide to the tissue we performed an immunohistochemical analysis. Figure 3 shows the presence of the peptide for the two concentrations tested *in vivo* (1 and 10 μM) within the hippocampus. Despite the fact that we do not know the exact amounts of Aβ reaching the tissue, there was a visible positive correlation between the concentration administered and the signal of Aβ immunoreactivity in the tissue. Moreover, immunohistochemical analysis shows that no evident signs of apoptosis were observed within the area of amyloid diffusion as shown by Hoechst staining.

Figure 3

Immunohistochemical analysis showing the presence of beta-amyloid (Aβ) in hippocampal tissue after the perfusion of the peptide at two different concentrations. Coronal section indicating the location of the microdialysis probe (hippocampus, black **...**

The choice of the concentration of the nicotinic cholinergic agonists to be delivered *in vivo* was derived from previous data demonstrating that the administration by microdialysis of 50 mM nicotine was able to significantly increase the levels of GLY in hippocampal extracellular compartment (Toth, 1996; Fedele et al., 1998; Zappettini et al., 2011). As previously shown in our

experimental conditions 40 min-long administration of 50 mM nicotine was able to greatly enhance GLY release from basal values. Figure 4A show that Aβ 1–40 significantly reduced (at 10 μM but not at 1 μM) the nicotine-evoked *in vivo* release of GLY. Aβ 40–1 did not produce any inhibitory effect used at 10 μM concentration. The GLY overflow stimulated by veratridine was unaltered in presence of Aβ 1–40 (Figure 4B). Then we compared the effects Aβ 1–40 after exposure to the selective nAChRs agonists, 5IA85380, and PHA543613. As shown in Figure 4C, Aβ 1–40 (at 10 μM but not at 1 μM) significantly inhibited the PHA543613 (1 mM)-elicited GLY overflow while was ineffective on the GLY overflow evoked by 5IA85380 (1 mM).

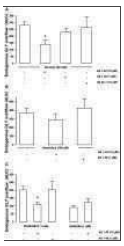


Figure 4

The figure shows the *in vivo* effect of Aβ 1–40 on the nicotine-, veratrine-, PHA543613-, and 5IA85380-induced overflow of GLY, in rat hippocampus [(A–C) respectively]. Aβ 1–40 (10 μM but not 1 μM) **...**

Discussion

Go to:

It is expected that cognitive deficits and memory impairments in AD patients could be related, at least in part, to Aβ mediated decrease of cholinergic function (Wang et al., 2009a,b, 2010; Parri et al., 2011). However it is still unclear whether these impairments are a consequence of a loss of cholinergic neurons and a decrease of nAChRs or of a direct molecular interaction of Aβ with nAChRs leading to a dysregulation of receptor function or of both mechanisms. To shed some light on these mechanisms we investigated in the present study as well as in previous researches whether Aβ, at concentrations not producing acutely neurotoxicity, is able to disrupt the cholinergic control of neurotransmitter release. To do this we took advantage of the fact that both α4β2, and α7 nAChRs receptors are known to be expressed in rat hippocampal interneurons (McQuiston and Madison, 1999; Sudweeks and Yakel, 2000; Yakel and Shao, 2004), and it is well-known that they have a positive role in regulating cognitive function (Picciotto et al., 1995; Levin and Simon, 1998). Being aware that several neurotransmitters that play important roles in cognitive functions could be affected either directly or indirectly by Aβ, we focused our attention on GLY. Indeed increasing evidence demonstrate that GLY is an important aminoacid at hippocampal level which has a dual role (a) acting as an inhibitory neurotransmitter when interacting with the strychnine-sensitive receptors and (b) playing a fundamental stimulatory role when co-activating excitatory *N*-Methyl-D-aspartic acid receptors together with glutamate (Johnson and Ascher, 1987; Hirai et al., 1996; Luccini et al., 2008; Kubota et al., 2010; Zappettini et al., 2011). The changes in the GLY release may therefore directly interfere with glutamate neurotransmission, which plays an important role in the processes of learning and memory in this brain area.

We here report that the cholinergic modulation of GLY release at the presynaptic level on hippocampal nerve endings was modulated not only by stimulatory α4β2 and α7 nAChRs as previously reported (Zappettini et al., 2011) but also by an inhibitory, mAChR subtype. We do not know whether both nicotinic and muscarinic receptors are present on all nerve endings or they are peculiar of a specific neuronal population and/or their physiological importance in the intact tissue. It is quite interesting however that the two modulatory mechanisms display a different sensitivity to Aβ. The stimulatory effects of both α4β2 and α7 nAChRs were partially blocked by nanomolar concentration of Aβ 1–40 which was on the contrary inactive on the mAChRs inhibiting GLY release. This observation allows to speculate that an excess of Aβ (the range of active concentrations was between 10 and 100 nM *in vitro* and 10 μM *in vivo*) as it may happen because of the disease may dysregulate the cholinergic modulation of hippocampal activity leading to a disproportionate inhibition since it leaves unaffected the muscarinic inhibitory control and impairs the nicotinic stimulatory one. All this occurs in absence of Aβ acutely induced neuronal damage.

The decreased release of GLY may have several functional consequences. Some of them may be relevant to the development of AD pathology. As an example, a decrease of GLY release may reduce the tonic inhibition exerted through the activation of GLY receptors (Mori et al., 2002; Petrini et al., 2004; Farrant and Nusser, 2005) normally providing neuroprotection under pathological conditions, when extracellular GLY levels are elevated (Baker et al., 1991; Saransaari et al., 1997; Zhao et al., 2005; Saransaari and Oja, 2008). A second relevant event caused by a reduced glycine release may consist in a decrease of *N*-Methyl-D-aspartic acid receptors co-activation. At this regard it is important to recall that intracerebroventricular injection of Aβ 1–40 significantly suppress high frequency stimulation-induced LTP (Chen et al., 2006; Wu et al., 2008).

The exact nature of the Aβ interaction with nAChR subtypes is so far not well understood.

Interestingly in our study both the α7 and the α4β2 nAChR subtypes which almost equally contribute to the stimulation of GLY release, were functionally inhibited *in vitro* apparently in a similar extent by Aβ concentrations in the nanomolar range (Figure 2B). However we do not know whether Aβ might have a similar mechanism of action on the two different nAChR subtypes. The inhibitory effect on both the α7 and the α4β2 nAChR was incomplete with a maximal inhibition of about 30–40%. A recently described allosteric binding pocket located within the trans membrane domain of the α7 and of non-α7 nAChRs might provide a potential structure-function mechanism to explain the inhibitory effects of Aβ (Young et al., 2008; Gill et al., 2011, 2012).

Indeed Aβ at concentration in the upper nanomolar range (10 nM) produced full effect while did not produced any effect at 1 nM concentration. Ten nanomolars Aβ beyond the estimated normal concentrations in human CSF but may be caused by the altered precursor protein processing as it occurs along with the disease (Reaume et al., 1996) or be related to defects in the removal of the peptide from the extracellular space as it may occur in the disease in association with the ApoE ε4 genotype (Cramer et al., 2012).

Of course talking about cholinergic modulation of GLY release we have also to consider the possibility that Aβ might for instance interfere directly with the mechanisms which modulate the release of ACh. However our data show that Aβ was ineffective also on the inhibitory muscarinic autoreceptors which inhibit ACh release (Figure 2). We can therefore foresee that in an integrated system, where cellular networks and their functional relationships are completely preserved and several direct and indirect processes are simultaneously taking place in neurons, the effect of Aβ on the cholinergic modulation of GLY function may mostly depend on the interaction of Aβ with the nAChRs.

Our findings *in vivo* largely support this view even if Aβ was unable to inhibit the release of GLY elicited by the specific α4β2 nAChRs agonist while it exerted an inhibition (over 40%) of both nicotine and PHA543613 stimulated GLY release. On the other hand *in vivo* important differences were noticed at the baseline in the ability of the two specific nAChRs agonists to elicit GLY release as compare to *in vitro*. Indeed *in vitro* both α7 and α4β2 nAChRs agonists were able to stimulate to the same extent GLY release; while *in vivo* the release elicited by 5IA85380 was less than a half that obtained in response to

PHA543613. *In vivo* to many variables may take place, and in spite of the above described discrepancy, there is a general good agreement of the effects *in vivo* and *in vitro* of Aβ on the release of various neurotransmitters (present work and references) including the observation that so far Aβ has never been able to modify the depolarization (veratridine or potassium)-elicited release of neurotransmitters, but only the one modulated by presynaptic receptors.

In conclusion our findings show that Aβ can modulate cholinergic evoked GLY release exclusively through the interaction with α7 and the α4β2 nAChR nicotinic receptors, acting probably through different mechanisms of action, but not through mAChRs. The present study may therefore provide further insight into the mechanism by which AB impairs synaptic plasticity and cholinergic function in AD brain.

Conflict of Interest Statement

Go to:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

Go to:

This work was supported by from the Italian Ministero Università Ricerca to Prof. Mario Marchi (Prot. No 2009R7WCZS_003), by Compagnia di San Paolo, by University of Genoa "Progetto Ricerca Ateneo," and by project AROMA (ALCOTRA 2007-2013) by the European Community Project PYRGI, project no. B51H10000000006, and by the project 7FP-KBBE BAMMBO and to Prof. Govoni (MIUR 2009). This work is supported by a grant of the Alzheimer's Association (NIRG-11-205183) to Elisa Mura. We wish to thank Dr. Cristina Lanni for technical support in immunohistochemical analysis, Maura Agate for editorial assistance and Dr. Silvia E. Smith, Ph.D. (University of Utah) for reviewing the manuscript.

References

Go to:

1. Baker A. J., Zornow M. H., Scheller M. S., Yaksh T. L., Skilling S. R., Smullin D. H., Larson A. A., Kuczenski R. (1991). Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. J. Neurochem. 57, 1370–1379. doi: 10.1111/j.1471-4159.1991.tb02096.x. [PubMed] [Cross Ref]
2. Chen L., Yamada K., Nabeshima T., Sokabe M. (2006). alpha7 Nicotinic acetylcholine receptor as a target to rescue deficit in hippocampal LTP induction in beta-amyloid infused rats. Neuropharmacology 50, 254–268. doi: 10.1016/j.neuropharm.2005.09.018. [PubMed] [Cross Ref]
3. Cramer P. E., Cirrito J. R., Wesson D. W., Lee C. Y., Karlo J. C., Zinn A. E., Casali B. T., Restivo J. L., Goebel W. D., James M. J., Brunden K. R., Wilson D. A., Landreth G. E. (2012). ApoE-directed therapeutics rapidly clear β-amyloid and reverse deficits in AD mouse models. Science 335, 1503–1506. doi: 10.1126/science.1217697. [PubMed] [Cross Ref]
4. Di Chiara G. (1990). In-vivo brain dialysis of neurotransmitters. Trends Pharmacol. Sci. 11, 116–121. doi: 10.1016/0165-6147(90)90197-G. [PubMed] [Cross Ref]
5. Dickinson J. A., Hanrott K. E., Mok M. H., Kew J. N., Wonnacott S. (2007). Differential coupling of alpha7 and non-alpha7 nicotinic acetylcholine receptors to calcium-induced calcium release and voltage-operated calcium channels in PC12 cells. J. Neurochem. 100, 1089–1096. doi: 10.1111/j.1471-4159.2006.04273.x. [PubMed] [Cross Ref]
6. Farrant M., Nusser Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat. Rev. Neurosci. 6, 215–229. doi: 10.1038/nrn1625. [PubMed] [Cross Ref]
7. Fedele E., Varnier G., Ansaldo M. A., Raiteri M. (1998). Nicotine administration stimulates the in vivo N-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. Br. J. Pharmacol. 125, 1042–1048. doi: 10.1038/sj.bjp.0702130. [PMC free article] [PubMed] [Cross Ref]
8. Gill J. K., Dhankher P., Sheppard T. D., Sher E., Millar N. S. (2012). A series of α7 nicotinic acetylcholine receptor allosteric modulators with close chemical similarity but diverse pharmacological properties. Mol. Pharmacol. 81, 710–718. doi: 10.1124/mol.111.076026. [PMC free article] [PubMed] [Cross Ref]
9. Gill J. K., Savolainen M., Young G. T., Zwart R., Sher E., Millar N. S. (2011). Agonist activation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. Proc. Natl. Acad. Sci. U.S.A. 108, 5867–5872. doi: 10.1073/pnas.1104501108. [PMC free article] [PubMed] [Cross Ref]
10. Grilli M., Zappettini S., Raiteri L., Marchi M. (2009). Nicotinic and muscarinic cholinergic receptors coexist on GABAergic nerve endings in the mouse striatum and interact in modulating GABA release. Neuropharmacology 56, 610–614. doi: 10.1016/j.neuropharm.2008.10.014. [PubMed] [Cross Ref]
11. Hirai H., Kirsch J., Laube B., Betz H., Kuhse J. (1996). The glycine binding site of the N-methyl-D-aspartate receptor subunit NR1: identification of novel determinants of co-agonist potentiation in the extracellular M3-M4 loop region. Proc. Natl. Acad. Sci. U.S.A. 93, 6031–6036. doi: 10.1073/pnas.93.12.6031. [PMC free article] [PubMed] [Cross Ref]
12. Johnson J. W., Ascher P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325, 529–531. doi: 10.1038/325529a0. [PubMed] [Cross Ref]
13. Jürgensen S., Ferreira S. T. (2010). Nicotinic receptors, amyloid-beta, and synaptic failure in Alzheimer's disease. J. Mol. Neurosci. 40, 221–229. doi: 10.1007/s12031-009-9237-0. [PubMed] [Cross Ref]
14. Kubota H., Alle H., Betz H., Geiger J. R. (2010). Presynaptic glycine receptors on hippocampal mossy fibers. Biochem. Biophys. Res. Commun. 393, 587–591. doi: 10.1016/j.bbrc.2010.02.019. [PubMed] [Cross Ref]
15. Levin E., Simon B. (1998). Nicotinic acetylcholine involvement in cognitive function in animals. Psychopharmacology (Berl.) 138, 217–230. doi: 10.1007/s002130050667. [PubMed] [Cross Ref]
16. Lu Y., Grady S., Marks M. J., Picciotto M., Changeux J. P., Collins A. C. (1998). Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. J. Pharmacol. Exp. Ther. 287, 648. [PubMed]
17. Luccini E., Romei C., Raiteri L. (2008). Glycinergic nerve endings in hippocampus and spinal cord release glycine by different mechanisms in response to identical depolarizing stimuli. J. Neurochem. 105, 2179–2189. doi: 10.1111/j.1471-4159.2008.05309.x. [PubMed] [Cross Ref]
18. McQuiston A. R., Madison D. V. (1999). Nicotinic receptor activation excites distinct subtypes of interneurons in the rat hippocampus. J. Neurosci. 19, 2887–2896. [PubMed]
19. Mori M., Gähwiler B. H., Gerber U. (2002). Beta-alanine and taurine as endogenous agonists at glycine receptors in rat hippocampus in vitro. J. Physiol. (Lond.) 539, 191–200. doi: 10.1113/jphysiol.2001.013147. [PMC free article] [PubMed] [Cross Ref]
20. Mukhin A. G., Gündisch D., Horti A. G., Koren A. O., Tamagnan G., Kimes A. S., Chambers J., Vaupel D. B., King S. L., Picciotto M. R., Innis R. B., London E. D. (2000). 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand

for nicotinic acetylcholine receptors. Mol. Pharmacol. 57, 642–649. [PubMed]
21. Mura E., Lanni C., Preda S., Pistoia F., Sarà M., Racchi M., Schettini G., Marchi M., Govoni S. (2010a). Beta-amyloid: a disease target or a synaptic regulator affecting age-related neurotransmitter changes? Curr. Pharm. Des. 16, 672–683. doi: 10.2174/138161210790883723. [PubMed] [Cross Ref]
22. Mura E., Preda S., Govoni S., Lanni C., Trabace L., Grilli M., Lagomarsino F., Pittaluga A., Marchi M. (2010b). Specific neuromodulatory actions of amyloid-beta on dopamine release in rat nucleus accumbens and caudate putamen. J. Alzheimers Dis. 19, 1041–1053. [PubMed]
23. Mura E., Zappettini S., Preda S., Biundo F., Lanni C., Grilli M., Cavallero A., Olivero G., Salamone A., Govoni S., Marchi M. (2012). Dual effect of beta-amyloid on α7 and α4β2 nicotinic receptors controlling the release of glutamate, aspartate and GABA in rat hippocampus. PLoS ONE 7, e29661. doi: 10.1371/journal.pone.0029661. [PMC free article] [PubMed] [Cross Ref]
24. Nakamura Y., Iga K., Shibata T., Shudo M., Kataoka K. (1993). Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. Glia 9, 48–56. doi: 10.1002/glia.440090107. [PubMed] [Cross Ref]
25. Ondrejcak T., Klyubin I., Hu N. W., Barry A. E., Cullen W. K., Rowan M. J. (2010). Alzheimer's disease amyloid beta-protein and synaptic function. Neuromolecular Med. 12, 13–26. doi: 10.1007/s12017-009-8091-0. [PubMed] [Cross Ref]
26. Parri H. R., Hernandez C. M., Dineley K. T. (2011). Research update: alpha7 nicotinic acetylcholine receptor mechanisms in Alzheimer's disease. Biochem. Pharmacol. 82, 931–942. doi: 10.1016/j.bcp.2011.06.039. [PubMed] [Cross Ref]
27. Paxinos G., Watson C. (1986). The Rat Brain in Stereotaxic Coordinates, 2nd Edn. New York: Academic Press.
28. Petrini E. M., Marchionni I., Zacchi P., Sieghart W., Cherubini E. (2004). Clustering of extrasynaptic GABA(A) receptors modulates tonic inhibition in cultured hippocampal neurons. J. Biol. Chem. 279, 45833–45843. doi: 10.1074/jbc.M407229200. [PubMed] [Cross Ref]
29. Picciotto M., Zoli M., Léna C., Bessis A., Lallemand Y., Le Novère N., Vincent P., Pich E., Brûlet P., Changeux J. (1995). Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. Nature 374, 65–67. doi: 10.1038/374065a0. [PubMed] [Cross Ref]
30. Preda S., Govoni S., Lanni C., Racchi M., Mura E., Grilli M., Marchi M. (2008). Acute beta-amyloid administration disrupts the cholinergic control of dopamine release in the nucleus accumbens. Neuropsychopharmacology 33, 1062–1070. doi: 10.1038/sj.npp.1301485. [PubMed] [Cross Ref]
31. Puzzo D., Privitera L., Leznik E., Fà M., Staniszewski A., Palmeri A., Arancio O. (2008). Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J. Neurosci. 28, 14537–14545. doi: 10.1523/JNEUROSCI.2692-08.2008. [PMC free article] [PubMed] [Cross Ref]
32. Raiteri L., Raiteri M. (2000). Synaptosomes still viable after 25 years of superfusion. Neurochem. Res. 25, 1265–1274. doi: 10.1023/A:1007648229795. [PubMed] [Cross Ref]
33. Raiteri M., Leardi R., Marchi M. (1984). Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain. J. Pharmacol. Exp. Ther. 228, 209. [PubMed]
34. Reaume A. G., Howland D. S., Trusko S. P., Savage M. J., Lang D. M., Greenberg B. D., Siman R., Scott R. W. (1996). Enhanced amyloidogenic processing of the beta-amyloid precursor protein in gene-targeted mice bearing the Swedish familial Alzheimer's disease mutations and a "humanized" Abeta sequence. J. Biol. Chem. 271, 23380–23388. doi: 10.1074/jbc.271.38.23380. [PubMed] [Cross Ref]
35. Romei C., Di Prisco S., Raiteri M., Raiteri L. (2011). Glycine release provoked by disturbed Na^+, Na^+ and Ca^{2+} homeostasis in cerebellar nerve endings: roles of Ca^{2+} channels, Na^+/Ca^{2+} exchangers and GlyT2 transporter reversal. J. Neurochem. 119, 50–63. doi: 10.1111/j.1471-4159.2011.07401.x. [PubMed] [Cross Ref]
36. Romei C., Luccini E., Raiteri M., Raiteri L. (2009). GABA(B) presynaptic receptors modulate glycine exocytosis from mouse spinal cord and hippocampus glycinergic nerve endings. Pharmacol. Res. 59, 154–159. doi: 10.1016/j.phrs.2008.12.004. [PubMed] [Cross Ref]
37. Russo C., Marchi M., Andrioli G. C., Cavazzani P., Raiteri M. (1993). Enhancement of glycine release from human brain cortex synaptosomes by acetylcholine acting at M4 muscarinic receptors. J. Pharmacol. Exp. Ther. 266, 142. [PubMed]
38. Saransaari P., Oja S. S. (2008). Effects of inhibitory amino acids on adenosine release in the mouse hippocampus. Proc. West. Pharmacol. Soc. 51, 15–17. [PubMed]
39. Saransaari P., Oja S. S., Borkowska H. D., Koistinaho J., Hilgier W., Albrecht J. (1997). Effects of thioacetamide-induced hepatic failure on the N-methyl-D-aspartate receptor complex in the rat cerebral cortex, striatum, and hippocampus. Binding of different ligands and expression of receptor subunit mRNAs. Mol. Chem. Neuropathol. 32, 179–193. doi: 10.1007/BF02815175. [PubMed] [Cross Ref]
40. Sudweeks S. N., Yakel J. L. (2000). Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. J. Physiol. (Lond.) 527(Pt 3), 515–528. doi: 10.1111/j.1469-7793.2000.00515.x. [PMC free article] [PubMed] [Cross Ref]
41. Toth E. (1996). Effect of nicotine on the level of extracellular amino acids in the hippocampus of rat. Neurochem. Res. 21, 903–907. doi: 10.1007/BF02532339. [PubMed] [Cross Ref]
42. Uteshev V., Meyer E., Papke R. (2003). Regulation of neuronal function by choline and 4OH-GTS-21 through alpha 7 nicotinic receptors. J. Neurophysiol. 89, 1797–1806. doi: 10.1152/jn.00943.2002. [PubMed] [Cross Ref]
43. Wang H. Y., Bakshi K., Shen C., Frankfurt M., Trocmé-Thibierge C., Morain P. (2010). S 24795 limits beta-amyloid-alpha7 nicotinic receptor interaction and reduces Alzheimer's disease-like pathologies. Biol. Psychiatry 67, 522–530. doi: 10.1016/j.biopsych.2009.09.031. [PubMed] [Cross Ref]
44. Wang H. Y., Stucky A., Liu J., Shen C., Trocme-Thibierge C., Morain P. (2009a). Dissociating beta-amyloid from alpha 7 nicotinic acetylcholine receptor by a novel therapeutic agent, S 24795, normalizes alpha 7 nicotinic acetylcholine and NMDA receptor function in Alzheimer's disease brain. J. Neurosci. 29, 10961–10973. doi: 10.1523/JNEUROSCI.1255-09.2009. [PubMed] [Cross Ref]
45. Wang Y., Zhang G., Zhou H., Barakat A., Querfurth H. (2009b). Opposite effects of low and high doses of Abeta42 on electrical network and neuronal excitability in the rat prefrontal cortex. PLoS ONE 4, e8366. doi: 10.1371/journal.pone.0007486. [PMC free article] [PubMed] [Cross Ref]
46. Wu M. N., He Y. X., Guo F., Qi J. S. (2008). Alpha4beta2 nicotinic acetylcholine receptors are required for the amyloid beta protein-induced suppression of long-term potentiation in rat hippocampal CA1 region in vivo. Brain Res. Bull. 77, 84–90. doi: 10.1016/j.brainresbull.2008.06.005. [PubMed] [Cross Ref]
47. Yakel J., Shao Z. (2004). Functional and molecular characterization of neuronal nicotinic ACh receptors in rat hippocampal interneurons. Prog. Brain Res. 145, 95–107. doi: 10.1016/S0079-6123(03)45006-1. [PubMed] [Cross Ref]

48. Young G. T., Zwart R., Walker A. S., Sher E., Millar N. S. (2008). Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. Proc. Natl. Acad. Sci. U.S.A. 105, 14686–14691. doi: 10.1073/pnas.0804372105. [PMC free article] [PubMed] [Cross Ref]
49. Zappettini S., Grilli M., Salamone A., Fedele E., Marchi M. (2010). Pre-synaptic nicotinic receptors evoke endogenous glutamate and aspartate release from hippocampal synaptosomes by way of distinct coupling mechanisms. Br. J. Pharmacol. 161, 1161–1171. doi: 10.1111/j.1476-5381.2010.00958.x. [PMC free article] [PubMed] [Cross Ref]
50. Zappettini S., Mura E., Grilli M., Preda S., Salamone A., Olivero G., Govoni S., Marchi M. (2011). Different presynaptic nicotinic receptor subtypes modulate in vivo and in vitro the release of glycine in the rat hippocampus. Neurochem. Int. 59, 729–738. doi: 10.1016/j.neuint.2011.06.022. [PubMed] [Cross Ref]
51. Zhao P., Qian H., Xia Y. (2005). GABA and glycine are protective to mature but toxic to immature rat cortical neurons under hypoxia. Eur. J. Neurosci. 22, 289–300. doi: 10.1111/j.1460-9568.2005.04222.x. [PubMed] [Cross Ref]

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.9

Neurochemistry International 62 (2013) 354–359

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Rapid communication

Effects of the neoclerodane Hardwickiic acid on the presynaptic opioid receptors which modulate noradrenaline and dopamine release in mouse central nervous system

Anna Pittaluga [a,c], Guendalina Olivero [a], Silvia Di Prisco [a], Elisa Merega [a], Angela Bisio [b], Giovanni Romussi [b], Massimo Grilli [a], Mario Marchi [a,c,*]

[a] Department of Pharmacy, Pharmacology and Toxicology Section, University of Genoa, Italy
[b] Department of Pharmacy, University of Genoa, Italy
[c] Center of Excellence for Biomedical Research, University of Genoa, Italy

ARTICLE INFO

Article history:
Received 5 October 2012
Received in revised form 15 January 2013
Accepted 18 January 2013
Available online 26 January 2013

Keywords:
Dopamine release
Hardwickiic acid
Noradrenaline release
Opioid receptors
Salvinorin A
Nerve endings

ABSTRACT

We have comparatively investigated the effects of Hardwickiic acid and Salvinorin A on the K^+-evoked overflow of [^{3}H]noradrenaline ([^{3}H]NA) and [^{3}H]dopamine ([^{3}H]DA) from mouse hippocampal and striatal nerve terminals, respectively. The K^+-evoked overflow of [^{3}H]DA was inhibited in presence of Salvinorin A (100 nM) but not in presence of Hardwickiic acid (100 nM). Hardwickiic acid (100 nM) mimicked Salvinorin A (100 nM) in facilitating K^+-evoked hippocampal [^{3}H]NA overflow and the two compounds were almost equipotent. Facilitation of [^{3}H]NA overflow caused by 100 nM Hardwickiic acid was prevented by the κ-opioid receptor (KOR) antagonist norbinaltorphimine (norBNI, 100 nM) and by the selective δ-opioid receptor (DOR) antagonist naltrindole (100 nM), but was not altered by 100 nM D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), a selective μ-opioid receptor (MOR) antagonist. We conclude that Hardwickiic acid modulates hippocampal [^{3}H]NA overflow evoked by a mild depolarizing stimulus by acting at presynaptic opioid receptor subtypes.

1. Introduction

Selective κ-opioid receptor (KOR) ligands are molecules attractive for their potential use as therapeutics for the cure of central nervous system (CNS) diseases including mood disorders, drug abuse, anxiety, hypoxia and pain (Carlezon et al., 2009; Wu et al., 2012).

Among the drugs showing agonist profile at this receptor subtype, Salvinorin A, a neoclerodane terpenoid from *Salvia divinorum* leaves, has attracted the interest of scientist representing a natural, non alkaloid, selective ligand for this molecular target. The unique structural and biological characteristics of this compound make it an interesting probe to exploring opioid pharmacology. Inasmuch, it can represent a potential template to obtain new semi-synthetic molecule(s) [see for instance the recent case of the semi-synthetic compound herkinorin (Butelman et al., 2008)], possibly devoid of allucinogenic properties, to be tested for their efficacy and potency in human therapies.

The interaction of Salvinorin A at presynaptic KOR receptors controlling the release of catecholamines from nerve terminals isolated from the hippocampus and the striatum of adult mice has been recently investigated (Grilli et al., 2009). These observations indicate that Salvinorin A could bind with high affinity and efficacy to pure KOR receptors presynaptically located on striatal dopaminergic nerve endings, whose activation causes a significant reduction of the Ca^{2+}-dependent release of [^{3}H]dopamine ([^{3}H]DA) caused by a mild depolarizing stimulus (12 mM KCl; Sandor et al., 1992; Svingos et al., 1999; Grilli et al., 2009). These findings are well in agreement with other data present in literature (Roth et al., 2002; Butelman et al., 2009). Unexpectedly, the inhibitory effect caused by Salvinorin A on DA overflow turns to facilitation when studying this compound on the 12 mM KCl-induced release of [^{3}H]noradrenaline ([^{3}H]NA) from hippocampal nerve terminals. Based on the pharmacological profile described using selective opioid receptor (OR) ligands, we proposed that facilitation of NA overflow relied on the activation of presynaptic OR subtypes having

Abbreviations: CNS, central nervous system; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; DA, dopamine; DMSO, dimethyl sulfoxide; DOR, δ-opioid receptor; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NA, noradrenaline; nor BNI, norbinaltorphimine; OR, opioid receptor; PTx, pertussis toxin.

* Corresponding author. Address: Department of Pharmacy, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4, 16148 Genova, Italy. Tel.: +39 010 3532657; fax: +39 010 3993360.
E-mail address: marchi@pharmatox.unige.it (M. Marchi).

0197-0186/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2013.01.016

different pharmacological profile from those located on dopaminergic terminals (Grilli et al., 2009).

Interestingly, semi-synthetic derivatives of Salvinorin A [i.e. herkinorin (Butelman et al., 2008), 12-epi-Salvinorin A (Béguin et al., 2012), C-12 triazole derivatives (Yang et al., 2009)] as well as new neoclerodane diterpenoid congeners of Salvinorin A from *Salvia splendens* (Fontana et al., 2008) were found to retain opioid activity, although structural modifications of the Salvinorin A scaffold correlated with changes in efficacy and potency of the new derivatives at opioid receptor subtypes.

The natural neoclerodane molecule Hardwickiic acid has been isolated from the surface exudate of aerial part of *Salvia wagneriana* and identified by comparison of their spectral data with those previously reported in literature and on the basis of ^{1}H–^{1}H-COSY, TOCSY, HSQC and HMBC correlations (Bisio et al., 2004). This natural substance is a clerodane diterpene with a modified Salvinorin A scaffold lacking substituents at C-1, C2, the 17, 12-olide ring, with double bond in a ring and a free 18 carboxylic group (Fig. 1). Taking into account the structural similarity between Salvinorin A and the Hardwickiic acid, we speculated that this compound could mimic Salvinorin A at presynaptic opioid receptors in CNS. To investigate this hypothesis, experiments were carried out to determine the effect of the latter natural compound on the presynaptic ORs modulating the release of DA and NA in CNS and compared its activity with that of Salvinorin A. Interestingly, we found that Hardwickiic acid modulates hippocampal NA overflow evoked by a mild depolarizing stimulus by acting at a specific presynaptic ORs. The pharmacological profile of the receptor involved in the effect of Hardwickiic acid suggested the existence, on noradrenergic terminals, of both KOR and DOR receptor subtypes (κ-δ receptor). Based on our functional results, we proposethat Hardwickiic acid could bind the DOR subunit component of the presynaptic κ-δ receptor heterodimers.

2. Materials and methods

2.1. Animals and brain tissue preparation

Adult male mice (Swiss; 20–25 g) were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) on a 12 h light/dark schedule (7.00 a.m.–7.00 p.m.). Food and water were freely available. The animals were killed by decapitation and then the hippocampus and the striatum rapidly removed. The experimental procedures were approved by the Department Ethical Committee, in accordance with the European legislation (European Communities Council directive of 24 November 1986, 86/609/EEC). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health.

Crude synaptosomes were prepared by homogenizing brain tissues in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was first centrifuged at 1000*g* for 5 min to remove nuclei and cellular debris, while synaptosomes were isolated by centrifugation at 13000*g* for 20 min. In a set of experiments, the tissue was homogenized in buffered sucrose containing 5 nM Pertussis Toxin (PTx), in order to entrap this agent into subsequently isolated synaptosomes (Di Prisco et al., 2012). This experimental approach (we refer to as entrapping technique, Grilli et al., 2009) was previously shown to successfully entrap compounds into subsequently isolated synaptosomes and allows to avoid excessively long incubations with PTx.

The synaptosomal pellets were always resuspended in a physiological solution (standard medium) with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 10; HEPES 10; pH adjusted to 7.2–7.4 with NaOH.

2.2. Superfusion of isolated nerve endings

The experimental technique used (referred to as the up–down superfusion of a monolayer of synaptosomes) is considered an approach of choice to investigate the existence and the functional role of presynaptic receptors (Grilli et al., 2012). The continuous superfusion of the synaptosomal monolayer assures the removal of any endogenous compounds released by a single particle, therefore impeding auto or hetero-regulation of synaptic functional events. Furthermore, the selective labelling with radioactive markers allows to monitor the release of defined neurotransmitters from specific synaptosomal families, including those that represent a minimal percentage (less than 1%) of the total synaptosomal population, like the hippocampal noradrenergic and serotonergic ones (Grilli et al., 2009). It's worth reminding that when monitoring the release of preloaded tritiated transmitters, the spontaneous release of these molecules is expected to decrease during time because of the continuous depletion of the intravesicular store of radioactive molecules. Inasmuch, this experimental approach is also considered a method of choice to investigate the functional consequences

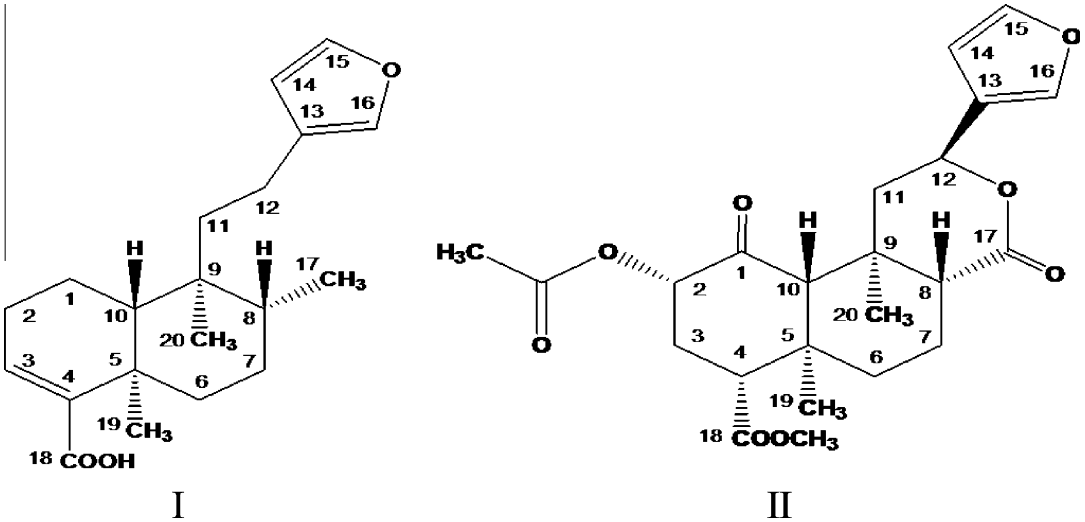


Fig. 1. Chemical formulas. I: Hardwickiic acid; II: Salvinorin A.

due to activation of ionotropic and metabotropic receptors located presynaptically (Grilli and Marchi, 2010; Summa et al., 2011).

Hippocampal synaptosomes were labeled with [^{3}H]NA (final concentration 30 nM), in the presence of 0.1 μM 6-nitroquipazine, a selective 5-HT uptake inhibitor, to avoid false labelling of serotonergic nerve endings. Striatal synaptosomes were labeled with [^{3}H]DA (final concentration 50 nM) in the presence of 0.1 μM 6-nitroquipazine and 0.1 μM desimipramine (to avoid false labeling of serotonergic and noradrenergic terminals). After the labelling period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a Superfusion System (Ugo Basile, Comerio, Varese, Italy) maintained at 37 °C and synaptosomes were then superfused at 0.5 ml/min with standard a physiological solution as above.

When studying the effects on the release of [^{3}H]NA and [^{3}H]DA evoked by high K^+, synaptosomes were transiently (90 s) exposed, at t = 39 min, to 12 mM KCl-containing medium (NaCl substituting for an equimolar concentration of KCl). Salvinorin A and Hardwickiic acid were added concomitantly to the depolarizing stimulus. In a set of experiments aimed at investigating whether the Hardwickiic acid could antagonize the Salvinorin A-mediated effects, this compound was added starting from 8 min before the stimulus was applied. When indicated, opioid receptor antagonists were added starting from 8 min before stimulus. Four three minutes fractions (namely b1–b4) were collected starting from t = 36 min of superfusion. Fractions collected and superfused synaptosomes were counted for measuring [^{3}H]NA and [^{3}H]DA content. The amount of [^{3}H]NA and [^{3}H]DA released into each superfusate fraction was expressed as percentage of the total synaptosomal [^{3}H]NA and [^{3}H]DA content, respectively present at the start of the fraction collected (fractional efflux). The K^+-induced [^{3}H]NA and [^{3}H]DA overflow was expressed as "induced overflow", and was estimated by subtracting the content of [^{3}H]NA or [^{3}H]DA into the first and the fourth fractions collected (basal release) from that found into the b2 and b3 fractions collected during and after the depolarization pulse [evoked release = (b2 + b3)−(b1 + b4)].

2.3. Statistical analysis

Analysis of variance was performed by one-way analysis of variance ANOVA followed by Dunnett's test for multiple comparisons (software Graph Pad Prism version 4.03). Data were considered significant for $p < 0.05$ at least. Appropriate controls with antagonist were always run in parallel.

2.4. Chemicals

1-[7,8 ^{3}H]-noradrenaline ([^{3}H]NA, specific activity 39 Ci/mmol), [7,8-^{3}H]-dopamine ([^{3}H]DA, specific activity 43 Ci/mmol) were purchased from Amersham Radiochemical Center (Buckinghamshire, UK); Salvinorin A (2*S*, 4*aR*, 6*aR*, 7*R*, 9*S*, 10*aS*, 10*aS*, 10*bR*)-9-(acetyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*h*-napht o[2,1-c]pyran-7-carboxylic acid methyl ester, purity >99%) was purchased from Ascent Scientific (Weston Super-Mare, UK). D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), naltrindole-HCl, norbinaltorphimine (norBNI) and Pertussis Toxin were purchased from Sigma Chemical Co (St. Louis, MO, USA). Salvinorin A was solubilized in dimethyl sulfoxide (DMSO, final concentration 10 mM), subsequently diluted with standard physiological medium to 100 μM. Hardwickiic acid was provided by Dr. G. Romussi.

3. Results

Aimed at comparing the central effects of the natural terpenoid Hardwickiic acid with those elicited by Salvinorin A, we investigated the effects of the two compounds on the overflow of [^{3}H]NA and [^{3}H]DA elicited by a transient (90 s) exposure of synaptosomes to 12 mM KCl. It has been already reported that this mild depolarizing stimulus can evoke a Ca^{2+}-dependent exocytotic release of both neurotransmitters (Raiteri et al., 1982; Maura et al., 1985). Fig. 2A shows that Hardwickiic acid (100 nM) mimicked Salvinorin A (100 nM) in facilitating K^+-evoked [^{3}H]NA overflow. Hardwickiic acid and Salvinorin A were almost equipotent in their effect (100 nM Hardwickiic acid: +34.14 ± 3.76; 100 nM Salvinorin A: +38.45 ± 2.88, results reported as percent of increase). Moreover, at the maximum concentration applied (100 nM), the two terpenoids added together did not produce any further increase of the [^{3}H]NA evoked overflow (Fig. 2A; 100 nM Hardwickiic acid plus 100 nM Salvinorin A: +36.90 ± 4.01 reported as percent of change), nor Hardwickiic acid prevented the Salvinorin A-induced facilitation of [^{3}H]NA exocytosis, compatible with the idea that the two natural drugs could share a common mechanism of action. Noticeably, in the experiments where Hardwickiic acid was added starting from 8 min before the K^+ stimulus, the natural clerodane did not affect, on its own, the spontaneous release of tritium before the depolarizing stimulus was applied (+100 nM Hardwickiic acid: +0.83 ± 1.27, results reported as percent of increase), suggesting that membrane depolarization is an essential prerequisite to detect the Hardwickiic acid-mediated modulation of amine release.

Differently from what observed studying [^{3}H]NA overflow, the 12 mM K^+-evoked overflow of [^{3}H]DA from striatal terminals was significantly inhibited in presence of Salvinorin A as previously described (Fig. 2B; 100 nM Salvinorin A: −29.65 ± 3.32, results reported as percent of change, but see Grilli et al., 2009). On the contrary Hardwickiic acid, applied at the same concentration used on [^{3}H]NA overflow (100 nM), did not modify the K^+-evoked overflow of [^{3}H]DA nor it prevented the Salvinorin A-induced inhibition of [^{3}H]DA overflow (Fig. 2B, +100 nM Hardwickiic acid: +6.97 ± 2.85, results reported as percent of change). It has been already reported that Salvinorin A, at the concentrations used in this paper, could not modify the spontaneous release of [^{3}H]NA and [^{3}H]DA from mice hippocampal and striatal nerve endings (Grilli et al., 2009). No significant changes in the spontaneous release of dopamine could be observed when synaptosomes were exposed to 100 nM Hardwickiic acid (data not shown).

The lack of effect of Hardwickiic acid on the K^+-evoked [^{3}H]DA overflow might suggest that this terpenoid is able to discriminate between the two different opioid receptor subtypes involved in the presynaptic control of NA and DA release in mouse CNS (Grilli et al., 2009). To further confirm this hypothesis we investigated the effects of some specific OR antagonists on the Hardwickiic acid-induced stimulatory effect on NA terminals. The effects of norbinaltorphimine (norBNI), a selective KOR antagonist, of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), a selective μ opioid receptor (MOR) antagonist, and of naltrindole, a selective DOR antagonist, were investigated on the Hardwickiic acid-induced potentiation of the K^+-evoked NA overflow. Fig. 2C shows that facilitation of [^{3}H]NA overflow caused by 100 nM Hardwickiic acid was prevented by 100 nM norBNI and 100 nM naltrindole but was not significantly affected by 100 nM CTAP clearly demonstrating a presynaptic effect of this molecule on OR which modulate [^{3}H]NA release. At the concentration applied these three antagonists did not modify by their own the 12 mM KCl-evoked [^{3}H]NA release. Finally, well consistent with the involvement of Pertussis sensitive (PTx) G protein coupled opioid receptors in the Hardwickiic acid-mediated facilitation of [^{3}H]NA exocytosis, we found that entrapped PTx significantly reduced the K^+-evoked [^{3}H]NA exocytosis elicited by 12 mM K^+ from hippocampal synaptosomes (control synaptosomes: 6.01 ± 0.48; PTx entrapped synaptosomes: 4.63 ± 0.36 $p < 0.05$; $n = 3$, data expressed as induced overflow). Facilitation of the K^+-evoked [^{3}H]NA release induced by 100 nM

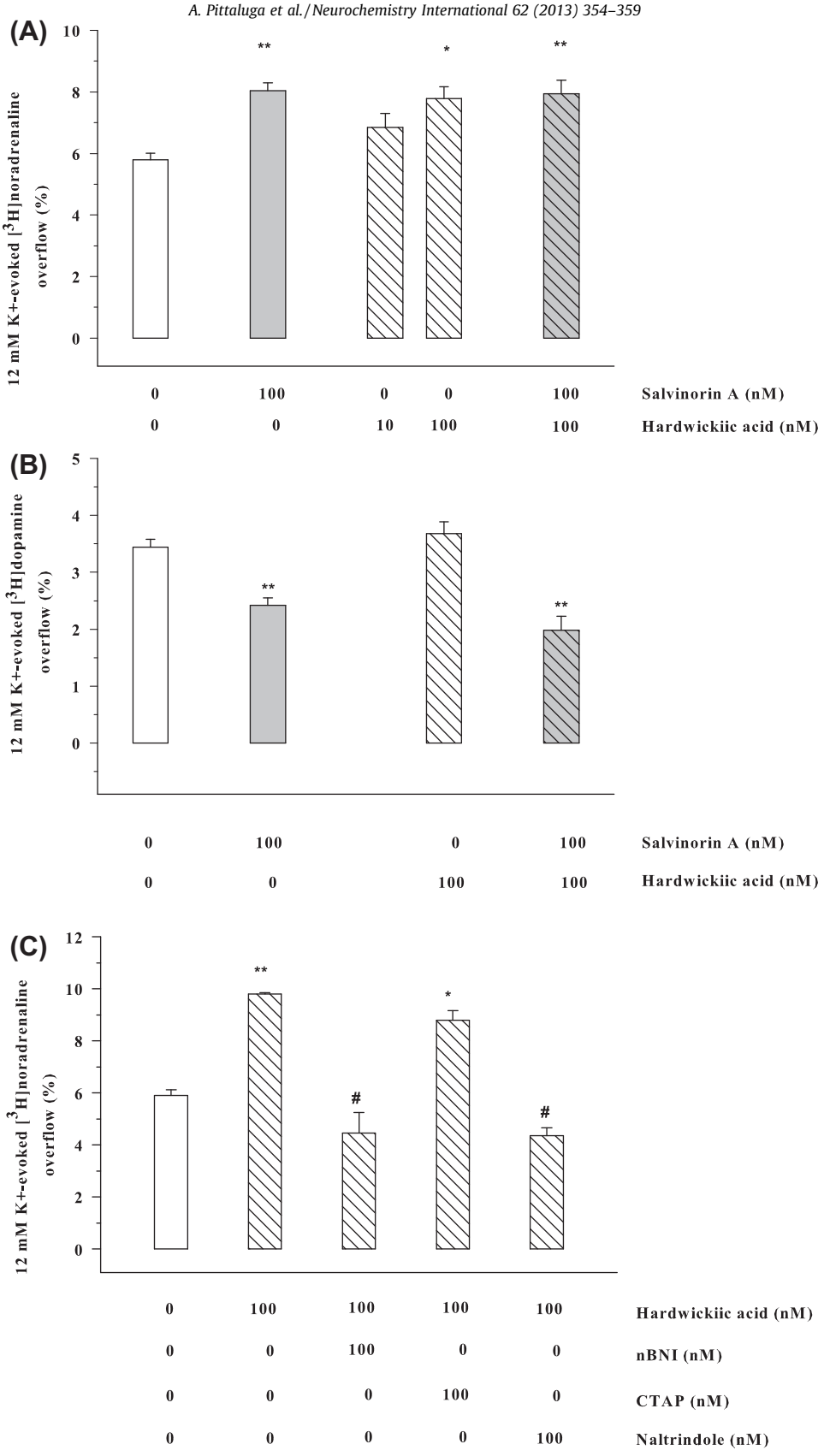


Fig. 2. Effects of Hardwickiic acid and Salvinorin A on the 12 mM K^+-evoked overflow of [^{3}H]NA (panel A) and [^{3}H]DA (panel B) from mice hippocampal and striatal synaptosomes, respectively. Synaptosomes preloaded with [^{3}H]NA or [^{3}H]DA were exposed to the depolarizing stimulus in the absence or in the presence of Salvinorin A, Hardwickiic acid or both. Results were expressed as induced overflow. Data are means ± SEM of four experiments run in triplicate (three superfusion chambers for each experimental condition). $^*p < 0.05$ vs 12 mM K^+; $^{**}p < 0.01$ vs 12 mM K^+. Effects of selective antagonists at MOR, DOR and KOR subtypes, norBNI (100 nM), CTAP (100 nM) and naltrindole (100 nM) on the Hardwickiic acid-induced facilitation of the 12 mM K^+-evoked overflow of [^{3}H]NA from mice hippocampal synaptosomes (panel C). Synaptosomes preloaded with [^{3}H]NA were exposed to the depolarizing stimulus in the absence or in the presence of Hardwickiic acid (100 nM). Antagonists were added eight minutes before agonists and were present throughout the superfusion period. Results were expressed as induced overflow. Data are means ± SEM of four experiments run in triplicate. $^*p < 0.05$ vs 12 mM K^+; $^{**}p < 0.01$ vs 12 mM K^+; $^{\#}p < 0.05$ vs 12 mM K^+ plus 100 nM Hardwickiic acid.

Hardwickiic acid could not be further observed (control synaptosomes, +100 nM Hardwickiic acid: 8.63 ± 0.78, $p < 0.05$ versus respective control; PTx entrapped synaptosomes, +100 nM Hardwickiic acid: 4.38 ± 0.54 *n.s.* versus respective control, $n = 3$, data expressed as induced overflow).

4. Discussion

The ORs in the CNS are heterogeneous and their pharmacological characteristics indicate that they can be classified as MOR, KOR, DOR and σ-opioid receptors which are no longer considered OR but rather target sites for phencyclidine and its analogs (Vizi, 1979; Vizi et al., 1991; Trescot et al., 2008). The main result of our study is that Hardwickiic acid, a non peptide natural neoclerodane molecule, has a role as presynaptic modulator of [^{3}H]NA overflow acting on a specific OR present on mouse hippocampal nerve endings. The finding that the Hardwickiic acid-induced increase of KCl-evoked [^{3}H]NA overflow was sensitive to norBNI and to naltrindole, but insensitive to CTAP (Fig. 2C) excludes the involvement of MORs being compatible with the idea that this compound might mimic Salvinorin A at κ-δ heterodimer (Sandor et al., 1992; Grilli et al., 2009). Indeed the involvement of these receptors has been proposed in the Salvinorin A-induced facilitation of [^{3}H]NA overflow while Salvinorin A-induced inhibition of [^{3}H]DA overflow was mediated by pure KORs (Sandor et al., 1992; Grilli et al., 2009). Since Hardwickiic acid did not mimic Salvinorin A in inhibiting [^{3}H]DA overflow (Fig. 2B), the possibility exists that this compound might act as an orthosteric agonist at the DOR component of presynaptic κ-δ heterodimer.

Orthosteric binding sites of receptor heterodimers usually cooperate to mediate receptor activity, so that the binding of orthosteric ligand at one binding site can alter the conformation of the second binding site modulating ligand-mediated responses (Milligan and Smith, 2007). If this is the case, pure KOR as well as DOR agonist (s) could favour κ-δ heterodimer-mediated effects (i.e. the facilitation of [^{3}H]NA overflow), that in turn could be prevented by either KOR or DOR orthosteric antagonists (i.e. norBNI and naltrindole, respectively), as indeed previously observed (Grilli et al., 2009). According to this view, we propose that Salvinorin A and Hardwickiic acid, acting, respectively as a KOR and DOR orthosteric agonists (Wu et al., 2012), could facilitate [^{3}H]NA overflow by acting at norBNI and naltrindole-sensitive presynaptic κ-δ receptor heterodimers (Grilli et al., 2009). As to the molecular events involved in the Hardwickiic acid-induced facilitation of noradrenaline overflow, the lack of efficacy of the natural compound on PTx synaptosomes suggests that this event could rely on intraterminal enzymatic pathway that positively reverberates on amine exocytosis. Increased cytosolic Ca^{2+} ions bioavailability due to phospholipase C-induced inositol 1,4,5 tryphosphate-mediated mobilization of calcium ions from intraterminals stores (Di Prisco et al., 2012) as well as increased calcium ions influx due to adenylyl cyclase-mediated opening of voltage operated calcium channels located on presynaptic synaptosomal plasma membranes (Grilli et al., 2004) could have a role: further studies are needed to address this aspect.

Based on the present findings, the possibility that Hardwickiic acid might represent the prototype of a new class of non peptidergic selective ligands of DOR subtypes should be considered and deserve future investigations. Since previous studies have shown that the binding of Salvinorin A to DOR receptor subtype is negligible (Wang et al., 2005), it is possible to speculate that the structural modification to the terpenoid structure in C2 and C4 might confer affinity to Hardwickiic acid for DOR receptor subtypes and it might account for the scarce interaction with KOR receptor subtypes. Accordingly, substitutes in the C2 and C4 positions were responsive of the interaction of Salvinorin A with tyrosine and isoleucine residues within the KOR binding pocket domain (Yan et al., 2005), while the C-ring opened compounds, such as Hardwickiic acid, showed reduced human KOR binding affinities (Lee et al., 2005).

There is increasing evidence suggesting a role for the opioid system in the control of pathophysiology of several neurological disorders. Among them, it seems that DOR modulation plays a major role in neuroprotection in both normoxic and hypoxic environments (Zhang et al., 2002). Consistent with this view, previous studies have shown that DOR activation attenuated the disruption of K^+ homeostasis induced by hypoxia in the cortex (Chao et al., 2007). Moreover, enkephalines, which are part of the endogenous opioid system, enhanced learning and memory, synaptic plasticity and emotional behaviours also by activating DOR (Nandhu et al., 2010). The availability of new non peptide molecule, such as Hardwickiic acid, active as selective DOR agonist could be therefore very important for the better understanding of the role of these receptors in the CNS and fundamental to guide the design of new ligands in order to develop drugs useful for therapeutic interventions in several CNS pathologies. Taking into account the importance of natural substances in medicine, for their possible therapeutic use, new molecules such as Hardwickiic acid, surely deserve further studies to unveil their in vivo pharmacological and toxicological profiles.

Acknowledgments

This work was supported by grants from Italian MIUR (Ministero dell'Istruzione, dell'Universita' e della Ricerca) Project n. 2009R7WCZS_003 to MM and Project n. 2009P7WHNR_003 to AP; from University of Genoa 'Progetto Ricerca Ateneo' 2011 to MM and AP; from ALCOTRA (Alpi Latine Cooperazione Transfrontaliera) – Project n. 231 «Developpement commercial des potentialite's du genre salvia: nouvelles productions» to MM and from the European Community Project PYRGI n. B51H10000000006 to AP. We wish to thank Maura Agate for careful editorial assistance and Silvia E. Smith, Ph.D. University of Utah, for reviewing the manuscript.

References

Béguin, C., Potuzak, J., Xu, W., Liu-Chen, L.Y., Streicher, J.M., Groer, C.E., Bohn, L.M., Carlezon, W.A., Cohen, B.M., 2012. Differential signaling properties at the kappa opioid receptor of 12-epi-salvinorin A and its analogues. Bioorg. Med. Chem. Lett. 22, 1023–1026.

Bisio, A., De Tommasi, N., Romussi, G., 2004. Diterpenoids from Salvia wagneriana. Planta Med. 70, 452–457.

Butelman, E.R., Rus, S., Simpson, D.S., Wolf, A., Prisinzano, T.E., Kreek, M.J., 2008. The effects of herkinorin, the first mu-selective ligand from a salvinorin A-derived scaffold, in a neuroendocrine biomarker assay in nonhuman primates. J. Pharmacol. Exp. Ther. 327, 154–160.

Butelman, E.R., Prisinzano, T.E., Deng, H., Rus, S., Kreek, M.J., 2009. Unconditioned behavioral effects of the powerful kappa-opioid hallucinogen salvinorin A in nonhuman primates: fast onset and entry into cerebrospinal fluid. J. Pharmacol. Exp. Ther. 328, 588–597.

Carlezon, W.A., Béguin, C., Knoll, A.T., Cohen, B.M., 2009. Kappa-opioid ligands in the study and treatment of mood disorders. Pharmacol. Ther. 123, 334–343.

Chao, D., Bazzy-Asaad, A., Balboni, G., Xia, Y., 2007. Delta-, but not mu-, opioid receptor stabilizes K^+ homeostasis by reducing Ca^{2+} influx in the cortex during acute hypoxia. J. Cell. Physiol. 212, 60–67.

Di Prisco, S., Summa, M., Chellakudam, V., Rossi, P.I., Pittaluga, A., 2012. RANTES-mediated control of excitatory amino acid release in mouse spinal cord. J. Neurochem. 121, 428–437.

Fontana, G., Savona, G., Rodríguez, B., Dersch, C.M., Rothman, R.B., Prisinzano, T.E., 2008. Synthetic studies of neoclerodane diterpenoids from Salvia splendens and evaluation of Opioid Receptor affinity. Tetrahedron 64, 10041–10048.

Grilli, M., Raiteri, L., Pittaluga, A., 2004. Somatostatin inhibits glutamate release from ouse cerebrocortical nerve endings trough presynaptic sst2 receptor linked to the adenylyl cyclase-protein linase a pathway. Neuropharmacology 46, 388–396.

Grilli, M., Marchi, M., 2010. Presynaptic nicotinic receptors modulating neurotransmitter release in the central nervous system: functional interactions with other coexisting receptors. Prog. Neurobiol. 92, 105–111.

Grilli, M., Neri, E., Zappettini, S., Massa, F., Bisio, A., Romussi, G., Marchi, M., Pittaluga, A., 2009. Salvinorin A exerts opposite presynaptic controls on neurotransmitter exocytosis from mouse brain nerve terminals. Neuropharmacology 57, 523–530.

Grilli, M., Summa, M., Salamone, A., Olivero, G., Zappettini, S., Di Prisco, S., Feligioni, M., Usai, C., Pittaluga, A., Marchi, M., 2012. In vitro exposure to nicotine induces endocytosis of presynaptic AMPA receptors modulating dopamine release in rat nucleus accumbens nerve terminals. Neuropharmacology 63, 916–926.

Lee, D.Y., Ma, Z., Liu-Chen, L.Y., Wang, Y., Chen, Y., Carlezon Jr., W.A., Cohen, B., 2005. New neoclerodane diterpenoids isolated from the leaves of Salvia divinorum and their binding affinities for human kappa opioid receptors. Bioorg. Med. Chem. 13, 5635–5639.

Maura, G., Gemignani, A., Raiteri, M., 1985. Alpha 2-adrenoceptors in rat hypothalamus and cerebral cortex: functional evidence for pharmacologically distinct subpopulations. Eur. J. Pharmacol. 116, 335–339.

Milligan, G., Smith, N.J., 2007. Allosteric modulation of heterodimeric G-protein-coupled receptors. Trends Pharmacol. Sci. 28, 615–620.

Nandhu, M.S., Naijil, G., Smijin, S., Jayanarayanan, S., Paulose, C.S., 2010. Opioid system functional regulation in neurological disease management. J. Neurosci. Res. 88, 3215–3221.

Raiteri, M., Marchi, M., Maura, G., 1982. Presynaptic muscarinic receptors increase striatal dopamine release evoked by 'quasi-physiological' depolarization. Eur. J. Pharmacol. 83, 127–129.

Roth, B.L., Baner, K., Westkaemper, R., Siebert, D., Rice, K.C., Steinberg, S., Ernsberger, P., Rothman, R.B., 2002. Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist. Proc. Natl. Acad. Sci. USA 99, 11934–11939.

Sandor, N.T., Lendvai, B., Vizi, E.S., 1992. Effect of selective opiate antagonists on striatal acetylcholine and dopamine release. Brain Res. Bull. 29, 369–373.

Summa, M., Di Prisco, S., Grilli, M., Marchi, M., Pittaluga, A., 2011. Hippocampal AMPA autoreceptors positively coupled to NMDA autoreceptors traffic in a constitutive manner and undergo adaptative changes following enriched environment training. Neuropharmacology 61, 1282–1290.

Svingos, A.L., Colago, E.E., Pickel, V.M., 1999. Cellular sites for dynorphin activation of kappa-opioid receptors in the rat nucleus accumbens shell. J. Neurosci. 19, 1804–1813.

Trescot, A.M., Datta, S., Lee, M., Hansen, H., 2008. Opioid pharmacology. Pain Phys. 11, S133–S153.

Vizi, E.S., 1979. Presynaptic modulation of neurochemical transmission. Prog. Neurobiol. 12, 181–290.

Vizi, E.S., Kiss, J., Elenkov, I.J., 1991. Presynaptic modulation of cholinergic and noradrenergic neurotransmission: interaction between them. News Physiol. Sci. 6, 119–123.

Wang, Y., Tang, K., Inan, S., Siebert, D., Holzgrabe, U., Lee, D.Y., Huang, P., Li, J.G., Cowan, A., Liu-Chen, L.Y., 2005. Comparison of pharmacological activities of three distinct kappa ligands (Salvinorin A, TRK-820 and 3FLB) on kappa opioid receptors in vitro and their antipruritic and antinociceptive activities in vivo. J. Pharmacol. Exp. Ther. 312, 220–230.

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G.W., Vardy, E., Liu, W., Thompson, A.A., Huang, X.P., Carroll, F.I., Mascarella, S.W., Westkaemper, R.B., Mosier, P.D., Roth, B.L., Cherezov, V., Stevens, R.C., 2012. Structure of the human κ-opioid receptor in complex with JDTic. Nature 485, 327–332.

Yan, F., Mosier, P.D., Westkaemper, R.B., Stewart, J., Zjawiony, J.K., Vortherms, T.A., Sheffler, D.J., Roth, B.L., 2005. Identification of the molecular mechanisms by which the diterpenoid salvinorin A binds to kappa-opioid receptors. Biochemistry 44, 8643–8651.

Yang, L., Xu, W., Chen, F., Liu-Chen, L.Y., Ma, Z., Lee, D.Y., 2009. Synthesis and biological evaluation of C-12 triazole and oxadiazole analogs of salvinorin A. Bioorg. Med. Chem. Lett. 19, 1301–1304.

Zhang, J., Gibney, G.T., Zhao, P., Xia, Y., 2002. Neuroprotective role of delta-opioid receptors in cortical neurons. Am. J. Physiol. Cell. Physiol. 282, C1225–C1234.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.10

Research Article

Received: 8 August 2011 Revised: 10 December 2011 Accepted: 4 April 2012 Published online in Wiley Online Library: 16 May 2012

(wileyonlinelibrary.com) DOI 10.1002/jsfa.5724

Effect of maturation and cold storage on the organic acid composition of myrtle fruits

Maurizio Mulas,[a]* Angela Fadda[b] and Alberto Angioni[c]

Abstract

BACKGROUND: The effect of maturation and senescence on the chemical composition of two myrtle cultivars was studied in mature, overripe and cold-stored fruits in order to find the most appropriate harvesting period and best storage technology for industrial purposes.

RESULTS: After cold storage at 10 °C for 15 days, berry weight loss ranged from 12.5 to 18.4%, with the highest losses in less mature fruits. Titratable acidity decreased during maturation and cold storage in both cultivars. Reducing and total sugars increased during maturation. Anthocyanin concentration increased during maturation but decreased in overripe berries. The major organic acids in myrtle fruits were quinic, malic and gluconic acids. In fresh and cold-stored fruits, malic acid rose to 3 g kg^{-1} and decreased thereafter. Quinic acid peaked at 90 or 120 days after bloom and decreased thereafter to reach low concentrations in mature fruits.

CONCLUSION: Cold storage for 15 days at 10 °C does not affect myrtle fruit quality for liqueur production. Anthocyanin concentration is the best indicator of harvest time for industrial purposes. Gluconic acid concentration is high in mature, overripe and cold-stored berries. This parameter can be used as a marker of the onset of fruit senescence.

Keywords: *Myrtus communis*; postharvest storage; anthocyanins; total phenols; gluconic acid

INTRODUCTION

Myrtle (*Myrtus communis* L.) is a widespread Mediterranean shrub widely known in folk medicine for its anti-inflammatory and antiseptic properties.[1,2] Despite various applications of this species, in Sardinia, myrtle leaves and berries are mainly used for the production of a liqueur.[3–5] Owing to its organoleptic properties,[6–8] myrtle liqueur is one of the most typical Sardinian products and is now exported all over the world. For liqueur production, berries are harvested in December when fully pigmented and are then processed immediately. Usually, the hydroalcoholic extracts obtained are stored for no longer than 1 year, since most of the compounds responsible for the organoleptic properties are poorly stable, decreasing at the end of 1 year of storage.[9,10] This, together with the increasing success of the liqueur and the availability of the raw material for only short periods, has increased the need to improve storage strategies to maintain the quality of fresh product. Studies have been focused mainly on the effect of different technologies applied during maceration to retain myrtle liqueur quality,[6,9] while little is known about the best storage conditions to be applied to keep berry quality for long periods, thus delaying the processing season. Angioni *et al.*[11] reported that storage at 2 °C for 3 months appeared to be an effective way to preserve myrtle berry quality, while frozen storage allowed the maintenance of good-quality berries for up to 6 months of storage.[12] Moreover, Montoro *et al.*[10] reported that berry extracts stored for 8–12 months at ambient temperature in the dark produced myrtle liqueur of poor quality compared with that obtained with fresh berries. Myrtle chemical composition during fruit development has been studied previously,[13–15] but no data could be found in the literature correlating the changes in chemical characteristics of myrtle berries during maturity and harvest time with storage. However, the possibility of using organic acid concentration as an indicator of senescence of myrtle berries has been reported previously.[11,12] Understanding the various changes in fruit biochemistry that occur during ripening and after storage will help to find the best period for harvest and to provide indicators of the freshness of berries used in the infusion. The aim of this work was to study the effect of harvest time and storage on the quality traits of myrtle berries from two different cultivars. Specifically, we investigated the concentrations of major organic acids, total anthocyanins, total phenols and tannins as well as other basic physicochemical properties of fresh and processed berries, such as pH, fruit weight loss, titratable acidity and reducing sugars, in order to find the most appropriate harvesting period and best storage conditions for industrial purposes and to provide indicators of fruit senescence.

* *Correspondence to: Maurizio Mulas, Department of Economics and Tree Systems, University of Sassari, Via De Nicola 9, I-07100 Sassari, Italy. E-mail: mmulas@uniss.it*

a *Department of Economics and Tree Systems, University of Sassari, Via De Nicola 9, I-07100 Sassari, Italy*

b *Institute of Sciences of Food Production. National Research Council. Traversa La Crucca 3, Località Baldinca Li Punti, I-07100 Sassari, Italy*

c *Department of Pharmaceutical Chemistry and Technology, University of Cagliari, Via Ospedale 72, I-09124 Cagliari, Italy*

EXPERIMENTAL

Chemicals

All reagents and solvents were of analytical grade unless specified otherwise and were used without further purification. Methanol and ethanol (950 mL L^{-1}), Folin–Ciocalteu reagent, sodium carbonate, catechin and malvidine were purchased from Aldrich (Milan, Italy), while absolute ethanol was obtained from Carlo Erba (Rodano, Italy).

Fruit material

The experiment was carried on 'Barbara' and 'Daniela' myrtle cultivars, previously described and tested for horticultural and technological quality.[16,17] Berries were randomly harvested from 7-year-old plants grown in an experimental orchard located in Alghero (40°39′ N, 8°21′ E, 39 m above sea level). Myrtle berries were collected starting from 90 days after full bloom till 210 days after full bloom, with sampling every 30 days, covering the period between fruit maturity (from 120 to 180 days) and senescence (from 180 to 210 days).

At each sampling date, six berry replicates of 100 g each were collected for each cultivar. Three replicates were immediately lyophilised and used for chemical composition assessment of fresh fruits, while the other three replicates were stored at 10 °C and 90% relative humidity (RH) for 15 days. Temperature and time corresponded to the most common conditions of postharvest storage empirically applied to myrtle berries before processing for liqueur production. After cold storage, fruits were weighed for weight loss assessment, lyophilised and analysed for chemical composition assessment. All data were expressed on a dry weight (DW) basis.

Titratable acidity, reducing and total sugars

Titratable acidity was measured in cold-stored and fresh fruits by titrating 10 g of ground fruits (blended with 40 mL of distilled water) with 0.1 mol L^{-1} NaOH to pH 8.2. Results were expressed as g malic acid kg^{-1}.

Sugars were analysed by diluting 10 g of ground fruits with 50 mL of a saturated solution of calcium carbonate and leaving overnight. Clarification of the solution was performed by adding 10 mL of lead acetate and subsequently 10 mL of sodium oxalate. The solution was then filtered and used to determine reducing and total sugars. Reducing and total sugars were assessed according to the Fehling method and results were expressed as g kg^{-1}. Briefly, a standard solution with 5 mL of Fehling A, 5 mL of Fehling B and 40 mL of distilled water was heated and titrated by adding the sugar solution previously prepared, until complete colour turning. Total sugar content was analysed according to the Fehling method after inversion of non-reducing sugars in an acid environment. Inversion was performed by adding 5 mL of HCl and heating the sugar solution for 15 min in a thermostatic bath at 67–70 °C. After rapid cooling, several drops of phenolphthalein and NaOH were added until the solution turned pink. Before the determination with Fehling solution as described above, several drops of 0.5 mol L^{-1} acetic acid were added to clarify the sugar solution.

Total phenols, tannins and anthocyanins

Phenolic compounds were extracted according to Franco *et al.*,[3] with some minor differences, and determined spectrophotometrically with a Cary 1E spectrophotometer (Varian, Palo Alto, CA, USA). Briefly, 10 g of lyophilised sample (fresh or stored fruits) was weighed into a screw-capped flask and diluted with 100 mL of acidified methanol (1 mL L^{-1} HCl). The flask was stored in the dark for 1 h. The extract was then filtered, made up to a volume of 100 mL with acidified methanol and stored at 4 °C in the dark until analysis. For total anthocyanin content determination, 1 mL of methanolic extract (diluted according to the expected absorbance value) was added to a reaction solution containing 1 mL of acidified ethanol and 10 mL of 0.5 mol L^{-1} HCl. After 30 min of incubation the absorbance at 525 nm was read against a blank prepared with 1 mL of methanolic extract, 1 mL of acidified ethanol and 10 mL of a buffer solution at pH 3.5. Total anthocyanin content was expressed as g malvidine kg^{-1}.

Tannins were assayed in a reaction solution containing 4 mL of diluted (1 : 25 v/v) methanolic extract, 2 mL of ethanol and 4 mL of vanillin solution (10 mL L^{-1} vanillin in 700 mL L^{-1} H_2SO_4). Samples were compared with a control containing 4 mL of H_2SO_4 instead of vanillin solution, and the absorbance at 500 nm was detected. Tannin concentrations (g kg^{-1}) were calculated from a calibration curve constructed using pure catechin solutions of known concentrations.

Total phenolic content was measured by the Folin–Ciocalteu colorimetric method.[3] The absorbance at 750 nm was read and total phenols were expressed as g kg^{-1}.

Extraction and determination of organic acids in myrtle fruit

Fruit samples (2.5 g) were homogenised in methanol (20 mL) and left in the dark for 16 h. Each mixture was then filtered and 500 μL of the extract was dried under nitrogen flow and redissolved in 1 mL of ultrapure water. Before analysis using a high-performance liquid chromatograph (HPLC) with a diode array detector (DAD), the solutions were filtered through Acrodisc PTFE 0.45 μm filters (Sigma-Aldrich, Milan, Italy).

The extracts were separated and detected using an HPLC (1100 Series, Agilent, Waldbronn, Germany) equipped with an online degasser (G1322A), quaternary pump (G1311A) and autosampler (G1313A) and coupled with a DAD (G1315A). Isocratic separation of the compounds was done on an Allure Organic Acids column (5 μm, 4.6 mm × 300 mm; Restek; Superchrom, Milan, Italy). Formic acid (0.85 mL L^{-1}) at flow rate of 0.4 mL min^{-1} was used as mobile phase. A calibration graph was constructed using the external standard method by measuring peak areas *versus* concentrations. Good linearity was achieved in the 0.008–1 g L^{-1} range, with correlation coefficients between 0.9989 and 0.9993. The injection volume was 50 μL. Data were expressed as g kg^{-1} dry fruit.

Statistical analysis

Analysis of variance was carried out using the microcomputer statistical program MSTAT-C (Michigan State University, East Lansing, MI, USA). Mean separation was performed by application of Duncan's multiple range test.

RESULTS AND DISCUSSION

Fruit weight loss

Myrtle berry fruit weight loss showed a similar behaviour for the two cultivars during maturation and cold storage, with higher values at the first harvest and thereafter a decrease at the second harvest and almost steady values till the end of the experiment (Fig. 1). In 'Barbara' fruits the lowest weight loss (13.34%) was measured in berries harvested 150 days after full bloom, while in 'Daniela' fruits the lowest value (12.73%) was reached at 210 days after full bloom. Fadda and Mulas[14] reported an increase in dry matter during fruit development and thus a reduction in fruit water

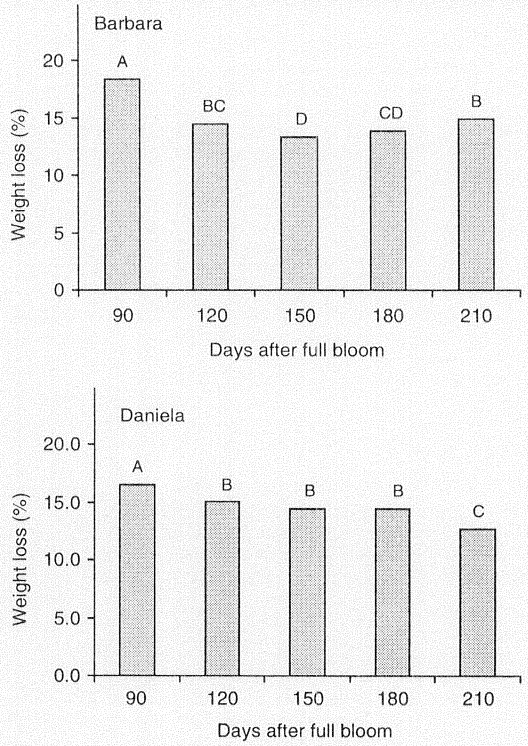


Figure 1. Weight loss of myrtle berries after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

content. On the other hand, studies on other fruit species have highlighted an increase in the amount of epicuticular waxes during fruit development,[18,19] thus reducing the transpiration rate. For these reasons, the decrease in weight loss may be associated with a lower water content of berries during ripening and with a diminished transpiration rate due to the increase in the epicuticular layer.

pH, titratable acidity, reducing and total sugars

Throughout ripening, pH values increased significantly in fresh fruits, ranging from 4.68 to 5.21 and from 4.48 to 5.26 in 'Barbara' and 'Daniela' cultivars respectively (Tables 1 and 2), while no differences were found between fresh and cold-stored fruits of both cultivars. The pH values in mature fruits are in agreement with previously reported data on myrtle berry fresh fruits.[5,11] On the other hand, a report on myrtle berry storage for 2 or 3 months at 2 °C showed a significant decrease in pH.[11] The authors suggested that the decrease in pH might be caused by an increase in gluconic acid and a decrease in other acids. Similar behaviour was recorded in frozen berries after 6 months, with lower pH values and higher amounts of organic acids.[12] High concentrations of organic acids and low pH are very important for fruit preservation and can be used as a marker of fruit quality.[11,12]

Titratable acidity (TA), expressed as g malic acid kg^{-1}, decreased significantly from 10.3 to 5.7 g kg^{-1} in 'Barbara' fruits during maturation, while in 'Daniela' fruits it decreased at a slow rate from 90 to 210 days after bloom (Tables 1 and 2). These results, comparable to those reported previously on the same cultivars, confirm the high variability of TA and highlight the low reliability of TA as a maturity index.[14] Storage produced only a slight further decrease in TA in both cultivars.

Reducing sugars increased during maturation, but no significant effects were observed as a consequence of postharvest storage (Tables 1 and 2). In 'Daniela' fruits, reducing sugars increased significantly up to 120 days after bloom and then levelled off until maturity. In 'Barbara' fruits an increase in reducing sugars was observed up to 180 days after bloom (74.1 g kg^{-1}), followed by a relatively constant level until overmaturation. From the third harvest, 'Barbara' fruits showed higher levels of reducing sugars than 'Daniela' fruits. Fifteen days of storage at 10 °C did not produce any significant modification in reducing sugar content. A previous study reported the progressive accumulation of reducing sugars in myrtle fruits after 3 months of cold storage at 2 °C, attributable to an increase in fructose content.[11] A slight increase in fructose concentration was found in strawberries after short-term cold storage (6 days at 6 °C).[20] In 'Fortune' mandarins, fructose and glucose showed a different behaviour, strictly related to the harvesting period and storage treatments.[21] In contrast, Singh *et al.*[22] reported that fruit maturity did not influence fructose and glucose concentrations in Japanese plums up to 7 weeks of storage at 0 °C. Therefore in myrtle fruits the different behaviour found in comparison with literature data might be related to the different storage conditions applied.

Total sugar concentrations ranged from 14.8 to 124.5 g kg^{-1} and from 16.0 to 121.6 g kg^{-1} in 'Barbara' and 'Daniela' fresh

Table 1. Chemical composition (g kg^{-1} DW) of myrtle berries of cultivar 'Barbara' during maturation and after 15 days of cold storage at 10 °C and 90% RH

Fruit treatment	Days after full bloom	pH	Titratable acidity	Reducing sugars	Total sugars	Total phenols	Tannins	Anthocyanins
Fresh	90	4.68g	10.3b	11.3e	14.8h	72.76ab	0.216a	0.13e
	120	4.81f	12.0a	38.8d	52.4f	71.31abc	0.158bcd	5.40d
	150	5.04d	8.3d	61.3c	122.9cd	65.91bc	0.195ab	8.43b
	180	5.16c	6.7ef	74.1ab	131.8bc	50.45d	0.167abc	9.44a
	210	5.21b	5.7fg	65.9bc	124.5bc	49.83d	0.101de	8.94ab
Stored	90	4.69g	9.0c	11.5e	24.1g	74.78a	0.040f	0.33e
	120	4.96e	11.0b	44.8d	84.1e	67.72abc	0.138bcd	7.03c
	150	5.15c	7.0e	67.2bc	133.1b	63.38c	0.125cde	8.99ab
	180	5.16c	5.7fg	60.7bc	115.0d	37.31e	0.101de	8.54b
	210	5.37a	5.7g	77.8a	144.7a	36.16e	0.077ef	7.69c

Values in a column with the same letter are not statistically different at $P \leq 0.05$ by Duncan's multiple range test.

Table 2. Chemical composition (g kg^{-1} DW) of myrtle berries of cultivar 'Daniela' during maturation and after 15 days of cold storage at 10 °C and 90% RH

Fruit treatment	Days after full bloom	pH	Titratable acidity	Reducing sugars	Total sugars	Total phenols	Tannins	Anthocyanins
Fresh	90	4.48g	8.0a	12.5c	16.0e	80.48a	0.165abc	0.14f
	120	4.90f	7.3bc	35.4b	79.1d	70.53b	0.201a	7.36cd
	150	5.10de	8.0a	38.6ab	100.2c	60.74d	0.146abc	8.15abc
	180	5.20cd	7.0bcd	40.5ab	94.1c	63.13cd	0.129c	8.10ab
	210	5.26a	6.7de	40.9ab	121.6a	46.77e	0.115c	7.71bc
Stored	90	4.58g	6.7cd	11.6c	17.5e	78.21a	0.136bc	0.16f
	120	5.03e	6.7bcd	39.0ab	83.7d	68.42bc	0.193ab	8.75a
	150	5.19cd	7.3b	36.2ab	82.1d	64.25bcd	0.119c	6.62de
	180	5.23bc	6.7de	42.3a	121.9a	43.09e	0.111c	7.69be
	210	5.32ab	5.7e	39.7ab	113.9b	41.44e	0.104c	6.26e

Values in a column with the same letter are not statistically different at $P \leq 0.05$ by Duncan's multiple range test.

fruits respectively (Tables 1 and 2). Total sugar concentration was not influenced by cold storage, following a similar trend to that during maturation and levelling off after 150 days till fruits became overripe. Some differences observed were probably due to the water loss of fruits.

Anthocyanin pigments

As already demonstrated, anthocyanin concentration increases during myrtle development and ripening.[14] Our results showed important increases in anthocyanin content from 0.13 to 5.40 g kg^{-1} and from 0.14 to 7.36 g kg^{-1} in 'Barbara' and 'Daniela' fruits respectively between 90 and 120 days after full bloom (Tables 1 and 2). In both cultivars the anthocyanin content reached its highest value at 150 days and then remained almost unchanged, even in overripe fruits.

Cold storage produced a slight rise in the anthocyanin content of nearly mature berries at 120 days after full bloom, as has been reported for other pigmented fruits,[23,24] while in cold-stored overripe fruits the anthocyanin concentration decreased after 15 days at 10 °C. Similar results were obtained by Wang *et al.*[25] During storage, raspberry fruits at 50–80% maturity accumulated levels of anthocyanins higher than those of ripe fruits. Our results suggest that storage conditions may produce a rise in anthocyanin content if fruits are stored when anthocyanin accumulation is still taking place. Similar outcomes were achieved in highbush blueberries by Kalt *et al.*,[26] who suggested that there may be a threshold level of ripeness after which anthocyanin synthesis can continue either on or off the plant.

This could also explain the differences observed between the two cultivars. The anthocyanin content of 'Barbara' rose in cold-stored fruits until the third sampling date, while that of 'Daniela' stopped rising in cold-stored fruits at the first harvesting date when the accumulation of anthocyanins in fresh fruits had already finished. In raspberry fruits the differences in anthocyanin content at the different maturity stages were not appreciable after 4 days of storage, even in less mature fruits.[26] In contrast, immature myrtle fruits harvested at 90 and 120 days after full bloom and cold stored for 15 days at 10 °C never attained an anthocyanin content as high as that of ripe fruits, whereas fruits harvested later accumulated anthocyanins during storage at the same level as ripe fruits.

Anthocyanins are key compounds in myrtle liqueur quality.[14,27] Most of the liqueur's organoleptic properties are strongly associated with anthocyanin content. These compounds, however, show very limited stability during berry infusion or liqueur storage.[9,10] After 1 year of storage the anthocyanin concentration in the liqueur decreased significantly, completely disappearing after 4 months of storage in open bottles.[9] The stability of anthocyanins is strongly related to acidity. It is well known that anthocyanin pigments undergo reversible structural transformation when acidity decreases.[28] Based on these issues, harvesting and processing berries with high anthocyanin content is of great importance for industrial purposes.

Although fruit antioxidant activity was not measured in this work, other studies on myrtle berries and other small fruits demonstrated that anthocyanins were not positively correlated with antioxidant activity,[11,26] which was dependent on the phenolic composition. Therefore harvesting berries with high phenolic content may provide a product with high antioxidant activity, which is an important parameter for liqueur preservation.

Tannins and phenols

As reported previously,[14] tannin concentration decreased during fruit development (Tables 1 and 2). This behaviour is of great importance, since in myrtle liqueur an excess of tannins is unpleasant while a moderate content contributes to the typical myrtle flavour.

The two cultivars showed different starting concentrations, 0.216 and 0.165 g kg^{-1} in 'Barbara' and 'Daniela' fruits respectively. Moreover, the rate of decrease was different, making it difficult to identify the harvesting period using tannin concentration. The effect of storage conditions on tannin concentration was negligible in both cultivars.

Total phenols occurred at high levels during development and declined over fruit maturation (Tables 1 and 2). Storage conditions did not produce any significant effect on total phenolic content in either cultivar. These results suggest that during ripening and cold storage there may be a shift in the production of phenolics towards anthocyanin synthesis.

Organic acids

The nature and concentration of organic acids are important factors influencing the organoleptic properties of fruits.[29] The major organic acids in myrtle fruits were quinic, malic and gluconic acids (Figs 2–4), while fumaric and shikimic acids showed lower concentrations (Figs 5 and 6). Our results are not completely in agreement with other data reported on spontaneous myrtle

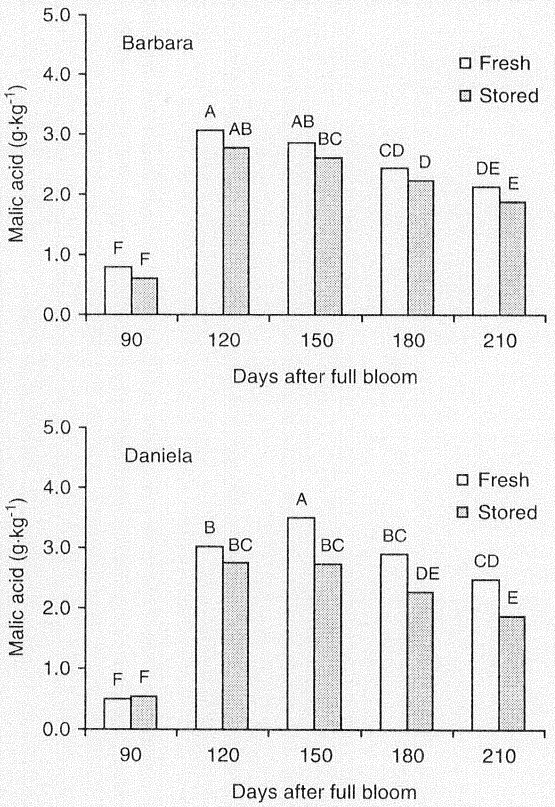


Figure 2. Malic acid content in myrtle berries during maturation and after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

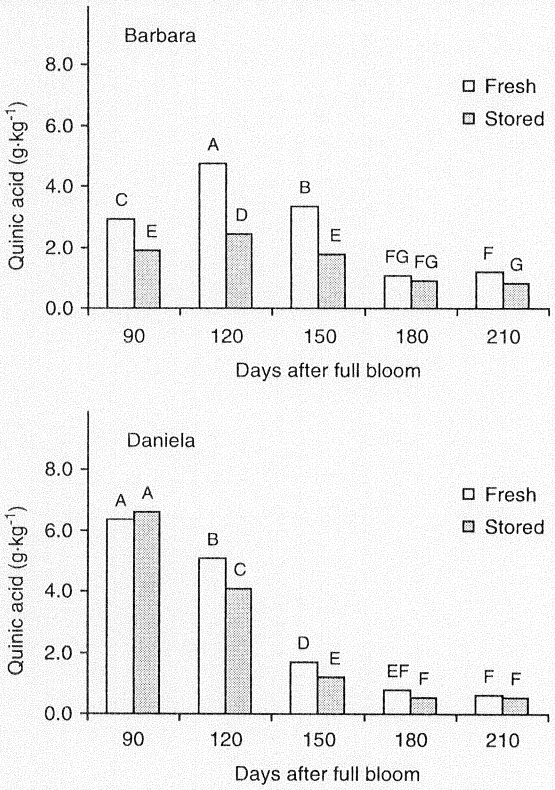


Figure 3. Quinic acid content in myrtle berries during maturation and after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

of Sardinia[3] and are completely different from determinations made on myrtle fruits from Turkey.[15] Franco *et al.*[3] found that malonic acid was the major organic acid in myrtle fruits and derived liqueurs, while Hacseferoullar *et al.*[15] reported tartaric acid as the major organic acid present in myrtle fruits. In the first case the different methodology applied may justify the different results, while in the second case a high genetic variability between Sardinian and Turkish cultivars could explain the different results.

Malic acid is a key intermediate in crassulacean acid metabolism and is a substrate in respiration. In myrtle berries, malic acid increased sharply from the first to the second sampling date and then decreased during maturation and ripening. Similarly, in some fruits such as apples and grapes, malic acid accumulates in the first stage of maturation and then decreases owing to respiration.[30,31] In stored fruits the pattern of malic acid levels followed the same trend observed for fresh berries, though the amounts recorded were lower in both cultivars (Fig. 2). Similar results were obtained after cold storage at 2 °C for 3 months.[11] In contrast, a decline in malic acid concentration during storage of apples, peaches and Japanese plums has already been reported.[22,30,32–34] Quinic acid was the major organic acid at the first harvest (Fig. 3). The two cultivars presented different concentration patterns. 'Barbara' showed an increase from the first to the second harvest, after which the concentration decreased constantly to 180 days after bloom and then remained constant. 'Daniela' showed its highest value (6.0 g kg^{-1}) at the first sampling date, after which the concentration decreased progressively till 210 days after bloom. These results are in agreement with similar experiments on other fruits such as peaches and citrus fruits.[34,35] A possible explanation for this behaviour could lie in the role of quinic acid in flavonoid biosynthesis. Weinstein *et al.*[36] provided evidence of the conversion of quinic acid to shikimic acid, a key compound in the phenylpropanoid pathway. Our results show that the decrease in quinic acid is concomitant with an increase in anthocyanin content, pointing out a shift in metabolic activity towards the biosynthesis of anthocyanins. Cold-stored fruits showed a similar pattern to fresh fruits, but with lower values at all harvesting dates. This is in accordance with previous storage studies at 2 °C for 3 months.[11]

At the first harvest, gluconic acid showed undetectable values in both cultivars (Fig. 4). In 'Daniela' fruits it accumulated sharply at the onset of ripening, increasing at 120 days after bloom to 10.1 g kg^{-1} and changing thereafter, whereas in 'Barbara' fruits it increased steadily and peaked at the fifth sampling date, reaching a concentration of 12.9 g kg^{-1}. Cold storage resulted in a different behaviour of gluconic acid compared with the other organic acids: 'Barbara' berries showed a further increase at all harvesting dates, while in 'Daniela' fruits the harvesting date did not affect the gluconic acid concentration during storage. Differences between the two myrtle cultivars may be justified because 'Barbara' is slightly earlier and shows a more homogeneous maturation than 'Daniela'. The accumulation of gluconic acid in mature, cold-stored and frozen-stored berries is of great importance, as it can be considered as a marker of the onset of fruit senescence.[11,12]

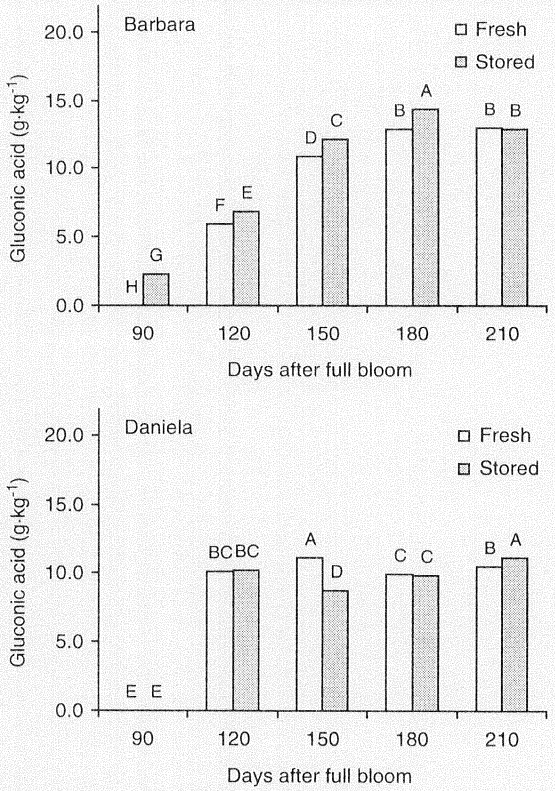


Figure 4. Gluconic acid content in myrtle berries during maturation and after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

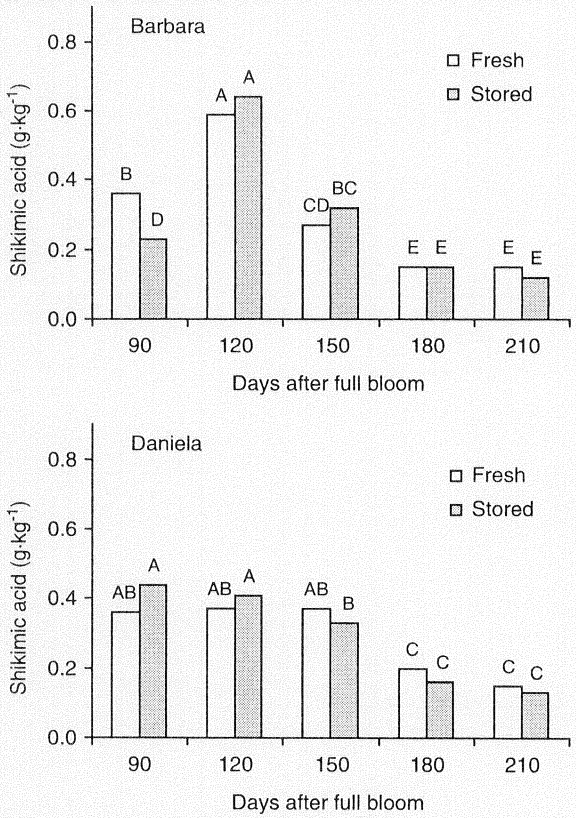


Figure 5. Shikimic acid content in myrtle berries during maturation and after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

An increase in gluconic acid has been associated with core breakdown in pears.[37] The authors ascribe such a rise to an imbalance in the metabolic pathways in which gluconic acid is involved. It is reasonable to think a similar imbalance could also occur in senescent tissues.[38] This was supported by the fact that, in sound pears, gluconic acid was present only in traces. Equally, no gluconic acid was present in myrtle fruits harvested 90 days after full bloom, while it was detected throughout maturation and in cold-stored fruits.

Shikimic and fumaric acids were present in myrtle fruits in very small amounts, with concentrations ranging from 0.3 to 0.6 g kg^{-1} and from 0.020 to 0.059 g kg^{-1} in overripe fresh and cold-stored fruits respectively (Figs 5 and 6). Shikimic acid peaked at 120 days after full bloom in both cultivars and decreased at subsequent sampling dates. This trend may be justified by the active biosynthesis of pigments and aroma volatiles by the shikimic acid pathway.[39,40] Fumaric acid concentration did not vary significantly during maturation and peaked at 180 days after full bloom in both cultivars. The evolution of this minor compound is quite similar to that observed in damson plum fruits, where malic and quinic acids are the major organic acids.[41]

CONCLUSION

In stored fruits the highest anthocyanin accumulation was observed in the first stages of fruit ripening when anthocyanin biosynthesis was still taking place and weight loss was nearly 15%. These results suggest that the practice of storing unripe berries is not advisable for liqueur producers. On the contrary, harvesting berries that are not fully ripe could provide fruits able to attain levels of anthocyanins during cold storage comparable to those found in berries harvested fully mature. Furthermore, berries that are not fully ripe have a higher total phenolic content than ripe fruits and a higher content of tannins, which are maintained during cold storage.

Organic acids in fruits have a fundamental role as nutritional components with good antioxidant properties and may have a role as potential chemical stabilisers of juices or other processed foods. The changes in chemical composition during maturation and cold storage highlight the importance of gluconic acid for myrtle quality assessment. This is further supported by the rise in gluconic acid content observed in hydroalcoholic extracts obtained from myrtle berries after cold storage at 2 °C.[11] These results lead us to suggest the use of gluconic acid as a marker for assessing the quality of myrtle fruits and liqueurs; in addition, organic acid composition could be used as a further indicator of the geographic origin of myrtle berries.

ACKNOWLEDGEMENT

The research was supported by the PYRGI project of the European Union (Special Grant Italy–France 'Marittimo').

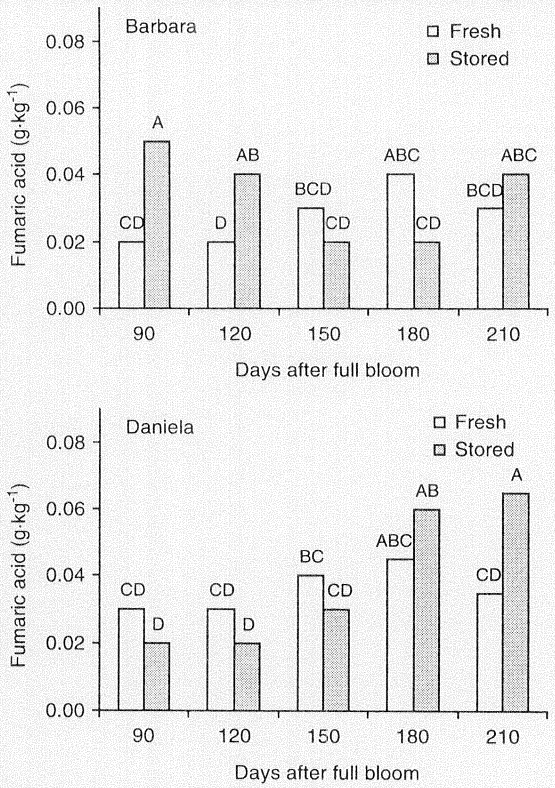


Figure 6. Fumaric acid content in myrtle berries during maturation and after 15 days of cold storage at 10 °C and 90% RH. Mean separation was performed separately for each cultivar using Duncan's multiple range test. Values with the same letter are not statistically different at $P \leq 0.05$.

REFERENCES

1 Mulas M, Francesconi AHD and Perinu B, Myrtle (*Myrtus communis* L.) as a new aromatic crop: cultivar selection. *J Herbs Spices Med Plants* **9**:127–131 (2002).

2 Tuberoso CIG, Rosa A, Bifulco E, Melis MP, Atzeri A, Pirisi FM, *et al*, Chemical composition and antioxidant activities of *Myrtus communis* L. berries extracts. *Food Chem* **123**:1242–1251 (2010).

3 Franco MA, Versini G, Mattivi F, Dalla Serra A, Vacca V and Manca G, Analytical characterization of myrtle berries, partially processed products and commercially available liqueurs. *J Commod Sci* **41**:143–267 (2002).

4 Tuberoso CIG, Barra A, Angioni A, Sarritzu E and Pirisi FM, Chemical composition of volatiles in Sardinian myrtle (*Myrtus communis* L.) alcoholic extracts and essential oils. *J Agric Food Chem* **54**:1420–1426 (2006).

5 Tuberoso CIG, Melis M, Angioni A, Pala M and Cabras P, Myrtle hydroalcoholic extracts obtained from different selections of *Myrtus communis* L. *Food Chem* **101**:806–811 (2007).

6 Tuberoso CIG, Barra A and Cabras P, Effect of different technological processes on the chemical composition of myrtle (*Myrtus communis* L.) alcoholic extracts. *Eur Food Res Technol* **226**:801–808 (2008).

7 Mulas M and Melis RAM, Influence of growing area, year, season, and cultivar on the composition of myrtle leaves and infusions. *HortScience* **43**:549–553 (2008).

8 Mulas M and Melis RAM, Essential oil composition of myrtle (*Myrtus communis*) leaves. *J Herbs Spices Med Plants* **17**:21–34 (2011).

9 Vacca V, Piga A, Del Caro A, Fenu PAM and Agabbio M, Changes in phenolic compounds, colour and antioxidant activity in industrial red myrtle liqueurs during storage. *Z Naturforsch C* **47**:442–447 (2003).

10 Montoro P, Tuberoso CIG, Piacente S, Perrone A, De Feo V, Cabras P, *et al*, Stability and antioxidant activity of polyphenols in extract of *Myrtus communis* L. berries used for the preparation of myrtle liqueur. *J Pharmaceut Biomed Anal* **41**:1614–1619 (2006).

11 Angioni A, Pirisi F, Caboni P, D'Aquino S, Fadda A and Schirra M, Effects of cold storage on quality traits of Sardinian myrtle (*Myrtus communis* L.) berries and their alcoholic extracts. *J Agric Sci Technol B* **1**:790–798 (2011).

12 Angioni A and Schirra M, Long-term frozen storage impact on the antioxidant capacity and chemical composition of Sardinian myrtle (*Myrtus communis* L.) berries. *J Agric Sci Technol B* **1**:1168–1175 (2011).

13 Aidi Wannes W, Mhamdi B and Marzouk B, Variations in essential oils and fatty acid composition during *Myrtus communis* var. *italica* fruit maturation. *Food Chem* **112**:621–626 (2009).

14 Fadda A and Mulas M, Chemical profile changes during myrtle (*Myrtus communis* L.) fruit development and ripening. *Sci Hort* **125**:477–485 (2010).

15 Hacseferoullar H, Özcan MM, Arslan D and Ünver A, Biochemical compositional and technological characterizations of black and white myrtle (*Myrtus communis* L.) fruits. *J Food Sci Technol* **49**:82–88 (2012).

16 Mulas M, Francesconi AHD, Perinu B and Fadda A, 'Barbara' and 'Daniela': two cultivars for myrtle berries production. *Acta Hort* **576**:169–175 (2002).

17 Mulas M and Cani MR, Germplasm evaluation of spontaneous myrtle (*Myrtus communis* L.) for cultivar selection and crop development. *J Herbs Spices Med Plants* **6**:31–49 (1999).

18 El-Otmani M and Coggins CW, Fruit development and growth regulator effects on normal alkanes of 'Washington' navel oranges fruit epicuticular wax. *J Agric Food Chem* **33**:656–663 (1985).

19 Bally ISE, Changes in the cuticular surface during the development of mango (*Mangifera indica* L.) cv. Kensington Pride. *Sci Hort* **79**:13–22 (1999).

20 Cordenunsi BR, Nascimento JRO and Lajolo FM, Physico-chemical changes related to quality of five strawberry fruit cultivars during cool storage. *Food Chem* **53**:167–173 (2003).

21 Holland N, Menezes HC and Lafuente MT, Carbohydrates as related to the heat induced chilling tolerance of respiratory rate of 'Fortune' mandarin fruit harvested at different maturity stages. *Postharv Biol Technol* **25**:181–191 (2002).

22 Singh SP, Singh Z and Swinny EE, Sugars and organic acids in Japanese plums (*Prunus salicina* Lindell) as influenced by maturation, harvest date, storage temperature and period. *Int J Food Sci Technol* **44**:1973–1982 (2009).

23 Cabrita L, Fossen T and Andersen OM, Colour stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chem* **68**:101–107 (2000).

24 Lo Piero AR, Puglisi I, Rapisarda P and Petrone G, Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. *J Agric Food Chem* **53**:9083–9088 (2005).

25 Wang SY, Chen CT and Wang CY, The influence of light and maturity on fruit quality and flavonoid content of red raspberries. *Food Chem* **112**:676–684 (2009).

26 Kalt W, Lawand C, Ryan DAJ, McDonald JE, Donner H and Forney CF, Oxygen radical absorbing capacity, anthocyanin and phenolic content of highbush blueberries (*Vaccinium corymbosum* L.) during ripening and storage. *J Am Soc Hort Sci* **128**:917–923 (2003).

27 Montoro P, Tuberoso CIG, Perrone A, Piacente S, Cabras P and Pizza C, Characterisation by liquid chromatography–electrospray tandem mass spectrometry of anthocyanins in extracts of *Myrtus communis* L. berries used for the preparation of myrtle liqueurs. *J Chromatogr A* **1112**:232–240 (2006).

28 Connor AM, Luby JJ, Hancock JF, Berkheimer S and Hanson E, Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *J Agric Food Chem* **50**:893–898 (2002).

29 Lee HS, HPLC method for separation and determination of nonvolatile organic acids in orange juice. *J Agric Food Chem* **41**:1991–1993 (1993).

30 Ackermann J, Fischer M and Amadò R, Changes in sugars, acids, and amino acids during ripening and storage of apples (cv. Glockenapfel). *J Agric Food Chem* **40**:1131–1134 (1992).

31 Lakso AN and Kliewer WM, The influence of temperature on malic acid metabolism in grape berries. *Plant Physiol* **56**:370–372 (1975).

32 Chapman Jr GW and Horvat RJ, Changes in nonvolatile acids, sugars, pectin, and sugar composition of pectin during peach (cv. Monroe) maturation. *J Agric Food Chem* **38**:383–387 (1990).

33 Robertson JA, Meredith FI, Horvat RJ and Senter SD, Effect of cold storage and maturity on the physical and chemical characteristics

and volatile constituents of peaches (cv. Cresthaven). *J Agric Food Chem* **38**:620–624 (1990).
34 Chapman Jr GW, Horvat RJ and Forbus Jr WR, Physical and chemical changes during the maturation of peaches (cv. Majestic). *J Agric Food Chem* **39**:867–870 (1991).
35 Albertini MV, Carcouet E, Pailly O, Gambotti C, Luro F and Berti L, Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. *J Agric Food Chem* **54**:8335–8339 (2006).
36 Weinstein LH, Porter CA and Laurencot Jr HJ, Evidence for the conversion of quinic acid to shikimic acid in roses. *Nature* **183**:326 (1959).
37 Pedreschi R, Franck C, Lammertyn J, Erban A, Kopka J, Hertog M, *et al*, Metabolic profiling of 'Conference' pears under low oxygen stress. *Postharv Biol Technol* **51**:123–130 (2009).
38 Pandey S, Ranade SA, Nagar PK and Kumar N, Role of polyamines and ethylene as modulators of plant senescence. *J Biosci* **25**:291–299 (2000).
39 Šturm K and Štampar F, Seasonal variation of sugars and organic acids in apple (*Malus domestica* Borkh.) in different growing systems. *Phyton* **39**:91–96 (1999).
40 Kumar A and Ellis E, The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. *Plant Physiol* **127**:230–239 (2001).
41 García-Mariño N, de la Torre F and Matilla AJ, Organic acids and soluble sugars in edible and nonedible parts of damson plum (*Prunus domestica* L. subsp. *insititia* cv Syriaca) fruits during development and ripening. *Food Sci Technol Int* **14**:187–193 (2008).

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.11

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Neuropharmacology xxx (2012) 1–10

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Presynaptic mGlu7 receptors control GABA release in mouse hippocampus

Maria Summa [a], Silvia Di Prisco [a], Massimo Grilli [a,c], Cesare Usai [b], Mario Marchi [a,c], Anna Pittaluga [a,c,*]

[a] Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genoa, viale Cembrano 4, 16148 Genoa, Italy
[b] Institute of Biophysics, National Research Council, via De Marini 6, 16149 Genoa, Italy
[c] Center of Excellence for Biomedical Research, University of Genoa, viale Benedetto XV, 16132 Genoa, Italy

ARTICLE INFO

Article history:
Received 27 December 2011
Received in revised form
5 April 2012
Accepted 19 April 2012

Keywords:
Hippocampal synaptosomes
GABA
mGlu7 receptors
$GABA_B$ receptors
AMN082
MMPIP

ABSTRACT

The functional role of presynaptic release-regulating metabotropic glutamate type 7 (mGlu7) receptors in hippocampal GABAergic terminals was investigated. Mouse hippocampal synaptosomes were preloaded with [^{3}H]D-γ-aminobutyric acid ([^{3}H]GABA) and then exposed in superfusion to 12 mM KCl. The K^+-evoked [^{3}H]GABA release was inhibited by the mGlu7 allosteric agonist N,N′-dibenzyhydryl-ethane-1,2-diamine dihydrochloride (AMN082, 0.001–10 μM), as well as by the group III mGlu receptor agonist L-(+)-2-amino-4-phosphonobutyric acid [(L)-AP4, 0.01–1 mM]. The mGlu8 receptor agonist (S)-3,4-dicarboxyphenylglycine [(S)-3,4-DCPG, 10–100 nM] was ineffective. AMN082 and (L)-AP4-induced effects were recovered by the mGlu7 negative allosteric modulator (NAM) 6-(4-methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one hydrochloride (MMPIP). AMN082 also inhibited in a MMPIP-sensitive manner the K^+-evoked release of endogenous GABA. AMN082 and the adenylyl cyclase (AC) inhibitor MDL-12,330A reduced [^{3}H]GABA exocytosis in a 8-Br-cAMP-sensitive. AMN082-inhibitory effect was additive to that caused by (−)baclofen, but insensitive to the $GABA_B$ antagonist 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl) phosphinic acid (CGP52432). Conversely, (−)baclofen-induced inhibition of GABA exocytosis was insensitive to MMPIP. Finally, the forskolin-evoked [^{3}H]GABA release was reduced by AMN082 or (−)baclofen but abolished when the two agonists were added concomitantly. Mouse hippocampal synaptosomal plasmamembranes posses mGlu7 receptor proteins; confocal microscopy analysis unveiled that mGlu7 proteins colocalize with syntaxin-1A (Stx-1A), with vesicular GABA transporter (VGAT)-proteins and with $GABA_B$ receptor subunit proteins. We propose that presynaptic inhibitory mGlu7 heteroreceptors, negatively coupled to AC-dependent intraterminal pathway, exist in mouse hippocampal GABA-containing terminals, where they colocalize, but do not functionally cross-talk, with $GABA_B$ autoreceptors.

This article is part of a Special Issue entitled 'mGluR'.

1. Introduction

Glutamate and γ-aminobutyric acid (GABA) are the two major neurotransmitters in the mammalian central nervous system (CNS), where they mediate excitatory and inhibitory transmission, respectively. Under normal conditions, these amino acids coexist in a physiological functional cross-talk which ensures homeostasis in the brain. Disruption of this balance could lead to a shift from

Abbreviations: GABA, γ-aminobutyric acid; mGlu, metabotropic glutamate receptor; NMDA-aspartate, N-methyl-D; 5-HT, 5-hydroxytryptamine; CNS, central nervous system; AMN082, N,N′-dibenzyhydryl-ethane-1,2-diamine dihydrochloride; MMPIP, 6-(4-methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one hydrochloride; (L)-AP4-(+)-2-amino-4-phosphonobutyric acid, L; (−)baclofen, S(−)-β-(aminomethyl)-4-chlorobenzenepropanoic acid; CGS19755, cis-4-[phosphomethyl]-piperidine-2-carboxylic acid; (S)-3,4-DCPG, (S)3,4-dicarboxyphenylglycine; CGP52432, 3-[[(3,4-dichlorophenyl)methyl]aminopropyl] diethoxymethyl phosphinic acid; WAY-100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide; SB 200646A, N-(1-Methyl-1H-indol-5-yl)-N′-3-pyridinylurea; DMIimipramine, desmethyl; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; MDL-12,330A, cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride; Stx-1A, syntaxin-1A; VGAT, anti vesicular GABA transporter; GAD, glutamate decarboxylase; NAM, negative allosteric modulator; ECL, enhanced chemiluminescence; mGlu7 receptor, metabotropic glutamate type 7 receptor; mGlu8 receptor, metabotropic glutamate type 8 receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tris, Tris-(hydroxymethyl)-amino methane; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

* Corresponding author. Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genoa, viale Cembrano 4, 16148 Genoa, Italy. Tel.: +39 010 3532120; fax: +39 010 3993360.
E-mail address: pittalug@pharmatox.unige.it (A. Pittaluga).

0028-3908/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2012.04.020

health to disease, accounting for the onset of neuropsychiatric and neurological symptoms as well as neurodegenerative processes (Danysz et al., 1995; Schoepp, 2001; Niswender and Conn, 2010).

Besides their quick actions at ionotropic receptors, glutamate and GABA can activate metabotropic receptors generally endowed with modulatory functions, i.e. the metabotropic glutamate (mGlu) receptors and the $GABA_B$ receptors, respectively (Raiteri, 2008). These receptors exist presynaptically and include auto and heteroreceptors whose activation control neurotransmitter release. In particular, at the hippocampal level, the GABAergic control of glutamate transmission mainly involves the activation of presynaptic $GABA_B$ heteroreceptors negatively coupled to adenylyl cyclase activity (Pittaluga et al., 1987; Bonanno and Raiteri, 1993). Similarly, glutamate synaptically released upon depolarization exerts a feedback control of GABA release by acting on metabotropic heteroreceptors (Desai et al., 1994; Gereau and Conn, 1995; Schoepp, 2001) presynaptically located on GABAergic nerve terminals.

Metabotropic glutamate receptors are divided into three groups: group I (mGlu1 and mGlu5), group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7 and mGlu8), based upon sequence homology, intracellular signal transduction mechanisms and pharmacological properties (Pin and Duvoisin, 1995; Conn and Pin, 1997; Pin and Ascher, 2002; Nicoletti et al., 2010). Recently, the mGlu receptors, especially the group I and group II mGlu receptors, have become attractive therapeutic targets for drug development for the treatment of CNS diseases (Spooren et al., 2003; Alexander and Godwin, 2006; Witkin et al., 2007; Marek, 2010), while group III mGlu receptors were the least studied due to the lack of selective pharmacological tools. The development of N,N′-dibenzyhydryl-ethane-1,2-diamine dihydrochloride (AMN082, Mitsukawa et al., 2005) and of 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one hydrochloride (MMPIP, Suzuki et al., 2007), with the first being the allosteric agonist and the second being the negative allosteric modulator (NAM) of the mGlu7 receptor subtype, however, has made pharmacological and neurochemical studies of mGlu7 receptor-mediated functions in the CNS feasible.

Data in literature has provided convincing evidence of the existence of presynaptic mGlu7 autoreceptors controlling glutamate release in CNS (Millán et al., 2002a,b; Martin et al., 2008, 2010). On the contrary, the existence of presynaptic mGlu7 heteroreceptors controlling GABA release in the hippocampus has been inferred on the basis of histochemical and functional observations (Shigemoto et al., 1997; Somogyi et al., 2003; Wierońska et al., 2010), but a direct demonstration is still lacking. We have tested this hypothesis by investigating the impact of the above-mentioned selective ligands on the release of GABA (measured as release of preloaded [^{3}H]GABA as well as of endogenous GABA) elicited by a mild depolarizing stimulus from adult mouse hippocampal synaptosomes. Our findings confirm that mouse hippocampal GABAergic terminals possess presynaptic mGlu7 heteroreceptors, whose activation inhibits GABA exocytosis. Our conclusions are supported by immunocytochemical data showing that VGAT-positive, Stx-1A containing mouse hippocampal nerve endings are endowed with mGlu7 receptor proteins. These receptors are negatively coupled to adenylyl cyclase activity and do not cross-talk with presynaptic $GABA_B$ autoreceptors.

2. Materials and methods

2.1. Animals

Adult mice (Swiss; 20–25 g) were killed by decapitation, the hippocampus was rapidly removed and purified synaptosomes were prepared. The experimental procedures were approved by Italian legislation on animal experimentation (protocol number n° 29823-10). Experiments were performed in accordance with the U.K. Animals guidelines (Scientific Procedures Act, 1986 and associated guidelines), with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357).

2.2. Preparation of synaptosomes

Purified isolated nerve endings (synaptosomes) were prepared according to Dunkley et al. (1986), with some modifications. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (Tris, final concentration 0.01 M) using a glass/Teflon tissue grinder (clearance 0.25 mm); the homogenate was centrifuged at 1000× g for 5 min to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll gradient (2%, 6%, 10% and 20% v/v in Tris-buffered sucrose), and centrifuged at 33,500× g for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was subsequently collected and washed by centrifugation. The synaptosomal pellet was always resuspended in a physiological solution (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4.

2.3. Experiments of release

Synaptosomes were incubated for 15 min at 37 °C in a rotary water bath in the absence (experiments of endogenous GABA release) or in the presence of [^{3}H]GABA, (final concentration 34 nM). After the labelling period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a superfusion system (Raiteri and Raiteri, 2000; Ugo Basile, Comerio, Varese, Italy) and were maintained at 37 °C. Synaptosomes were superfused at 0.5 ml/min with standard physiological solution for a total period of 48 min (of superfusion). When studying the effect of mGlu receptor agonists on the release of endogenous GABA or preloaded [^{3}H]GABA evoked by high K^+ or by the adenylyl cyclase activator forskolin, synaptosomes were transiently (90 s) exposed, at $t = 39$ min, to K^+ containing medium (12 mM, NaCl substituting for an equimolar concentration of KCl), or to forskolin. NAM and antagonists were always added starting at $t = 30$ min of superfusion. Fractions were collected according to the following scheme: two 3-min fractions (basal release), one before ($t = 36$–39 min) and one after ($t = 45$–48 min) a 6-min fraction ($t = 39$–45 min; evoked release). Superfusion was always performed with media containing 50 μM amino-oxyacetic acid to avoid metabolism of GABA. Fractions collected and superfused synaptosomes were counted for radioactivity. In the multiple stimulation experiments, two consecutive pulses of 12 mM KCl in the absence or in the presence of AMN082, were applied for 90 s starting at $t = 39$ min (S1) and at, $t = 61$ min (S2). Synaptosomes were superfused with standard medium before and after each stimulus. Fractions were collected according to the following scheme: first stimulus (S1), as previously described, second stimulus (S2) two 3-min fractions (basal release), one before ($t = 59$–61 min) and one after ($t = 67$–69 min) a 6-min fraction ($t = 61$–67 min; evoked release). In parallel experiments carried out to evaluate whether presynaptic mGlu7 heteroreceptors can desensitize (Pelkey et al., 2007), synaptosomes were pre-exposed to AMN082 from t 20–30 min of superfusion, then substituted until $t = 39$ min with standard medium and then rechallenged with the depolarizing stimulus in the absence or in the presence of the agonist. Synaptosomal protein contents were determined according to Bradford (1976). The amount of endogenous amino acid from synaptosomes in each superfusate fractions was expressed as picomoles per milligram of protein (pmol mg^{-1} protein). The K^+-induced overflow of endogenous GABA from synaptosomes was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2). The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). When studying the effect of mGlu receptor agonists on the K^+-evoked and on the forskolin-induced [^{3}H]GABA release, drug effects were estimated by subtracting the neurotransmitter content into the fractions corresponding to the basal release from those corresponding to the evoked release.

2.4. Endogenous amino acid determination

Collected fractions and superfused synaptosomes were analyzed for the endogenous neurotransmitter content. Endogenous GABA was measured by high performance liquid chromatography analysis after precolumn derivatization with *o*-phthalaldehyde and separation on a C18 reverse-phase chromatographic column (10 × 4.6 mm, 3 μm; at 30 °C; Chrompack, Middleburg, The Netherlands) coupled with fluorimetric detection (excitation wavelength, 350 nm; emission wavelength, 450 nm). Buffers and the gradient program were described elsewhere (see Luccini et al., 2007). Homoserine was used as internal standard.

2.5. Immunoblotting

Synaptosomes were washed twice with standard medium and then collected by centrifugation (14,000 *g*, 15 min at 4 °C). The pellet was lysed in ice-cold Tris-buffered saline solution (0.02 M Tris/HCl, 0.01 M NaCl, protease inhibitor cocktail, pH

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

7.4) and quantified for protein content with Lowry Assay. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide), and then transferred onto polyvinylidene fluoride membranes. Membranes were saturated with a Tris-buffered saline solution (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4) containing 5% (w/v) non-fat dried milk for 120 min at room temperature and then incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-mGlu7 (1:500), mouse anti-β-actin (1:2000). After extensive wash, membranes were incubated for 1 h at 20 °C with the appropriate horseradish peroxidase-linked secondary antibodies (1:2000) and immunoreactivity was detected using an enhanced chemiluminescence Western blotting detection system.

2.6. Immunocytochemical analysis in mouse hippocampal nerve terminals

Purified mouse hippocampal synaptosomes were prepared as previously described. For immunocytochemical analysis, 50 μg of synaptosomal proteins were placed onto coverslips, previously coated with poly-L-lysine, fixed with 2% paraformaldehyde for 15 min and washed with phosphate-buffered saline (PBS). After extensive washes with 0.5% bovine serum albumin (BSA) PBS, synaptosomes were permeabilized with 0.05% Triton X-100 PBS for 5 min and exposed to rabbit anti-mGlu7 receptor (1:500), to the mouse anti-Stx-1A (1:10,000), to guinea pig anti-VGAT (1:1000) or to guinea pig anti-$GABA_B$ (1:2000) as indicated. Synaptosomes were then washed with 0.5% BSA PBS and were incubated for 30 min at room temperature with Alexa Fluor 488 (green)-labelled goat anti-guinea pig IgG antibodies, Alexa Fluor 488 (green)-labelled donkey anti-mouse, Alexa Fluor 647 (red)-labelled donkey anti-mouse and Alexa Fluor 633 (red)-labelled goat anti-rabbit antibodies (1:500 for all), as appropriate. Fluorescence image acquisition was performed by a multi-channel Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543 and 633 nm excitation lines. Images (512 × 512 pixels, 12 bit) were taken through a plan-apochromatic oil immersion objective 63×/NA1.4. Light collection configuration was optimized according to the combination of chosen fluorochromes and sequential channel acquisition was performed to avoid cross-talk phenomena. Leica LasAF software package was used for acquisition, storage and visualization. Each coverslip was analyzed by counting at least three different fields.

2.7. Calculations

Analysis of variance was performed by ANOVA followed by Dunnett's test or Newman Keuls multiple-comparisons test as appropriate. Direct comparisons were performed by Student's *t*-test. Data were considered significant for $p < 0.05$ at least. Appropriate controls with antagonists or enzyme inhibitors were always run in parallel. The quantitative estimation of colocalized proteins in immunocytochemical studies was performed calculating the colocalization coefficients (Manders et al., 1992) from the red- and green-channel scatterplot. Co-localization coefficients express the fraction of colocalizing molecular species in each component of a dual-colour image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition. If two molecular species are colocalized, the overlay of their spatial distributions has a correlation value higher than what would be expected by chance alone. Costes et al. (2004) developed an automated procedure to evaluate correlation between the green and red channels with a significance level > 95%. The same procedure automatically determines an intensity threshold for each colour channel based on a linear least-square fit of the green and red intensities in the image's 2D correlation cytofluorogram. Costes approach (2004) was implemented by using macro routines integrated as plug-ins (WCIF Co-localization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.46b image-analysis software (Wayne Rasband, NIH, USA).

2.8. Chemicals

[^{3}H]D-γ-aminobutyric acid ([^{3}H]GABA, specific activity 35.0 Ci/mmol) was from Perkin Elmer (Massachusetts, USA). N,N′-dibenzyhydryl-ethane-1,2-diamine (AMN082), 6-(4-Methoxyphenyl) 5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP), L-(+)-2-Amino-4-phosphonobutyric acid (L)-AP4, N-methyl-D-aspartate (NMDA), cis-4-[phosphomethyl]-piperidine-2-carboxylic acid (CGS19755), (S)-3,4-Dicarboxyphenylglycine ((S)-3,4-DCPG), 3-[[(3,4-dichlorophenyl)methyl]aminopropyl] diethoxymethyl phosphinic acid (CGP52432), N-(1-Methyl-1H-indol-5-yl)-N′-3-pyridinylurea (SB, 200646A) were from Tocris Bioscience (Bristol, UK). N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY-100635) S(−)-β-(aminomethyl)-4-chlorobenzenepropanoic acid ((−)baclofen), (aminooxy)acetic acid hemihydrochloride, β-actin monoclonal mouse IgG1, cis-N-(2-Phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL-12,330A), 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), desmethylimipramine (DMI), horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies, o-phthalaldehyde, forskolin, anti-$GABA_B$ receptor antibody in guinea pig (whole antiserum) and protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-mGlu7 polyclonal rabbit was from ABCAM (Cambridge Science Park, Cambridge, UK), anti-syntaxin-1A monoclonal mouse IgG was obtained from GeneTex (Irvine, CA, USA). Anti-VGAT was obtained from Chemicon (CA, USA).

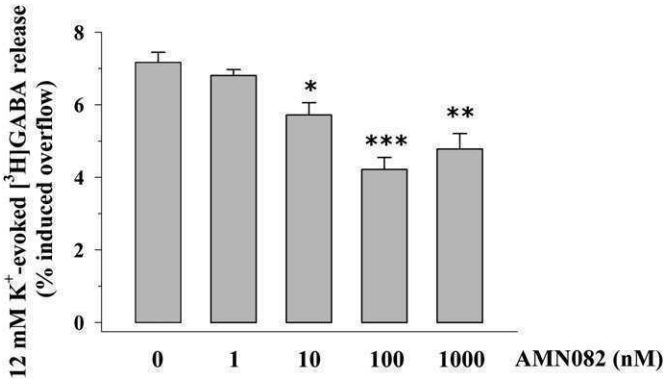


Fig. 1. Effects of AMN082 on the high K^+-evoked release of [^{3}H]GABA from mouse hippocampal GABAergic nerve terminals. Mouse hippocampal synaptosomes preloaded with the radioactive tracer were exposed in superfusion to 12 mM KCl in the absence or in the presence of AMN082 (concentration as indicated). Results are expressed as induced overflow. Data are means ± SEM of 3–15 experiments run in triplicate (three superfusion chambers for each experimental conditions). *$p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; **$p < 0.01$ vs K^+-evoked release of [^{3}H]GABA; ***$p < 0.001$ vs K^+-evoked release of [^{3}H]GABA.

Western blotting detection system was purchased from EuroClone (Milan, Italy). Anti-guinea pig in goat Alexa Fluor 488-conjugated, anti-mouse in donkey Alexa Fluor 488-conjugated, Alexa Fluor 647 (red)-labelled donkey anti-mouse and anti-rabbit in goat Alexa Fluor 633-conjugated secondary antibodies were purchased from Molecular Probes (Alfagene, OR, USA).

3. Results

Mouse hippocampal synaptosomes were preloaded with [^{3}H]GABA and then superfused according to an experimental approach developed in our laboratory, the up-down superfusion of a thin layer of synaptosomes (Raiteri et al., 1974). In this condition, transient (90 s) exposure of nerve terminals to a mild depolarizing stimulus (12 mM KCl) is known to elicit the Ca^{2+}-dependent, exocytotic-like release of preloaded [^{3}H]GABA (Pittaluga et al., 1987; Bonanno and Raiteri, 1993). The concomitant addition of the selective mGlu7 allosteric agonist AMN082 (0.001–10 μM,) to the depolarizing stimulus caused a significant reduction of [^{3}H]GABA exocytosis. The effect occurred in a concentration-dependent

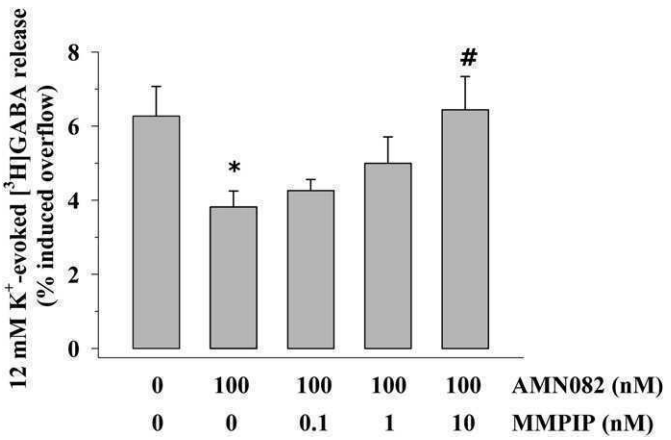


Fig. 2. Effect of the mGlu7 negative allosteric modulator MMPIP on the release of [^{3}H]GABA release evoked by high K^+ in the presence of AMN082 from mouse hippocampal GABAergic nerve terminals. Mouse hippocampal synaptosomes preloaded with the radioactive tracer were exposed in superfusion to 12 mM KCl in the absence or in the presence of 100 nM AMN082. MMPIP (concentration as indicated) was added starting from eight minutes before the depolarizing stimulus. Results are expressed as induced overflow. Data are means ± SEM of 3 experiments run in triplicate. *$p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; #$p < 0.05$ vs K^+/100 nM AMN082-evoked release of [^{3}H]GABA.

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

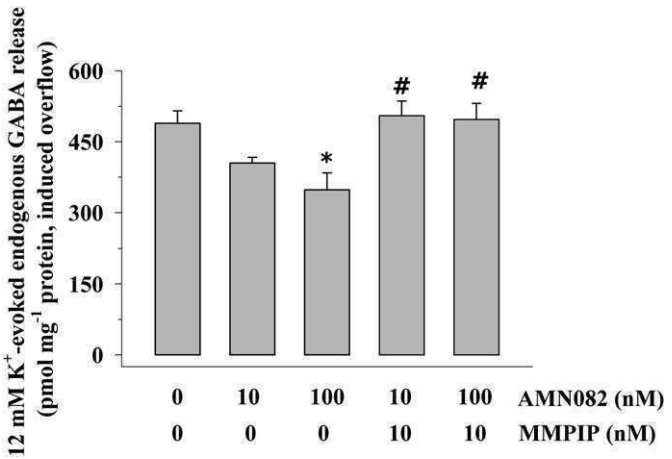


Fig. 3. Effects of mGlu7 receptor agonist AMN082 and of the negative allosteric modulator MMPIP on the K^+-evoked release of endogenous GABA from mouse hippocampal synaptosomes. Mouse hippocampal synaptosomes were exposed in superfusion to AMN082 (concentration as indicated) in the absence or in the presence of 10 nM MMPIP Results are expressed as pmol mg^{-1} protein and are calculated as endogenous GABA induced overflow. Data are means ± SEM of 3 experiments run in triplicate $^*p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; $^{\#}p < 0.05$ vs K^+/100 nM AMN082-evoked release of [^{3}H]GABA.

fashion, with the maximum inhibitory effect being observed when synaptosomes were exposed to 100 nM AMN082 (Fig. 1). The agonist alone failed to affect the spontaneous release of [^{3}H]GABA (not shown).

The compound MMPIP is listed as a selective mGlu7 receptor NAM, although recent evidence suggests a context-dependent antagonist profile for this compound (see Niswender and Conn, 2010). The NAM MMPIP prevented in a concentration-dependent fashion (0.1–10 nM) the inhibitory effect 100 nM AMN082 exerted on the 12 mM K^+-evoked release of [^{3}H]GABA (Fig. 2). The antagonist was extremely potent in doing that, with the maximum reversal being reached when the antagonist was added at 10 nM. At the concentration applied, MMPIP failed to affect on its own the spontaneous and the 12 mM K^+-evoked release of [^{3}H]GABA (not shown). In parallel experiments, the impact of AMN082 and of MMPIP on the 12 mM KCl-evoked release of endogenous GABA was monitored. Similarly to what was observed in the experiments of [^{3}H]GABA release, nanomolar (10–100 nM) AMN082 reduced the K^+-evoked exocytosis of endogenous GABA. The inhibitory effect was prevented

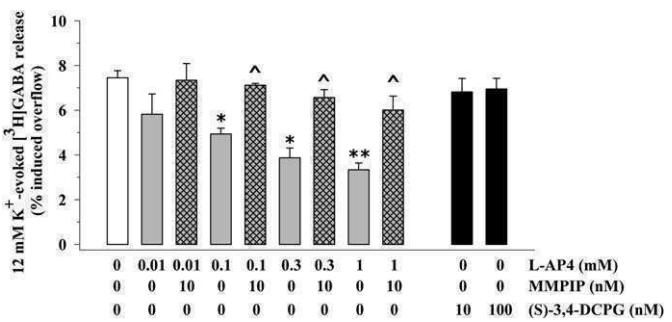


Fig. 4. Effects of group III mGlu receptor agonists on the K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes: antagonism by MMPIP. Mouse hippocampal synaptosomes preloaded with the radioactive tracer were exposed in superfusion to 12 mM KCl in the absence or in the presence of (L)-AP4 or (S)-3,4-DCPG (concentration as indicated). MMPIP (10 nM) was added starting from eight minutes before the depolarizing stimulus. Results are expressed as induced overflow. Data are means ± SEM of 3 experiments run in triplicate. $^*p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; $^{**}p < 0.01$ vs K^+-evoked release of [^{3}H]GABA; $^{\wedge}p < 0.05$ vs K^+/(L)-AP4-evoked release of [^{3}H]GABA.

Table 1
AMN082-induced inhibition of [^{3}H]GABA release does not involve the binding to presynaptic 5-HT_{1A}, 5-$HT_{2B/2C}$ and NMDA receptors.

	12 mM KCl-evoked [^{3}H]GABA overflow	(12 mM KCl/100 nM AMN082)-evoked [^{3}H]GABA overflow
Control	6.31 ± 0.10	4.26 ± 0.24[a]
+1 μM SB 200646A	5.71 ± 0.22	3.98 ± 0.32[a]
+10 μM WAY 100635	5.48 ± 0.42	3.84 ± 0.51[a]
+10 μM CGS19755	5.35 ± 0.47	3.84 ± 0.56[a]

Mouse hippocampal synaptosomes were exposed in superfusion to 12 mM KCl in the absence or in the presence of 100 nM AMN082. Antagonists (concentration as indicated) were added starting from eight min before the depolarizing stimulus. Results are expressed as induced overflow. Data are means ± SEM of 6 experiments run in triplicate (three superfusion chambers for each experimental condition).
[a] $p < 0.05$ vs control.

by 10 nM MMPIP (Fig. 3). Once again, MMPIP alone failed to affect on its own the K^+-evoked release of tritium (not shown).

In order to confirm the pharmacological profile of the presynaptic mGlu heteroreceptor controlling GABA exocytosis, we investigated the impact of (L)-AP4, a broad spectrum group III mGlu receptor agonist, and of (S)-3,4-DCPG, a group III mGlu receptor agonist showing a preferential activity at mGlu8 receptor subtype (Duty, 2010 and references therein), on the 12 mM K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes. (L)-AP4 (0.01–1 mM) inhibited in a concentration-dependent fashion the K^+-evoked tritium exocytosis, while (S)-3,4-DCPG (10–100 nM) was inactive (Fig. 4). The mGlu7 negative allosteric modulator MMPIP (10 nM) prevented the (L)-AP4-induced inhibition of the 12 mM K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes (Fig. 4).

Experiments were then dedicated to investigate whether the AMN082-induced changes to [^{3}H]GABA release could rely on the binding at 5-hydroxytryptamine (5-HT) and N-methyl-D-aspartate (NMDA) receptors, since these receptors were reported to represent potential targets of the mGlu7 agonist (Pałucha-Poniewiera et al., 2010; Risso Bradley et al., 2012). To this aim, the effects of N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY-100635), a selective antagonist at 5-HT_{1A} receptor subtype, of N-(1-Methyl-1H-indol-5-yl)-N′-3-pyridinylurea (SB, 200646A), a selective antagonist at 5-$HT_{2B/2C}$ (Maura et al., 2000 and references therein) and of the NMDA receptor antagonist cis-4-[Phosphomethyl]-piperidine-2-carboxylic acid (CGS19755) were investigated The three antagonists failed to significantly modify the release of [^{3}H]GABA elicited by 12 mM K^+, nor did they modify the 100 nM AMN082-induced inhibition of tritium overflow (Table 1). Finally, AMN082 was proposed to have appreciable affinity for noradrenaline transporter (NET, Sukoff Rizzo et al., 2011). The NET protein, however, was not involved in the AMN082-mediated control of GABA exocytosis since the selective NET blocker DMI, inactive on its own on the K^+-evoked GABA exocytosis (12 mM K^+/10 nM AMN082: 4.89 ± 0.36; +10 nM DMI:

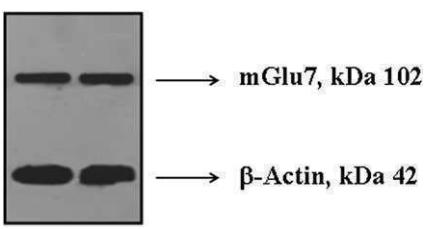


Fig. 5. Western blot analysis of mGlu7 receptor protein in synaptosomal lysate from the hippocampus of adult mice. The blot is representative of 4 different analyses and illustrates the mGlu7 receptor protein expression in mouse hippocampal synaptosomes. β-actin was used as internal control. Protein weights are expressed in kDa.

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

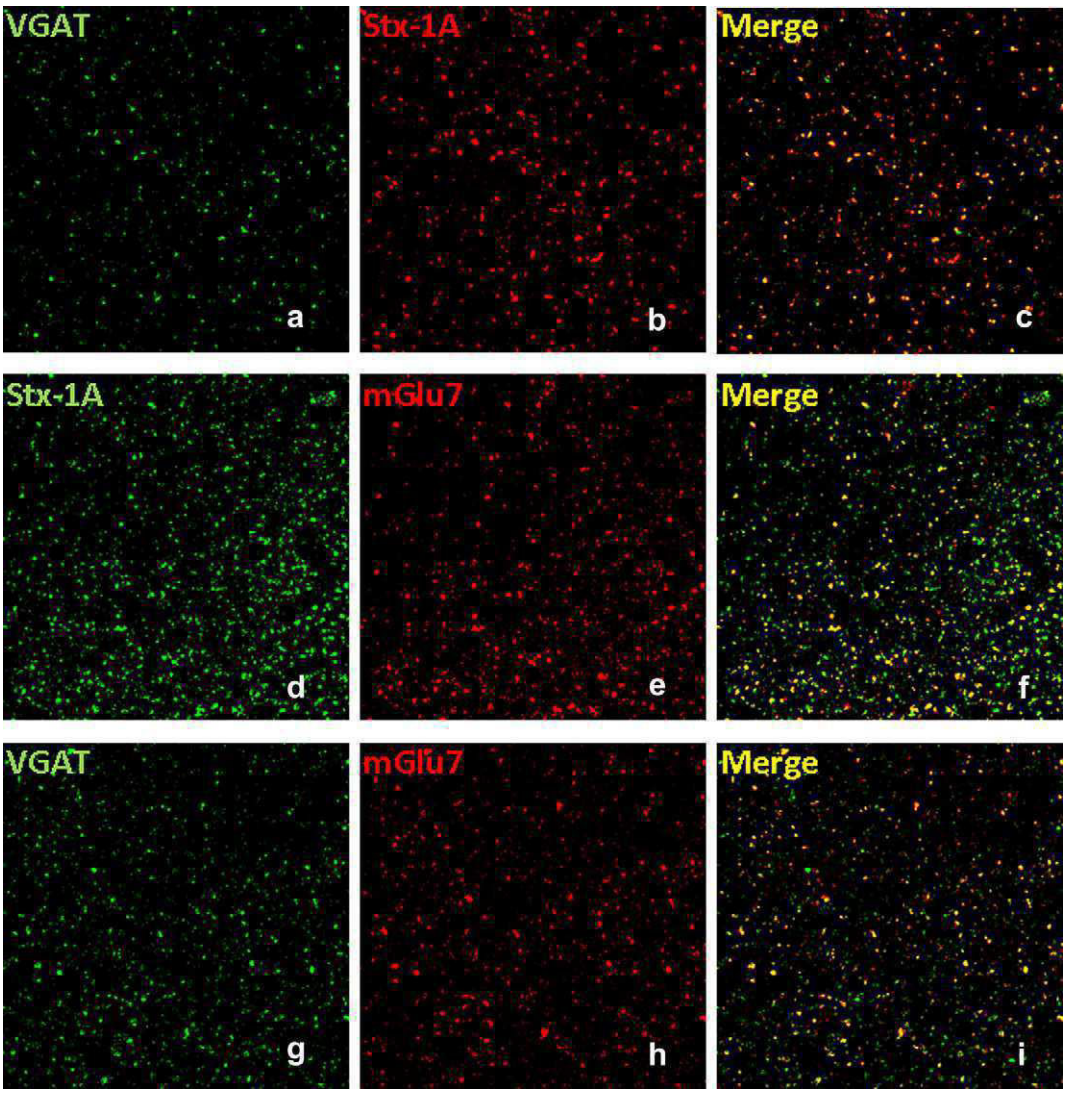


Fig. 6. mGlu7 receptor proteins locate presynaptically on GABA-containing nerve terminals isolated from the hippocampus of adult mice: representative double-labelling images of anti-mGlu7, anti-Stx-1A (here used as selective marker of nerve terminals) and anti-VGAT (here used as marker of nerve terminals where GABA could be taken up and stored in vesicles). The images are representative of 5 different analyses.

5.01 ± 0.33, *n.s.* results expressed as induced overflow, data from 3 experiments run in triplicate), failed to modify the 100 nM AMN082-induced inhibition of tritium overflow (12 mM K^+/10 nM AMN082: 3.12 ± 0.16; +10 nM DMI: 3.64 ± 0.24, *n.s.* results expressed as induced overflow, data from 3 experiments run in triplicate).

The results so far described seemed consistent with the presence of mGlu7 heteroreceptors on GABAergic hippocampal nerve terminals. In order to confirm this hypothesis, we investigated whether mouse hippocampal synaptosomal plasmamembranes could be endowed with mGlu7 receptor proteins. Fig. 5 shows that mGlu7 immunoreactivity with appropriate mass was observed in synaptosomal membranes isolated from mouse hippocampal synaptosomes.

Considering that the above procedure does not permit discrimination between families of nerve terminals, it was important to ascertain whether mGlu7 receptor immunoreactivity could be detected in GABAergic nerve endings. As a first approach, we tried to identify GABAergic terminals by using an antibody raised against the enzyme glutamate decarboxylase (GAD). Unfortunately, unsormountable interferences between anti GAD and anti-mGlu7 antibodies did not allow this confocal analysis (not shown). GABAergic nerve endings were therefore identified by using an antibody raised against the vesicular GABA transporter (VGAT) and by monitoring the colocalization of this protein with Syntaxin-1A, here used as a presynaptic marker. The mouse hippocampal synaptosomal preparations efficiently stained for VGAT (Fig. 6a, green) and Stx-1A (Fig. 6b, red); merging these images revealed

Table 2
Effect of repeated exposure to AMN082 on the agonist-induced inhibition of [^{3}H] GABA release from mouse hippocampal synaptosomes.

	S1		S2	
	% induced overflow	% of change vs S1	% induced overflow	% of changes vs S2
12 mM K^+	3.60 ± 0.22		2.82 ± 0.27	
12 mM K^+/100 nM AMN082	1.75 ± 0.15[a]	−45.48%	1.35 ± 0.09[a]	−52.12%

Synaptosomes were preloaded with [^{3}H]GABA and exposed in superfusion to two consecutive depolarizing stimuli. When indicated, 100 nM AMN082 was added concomitantly to the 12 mM KCl solution and substituted after 90 s with standard superfusion medium. The ratio S2/S1 amounted to −21.66%, consistent with the depletion of radioactive intraterminal store.

[a] $p < 0.05$ vs respective control.

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

that a significant percentage of Stx-1A-positive particles (59 ± 5%, data from five different image couples) were also VGAT-positive (Fig. 6c). Notably, the high percentage of VGAT expression in Stx-1A-positive particles might be well consistent with the widespread expression of VGAT proteins. Indeed, beside the pure GABAergic synaptosomal subpopulation, this transporter also locates in GABA/glycine nerve endings (Chaudhry et al., 1998) as well as in hippocampal mossy fibre terminals, where it colocalizes with the vesicular glutamate transporters type 1 (Zander et al., 2010). The confocal microscopy analyses show that the mouse hippocampal synaptosomal preparations efficiently stained for mGlu7 receptor (Fig. 6e and h, red), and a significant percentages of the Stx-1A-

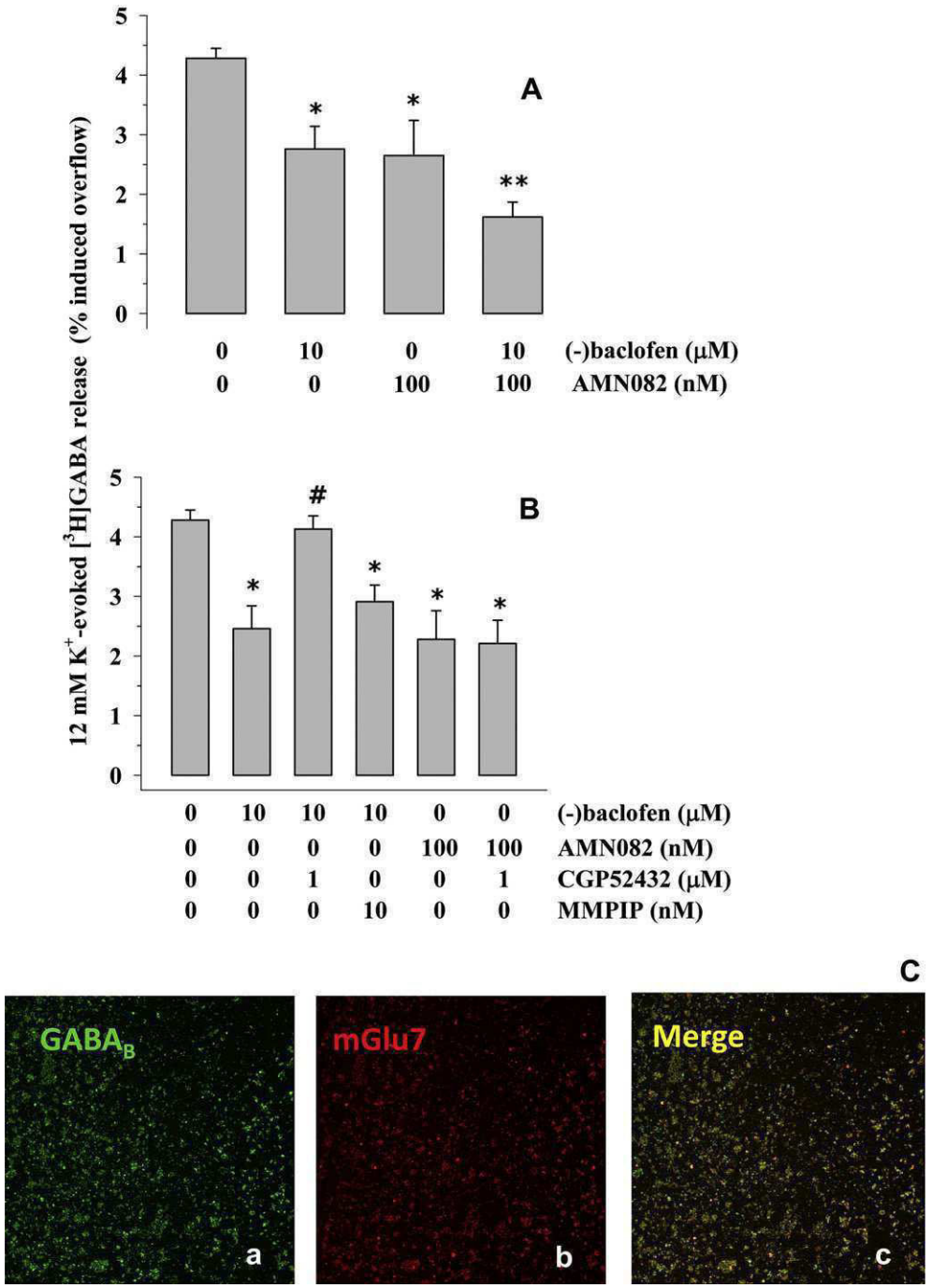


Fig. 7. Effects of AMN082 and (−)baclofen on the K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes: antagonism by CGP52432 and MMPIP. *Panel A*: Mouse hippocampal synaptosomes preloaded with the radioactive tracer were exposed in superfusion to 12 mM KCl. When indicated, (−)baclofen (10 μM) and AMN082 (100 nM) were added alone or together, contemporary to the depolarizing stimulus *Panel B*: Effects of CGP52432 and MMPIP on the inhibitory effects exerted by (−)baclofen and AMN082 on the K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes. CGP52432 (1 μM) and MMPIP (10 nM) were added starting from eight minutes before the depolarizing stimulus. Results are expressed as induced overflow. Data are means ± SEM of 3 experiments run in triplicate. $^*p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; $^{**}p < 0.05$ vs K^+-evoked release of [^{3}H]GABA; $^{\#}p < 0.05$ vs K^+/1 μM (−)baclofen-evoked release of [^{3}H]GABA. *Panel C*: mGlu7 receptor proteins colocalize with $GABA_B$ receptor subunits on nerve terminals isolated from the hippocampus of adult mice: representative double-labelling images of anti-mGlu7 and anti-$GABA_B$ immunoreactivities. The images are representative of 4 different analysis.

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

positive terminal particles (Fig. 6d, green, 68 ± 5%) and of the VGAT-positive particles (Fig. 6g, green, 68 ± 6%) were positive for mGlu7 receptor.

mGlu7 receptors were reported to undergo desensitization (Pelkey et al., 2007 and references therein) shortly after a brief pre-exposure to agonist. To investigate whether this phenomenon also could take place in our experimental conditions, hippocampal synaptosomes were challenged with two consequent depolarizing stimuli (12 mM K^+, here referred to as S1 and S2) in the absence or in the presence of 100 nM AMN082. Table 2 shows that significant changes of the AMN082-induced inhibitory effect could not be observed following repeated application of the depolarizing stimulus in the presence of the agonist.

Changes in functional consequence due to mGlu7 receptors activation were also reported to occur following prolonged pre-exposure (10 min) of nerve terminals to the agonist (Martin et al., 2010). Synaptosomes pretreatment with the mGlu7 agonist however did not caused significant modifications to the [^{3}H]GABA exocytosis elicited by 12 mM K^+ in the absence or in the presence of 100 nM AMN082 (control synaptosomes: 12 mM K^+: 3.11 ± 0.18; 12 mM K^+: 1.89 ± 0.22, $p < 0.05$, $n = 3$; AMN082 pretreated synaptosomes: 12 mM K^+: 2.89 ± 0.21; 12 mM K^+: 1.76 ± 0.19, $p < 0.05$, $n = 3$).

Hippocampal GABAergic terminals possess presynaptic $GABA_B$ autoreceptors, whose activation inhibits GABA exocytosis. Consistently with previous observations (Pittaluga et al., 1987), 10 μM (−) baclofen inhibited the 12 mM K^+-evoked release of [^{3}H]GABA. The inhibitory effect caused by (−)baclofen was additive to that evoked by 10 nM AMN082 (Fig. 7A). The (−)baclofen-induced inhibition of12 mM K^+-evoked release of [^{3}H]GABA was reverted by the $GABA_B$ antagonist 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl) phosphinic acid (CGP52432) (Bonanno and Raiteri, 1993), but was insensitive to the mGlu7 antagonist MMPIP (10 nM). Conversely, the AMN082-induced inhibition of 12 mM K^+-evoked release of [^{3}H]GABA was prevented by MMPIP (see Fig. 2) but was insensitive to CGP52432 (1 μM). At the concentration applied CG52432 did not modify on its own the 12 mM K^+-evoked release of [^{3}H]GABA. Confocal analyses unveiled that the mouse hippocampal synaptosomal preparations efficiently stained for the $GABA_B$ receptor proteins. Analysis of four different image couples indicated that 43 ± 5% of the $GABA_B$-positive particles were also mGlu7 positive (Fig. 7C).

Finally, Fig. 8A shows that exposure of mouse hippocampal synaptosomes preloaded with [^{3}H]GABA to 10 μM forskolin caused a significant release of radioactive tracer. The forskolin-evoked release of tritium was halved by the concomitant addition of 100 nM AMN082 as well as by 10 μM (−)baclofen (Fig. 8A). Again, the inhibitory effect caused by (−)baclofen was additive to that evoked by 10 nM AMN082 (Fig. 8A). AC-mediated intraterminal events participated to the 12 mM K^+-evoked GABA exocytosis, as suggested by the finding that i) the AC inhibitor MDL-12,330A (10 μM) significantly inhibited the 12 mM K^+-evoked tritium exocytosis, and ii) the MDL-12,330A-induced inhibition of GABA exocytosis was totally recovered by the cAMP analogue, 8-Br-cAMP (10 μM, Fig. 8B). Interestingly, 8-Br-cAMP also restored the AMN082-induced inhibition of GABA exocytosis, further supporting the hypothesis that AMN082-induced effect involves reduction of AC-mediated cAMP production (Fig. 8B).

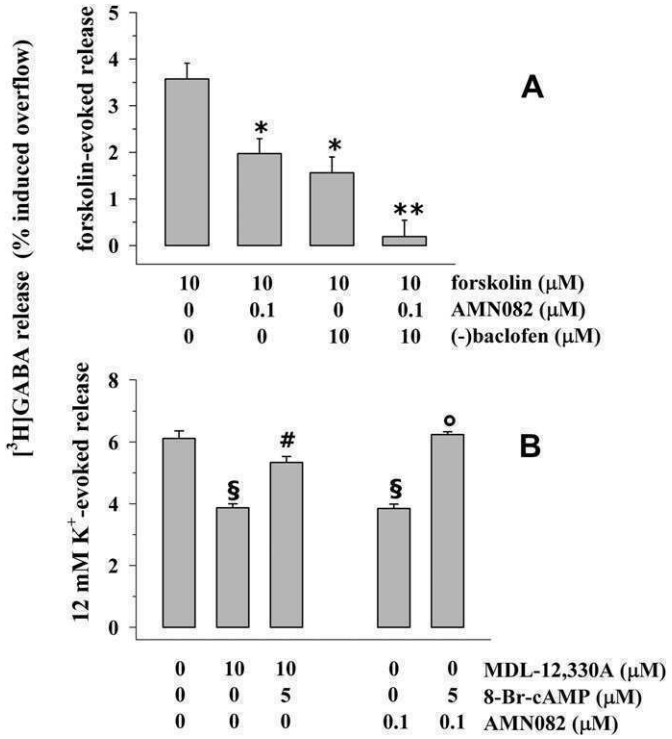


Fig. 8. AMN082-induced inhibition of [^{3}H]GABA exocytosis from mouse hippocampal synaptosomes relies on inhibition of adenylyl cyclase-dependent pathway: comparison with (−)baclofen. *Panel A*: Effects of (−)baclofen or AMN082 on the forskolin-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes. Mouse hippocampal nerve terminals were exposed in superfusion to 10 μM forskolin in the absence or in the presence of 5 μM (−)baclofen or 100 nM AMN082. Results are expressed as induced overflow. Data are means ± SEM of 3 experiments run in triplicate. $^*p < 0.05$ vs 10 μM forskolin-evoked release of [^{3}H]GABA; $^{**}p < 0.01$ vs 10 μM forskolin-evoked release of [^{3}H]GABA. *Panel B*: Effects of AMN082 and MDL-12,330A on the K^+-evoked release of [^{3}H]GABA from mouse hippocampal synaptosomes: antagonism by 8-Br-cAMP. Mouse hippocampal synaptosomes preloaded with [^{3}H]GABA were exposed in superfusion to 12 mM KCl in the absence or in the presence of 10 μM MDL-12,330A or 100 nM AMN082. When indicated, 5 μM 8-Br-cAMP was added concomitantly with agonists. Results are expressed as induced overflow. Data are means ± SEM of 3 experiments run in triplicate. $^§p < 0.05$ vs 12 mM K^+-evoked release of [^{3}H]GABA; $^{\#}p < 0.05$ 12 mM K^+/10 μM MDL-12,330A -evoked release of [^{3}H]GABA; $^\circ p < 0.05$ 12 mM K^+/100 nM AMN082-evoked release of [^{3}H]GABA.

4. Discussion

Previous studies provided evidence of the existence of presynaptic mGlu7 heteroreceptors on GABAergic interneurones in rodent hippocampus. mGlu7 immunoreactivity was found in somatostatin/GABA immunoreactive interneurones (Shigemoto et al., 1997) and in the presynaptic active zone of selected populations of GABAergic terminals (Somogyi et al., 2003), although contrasting evidence was also described (Bradley et al., 1996). Given their presynaptic location, mGlu7 receptors were predicted to inhibit GABA exocytosis, subsequently favouring glutamate transmission (Schoepp, 2001). Direct evidence supporting the presynaptic modulatory effect of mGlu7 receptors on GABA release in rodent hippocampus, however, is so far lacking.

In the present work, hippocampal isolated nerve terminals (synaptosomes) were used to investigate the possible mGlu7-mediated regulation of GABA release. Functional experiments were performed by monitoring neurotransmitter release from superfused monolayers of purified synaptosomes, a technique that is widely recognized as particularly appropriate to study release-regulating presynaptic receptors (for a review please see Raiteri and Raiteri, 2000). Briefly, after selective labelling with [^{3}H]GABA, identical portions of synaptosomal suspension are stratified at the bottom of parallel superfusion chambers to constitute less than one monolayer and then are up-down superfused. Several laboratories reported that, under these experimental conditions, the compounds just released are removed by the superfusion solution so hastily that they can not feed back on releasing terminals, nor on

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

neighbouring particles, thus avoiding indirect effects. Under this experimental condition, if mGlu7 agonist added concomitantly to the depolarizing stimulus inhibits [^{3}H]GABA exocytosis, one could conclude that presynaptic mGlu7 receptors are localized on GABAergic axon terminals and control negatively GABA exocytosis.

The present results demonstrate that the mGlu7 allosteric agonist, the compound AMN082, inhibited the K^+-evoked release of endogenous GABA as well as that of preloaded [^{3}H]GABA from hippocampal nerve terminals, with an apparent affinity well consistent with that firstly reported by Mitsukawa et al. (2005). This observation could be predictive of the involvement of mGlu7 receptor in the effect observed, although some recent observation could question this conclusion. In fact, both in vivo and in vitro studies had suggested that AMN082-mediated effects also could rely on non-mGlu7 receptor-mediated signalling. In particular, NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (Risso Bradley et al., 2012), as well as serotonergic receptor subtypes (Pałucha-Poniewiera et al., 2010) and NET (Sukoff Rizzo et al., 2011) were proposed to represent suitable targets of AMN082. In an attempt to ascertain whether the effects here described could be ascribed to activation of the above-mentioned receptors and/or transporter, experiments were carried out to assess whether antagonists at NMDA receptor complex as well as at 5-HT receptor subtypes and NET blocker could prevent AMN082-induced modification to GABA exocytosis. We focussed on these receptors since previous studies demonstrated the existence of presynaptic inhibitory NMDA and 5-HT receptors controlling GABA outflow in the hippocampus (Sequeira et al., 2001; Chadha et al., 2000). We did not investigate the impact of AMPA receptor antagonist, since AMPA receptors were reported to favour, not to inhibit, GABA outflow (Pittaluga et al., 1997). Our results demonstrate that neither the above-mentioned antagonists nor the NET blocker DMI could counteract the AMN082-induced inhibitory effect on GABA transmission that, on the contrary, was totally abrogated by the mGlu7 negative allosteric modulator MMPIP. All together these observations support the view that AMN082-induced reduction of GABA exocytosis involves mGlu7 receptor. Accordingly, the widespread group III mGlu receptor agonist (L)-AP4, but not the selective mGlu8 agonist (S)-3,4-DCPG, mimicked AMN082 in inhibiting the release of [^{3}H]GABA evoked by high K^+ in a MMPIP-sensitive manner, also leading to exclude the presence of (L)-AP4-sensitive metabotropic glutamate receptors other than mGlu7 receptor subtype.

AMN082 was reported to promote the internalization of recombinant mGlu7 receptors in hippocampal neurones (Pelkey et al., 2007). Repeated application of the agonist however did not affect GABA exocytosis nor it modified the AMN082-mediated inhibitory effect, suggesting that presynaptic native mGlu7 heteroreceptors may undergo a limited, if ever, receptor internalization or desensitization. A shift from inhibition to facilitation of presynaptic mGlu7 autoreceptors was also described to follow a prolonged (10 min) exposure of terminals to agonist (Martin et al., 2010). Again, changes in mGlu7 receptor-mediated control of GABA exocytosis following prolonged exposure to agonist could not be detected. Although differences in the experimental approaches may account for the apparent discrepancy, the possibility that mGlu7 receptor on GABAergic terminals differs from those located on other terminal subpopulations, i.e. the glutamatergic one, in term of agonist-induced desensitization or coupling to intraterminal enzymatic pathways should be considered.

Thus, as a first conclusion, we propose that, in mouse hippocampus, glutamate can modulate the depolarization-evoked release of GABA by acting at inhibitory presynaptic receptors belonging to the mGlu7 receptor subtypes. Notably, the observation that endogenous GABA responds equally to previously taken up [^{3}H]GABA to the action of mGlu7 agonists could suggest that the radioactive tracer allows, in the present case, a reliable measure of the endogenous amino acid.

The functional results described here are consistent with data previously published reporting mGlu7 receptor immunoreactivity in rat hippocampal GABAergic terminals (Somogyi et al., 2003). Accordingly, mGlu7 immunoreactivity could be detected in mouse hippocampal synaptosomal plasmamembranes and the presence of mGlu7 receptor proteins was highlighted at the presynaptic level, in Stx-1A-positive terminals where GABA is actively stored in intraterminal VGAT-positive vesicles. GABA, however, does not represent the sole substrate of VGAT, since this protein is also responsible for the uptake and the storage of glycine at pure glycinergic synapses, as well as at GABA/glycine synapses (Chaudhry et al., 1998). Our observation therefore could imply that mouse hippocampal glycinergic terminals could also be endowed with presynaptic mGlu7 receptors. The hypothesis is plausible and surely deserves future investigation.

Hippocampal GABAergic terminals possess presynaptic $GABA_B$ autoreceptors, whose activation inhibits GABA exocytosis (Pittaluga et al., 1987). Reciprocal interaction between $GABA_B$ and mGlu7 receptors has been the object of previous investigation. In particular, mGlu7 and $GABA_B$ receptors were reported to be functionally coupled in hippocampal glutamatergic nerve terminals and to negatively control glutamate exocytosis (Martín et al., 2008). On the other hand, the second finding of the present study shows that presynaptic mGlu7 heteroreceptors and $GABA_B$ autoreceptors located on GABAergic nerve endings do not cross-talk. Our conclusion is supported by the findings that i) the inhibitory effects exerted by AMN082 and by (−)baclofen on GABA exocytosis after simultaneous activation of both mGlu7 and $GABA_B$ receptors were additive, corresponding to the sum of the inhibition of each agonists ($-62.15 \pm 4.30\%$, $n = 3$, $p < 0.01$), ii) mGlu7-mediated control of GABA exocytosis was insensitive to the presence of the $GABA_B$ receptor antagonist CGP52432, while MMPIP failed to affect the (−)baclofen-induced control of GABA overflow, and iii) the inhibitory effects exerted by AMN082 and (−)baclofen on the forskolin-evoked release of [^{3}H]GABA from hippocampal synaptosomes were additive. Although the possibility that the $GABA_B$ receptors and mGlu7 receptors might colocalize on the same nerve terminals cannot be excluded, our results seem best interpreted by assuming that mGlu7 and $GABA_B$ receptors are located on different subpopulation of mouse hippocampal GABAergic nerve terminals (as suggested by confocal analysis in Fig. 7C). Accordingly, Somogyi et al. (2003) demonstrated that mGlu7 immunoreactivity was mainly restricted to GABAergic inputs of interneurones that receive innervation from mGlu7-enriched glutamatergic terminals.

Finally, as to the transducing mechanism involved, we propose that mGlu7 heteroreceptors presynaptically located on GABAergic nerve endings might negatively couple to the intraterminal AC-cAMP enzymatic pathway. This enzymatic pathway is also located presynaptically where it participates to high K^+-evoked vesicular exocytosis of transmitters (Grilli et al., 2004 and reference therein) including GABA, as suggested by the finding that the AC selective inhibitor MDL-12-330A drastically reduces GABA in a 8-Br-cAMP-sensitive. If this is the case, the capability of AMN082 in reducing the release of GABA elicited by forskolin together with the efficacy of 8-Br-cAMP in preventing AMN082-mediated inhibition of K^+-evoked GABA overflow seems best interpreted by assuming that mGlu7 negatively control the AC-cAMP-mediated component of GABA exocytosis from hippocampal synaptosomes. Notably, the efficacy of MMPIP in reverting the AMN082-mediated inhibition of GABA exocytosis further supports the hypothesis that NAM activity is context-dependent (see Niswender and Conn, 2010). Whether mGlu7-inhibitory effect in GABAergic hippocampal terminals could

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

rely on the reduction of Voltage Operated Calcium Channel (VOCC)-mediated functions, as firstly proposed by Millán et al. (2002b) cannot be excluded. Indeed, of the AC isoforms known so far, AC1 and AC8 are Ca^{2+}-dependent enzymes, so that the AC-cAMP-mediated component of GABA exocytosis may represent a direct consequence of AC activation by Ca^{2+} entering the K^{+}-depolarized synaptosomes through VSCCs.

In conclusion, the present results support the existence of presynaptic mGlu7 heteroreceptors on a distinct subpopulation of mouse hippocampal GABAergic nerve terminals. These receptors are negatively coupled to adenylyl cyclase and inhibit GABA exocytosis and could have a relevant role in the reciprocal modulation GABA and glutamate exerts on their depolarization-evoked release at the hippocampal level. Thus, these receptors represent an important therapeutic target for the cure of neurological disorders that are typified by a marked unbalance between excitatory and inhibitory transmission including epilepsy (Sansig et al., 2001), anxiety and depression (Swanson et al., 2005; Wierońska and Pilc, 2009), and schizophrenia (Wierońska et al., 2010).

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

This work was supported by grants from Italian MIUR (Ministero dell'Istruzione, dell'Universita' e della Ricerca, Projects n. 2009P7WHNR_003), from University of Genova 'Progetto Ricerca Ateneo' and from ALCOTRA (Alpi Latine Cooperazione Transfrontaliera– Project n. 231 «Developpement commercial des potentialite' s du genre salvia: nouvelles productions); from the European Community (Project Strategia d'Impresa in Settori di Nicchia per l'Economia Agro-Industriale del Mediterraneo, Pyrgi, project n° B51H10000000006 and project Progetto 7FP-KBBE BAMMBO) We wish to thank Maura Agate for careful editorial assistance and Dr. Silvia E. Smith, PhD. (University of Utah, School of Medicine) for reviewing the manuscript.

References

Alexander, G.M., Godwin, D.W., 2006. Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy. Res. 71, 1–22.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bradley, S.R., Levey, A.I., Hersch, S.M., Conn, P.J., 1996. Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J. Neurosci. 16, 2044–2056.

Bonanno, G., Raiteri, M., 1993. Multiple $GABA_B$ receptors. Trends. Pharmacol. Sci. 14, 259–261.

Chadha, A., Sur, C., Atack, J., Duty, S., 2000. The 5HT1B receptor agonist, CP-93129, inhibits [^{3}H]-GABA release from rat globus pallidus slices and reverses akinesia following intrapallidal injection in the reserpine-treated rat. Br. J. Pharmacol. 130, 1927–1932.

Chaudhry, F.A., Reimer, R.J., Bellocchio, E.E., Danbolt, N.C., Osen, K.K., Edwards, R.H., Storm-Mathisen, J., 1998. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sites of glycinergic as well as GABAergic neurones. J. Neurosci. 18, 9733–9750.

Conn, P.J., Pin, J.P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol. Toxicol. 37, 205–237.

Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G., Lockett, S., 2004. Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys. J. 86, 3993–4003.

Danysz, W., Parsons, C.G., Bresink, I., Quack, G., 1995. Glutamate in CNS disorders. Drug. News. Perspect. 8, 261–276.

Desai, M.A., McBain, C.J., Kauer, J.A., Conn, P.J., 1994. Metabotropic glutamate receptor-induced disinhibition is mediated by reduced transmission at excitatory synapses onto interneurons and inhibitory synapses onto pyramidal cells. Neurosci. Lett. 181, 78–82.

Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kidd, G.J., Rostas, J.A., 1986. A rapid method for isolation of synaptosomes on Percoll gradients. Brain. Res. 372, 115–129.

Duty, S., 2010. Therapeutic potential of targeting group III metabotropic glutamate receptors in the treatment of Parkinson's disease. Br. J. Pharmacol. 161, 271–287.

Gereau 4th, R.W., Conn, P.J., 1995. Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. J. Neurosci. 15, 6879–6889.

Grilli, M., Raiteri, L., Pittaluga, A., 2004. Somatostatin inhibits glutamate release from mouse cerebrocortical nerve endings trough presynaptic sst2 receptor linked to the adenylyl cyclase-protein kinase A pathway. Neuropharmacology 46, 388–396.

Luccini, E., Musante, V., Neri, E., Raiteri, M., Pittaluga, A., 2007. N-methyl-D-aspartate autoreceptors respond to low and high agonist concentrations by facilitating, respectively, exocytosis and carrier-mediated release of glutamate in rat hippocampus. J. Neurosci. Res. 85, 3657–3665.

Manders, E.M., Stap, J., Brakenhoff, G.J., van Driel, R., Aten, J.A., 1992. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J. Cell. Sci. 103, 857–862.

Marek, G.J., 2010. Metabotropic glutamate2/3 (mGlu2/3) receptors, schizophrenia and cognition. Eur. J. Pharmacol. 639, 81–90.

Martín, R., Ladera, C., Bartolomé-Martín, D., Torres, M., Sánchez-Prieto, J., 2008. The inhibition of release by $mGlu_7$ receptors is independent of the Ca^{2+} channel type but associated to $GABA_B$ and adenosine A1 receptors. Neuropharmacology 55, 464–473.

Martin, R., Durroux, D., Ciruela, F., Torres, M., Pin, J.P., Sanchez-Prieto, J., 2010. The metabotropic glutamate receptor mGlu7 activates phospholipase C, translocate Munc-13-1 protein, and potentiates glutamate release at cerebrocortical nerve terminals. J. Biol. Chem. 285, 17907–17917.

Maura, G., Marcoli, M., Pepicelli, O., Rosu, C., Viola, C., Raiteri, M., 2000. Serotonin inhibition of the NMDA receptor/nitric oxide/cyclic GMP pathway in human neocortex slices: involvement of $5HT_{2C}$ and $5HT_{1C}$ receptors. Br. J. Pharmacol. 130, 1853–1858.

Millán, C., Luján, R., Shigemoto, R., Sánchez-Prieto, J., 2002a. The inhibition of glutamate release by metabotropic glutamate receptor 7 affects both [Ca^{2+}]c and cAMP: evidence for a strong reduction of Ca^{2+} entry in single nerve terminals. J. Biol. Chem. 277, 14092–14101.

Millán, C., Luján, R., Shigemoto, R., Sánchez-Prieto, J., 2002b. Subtype-specific expression of group III metabotropic glutamate receptors and Ca^{2+} channels in single nerve terminals. J. Biol. Chem. 277, 47796–47803.

Mitsukawa, K., Yamamoto, R., Ofner, S., Nozulak, J., Pescott, O., Lukic, S., Stoehr, N., Mombereau, C., Kuhn, R., McAllister, K.H., van der Putten, H., Cryan, J.F., Flor, P.J., 2005. A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. Proc. Natl. Acad. Sci. U S A 102, 18712–18717.

Nicoletti, F., Bockaert, J., Collingridge, G.L., Conn, P.J., Ferraguti, F., Schoepp, D.D., Wroblewski, J.T., Pin, J.P., 2010. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60, 1017–1041.

Niswender, C.M., Conn, P.J., 2010. Metabotropic glutamate receptors: physiology, pharmacology and disease. Annu. Rev. Pharmacol. Toxicol. 50, 295–322.

Pałucha-Poniewiera, A., Brański, P., Lenda, T., Pilc, A., 2010. The antidepressant-like action of metabotropic glutamate 7 receptor agonist N, N'-bis(diphenylmethyl)-1,2-ethanediamine (AMN082) is serotonin-dependent. J. Pharmacol. Exp. Ther. 334, 1066–1074.

Pelkey, K., Yaun, X., Lavezzari, G., Roche, K.W., Mc Bain, C.J., 2007. mGluR7 undergoes rapid internalization in response to activation by the allosteric agonist AMN082. Neuropharmacology 52, 108–117.

Pin, J.P., Duvoisin, R., 1995. The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34, 1–26.

Pin, J.P., Ascher, F., 2002. The metabotropic glutamate receptors: structure, activation mechanism and pharmacology. Curr. Drug. Targets. CNS. Neurol. Disord. 1, 297–317.

Pittaluga, A., Asaro, D., Pellegrini, G., Raiteri, M., 1987. Studies on [^{3}H]GABA and endogenous GABA release in rat cerebral cortex suggest the presence of autoreceptors of the $GABA_B$ type. Eur. J. Pharmacol. 144, 45–52.

Pittaluga, A., Bonfanti, A., Raiteri, M., 1997. Differential desensitization of ionotropic non-NMDA receptors having distinct neuronal location and function. Naunyn. Schmiedebergs. Arch. Pharmacol. 356, 29–38.

Raiteri, M., 2008. Presynaptic metabotropic glutamate and $GABA_B$ receptors. Handb. Exp. Pharmacol. 184, 373–407.

Raiteri, L., Raiteri, M., 2000. Synaptosomes still viable after 25 years of superfusion. Neurochem. Res. 25, 1265–1274.

Raiteri, M., Angelini, F., Levi, G., 1974. A simple apparatus for studying the release of neurotransmitters from synaptosomes. Eur. J. Pharmacol. 25, 411–414.

Risso Bradley, S., Uslaner, J.M., Flick, R.B., Lee, A., Groover, K.M., Huston, P.H., 2012. The mGluR7 allosteric agonist AMN082 produces antidepressant-like effects by modulating glutamatergic signalling. Pharmacol. Biochem. Behav. 101, 35–40.

Sansig, G., Bushell, T.J., Clarke, V.R., Rozov, A., Burnashev, N., Portet, C., Gasparini, F., Schmutz, M., Klebs, K., Shigemoto, R., Flor, P.J., Kuhn, R., Knoepfel, T., Schroeder, M., Hampson, D.R., Collett, V.J., Zhang, C., Duvoisin, R.M., Collingridge, G.L., van Der Putten, H., 2001. Increased seizure susceptibility in mice lacking metabotropic glutamate receptor 7. J. Neurosci. 21, 8734–8745.

Schoepp, D.D., 2001. Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. J. Pharmacol. Exp. Ther. 299, 12–20.

Sequeira, S.M., Malva, J.O., Carvalho, A.P., Carvalho, C.M., 2001. Presynaptic N-methyl-D-aspartate receptor activation inhibits neurotransmitter release through nitric oxide formation in rat hippocampal nerve terminals. Mol. Brain. Res. 89, 111–118.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P.J., Neki, A., Abe, T., Nakanishi, S., Mizuno, N., 1997. Differential presynaptic

localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J. Neurosci. 17, 7503–7522.
Somogyi, P., Dalezios, Y., Luján, R., Roberts, J.D., Watanabe, M., Shigemoto, R., 2003. High level of mGluR$_7$ in the presynaptic active zones of select populations of GABAergic terminals innervating interneurons in the rat hippocampus. Eur. J. Neurosci. 17, 2503–2520.
Spooren, W., Ballard, T., Gasparini, F., Amalric, M., Mutel, V., Schreiber, R., 2003. Insight into the function of group I and group II metabotropic glutamate (mGlu) receptors: behavioural characterization and implications for the treatment of CNS disorders. Behav. Pharmacol. 14, 257–277.
Sukoff Rizzo, S.J., Leonard, S.K., Gilbert, A., Dollings, P., Smith, D.L., Zhang, M.Y., Di, L., Platt, B.J., Neal, S., Dwyer, J.M., Bender, C.N., Zhang, J., Lock, T., Kowal, D., Kramer, A., Randall, A., Huselton, C., Vishwanathan, K., Tse, S.Y., Butera, J., Ring, R.H., Rosenzweig-Lipson, S., Hughes, Z.A., Dunlop, J., 2011. The metabotropic glutamate receptor 7 allosteric modulator AMN082: a monoaminergic agent in disguise? J. Pharmacol. Exp. Ther. 338, 345–352.
Suzuki, G., Tsukamoto, N., Fushiki, H., Kawagishi, A., Nakamura, M., Kurihara, H., Mitsuya, M., Ohkubo, M., Ohta, H., 2007. In vitro pharmacological characterization of novel isoxazolopyridone derivatives as allosteric metabotropic glutamate receptor 7 antagonists. J. Pharmacol. Exp. Ther. 323, 147–156.
Swanson, C.J., Bures, M., Johnson, M.P., Linden, A.M., Monn, J.A., Schoepp, D.D., 2005. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. Nat. Rev. Drug. Discov. 4, 131–144.
Wierońska, J.M., Pilc, A., 2009. Metabotropic glutamate receptors in the tripartite synapse as a target for new psychotropic drugs. Neurochem. Int. 55, 85–97.
Wierońska, J.M., Brański, P., Siwek, A., Dybala, M., Nowak, G., Pilc, A., 2010. GABAergic dysfunction in mGlu7 receptor-deficient mice as reflected by decreased levels of glutamic acid decarboxylase 65 and 67 kDa and increased reelin proteins in the hippocampus. Brain. Res. 1334, 12–24.
Witkin, J.M., Marek, G.J., Johnson, B.G., Schoepp, D.D., 2007. Metabotropic glutamate receptors in the control of mood disorders. CNS. Neurol. Disord. Drug. Targets 6, 87–100.
Zander, J.F., Münster-Wandowsky, A., Brunk, I., Gomez-Lira, G., Heinemann, U., Gutirrez, R., Laube, G., Ahnert-Hilger, G., 2010. Synaptic and vesicular coexistence of VGLUT and VGAT in selected excitatory and inhibitory synapses. J. Neurosci. 30, 7634–7645.

Please cite this article in press as: Summa, M., et al., Presynaptic mGlu7 receptors control GABA release in mouse hippocampus, Neuropharmacology (2012), doi:10.1016/j.neuropharm.2012.04.020

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26a.12

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territuriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

ACTA ABP BIOCHIMICA POLONICA

Vol. 60, No 1/2013
43–50
on-line at: www.actabp.pl

Regular paper

Composition of volatile in micropropagated and field grown aromatic plants from Tuscany Islands

Laura Pistelli[1]✉, Cecilia Noccioli[2], Francesca D'Angiolillo[1] and Luisa Pistelli[2]

[1]Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy; [2]Department of Pharmacy, University of Pisa, Pisa, Italy

Aromatic plant species present in the natural Park of Tuscany Archipelago are used as flavoring agents and spices, as dietary supplements and in cosmetics and aromatherapy. The plants are usually collected from wild stands, inducing a depletion of the natural habitat. Therefore, micropropagation of these aromatic plants can play a role in the protection of the natural ecosystem, can guarantee a massive sustainable production and can provide standardized plant materials for diverse economical purposes. The aim of this study is to compare the volatile organic compounds produced by the wild plants with those from *in vitro* plantlets using headspace solid phase micro-extraction (HS-SPME) followed by capillary gas-chromatography coupled to mass spectrometry (GC-MS). Typical plants of this natural area selected for this work were *Calamintha nepeta* L., *Crithmum maritimum* L., *Lavandula angustifolia* L., *Myrtus communis* L., *Rosmarinus officinalis* L., *Salvia officinalis* L. and *Satureja hortensis* L. Different explants were used: microcuttings with vegetative apical parts, axillary buds and internodes. Sterilization percentage, multiplication rate and shoot length, as well as root formation were measured. The volatile aromatic profiles produced from *in vitro* plantlets were compared with those of the wild plants, in particular for *C. maritimum*, *R. officinalis*, *S. officinalis* and *S. hortensis*. This study indicated that the micropropagation technique can represent a valid alternative to produce massive and sterile plant material characterised by the same aromatic flavour as in the wild grown plants.

Key words: VOC, *in vitro* shoot cultures, aromatic plants, HS-SPME, GC-MS, biodiversity

Received: 03 October, 2012; revised: 15 January, 2013; accepted: 21 February, 2013; available on-line: 25 February, 2013

INTRODUCTION

The Tuscany Archipelago is the largest marine (and land) Park in Europe, with more than 50 000 hectares of sea and about 20 000 hectares of land. The Archipelago includes seven main islands as well as some minor islands and rocks (Landi, 1989). The vegetation, influenced by the Mediterranean climate and by the insularity, is characterised by several aromatic species. Typical plants of the Mediterranean that can be observed for example on the Elba Island are adapted to grow near the sea, tolerating salt stress, drought, and rock side soils include *Crithmum maritimum* L*., Rosmarinus officinalis* L*., Myrtus* sp., *Spartium* sp*., Cistus* sp*., Lavandula* sp*., Helichrysum* spp. and *Juniperus* spp. (Rinaldi, 2001). These plants constitute an ecological niche for their typical aromatic characteristics and represent an ecological population to exploit and to preserve (Hamilton, 2004; Abraham, 2010). The aromatic plants are usually collected on their natural stands and used as spices in traditional food and cosmetics, in phytotherapy and aromatherapy. This practice harms the natural habitat, so the development of efficient protocols for rapid clonal propagation and the conservation of germplasm of selected species typical of these islands is badly needed. *In vitro* culture of aromatic and medicinal plants is a useful technique to produce rapidly and in small spaces a large amount of plant material, avoiding the damage and the extinction of natural field grown plants (Debnath *et al*., 2006; Lucchesini & Mensuali-Sodi, 2010; Ruffoni *et al*., 2010). Moreover, micropropagation represents a valid alternative for rapid clonal propagation and an improvement of the production and marketing of the selected plants. The micropropagation protocols are based on regeneration of plants starting from different organs (Lucchesini & Mensuali-Sodi, 2010). The *in vitro* culture technique can be also useful for the production of active compounds naturally present in aromatic plants and in particular enhancing the levels of some metabolites (Pistelli *et al*., 2010).

The plants are able to emit organic volatile compounds (VOC), but the intensity and profile of emission is dependent on the genetic variability and plasticity of phenotypes (Dicke & Loreto, 2010). VOC are mainly produced by plants for three main reasons: plants-plants interactions, signals for symbiotic organisms, and as insect attractants or repellents. Many VOCs produced by plants are constituents of aromatic essential oils, often used in the food and perfume industries as flavors and/or fragrances (Maffei *et al.,* 2011). Moreover, it is known that essential oils can have antibacterial, anti-inflammatory and other pharmaceutical uses (Maffei *et al.,* 2011).

Headspace solid phase micro-extraction (HS-SPME) followed by capillary gas-chromatography mass-spectrometry (GC-MS) is currently a widely used technique for the characterization of the composition of plant volatile fraction, due to its easy and fast management (Belliardo *et al*., 2006).

The aim of the present work was to investigate, for the first time, the chemical composition of the aroma scent emitted by *in vitro* plantlets (ivP) of several aromatic species, in comparison with the field-grown mother-plants (fgP), using HS-SPME. Seven typical species grown in the Tuscany Archipleago were chosen as plant

✉e-mail: pistelli@agr.unipi.it

Abbreviations: BA, benzyladenine; GC-MS, capillary gas-chromatography mass spectrometry; HS-SPME, headspace solid phase micro-extraction; IAA, indole-3-acetic acid; IBA, indolebutiric acid; MS0, basal Murashige and Skoog medium; NAA, naphtalene acetic acid; PAR, photosynthetically active radiation

material from different families: *Calamintha nepeta* (L.) Savi, *Lavandula angustifolia* Mill., *Rosmarinus officinalis* L., *Salvia officinalis* L. and *Satureja hortensis* L. belonging to Labiatae, *Crithmum maritimum* L. (Umbelliferae family) and *Myrtus communis* L. (Myrtaceae family).

MATERIALS AND METHODS

Plant Materials. *Calamintha nepeta* L., *Crithmum maritimum* L., *Lavandula angustifolia* Mill., *Myrtus communis* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., *Satureja hortensis* L., representatives of the plant species typical of the Tuscan herbs, were collected in spring 2011 in the Elba Island of the Tuscan Archipelago, and kept in pots in a greenhouse for acclimatization. A voucher specimen was authenticated by S. Maccioni (Università di Pisa, Dipartimento di Biologia) and deposited at the Botanical Garden of the University of Pisa.

Micropropagation. Different types of explants were used: apical portions of *R. officinalis* and *S. hortensis,* internodes with lateral buds for *C. nepeta, M. communis, L. angustifolia*, and *S. officinalis,* basal shoots of *C. maritimum.* The explants from the selected species were differently sterilised. *C. nepeta* internodes with lateral buds, 10 mm length, were submerged in 0.05% (v/v) Tween-20® for 20 min, followed by washing with sterile water for 5 min. The explants were then transferred in 50% (v/v) commercial hypochlorite for 10 min, and then washed with sterile water for 5 min (sterilization method called S1) (Grigoriadou & Maloupa, 2008)**.** Ten-millimetre long internodes of *M. communis, L. officinalis*, and *S. officinalis* and basal shoots of *C. maritimum* were treated with detergent 0.05% (v/v) Tween-20® for 20 min, then dipped in a 70% (v/v) ethanol solution for 1 min before surface sterilized in 25%(v/v) commercial sodium hypochlorite for 10 min, and washed with sterile water for 5 min (S2) (Ruffoni & Mascarello, 2009). The S3 method, adopted for the apical portions of *R. officinalis* and *S. hortensis*, comprised a preliminary incubation of the explants in 2% (w/v) sucrose and 0.01% (v/v) commercial sodium hypochlorite for 15 days in the dark (Ruffoni & Mascarello, 2009). After washing with water for 5 min, the explants were then sterilized with the S2 treatment. Shoot proliferation was obtained by culturing the sterile explants in different media. The constituents of the basal medium (MS0) were MS macro- and micronutrients, vitamins (Murashige & Skoog, 1962), 3% (w/v) sucrose, 0.8% (w/v) agar and 0.05% (v/v) "Plant preservative mixture" (PPM); the above medium with the addition of 0.5 mg/L benzyladenine (BA) was called M1. The shoot proliferation medium for *R. officinalis* (M2) contained an extra 0.017 mg/L indole-3-acetic acid (IAA). The shoot culture multiplication medium for *C. maritimum* (M3) contained MS0 medium with the addition of 0.5 mg/L BA and 0.46 mg/L NAA. The rooting phase was induced *in vitro* by transferring the shoots to rooting media according to the literature. The following rooting media were utilized: R1, composed of MS0 and 0.2 mg/L NAA for *C. nepeta, R. officinalis* and *L. angustifolia* (Andrade *et al*., 1999); R2 (MS0 with 1 mg/L IAA + 1.5% sucrose) was used for *M. communis* (Scarpa *et al*., 2000), R3 (MS0 + 0.1 mg/L IBA) for *C. maritimum* (Grigoriadou & Maloupa, 2008), and medium R4 (MS0 + 0.5 mg/L IBA) for *S. officinalis* and *S. hortensis* (Arikat *et al.,* 2004). All media were adjusted to pH 5.7 with 0.1 M KOH before autoclaving for 20 min at 120°C. Cultures were maintained in a growthchamber at 22 ± 1°C under 16 h light and 8 h dark photoperiod provided by cool white fluorescent tubes (Philips TLM 40W/33RS) with 80 μmoles m^{-2} s^{-1} PAR. Cultures were monitored regularly for shoot production. Vitality was recorded after one week; after four weeks, the number and length of shoots, and percentage of root formation were recorded and the plantlets were transferred to fresh medium.

SPME analyses. Emitted volatiles were analyzed using a Supelco SPME device coated with polydimethylsiloxane (PDMS, 100 μm) in order to sample the headspace of 10 g of fresh plant mass comprising either field grown plants or aerial parts without flowers of *in vitro* plantlets collected at the end of 4-week subculture. Each sample was introduced into a 30 ml glass conical flask and allowed to equilibrate for 30 min. After the equilibration time, the fiber was exposed to the headspace for 15 min at room temperature; once sampling was finished, the fiber was withdrawn into the needle and transferred to the injector of the GC and GC-MS system, where the fiber was desorbed.

Gas Chromatography-FID. GC analyses were performed using an HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60°C for 10 min, rising at 5°C/min to 220°C; splitless injection mode, injector temperature, 250°C; carrier gas, nitrogen (2 ml/min); detector, dual FID. The identification of the components was performed for the both columns by comparison of their retention time with those of pure authentic samples and by means of their linear retention indices (*lRI*) relative to a series of *n*-hydrocarbons. The percentage of the volatile constituents was obtained by FID peak-area normalization.

Gas Chromatography-Mass Spectrometry. GC-MS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: splitless injection mode, injector temperature, 250°C; oven temperature programmed from 60 °C to 240°C at 3°C/min; carrier gas, helium at 1 ml/min; injection, 0.2 μl (10% hexane solution). Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to a series of *n*-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built up from pure substances, components of known oils and MS literature data (Adams, 1995; Connolly & Hill, 1991; Jennings & Shibamoto, 1980; Massada, 1976; Stenhagen *et al.,* 1974; Swigar & Silverstein, 1981).

RESULTS AND DISCUSSION

Calamintha nepeta L., *Crithmum maritimum* L. *Lavandula angustifolia* Mill., *Myrtus communis* L., *Rosmarinus officinalis* L., *Salvia officinalis* L. and *Satureja hortensis* L., typical species of the Tuscan Archipelago, were selected for micropropagation with the aim to preserve the environment and to collect the germplasm of typical insular Mediterranean plants. The main type of explants used for the sterilization process were microcutting (vegetative apical portions), in some cases axillary buds or internodes. Various sterilization methods were used to select the best method for each explants. Table 1 shows the sterilization efficiency expressed as the explants'vitality after 7 days of culture. The explants of *C. maritimum* and *M. commu-*

Table 1. Vitality percentage, multiplication rate, shoot length, and root formation of micropropagated plants.
Mean values from five independent replicates ± S.D. are shown. Abbreviations represent respectively: S1, S2 and S3 (sterilization method), M1, M2 and M3 (proliferation media), R1, R2, R3 and R4 (rooting media). For details see the material and methods section.

Species	Explants	Vitality (%)	Shoot n°/explant	Shoot height (cm)	Roots (%)
Calaminta nepeta L.	internodal segments	50 (S1)	4 ± 0.5 (M1)	2.80 ± 0.43	100 (R1)
Crithmum maritimum L.	basal shoots	100 (S2)	3 ± 0.3 (M3)	2.72 ± 0.33	40 (R3)
Lavandula angustifolia Mill.	internodal segments	80 (S2)	3 ± 0.4 (M1)	1.84 ± 0.49	40 (R1)
Myrtus communis L.	internodal segments	100 (S2)	3 ± 0.5 (M1)	3.27 ± 0.51	79 (R2)
Rosmarinus officinalis L.	apical portions	80 (S3)	1 ± 0.1 (M2)	1.50 ± 0.50	0 (R1)
Salvia officinalis L.	internodal segments	10 (S2)	2 ± 0.3 (M1)	4.29 ± 0.41	100 (R4)
Satureja hortensis L.	apical portions	80 (S3)	1 ± 0.2 (M1)	2.90 ± 1.17	5 (R4)

nis gave the best results of sterilization, reaching 100% vitality, not contaminated, as indicated in the literature (Grigoriadou & Maloupa, 2008; Ruffoni & Mascarello, 2009). A good percentage of success, 80%, was also observed for apical portions of *R. officinalis* and *S. hortensis* as well as for internodes of *L. angustifolia*. The method S3 included a pre-treatment with sucrose for 15 days, already successfully demonstrated in other Mediterranean explants (Ruffoni & Mascarello, 2009). A lower percentage of vitality was observed for explants of *C. nepeta* (50%) and for *S. officinalis*, as known from the literature (Avato *et al.,* 2005). The best explants were propagated using different media, chosen for simplicity of use, and subcultured every 4 weeks. The multiplication of shoots and their length were evaluated at the end of the cycle of subculture (Table 1). M1 medium, with addition of only BA as growth regulator, exhibited good results for *M. communis, C. nepeta, L. angustifolia* and *S. officinalis*; this confirms that proliferation can be achieved also with a minimum addition of growth regulators, as often reported in the literature for some species (Scarpa *et al.*, 2000; Avato *et al.,* 2005; Ruffoni & Mascarello, 2009). However, for *C. maritimum* the highest number of shoots was obtained adding both auxin (NAA) and cytokinin (BA) to the MS0 medium (Grigoriadou & Maloupa, 2008). *S. hortensis* produced modest results of propagation when apical portions were used as explants, confirming the literature data for *S. obovata* (Arrebola, 1997). *R. officina-*

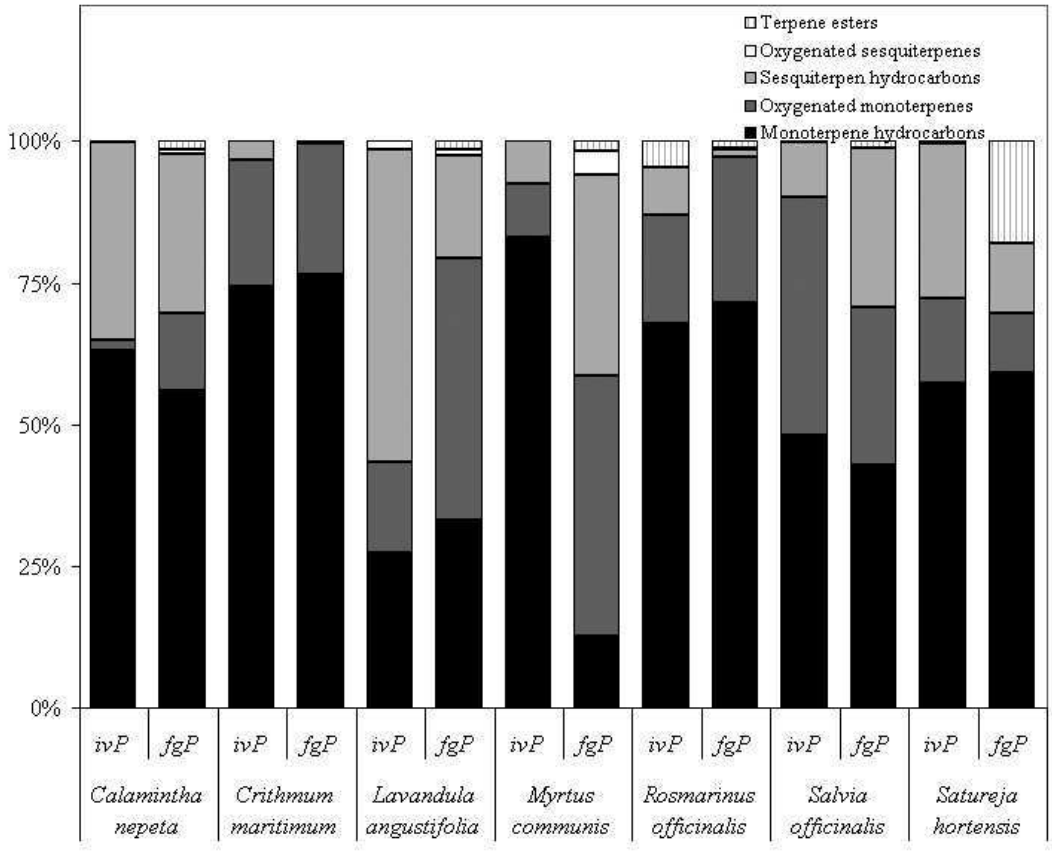


Figure 1. Terpene composition of the headspace of plants grown in the field (fgP) and *in vitro* (ivP).

Table 2. Main constituents identified in HS-SPME of examinated species

Compound			*C. nepeta*		*C. maritimum*		*L. angustifolia*		*M. communis*		*R. officinalis*		*S. officinalis*		*S. hortensis*	
		I*RI*	% ivP	% fgP	% ivP	% fgP	% ivP	% fgP	% ivP	% fgP	% ivP	% fgP	% ivP	% fgP	% ivP	% fgP
α-thujene	MH	932			1.2	0.3		0.4			0.3	0.9	0.3	0.8	1.5	1.4
tricyclene	MH	938							65.0							
α-pinene	MH	940	1.8	6.3	10.3	11.9	1.4	4.6	4.2	4.8	24.7	34.4	14.6	5.3	1.5	3.0
camphene	MH	955	1.8		0.4		0.6	1.6			13.4	19.7	8.5	1.9	0.1	3.3
β-thujene	MH	971			1.0											
sabinene	MH	978	1.2	1.4	15.0	1.5	0.5	0.4							0.2	0.2
β-pinene	MH	981	42.5	8.4			2.4	2.3			9.6	10.5	19.4	10.1	1.0	1.8
myrcene	MH	993		2.1	1.7	0.9	1.6	1.9			10.8	3.2	1.4	11.0	1.0	1.7
α-phellandrene	MH	1006					0.7	1.4			0.1				0.3	
δ-3-carene	MH	1012		0.3			6.9	11.3							0.1	
α-terpinene	MH	1019			0.3	0.4					0.4		0.1	0.3	3.4	2.5
o-cymene	MH	1026									0.5		0.1	1.1		
p-cymene	MH	1028			9.6	10.1	1.0	0.6		2.1		0.6			15.6	30.3
β-phellandrene	MH	1031					6.2									
limonene	MH	1032	15.0	34.8						2.5	4.0		2.3	4.3	1.2	
1,8-cineol	MH	1036					11.9	22.2		32.5	10.7	19.2	7.2	5.8		1.2
(*Z*)-β-ocimene	MH	1042			0.6			5.3						4.4		
(*E*)-β-ocimene	MH	1053						0.4						0.4		
γ-terpinene	MH	1062		0.4	30.9	49.3	0.4	0.1	2.9		1.5	0.2	0.4	1.1	29.0	11.7
p-mentha-3,8-diene	MH	1072							2.0							
cis-sabinene hydrate	MH	1072						0.5				0.2				
p-cymenene	MH	1080													0.4	0.8
isoterpinolene	MH	1086					0.5	0.8								
terpinolene	MH	1090	0.1	0.3	1.3		1.4	0.7			1.7	0.3	0.9	0.6		
linalool	MO	1102										0.5		0.4		0.5
nonanal	ALD	1104								1.7						
α-thujone	MO	1109											20.7	9.2		0.4
β-thujone	MO	1120											4.5	3.1		
cis-limonene oxide	MO	1137	0.2	0.4												
neo-alloocimene	MH	1142												0.6		
camphor	MO	1148						21.5			7.9	1.4	8.8	6.9		0.3
menthone	MO	1154	0.5	0.4												
menthofuran	MO	1164	0.5	0.7												
trans-pinocamphone	MO	1165											0.4			
borneol	MO	1169					1.8	1.1		0.9		3.5		1.4		2.0
cis-pinocamphone	MO	1177										0.3	0.2	0.3		
myrtenal	MO	1184		1.8												
α-terpineol	MO	1192					0.2	0.2		0.9		0.2				
1-dodecene	ALK	1192					1.8									
decanal	ALD	1206								1.4						
verbenone	MO	1214						0.2			0.3	0.1				
methyl thymol	MO	1235			0.4	0.3			7.2						0.2	11.9
pulegone	MO	1237		0.5												
methyl carvacrol	MO	1244			21.1	22.0			1.2						0.3	4.1
piperitone oxide	MO	1257		4.9												

bornyl acetate	TE	1287						1.2			4.5	1.2	0.2	0.8		1.4
thymol	MO	1293														5.5
lavandulyl acetate	TE	1289						0.2								
n-tridecane	ALK	1300					2.0									
carvacrol	MO	1301	0.5												14.4	
myrtenyl acetate	TE	1327								1.3						
δ-elemene	SH	1340												0.4		
piperitenone	MO	1342		0.4												
piperitenone oxide	MO	1363		4.1												
cyclosativene	SH	1371												0.7		
α-copaene	SH	1376	0.1	0.7										0.6		
β-bourbonene	SH	1383		1.1												
β-cubebene	SH	1390	0.9	0.3												
7-*epi*-sesquithujene	SH	1391					0.9									
β-elemene	SH	1392		0.5				0.3		6.9						
n-tetradecane	ALK	1400					0.5									
isocaryophyllene	SH	1406					0.4								0.2	
α-cedrene	SH	1409	0.6													
α-gurjunene	SH	1410					2.1	3.0						0.3		
caryophyllene	SH	1418	2.7	10.3		0.1	19.3	6.5	6.8	8.1	5.0	1.1	1.5	3.3	20.0	5.0
β-gurjunene	SH	1428	1.8	0.5			0.3							0.2		
trans-α-bergamotene	SH	1437		0.5	0.6		3.3								0.1	
cis-muurola-3,5-diene	SH	1448	13.9				0.9	0.7								
aromadendrene	SH	1445		0.4			0.3							2.0		
α-himachalene	SH	1453	0.2	0.4												
α-humulene	SH	1456		0.7			0.8	0.2		3.0	3.5	0.1	8.1	11.3	1.1	0.6
(*E*)-β-farnesene	SH	1460					1.7									
cis-muurola-4(14),5--diene	SH	1463	9.1				1.1	0.7								
γ-muurolene	SH	1477												1.4		
germacrene D	SH	1481	2.0	10.7	0.7	0.3	8.5	2.1						2.0		2.2
β-selinene	SH	1485	1.3				0.4			3.8						
α-zingiberene	SH	1495			0.6											
bicyclogermacrene	SH	1495	0.1				1.1	0.1						3.2	1.0	0.5
α-selinene	SH	1497								4.7						
n-pentadecane	ALK	1500					1.9									
(*E*,*E*)-α-farnesene	SH	1508					0.8									
β-bisabolene	SH	1509	0.1	0.5	0.3										4.0	3.5
trans-γ-cadinene	SH	1513	0.2				3.8	3.8						0.4		
trans-calamenene	SH	1522	1.1					0.1								
δ-cadinene	SH	1523		0.3			1.5							1.0		
β-sesquiphellandrene	SH	1524			0.5											
caryophyllene oxide	SO	1582						0.5		3.2		0.3				
1,10-*di*-*epi*-cubenol	SO	1614	0.2	0.4			0.4									
germacrene B	SH	1556			0.4	0.1										
diilapiol	PP	1623			0.6	2.3			3.4							
epi-α-cadinol	SO	1642					0.9	0.5								
pentyl decanoate	AE									13.6						
α-bisabolol	SO	1684		0.4												

n-heptadecane	ALK	1700					0.6			0.9						
n-octadecane	ALK	1800								0.9						
Monoterpene hydrocarbons	MH		62.4	54.0	72.3	74.4	23.6	32.8	74.1	9.4	67.2	70.0	48.0	41.9	55.3	56.7
Oxygenated monoterpenes	MO		1.7	13.2	21.5	22.3	13.9	45.5	8.4	34.3	18.9	25.4	41.8	27.3	14.4	9.9
Sesquiterpen hydrocarbons	SH		34.6	26.9	3.3	0.5	47.6	18	6.8	26.5	8.5	1.2	9.6	27.7	26.4	11.8
Oxygenated sesquiterpenes	SO		0.2	0.8			1.3	1.0		3.2		0.3				
Terpene esters	TE			1.5				1.5		1.3	4.5	1.2	0.2	1.2	0.5	17.4
Phenylpropanoids	PP				0.6	2.3			3.4							
Alkanes	ALK						7.4			1.8						0.2
Adeydes	ALD						0.2			3.1						0.5
Acids/esters	AE			0.3						13.6						
Total			98.4	94.9	97.5	99.5	92.8	97.4	92.7	93.2	98.9	97.9	99.6	96.6	96.6	95.8

I*RI*, Linear retention index. The components are listed in order of their elution on the DB-5 column. IvP, *in vitro* plants; fgP, field-grown plants. MH, monoterpene hydrocarbons; MO, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; SO, oxygenated sesquiterpenes; TE, terpene esters; ALD, aldehydes; ALK, alkanes/alkenes; PP, phenyl propanoids; AE, esters. Compounds present at less than 0.1% were excluded from the table and from analysis.

lis exhibited the lowest number of shoot proliferation even in a specific medium (M2), and no root formation was observed (R1 medium), confirming the refractivity of this species to proliferation (Misra & Chaturvedi, 1984). The shoot length is also shown in Table 1, and the varied growth ability of the explants confirmed the data already known from the literature. Notably, we devised a good method for the *in vitro* propagation of *C. nepeta*, not considered for micropropagation until now (Misra & Chaturvedi, 1984, Arrebola, 1997; Avato *et al.,* 2005; Grigoriadou & Maloupa, 2008). Root formation was then induced in order to produce aseptically whole plants to be easily transferred in diverse environments. Best results were obtained with *C. nepeta* and *S. officinalis*, whereas the percentage of produced roots was lower for other species.

The volatiles emitted from the analysed species and identified by GC-MS are reported in Table 2. In total, 71 and 78 compounds were identified in micropropagated (ivP) and field-grown (fgP) plants. The headspace analyses of these two growth conditions accounted for 92.7–99.6% and 93.2–99.5% of the total compositions, respectively. The volatile fractions were characterized mainly by hydrocarbons and oxygenated monoterpenes together with sesquiterpene hydrocarbons (Fig. 1). The SPME analysis of the two samples of *C. nepeta* showed a similar composition in monoterpenes (64.1 and 67.2% in ivP and fgP, respectively) and sesquiterpenes (34.1 and 26.9% in ivP and fgP, respectively), even if fgP exhibited higher percentages of oxygenated monoterpenes (13.2%) than ivP (1.7%). In fgP typical compounds of *Calamintha* spp. were detected, as limonene (34.8%), piperitone oxide and piperitenone oxide (4.9 and 4.1%, respectively), together with low amounts of pulegone and menthone, (0.5 and 0.4%, respectively), in accordance with literature data (De Pooter *et al.,* 1986). In contrast, ivP showed the presence of *cis*-muurola 3,5-diene (13.9%) and cis-muurola-4(14),5-diene (9.1%), not detected in fgP, and a high percentage of β-pinene (42.5%, against 8.4% in fgP). The headspaces of *C. maritimum* samples showed a similar composition, characterised by monoterpene hydrocarbons (72.3% in ivP and 74.4% in fgP) and oxygenated monoterpenes (21.5% in ivP and 22.3% in fgP), differing only by the percentages of sabinene (15.0% in ivP and 1.5% in fgP) and γ-terpinene (30.9% in ivP and 49.3 in fgP). The identified constituents are in agreement also with the composition of the essential oils reported in the literature (Özcan *et al.,* 2006), even if many reports on the essential oil composition of sea fennel grown in different parts of Mediterranean area showed differences in chemical constituents, suggesting different chemotypes of this species (Kulisic-Bilusic *et al.,* 2010). The two *L. angustifolia* samples showed qualitative differences in the composition of the volatiles emitted: the ivP headspace was characterised especially by sesquiterpene hydrocarbons (47.2%), while fgP exhibited high percentages of oxygenated and hydrocarbon monoterpenes (45.2% and 32.3%, respectively). 1,8-cineol, the compound that gives the particular flavour of the lavender fields, was detected in both samples (11.9% in ivP and 22.2% in fgP), while camphor was identified only in the headspace of fgP (21.5%). These results represent new data regarding the head space composition of the vegetative aerial part of *L. angustifolia*, since earlier reports focused on the flower aroma and/or the reproductive aerial parts (Kim *et al.,* 2002, Da Porto & Decorti 2008). SPME analyses of micropropagated *L. viridis* plants and field-grown mother-plant exhibited a different composition of the emitted volatiles (Gonçalves *et al.*, 2008). The headspace analysis obtained from field-grown *M. communis* showed a similar composition with the literature data (Flamini *et al.,* 2004), with the presence of 1,8-cineol (32.5%), α-pinene (4.8%), limonene (2.5%) and mirtenyl acetate (1.3%). In contrast, in the SPME analysis of ivP, tricyclene was revealed as the main constituent (65.0%), together with methyl thymol (7.2%), showing a very different composition from the field-grown plant. The SPME analysis of *R. officinalis* samples showed the presence of similar percentages of monoterpene hydrocarbons (67.0% in ivP and 70.0% in fgP), but quantitative differences in the percentages of oxygenated monoterpenes (18.9% in ivP and 25.2% in fgP) and of sesquiterpene hydrocarbons (8.5% in ivP and 1.2% in fgP). α-pinene (24.7% and 34.4% in ivP and fgP, respectively). 1,8-cineol (10.7% in ivP and 19.2% in fgP) and camphor (7.9% in ivP and 1.4% in fgP) represent the most important constituents of the essential

oil and aroma of *R. officinalis* (Katerinopoulos *et al.,* 2005; Zawirska-Wojtasiak & Wasowicz, 2009). The main compounds identified in the headspace of both samples of *S. officinalis* were α-thujone (20.7% and 9.2% in ivP and fgP, respectively), β-pinene (19.4% in ivP and 10.1% in fgP) and α-pinene (14.6% and 5.3% in ivP and fgP, respectively), together with camphor (8.8% in ivP and 6.9% in fgP) and α-humulene (8.1% in ivP and 11.3% in fgP), in agreement with the quali-quantitative composition reported for *S. officinalis* essential oil (Longaray Delamare *et al*., 2007; Santos-Gomes *et al.,* 2001). Despite the presence of compounds characteristic for *S. officinalis* in both analysed samples, the micropropagated plants showed higher content of oxygenated monoterpenes (41.8%) then the field-grown plants (27.1%), while the sesquiterpene hydrocarbons were present especially in the field-grown samples (26.8% and 9.6% in ivP and fgP, respectively). The headspace of the *S. hortensis* two samples was characterised mainly by γ-terpinene (29.0% in ivP and 11.7% in fgP), *p*-cymene (15.6% in ivP and 30.3% in fgP), carvacrol (14.4%, identified only in ivP) and methylthymol and thymol (11.9 and 5.5%, respectively, the both detected only in the field-grown plants), characteristic constituents of the essential oil from aerial parts. γ-terpinene and *p*-cymene are biogenetic precursors (*via* enzymatic hydroxylation) of the phenolic terpenes thymol and carvacrol, so there is a correlation between these compounds and their percentages are affected by harvesting time and location (Azaz *et al.,* 2005; Sefidkon *et al.,* 2006; Güllüce *et al.,* 2003; Abu-Lafi *et al.,* 2008).

Many VOCs produced by officinal and medicinal plants are commercialized as flavors and/or fragrances, and their use in the food and perfume industries has a long tradition. This work showed that the headspace composition of micropropagated plantlets of *C. maritimum*, *R. officinalis*, *S. officinalis* and *S. hortensis* is very similar to the corresponding field-grown plants, containing the same main constituents, although further investigations are requested for *C. nepeta*, *L. angustifolia* and *M. communis* in order to obtain *in vitro* plant material with aromatic profile more similar to the wild plants. These results indicated that micropropagation can represent a valid alternative to produce rapidly large amounts of plant material characterised by the same aromatic flavor as the wild grown plants. Other advantages of this technique are the protection of the natural ecosystem avoiding the damage of endemic plants. The emission of VOC by the micropropagated plants can represent a good market actractant for baby plants. Customers may use them as spices or as steril ornamental plants to be transported everywhere.

Acknowledgments

This work was supported by the Interreg IT-FR Marittimo Project "PYRGI, CUP B51H10000000006, Strategia d'impresa in settori di nicchia per l'economia agroindustriale del mediterraneo".

REFERENCES

Abraham Z (2011) Collection and conservation of medicinal and aromatic plants resources. *OAJMAP* **1**: 40–43.

Abu-Lafi S, Odeh I, Dewik H, Qabajah M, Hanus LO, Dembitsky VM (2008) Thymol and carvacrol production from leaves of wild palestinian *Majorana syriaca*. *Bioresour Technol.* **99**: 3914–3918.

Adams RP (1995) In *Identification of essential oil components by gas chromatography-mass spectroscopy*. Allured, Carol Stream, Illinois.

Andrade LB, Echeverrigaray S, Fracaro F, Pauletti GF, Rota L (1999) The effect of growth regulators on shoot propagation and rooting of common lavender (*Lavandula vera* DC). *Plant Cell Tissue Org Cult* **56**: 79–83.

Arikat NA, Jawad M, Karam NS, Shibli RA (2004) Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.) *Sci Hortic* **100**: 193–202.

Arrebola ML, Socorro O, Barceló-Muñoz A, Simón-Pérez E, Pliego-Alfaro F (1997) Micropropagation of *Satureja obovata* Lag. *Hort Sci* **32**: 1278–1280.

Avato P, Morone Fortunato I, Ruta C, D'Elia R (2005) Glandular hair's and essential oils in micropropagated plants of *Salvia officinalis* L. *Plant Sci* **169**: 29–36.

Azaz AD, Kürkcüoglu M, Satil F, Can Baser KH, Tümen G (2005) *In vitro* antimicrobial activity and chemical composition of some *Satureja* essential oils. *Flavour Fragr J* **20**: 587–591.

Belliardo F, Bicchi C, Cordero C, Liberto E, Rubiolo P, Sgorbini (2006) Headspace solid-phase microextraction in the analysis of the volatile fraction of aromatic and medicinal plants. *J Chrom Sci* **44**: 416–429.

Connolly JD, Hill RA (1991) In *Dictionary of terpenoids*. Chapman & Hall, London.

Da Porto C, Decorti D (2008) Analysis of the volatile compounds of flowers and essential oils from *Lavandula angustifolia* cultivated in northeastern italy by headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry. *Planta Med* **74**: 182–187.

De Pooter HL, De Buyck LF, Schamp NM (1986) The volatiles of *Calamintha nepeta* subsp. *glandulosa*. *Phytochemistry* **25**: 691-694.

Debnath M, Malik CP, Bisen PS (2006) Micropropagation: a tool for the production of high quality plant-based medicines. *Curr Pharm Biotechnol* **7**: 33–49.

Dicke M, Loreto F (2010) Induced plant volatiles: from genes to climate change. *Trends Plant Sci* **15**: 115-117.

Flamini G, Cioni PL, Morelli I, Maccioni S, Baldini R (2004) Phytochemical typologies in some populations of *Myrtus communis* L. on Caprione Promontory (East Liguria, Italy). *Food Chem* **85**: 599–604.

Gonçalves S, Serra H, Nogueira JMF, Almeida R, Custódio L, Romano A (2008) Headspace-SPME of *in vitro* shoot-cultures and micropropagated plants of *Lavandula viridis*. *Biol Plantarum* **52**: 133–136.

Grigoriadou K, Maloupa E (2008) Micropropagation and salt tolerance of *in vitro* grown *Crithmum maritimum* L. *Plant Cell Tiss Org Cult* **94**: 209–217.

Güllüce M, Sökmen M, Daferera D, Agar G, Özkan H, Kartal N, Polissiou M, Sökmen A, Sahin F (2003) *In vitro* antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. *J Agric Food Chem* **51**: 3958–3965.

Hamilton AC (2004) Medicinal plants, conservation and livelihoods. *Biodivers Conserv* **13**: 1477–1517.

Jennings W, Shibamoto T (1980) In *Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography*. Academic Press, New York.

Katerinopoulos HE, Pagona G, Afratis A, Stratigakis N, Roditakis N (2005) Composition and insect attracting activity of the essential oil of *Rosmarinus officinalis*. *J Chem Ecol* **31**: 111–122.

Kim NS, Lee DD (2002) Comparison of different extraction method for the analysis of fragrance from *Lavandula* species by gas chromatographymass spectrometry. *J Chromatogr A* **982**: 31–47.

Kulisic-Bilusic T, Blazevic I, Dejanovic B, Milos M, Pifat G (2010) Evaluation of the antioxidant activity of essential oils from caper (*Capparis spinosa*) and sea fennel (*Crithmum maritimum*) by different methods. *J Food Biochem* **34**: 286–302.

Landi S (1989) In *Flora e ambiente dell'isola d'Elba*. Editrice Azzurra, Cavalese (Tn).

Longaray Delamare AP, Moschen-Pistorello IT, Artico L, Atti-Serafini L, Echeverrigaray S (2007) Antibacterial activity of the essential oils of *Salvia officinalis* L. and *Salvia triloba* L. cultivated in South Brazil. *Food Chem* **100**: 603–608.

Lucchesini M, Mensuali-Sodi A (2010) Plant tissue culture-An opportunity for the production of nutraceuticals. *Adv Exp Med Biol* **698**: 185–202.

Maffei M, Gertsch J, Appendino G (2011) Plant volatiles: production, function and pharmacology. *Nat Prod Rep* **28**: 1359–1380.

Massada Y (1976) In *Analysis of essential oils by gas chromatography and mass spectrometry*. Wiley & Sons, New York.

Misra P, Chaturvedi HC (1984) Micropropagation of *Rosmarinus officinalis* L. *Plant Cell Tiss Org Cult* **3**: 163–168.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473–493.

Özcan MM, Pedro LG, Figueiredo AC, Barroso JG (2006) Constituents of the essential oil of sea fennel (*Crithmum maritimum* L.) growing wild in Turkey. *J Med Food* **9**: 128–130.

Pistelli L, Giovannini A, Ruffoni B, Bertoli A, Pistelli L. (2010) Hairy root cultures for secondary metabolites production. *Adv Exp Med Biol* **698**: 167–184.

Rinaldi G (2000) In *Flora dell'Arcipelago Toscano*. Edizioni Archipelagos, Portoferraio (Livorno).

Ruffoni B, Pistelli L, Bertoli A, Pistelli L (2010) Plant Cell Cultures: Bioreactors for Industrial Production. *Adv Exp Med Biol* **698**: 203–221.

Ruffoni B, Mascarello C (2009) Tecniche per la propagazione *in vitro* degli arbusti mediterranei. *Flortecnica* **6**: 51–52.
Santos-Gomes PC, Fernandes-Ferreira M (2001) Organ- and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated at two different sites. *J Agric Food Chem* **49**: 2908–2916.
Scarpa GM, Milia M, Satta M 2000. The influence of growth regulators on proliferation and rooting of *in vitro* propagated myrtle *Plant Cell Tiss Org Cult* **62**: 175–179.
Sefidkon F, Abbasi K, Khaniki GB (2006) Influence of drying and extraction methods on yield and chemical composition of the essential oil of *Satureja hortensis*. *Food Chem* **99**: 19–23.
Stenhagen E, Abrahamsson S, McLafferty FW (1974) In *Registry of mass spectral data*. Wiley & Sons, New York.
Swigar AA, Silverstein RM (1981) In *Monoterpenes*. Aldrich Chem. Comp., Milwaukee.
Zawirska-Wojtasiak R, Wasowicz E (2009) GC analysis of rosemary aroma isolated traditionally by distillation and by SPME. *J Essent Oil Res* **21**: 8–15.

DESCRIZIONE DEL PRODOTTO

All'interno della stessa componente 5 sono stati realizzati 12 poster scientifici presentati nell'ambito di convegni e congressi nazionali ed internazionali. Se ne riporta la lista relativamente agli anni 2010, 2011, 2012 e 2013.

ANNO 2010:

1) Prodotto 26b.1
   Autori: Grilli M., Summa M., Zappettini S., Di Prisco S., Salamone A., Bisio A., Romussi G., Cafaggi S., Pittaluga A. , Marchi M.
   Titolo: Studies on the effects of SW-8 on the release of some neurotransmitters from mouse brain nerve terminals
   Convegno: II Convegno Monotematico della SIF. Erbe Medicinali: dalla ricerca di base alla clinica. Messina, 29-30 Aprile 2010

ANNO 2011:

1) Prodotto 26b.2
   Autori: Olivero G., Di Prisco S., Summa M., Giacomelli E., Grilli M., Bisio A., Romussi G., Marchi M., Pittaluga A.
   Titolo: Salviae neoclerodane derivatives differently affect catecholamine release in mammals brains: comparison with Salvinorin A.
   Convegno: 35° Congresso Nazionale Società Italiana di Farmacologia. Bologna, 14-17 settembre 2011
2) Prodotto 26b.3
   Autori: Bisio A., Giacomelli E., Damonte G., Salis A., Fraternale F., Romussi G., Cafaggi S., Ricci D., De Tommasi N.
   Titolo: A news clerodane diterpenoid from the aerial part exudate of *Salvia chamaedryoides* Cav.
   Convegno: INTERNATIONAL PSE SYMPOSIUM - Phytochemicals in Nutrition and Health - 27-30 September 2011 - Giovinazzo (BARI), Italy
3) Prodotto 26b.4
   Autori: Bisio A., Damonte G., Giacomelli E., Mele G., Romussi G., Cafaggi S., De Tommasi N.
   Titolo: A new derivative of 12-oxo-Phytodienoic Acid from S*alvia adenophora* Fernald.
   Convegno: XX SILAE Congress - Fortaleza, Brazil - 19/22 September 2011
4) Prodotto 26b.5
   Autori: Olivero G., Di Prisco S., Summa M., Giacomelli E., Grilli M., Bisio A., Romussi G., Marchi M., Pittaluga A.
   Titolo: *Salviae neoclerodane* derivatives differently affect catecholamine release in mammals brains: comparison with Salvinorin A.

Convegno: 35° Congresso Nazionale Società Italiana di Farmacologia. Bologna, 14-17 settembre 2011.

5) Prodotto 26b.6
   Autori: Pistelli L., Flamini G., Cioni P.L., Melai B., Leonardi M.
   Titolo: Composition of essential oils of *Helychrysum italicum* (ROTH) subsp. *Italicum* from Elba Island.
   Convegno: Congresso Internazionale sulle Piante Aromatiche e Medicinali (CIPAM). Cagliari, 13-15 Aprile 2011.
6) Prodotto 26b.7
   Autori: Pistelli L., D'Angiolillo F., Noccioli C., Pistelli L.
   Titolo: Micropropagazione di specie aromatiche dell'Arcipelago Toscano
   Convegno: 2° Convegno Nazionale sulla micropropagazione – Un incontro tra gli operatori del settore e della ricerca. Sanremo, 7-9 novembre 2011

ANNO 2012:

1) Prodotto 26b.8
   Autori: Bisio A., Damonte G., Giacomelli E., Mele G., Fraternale D., Ricci D., Romussi G., De Tommasi N.
   Titolo: Oxylipin compounds from the aerial part exudate of *Salvia adenophora* Fernald.
   Convegno: PSE Meeting – Bio-Communications: Semiochemicals involving plants - Cadiz , Spain - September 10th-12th 2012
2) Prodotto 26b.9
   Autori: Bisio A., Damonte D., Giacomelli E., Mele G., Profumo A., Romussi G., De Tommasi N.
   Titolo: A new diterpenoid from *Salvia Adenophora* Fernald (Lamiaceae).
   Convegno: International Congress on Natural Products Research. New York, 28 luglio – 1 agosto 2012
3) Prodotto 26b.10
   Autori: Bisio A., Giacomelli E., Mele G., Damonte G., Fratenale D., Romussi G., Ricci D., De Tommasi N., 2012.
   Titolo: Phytotoxic activity and a new clerodane diterpenoid from the exudate of the aerial parts of *Salvia Buchananii* Hedge.
   Convegno: 107° Congresso SBI Onlus. Benevento, 18-22 settembre 2012
4) Prodotto 26b.11
   Autori: Bisio A, Damonte G., Fraternale D., Giacomelli E., Mele G., Romussi G., Ricci D., De Tommasi N.
   Titolo: Chemical Constituents of roots of *Salvia x jamensis* J. Compton grown in aeroponic culture.
   Congresso: Congresso Interdisciplinare sulle Piante Medicinali. Cetraro (CS), 31 Maggio – 2 Giugno 2012

5) Prodotto 26b.12
Autori: Tavarini S., Pieve B., Pistelli L., Angelini L.G.
Titolo: Valorizzazione di specie spontanee per la produzione di coloranti vegetali per produzioni innovative di nicchia e lo sviluppo locale sostenibile
Presentato a "La notte dei ricercatori" del 28 settembre 2012

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b
POSTER PRESENTATI IN OCCASIONE DI CONVEGNI NAZIONALI ED INTERNAZIONALI

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.1

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Studies on the effects of SW-8 on the release of some neurotransmitters from mouse brain nerve terminals

M. Grilli[a], M. Summa[a], S. Zappettini[a], S. Di Prisco[a], A. Salamone[a], A. Bisio[b], G. Romussi[b], S.Cafaggi[b], A. Pittaluga[a,c], M. Marchi[a,c]

[a] Department of Experimental Medicine, Pharmacology and Toxicology Section, Genova,
[b] Department of Chemistry and Pharmaceutical and Food Technology,
[c] Center of Excellence for Biomedical Research, University of Genoa, Italy

Granted by *Alpes Latines Coopération Transfrontalière France – Italie (Alpes) INTERREG III A 2000-2006*

SUMMARY

The effects of hautriwaic acid (SW-8), a clerodane diterpenoid obtained from Salvia wagneriana (Lamiaceae) (1) on the basal and the evoked release of preloaded [³H]noradenaline ([³H]NA) and [³H]serotonin ([³H]5-HT) from mouse hippocampal nerve terminals, were investigated. The experimental technique used (referred to as the up-down superfusion of a monolayer of synaptosomes) is considered an approach of choise to investigate the existence and the functional role of presynaptic receptors (2). In a previous work (2) we have found that Salvinorin A (0.1–1000 nM) failed to affect the basal release of amines, but inhibited the 12 mM K⁺-evoked, Ca²⁺-dependent, exocytotic-like release of [³H]5-HT and [³H]DA. At the same concentration, Salvinorin A facilitated the 12 mM K⁺-evoked, Ca²⁺-dependent, exocytotic-like release of [³H]NA. The broad spectrum κ-opioid receptor antagonist norbinaltorphimine (1–100 nM) antagonized the inhibition of [³H]5-HT and [³H]DA exocytosis as well as the facilitation of [³H]NA overflow induced by 100 nM Salvinorin A. Our new findings using the compound SW-8 show that this drug is able to mimic Salvinorin A on the 12 mM K⁺-evoked release of hippocampal [³H]NA and [³H]5-HT. Interestingly SW-8, when co-applied with Salvinorin A, blocks its inhibitory effect on the [³H]5-HT exocytosis and partially inhibits the Salvinorin A-induced facilitation of [³H]NA release.

Fig. 1. Concentration-effects relationship of Salvinorin A on the 12 mM K⁺-evoked release of neurotransmitters from superfused synaptosomes.

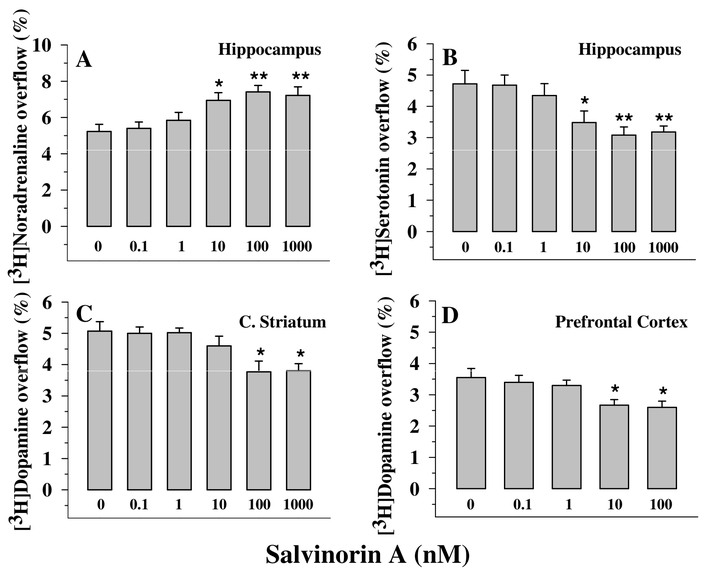


Fig. 2. Effects of pertussis toxin (PTx) on the Salvinorin A induced modification of neurotransmitter overflows evoked by K⁺ depolarization from superfused synaptosomes.

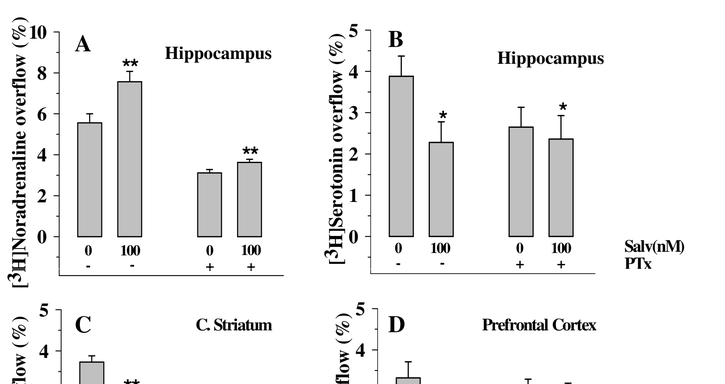


Fig. 3. Effects of norbinaltorphimine (norBNI) on the Salvinorin A induced modification of neurotrasmitter overflows evoked by K⁺ depolarization from superfused synaptosomes.

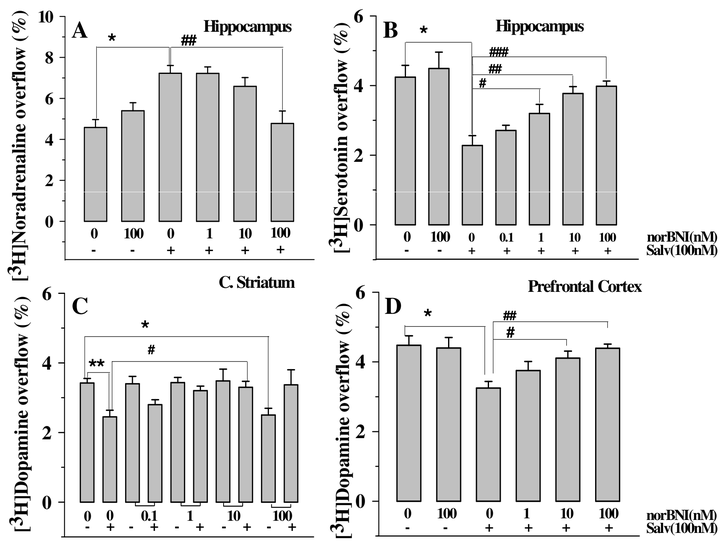


Table 2. U69593-induced inhibition of the 12 mM K+-evoked neurotransmitter release: effects of nBNI.

Brain area	Neurotransmitter	12mM KCl (% induced overflow)	12mM KCl 100nM U69593 (% induced overflow)	12mM KCl 100nM U69593 100 nM nBNI (% induced overflow)
Hippocampus	[³H]5-HT	4.02 ± 0.18	2.21 ± 0.23***	3.99 ± 0.21††
C. Striatum	[³H]DA	4.45 ± 0.14	3.06 ± 0.30*	4.17 ± 0.52†
PFc	[³H]DA	4.69 ± 0.25	3.44 ± 0.33**	4.39 ± 0.19†

Fig. 4. Effects of naltrindole (Naltr) on the Salvinorin A induced modification of neurotrasmitter overflows evoked by K⁺ depolarization from superfused synaptosomes.

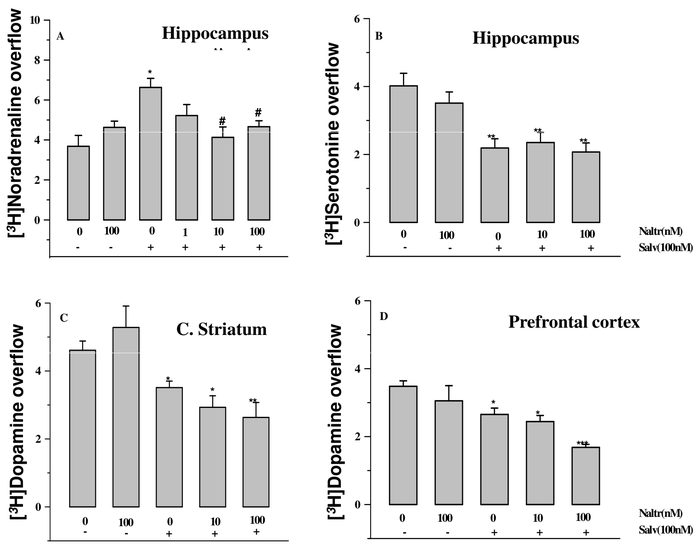


Fig. 5. Concentration-effect relationship of U69593 on the 12 mM K⁺-evoked release of neurotransmitter from superfused synaptosomes.

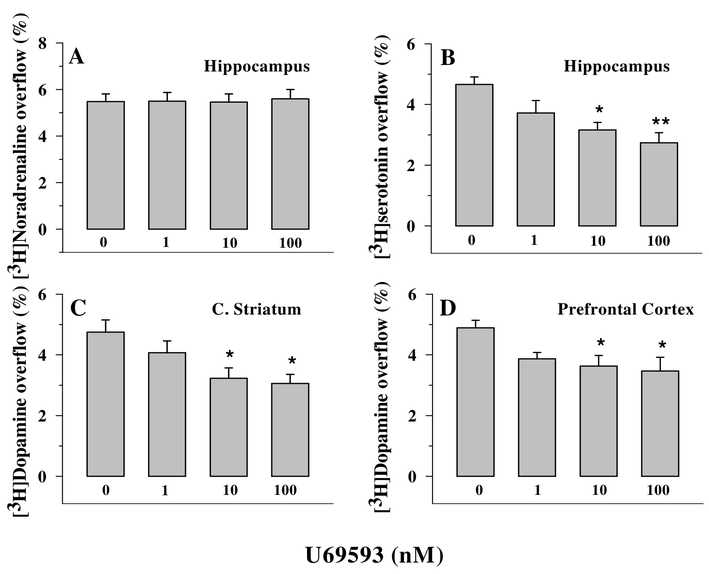


Fig. 6. Effects of SW-8 on the Salvinorin A induced modification on the 12 mM K⁺-evoked release of neurotransmitter from superfused synaptosomes.

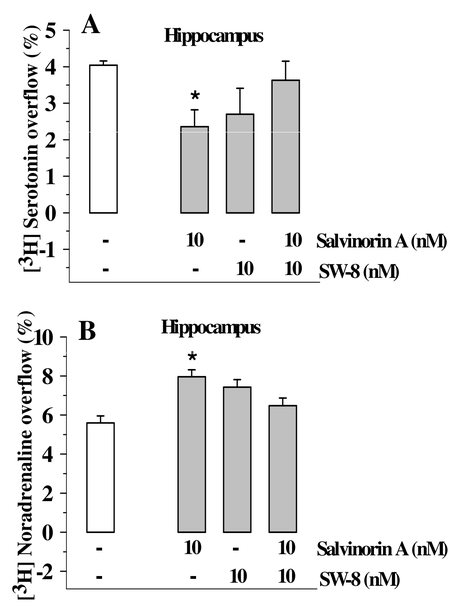


Table 1. Effects of CTAP on the Salvinorin A-induced modification of 12 mM K⁺-evoked neurotransmitter overflows from superfused synaptosomes.

Neurotransmitter Brain area	CTAP (nM)	12 mM K⁺-evoked release (% induced overflow)	(12 mM K⁺/100 nM Salvinorin A) - evoked release (% induced overflow)
[³H]NA hippocampus	0	4.10 ± .52	6.34 ± .51 *
	10	4.15 ± 0.61	6.83 ± .94*
	100	5.13 ± .21	7.10 ± .16*
[³H]5-HT hippocampus	0	4.31 ± .11	2.72 ± .11**
	10	3.58 ± .22	2.70 ± .26*
	100	4.04 ± .20	2.90 ± .26 **
[³H]DA c. striatum	0	4.74 ± .10	3.68 ± .15**
	10	4.26 ± .39	3.06 ± .12**
	100	5.04 ± .34	2.48 ± .18***
[³H]DA PFc	0	2.87 ± .21	2.08 ± .20*
	10	2.65 ± .11	1.65 ± .25*
	100	2.85 ± .25	1.61 ± .08**

CONCLUSIONS

Salvinorin A facilitates NA exocytosis from hippocampal terminals, while it inhibits the K⁺-evoked release of 5-HT from hippocampal terminals, as well as that of DA from striatal and PFc synaptosomes. The presynaptic modulation occurs in the nM range, indicative of the involvement of high-affinity binding sites. The inhibition of DA and 5-HT release as well as facilitation of NA exocytosis relies on the binding of Salvinorin A to presynaptic PTx-sensitive GPCRs, since enriching synaptosomes with the toxin impeded the modifications caused by the diterpene. The results obtained in the present investigation suggest the involvement of different receptor subtypes in the mechanism of action of Salvinorin A. The efficacy of U69593 in reducing DA and 5-HT exocytosis, together with the high potency of norBNI in preventing Salvinorin A-induced inhibition could be consistent with the existence of presynaptic k oligomers on serotonergic and dopaminergic terminals. The results obtained when studying the pharmacological profile of the receptor involved in the Salvinorin A-induced facilitation of noradrenaline release unveiled a complex scenario. The lack of efficacy of U69593 in facilitating NA exocytosis together with the low potency of norBNI in antagonizing the Salvinorin A-induced potentiation of NA overflow suggest that the activation of k oligomers cannot account for the effect observed.

On the other hand, the effectiveness of naltrindole in antagonizing Salvinorin A-induced potentiation of NA exocytosis (10 nM naltrindole caused an almost maximum inhibition) seems consistent with the existence of presynaptic d oligomers on mouse hippocampal terminals and with their involvement in the effect observed The presynaptic effects here described improve the knowledge of the central effects of Salvinorin A and confirm the role this natural compound plays in regulating central neurotransmission. Based on the present results, Salvinorin A appears to act as a broad spectrum agonist able to activate norBNI-sensitive opioid receptors having different pharmacological profile sited on catecholaminergic and indoleaminergic nerve endings. Interestingly the new compound.

Our new findings using the compound SW-8 show that this drug is able to mimic Salvinorin A on the 12 mM K⁺-evoked release of hippocampal [³H]NA and [³H]5-HT. Interestingly SW-8, when co-applied with Salvinorin A, blocks its inhibitory effect on the [³H]5-HT exocytosis and partially inhibits the Salvinorin A-induced facilitation of è [³H]NA release.

REFERENCES:

1) Bisio et al., Planta Med., 70, 452-7, 2004.
2) Grilli et al., Neuropharmacology, 57, 523–530, 2009

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.2

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Salviae neocloredane derivates differently affect catecholamine release in mammals brains: comparisons with Salvinorin A

G. Olivero[a], S. Di Prisco[a], M. Summa[a], E. Giacomelli[b], M. Grilli[a,c] A. Bisio[b], G. Romussi[b], M. Marchi[a,c], A. Pittaluga[a,c] ,

[a] Department of Experimental Medicine, Pharmacology and Toxicology Section, Genova,
[b] Department of Chemistry and Pharmaceutical and Food Technology ,
[c] Center of Excellence for Biomedical Research, University of Genoa, Italy

Granted by Alpes Latines Coopération Transfrontalière France – Italie (Alpes) INTERREG III A 2000-2006

SUMMARY

Salvinorin A is the main active compound found in Salvia Divinorum: it's a neoclerodane diterpene that acts like an highly selective and potent opioid receptor agonist and it remains one of the most potent naturally occuring hallucinogen. Because of its chemical features, this coumpound could represent the prototype of a new class of compounds useful to therapeutic treatment in ansia, depression, pain and drug seeking. In a previous work (2) we have found that Salvinorin A (0.1–1000 nM) failed to affect the basal release of amines, but inhibited the 12 mM K^+-evoked, Ca^{2+}-dependent, exocytotic-like release of [^{3}H]DA from mouse striatal nerve endings. At the same concentration, Salvinorin A facilitated the 12 mM K^+-evoked, Ca^{2+}-dependent, exocytotic-like release of [^{3}H]NA mouse hippocampal terminals. We used the experimental technique of superfusion of a monolayer of synaptosomes, which is considered an approach of choice to investigate the existence and the functional role of presynaptic receptors (2).

Recently we extended our investigation to new clerodane diterpenes isolated from *Salvia jamensis* (the compound SJ217), *Salvia wagneriana* (the compounds SW8 and SW22) (Bisio et al., 2004). Our results unveiled a complex scenario, suggesting that the three different compounds could discriminate between K opioid receptor subtypes.

Fig. 1. Concentration-effects relationship of Salvinorin A on the 12 mM K^+-evoked release of neurotransmitters from superfused synaptosomes.

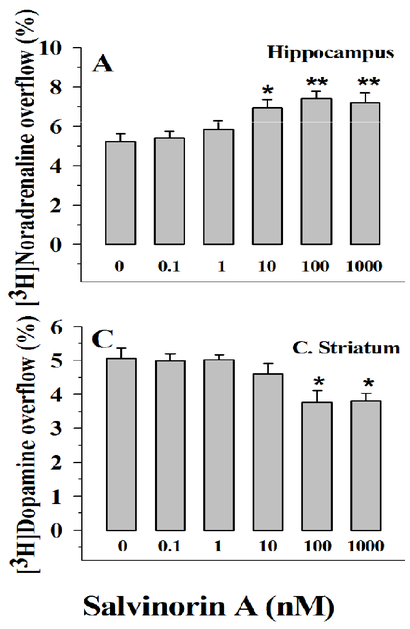


Fig. 2. Effects of pertussis toxin (PTx) on the Salvinorin A induced modification of neurotransmitter overflows evoked by K^+ depolarization from superfused synaptosomes.

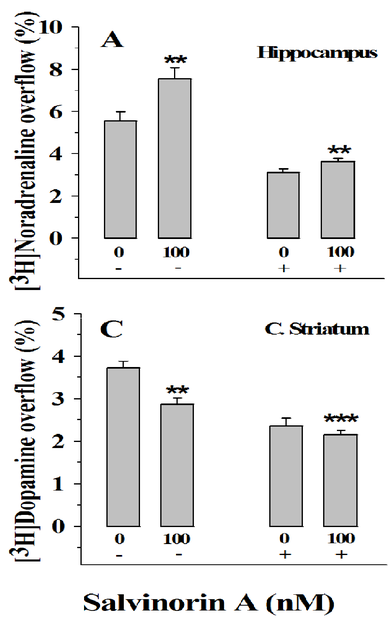


Fig. 4. Effects of naltrindole (Naltr) on the Salvinorin A induced modification of neurotransmitter overflows evoked by K^+ depolarization from superfused synaptosomes.

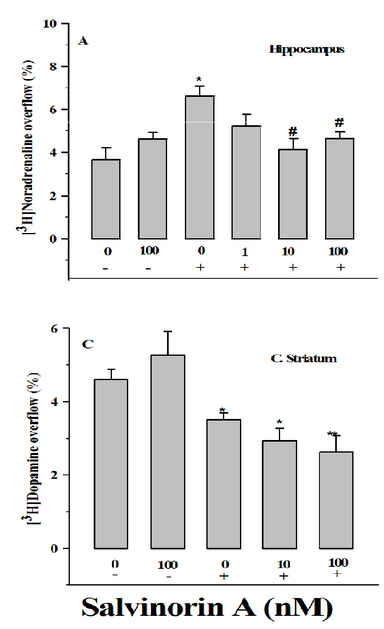


Fig. 5. Concentration-effect relationship of U69593 on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.

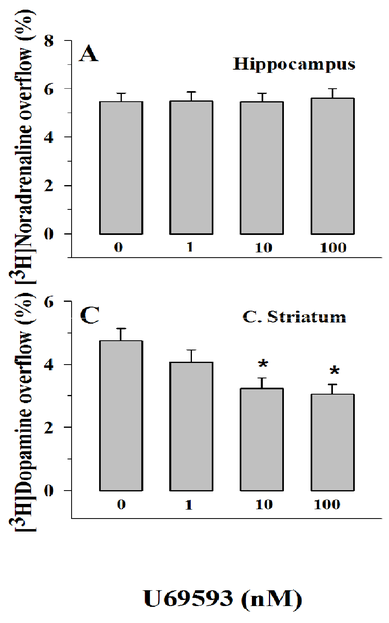


Fig. 6. Effects of SW 8 on the Salvinorin A induced modification on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.

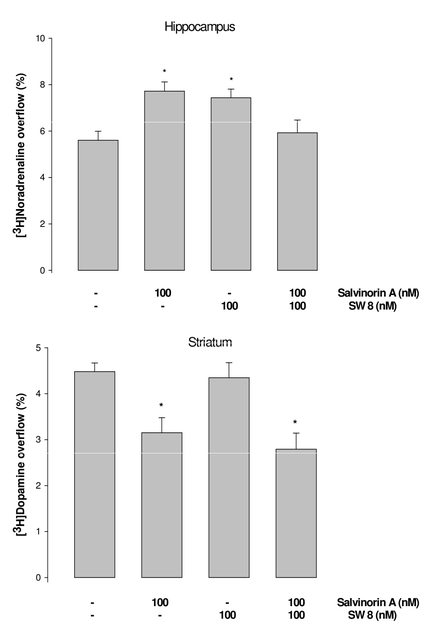


Fig. 6. Effects of SW 22 on the Salvinorin A induced modification on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.

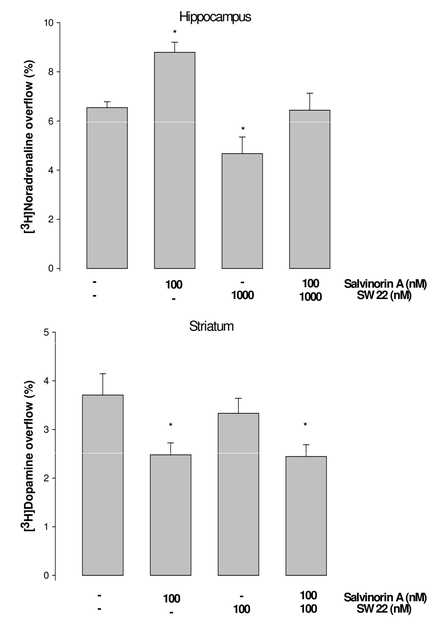
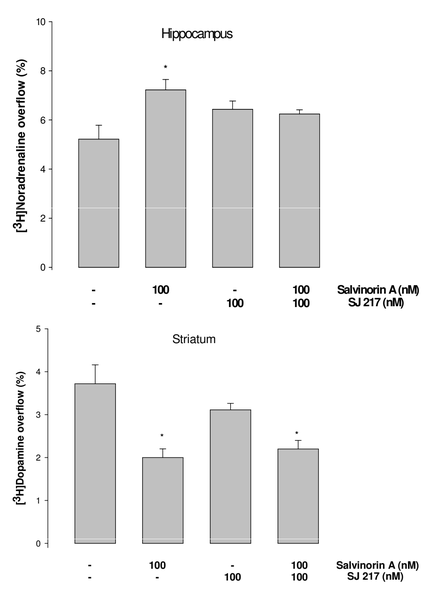
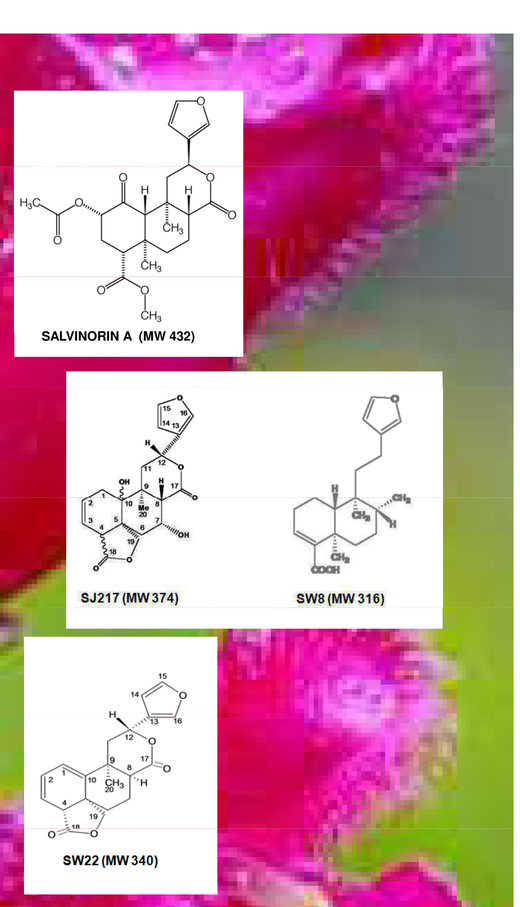


Fig. 7 Effects of SJ 217 on the Salvinorin A induced modification on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.





CONCLUSIONS

•Salvinorin A facilitates NA exocytosis from hippocampal terminals, while it inhibits the K^+-evoked release of DA from striatal synaptosomes: the diterpene acts on presynaptic PTx-sensitive GPCRs, since enriching synaptosomes with the toxin impeded its effects. The presynaptic modulation occurs in the nM range, indicative of the involvement of high-affinity binding sites.

•The opposite effects of Salvinorin A were found to rely on its binding to distinct receptor subtypes. Our results led us to conclude that κ and δ heterodimers presynaptically located on mouse hippocampal noradrenergic terminals may account for the Salvinorine A-induced facilitation of NA release, while the inhibition of DA release depends on the binding of the diterpene to κ homodimers on mouse striatal dopaminergic terminals.

•The presynaptic effects here described improve the knowledge of the central effects of Salvinorin A and confirm the role this natural compound plays in regulating central neurotransmission. Based on the present results, Salvinorin A appears to act as a broad spectrum agonist able to activate norBNI-sensitive opioid receptors having different pharmacological profile sited on catecholaminergic and indoleaminergic nerve endings.

•Our recent findings show that the new compounds SW8 and SJ217 are able to mimic Salvinorin A on the 12 mM K^+-evoked release of hippocampal [^{3}H]NA, while SW22 inhibits this release. SW8 and SW22, when co-applied with Salvinorin A, prevent its positive effect on the [^{3}H]NA exocytosis. As to the dopamine release is concerned, the three compounds fail to modify the 12 mM K^+-evoked release of dopamine. The compounds were also unable to counteract the inhbitory effect exerted by Salvinorin A on dopamine exocytosis.

•

•All together these results suggest that the new neocloredane derivatives could pharmacologically discriminate between k homodimers and k/ δ heterodimers . Future investigations are needed to explore this hypothesis.

REFERENCES:

1) Bisio et al., Planta Med., 70, 452-7, 2004.
2) Grilli et al., Neuropharmacology, 57, 523–530, 2009

Supported by Italian Ministero Università Ricerca, by European project ALCOTRA (Cooperazione territoriale europea Italia-Francia (Alpi) "AROMA"), by the European project PYRGI (progetto Italia-Francia "Marittimo" 2007-2013) and by Compagnia San Paolo

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.3

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

A new clerodane diterpenoid from the aerial part exudate of *Salvia chamaedryoides* Cav.

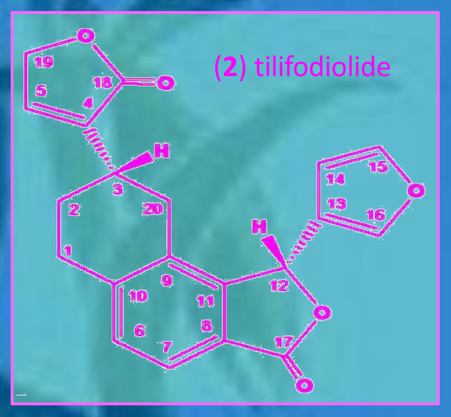
Angela Bisio[a], Emanuela Giacomelli[a], Gianluca Damonte[b], Annalisa Salis[b], Daniele Fraternale[c], Giovanni Romussi[a], Sergio Cafaggi[a], Donata Ricci[c], Nunziatina De Tommasi[d]

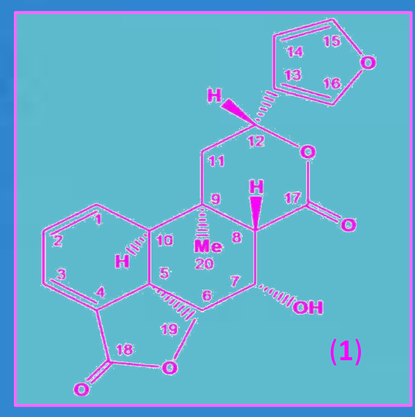
[a] *Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari, Università di Genova, Via Brigata Salerno 16147 Genova, Italy*
[b] *Dipartimento di Medicina Sperimentale e Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, Viale Benedetto XV 7, 16132, Genova, Italy*
[c] *Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura, Università di Urbino, Via Bramante 28, Urbino, Italy*
[d] *Dipartimento di Scienze Farmaceutiche,Università di Salerno, Via Ponte Don Melillo, 84084 Salerno, Italy*
bisio@dictfa.unige.it

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

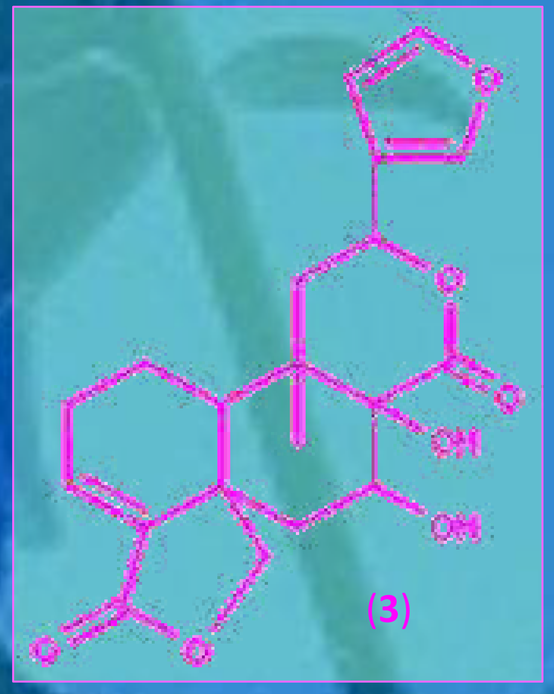
The secretion product of epidermal structures is involved in plant-plant and plant-environment interactions [1-2], and its phytotoxic chemicals are released into the environment by foliar leaching, volatilization or residue decomposition [3]. *Salvia chamaedryoides* Cav. [4], is a Mexican species whose aerial part exudate showed herbicide activity against *Papaver rhoeas* L. and *Avena sativa* L [5].

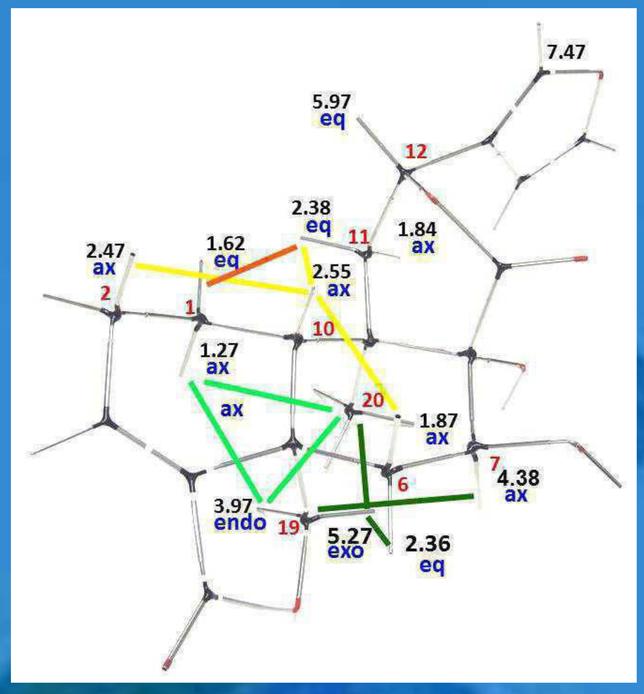
Plant Material. Fresh aerial parts of *S. chamaedryoides* Cav. were obtained from Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).





Extraction and isolation. For the isolation of leaf surface constituents, fresh aerial parts (4.9 kg) were immersed in CH_2Cl_2 for 20 s. After filtration, the extraction solvent was removed under reduced pressure obtaining 41 g of exudate material. 20.5 g of exudate were washed with *n*-hexane, and 17.33 g of the residue were chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 7:3 as an eluent; analytical TLC control), to give in order of elution six fraction groups: fraction group I (from 0 to 60 mL; 0.66 g) with waxy compounds, fraction group II (from 60 to 80 mL; 1.05 g), fraction group III (from 80 to 100 mL; 3.81 g), fraction group IV (from 100 to 120 mL; 13.58 g), fraction group V (from 120 to 160 mL; 2.83 g) and fraction group VI (from 160 to 240 mL; 0.45 g).
Fraction group IV was chromatographed on silica gel column (6.6 g portions, 65x4 cm; analytical TLC control) eluting with n-hexane-$CHCl_3$ (25:75, 26.5 L) afforded compound **1** (from 10.1 to 10.8 L), purified by means HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 50:50, R_T 22 min, 8.6 mg).
Fraction group V was chromatographed on silica gel column (50x4.5 cm; analytical TLC control) eluting with mixtures of n-hexane-$CHCl_3$ (25:75, 7.3 L; 10:90, 2.3 L). Elution with n-hexane-$CHCl_3$ 10:90 afforded compound **2** (from 1.2 to 1.6 L) purified by means HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 60:40, R_T 20 min, 3.7 mg). Elution with $CHCl_3$-CH_3OH 95:5 afforded among others the fraction group 165-168 (from 14.2 to 14.4 L). This fraction group was re-chromatographed on silica gel column (35x1 cm; analytical TLC control) eluting with mixtures of $CHCl_3$-CH_3OH ($CHCl_3$ 100%, 0.76 L; $CHCl_3$-CH_3OH 95:5, 0.32 L). Elution with $CHCl_3$ afforded compound **3** purified by means of HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 50:50, R_T 30 min, 4.0 mg). **2** has been identified as tilifodiolide as its ^1H- and ^{13}C-NMR data were largely consistent with those published in the literature [6].
Compounds **1** [7] and **3** have been identified by NMR, including TOCSY, COSY, HSQC, HMBC experiments, ESI-TRAP-MS and HR-MS analysis.



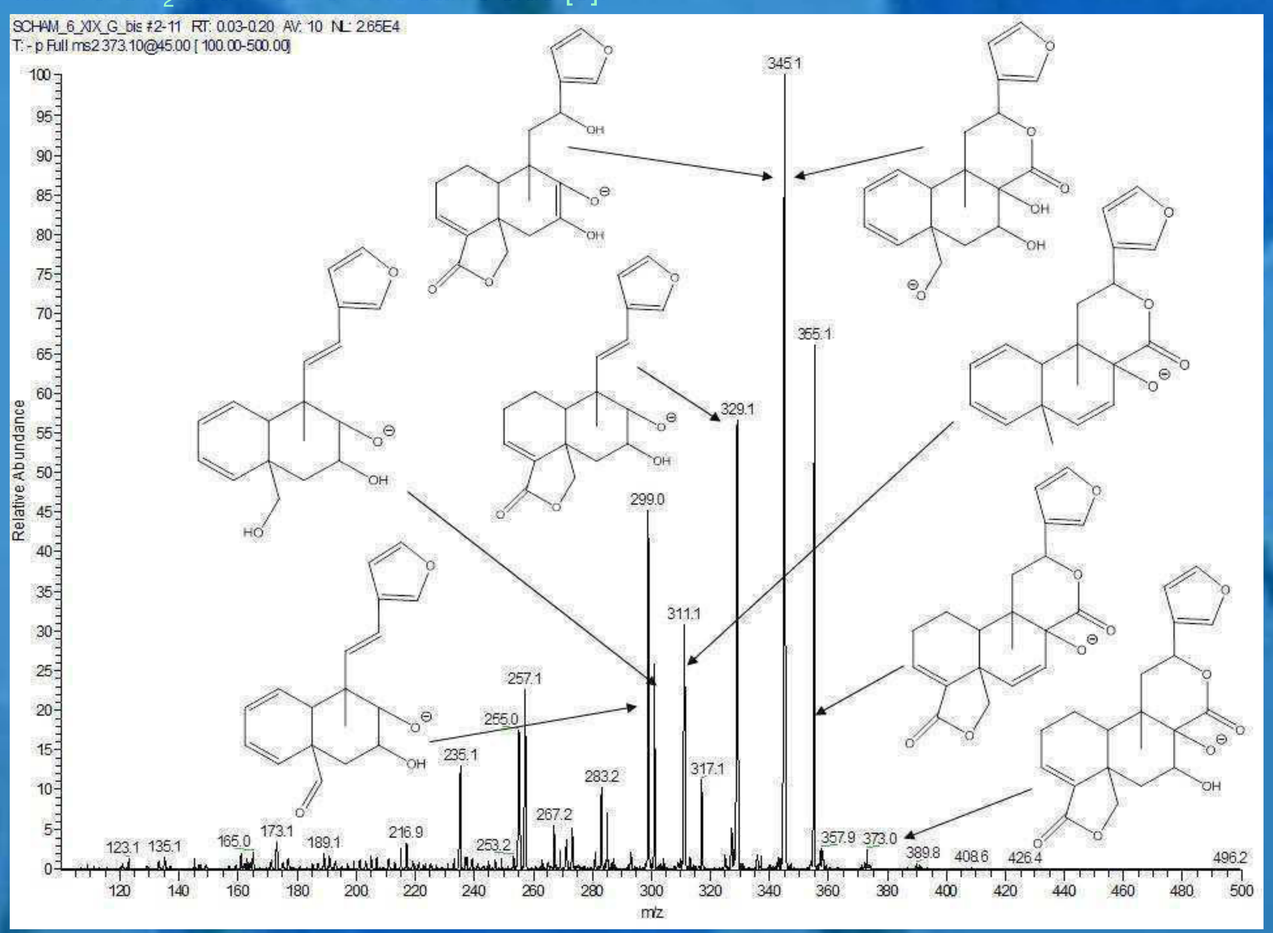


ROESY correlations 19-CH_2 /20-CH_3, 1-H *axial* (δ_H = 1.27)/20-CH_3 and 7-H /19- CH_2 showed that these groups are on the same side.
ROESY correlations 10-H/6-H at δ_H = 1.87, 10-H/2-H *axial* (δ_H = 2.47), and 10-H/11-H *equatorial* (δ_H = 2.38) showed that these protons are on the same opposite side and that the 6-H proton at δ_H = 1.87 is in the *axial* position.
ROESY correlation between 1-H *axial* (δ_H = 1.27) and the 19-H at δ_H = 3.97, which had the expected *W* coupling (2 Hz) with 6-H *axial* (δ_H = 1.82) showed that this proton in 19 is in the *endo* position and could be explained only A/B trans fused structure. The correlation between the 6-H at δ_H = 2.36 and the 19-H at δ_H = 5.27 showed that this proton in 19 is in the *exo* position.

Table 1. NMR spectral data for compound **3** (δ values, CDCl3, 13C-NMR at 150 MHz, 1H-NMR at 600 MHz).

C	^{13}C	^1H (J in Hz)	COSY correlations of the H	HMBC correlations of the C
1	19.0	1.29 dddd ax (16.0. 12.0. 12.0. 4.0)	1.64; 2.26; 2.44; 2.52;	2.26; 6.74
		1.64 m eq	2.26; 2.52	
2	27.6	2.26 m eq	1.29; 1.64; 2.44; 6.74	1.29; 1.64; 2.52; 6.74
		2.44 dddd ax (16.0. 12.0. 6.5. 2.8)	1.29; 6.74	
3	135.5	6.74 d	2.26; 2.44	1.64; 2.26; 2.44
4	138.3	-	-	2.26; 2.44; 5.25
5	44.3	-	-	1.29; 2.52; 4.35; 6.74
6	34.0	1.84 m ax (7.6. 12.0)	2.33; 4.35; 3.94	2.52; 3.94; 4.35; 5.25
		2.33 m eq	4,35	
7	72.0	4.35 m	1.84; 2.33	2,33
8	75.3	-	-	1.20; 1.81; 2.33; 2.52; 4.35
9	39.2	-	-	1.20; 1.64; 1.81; 2.35; 2.52; 4.35
10	45.0	2.52 dd ax (12.0. 2.0)	1.29; 1.64	1.20; 1.64; 1.81; 2.26; 2.33; 2.35; 2.44; 3.94; 5.25
11	41.1	1.81 m ax	2.35; 5.98	1.20; 5.98
		2.35 m eq	5,98	
12	72.1	5.98 t (8.0)	1.81; 2.35	1.81; 2.35; 6.43
13	125.8	-	-	1.81; 2.35; 5.98; 6.43; 7.46; 7.47
14	108.5	6.43 ns	7,46	5.98; 7.46; 7.47
15	144.1	7.46 ns	6,43	6.43; 7.47
16	139.7	7.47 ns	-	5.98; 6.43; 7.46
17	172.6	-	-	-
18	169.3	-	-	2.26; 5.25; 6.74
19	72.5	3.94 d endo (8.0. 2.0). 5.25 d eso (8.0)	3.94-5.25; 1.84	2.33; 2.52
20	19.9	1.20 s	-	1.81; 2.35; 2.52

The equatorial orientation of the hydroxy group at 7-C was indicated by the deshielding effect on H-19 *exo* (δ_H = 5.27) as compared to H-19 *endo* (δ_H = 3.97), that was consistent with 19-CH_2 and 7-OH on the same side [8].



References: [1] R.G. Kelsey, G.W. Reynolds, E. Rodriguez, **1984**, In: *Biology and chemistry of plant trichomes,* Rodriguez E., Healey P.L., Metha J., eds. New York: Plenum Press, 187-241; [2] E. Werker, **2000,** In : *Advances in Botanical Research*, Vol. 31. San Diego: Academic Press, 77-111; [3] C.H. Chou, **2006**, In: *Allelopathy – A Physiological Process with Ecological Implications*, Reigosa M.J., et al. Eds., Springer: Dordrecht, The Nederlands, 1-10; [4] C. Epling, **1940,** In: *Repertorium Specierum Novarum Regni Vegetabilis*, Vol.110. Fedde F., University of California Press: Berkley, California; [5] Bisio, A., Fraternale, D., Giacomini, M., Giacomelli, E., Pivetti, S., Russo, E., Caviglioli, G., Romussi, G., Ricci, D., De Tommasi, N. Crop Protection, 2010, 29, 1434-1446; [6] L. Rodriguez-Hahn, R. O'Reilly, B. Esquivel, E. Maldonado, A. Ortega, J. Càrdenas, R.A. Toscano, **1990**, *J. Org. Chem.*, **55**: 3522-3525; [7] Bisio, A., Fraternale, D., Damonte, G., Giacomelli, E., Salis, A., Ricci, D., Cafaggi, S., Romussi, G., De Tommasi, N., **2010**, SILAE, XIX Congresso italo-latinamericano di etnomedicina "Fernando Cabieses Molina", Villasimius (CA); [8] A. Givovich, A. San-Martín, M. Castillo, **1986**, *Phytochemistry* ***25***, 2829-2831.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.4

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

A New Derivative of 12-oxo-Phytodienoic Acid from *Salvia adenophora* Fernald.

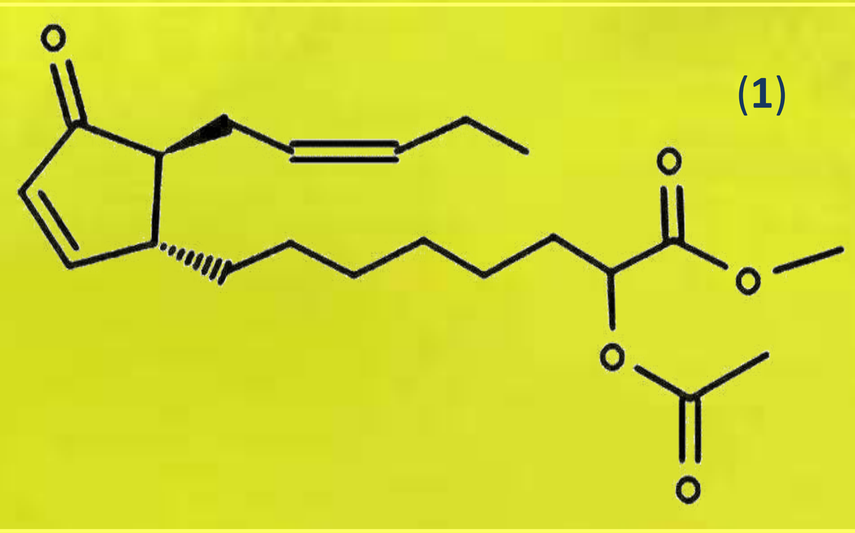
Bisio°A.[1], Damonte°G.[2], Giacomelli°E.[1], Mele°G.[1], Romussi°G.[1], Cafaggi°S.[1], De Tommasi°N.[3]

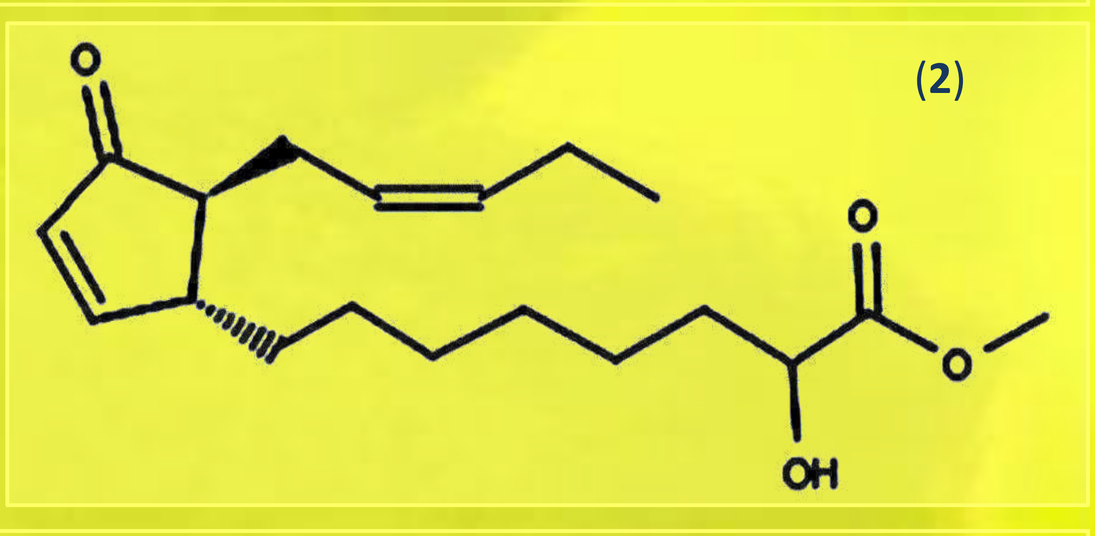
[1] *Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari, Università di Genova, Via Brigata Salerno 13, 16147 Genova, Italia,*
[2] *Dipartimento di Medicina Sperimentale e Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, Viale Benedetto XV 7, 16132, Genova, Italia,*
[3] *Dipartimento di Scienze Farmaceutiche,Università di Salerno, Via Ponte Don Melillo, 84084 Salerno, Italia.*

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

In course of a research devoted to the identification of natural pesticides in the surface exudates [1] of aromatic plants, we considered *Salvia adenophora* Fernald [2], which previously showed herbicide activity [3]. In this work we focused on the more lipophylic part of the exudate in order to isolate cyclic metabolites of the polyunsaturated fatty acids. As a matter of fact, due to their great structural variety, oxylipins may have various biological roles as antimicrobial, anti-insecticidal and antifungal compounds [4, 5]; these compounds have also shown effects of inhibition of root elongation, alteration of seedling growth and photosynthetic activity [6].

Plant Material. Fresh aerial parts of *S. adenophora* Fernald were obtained from Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

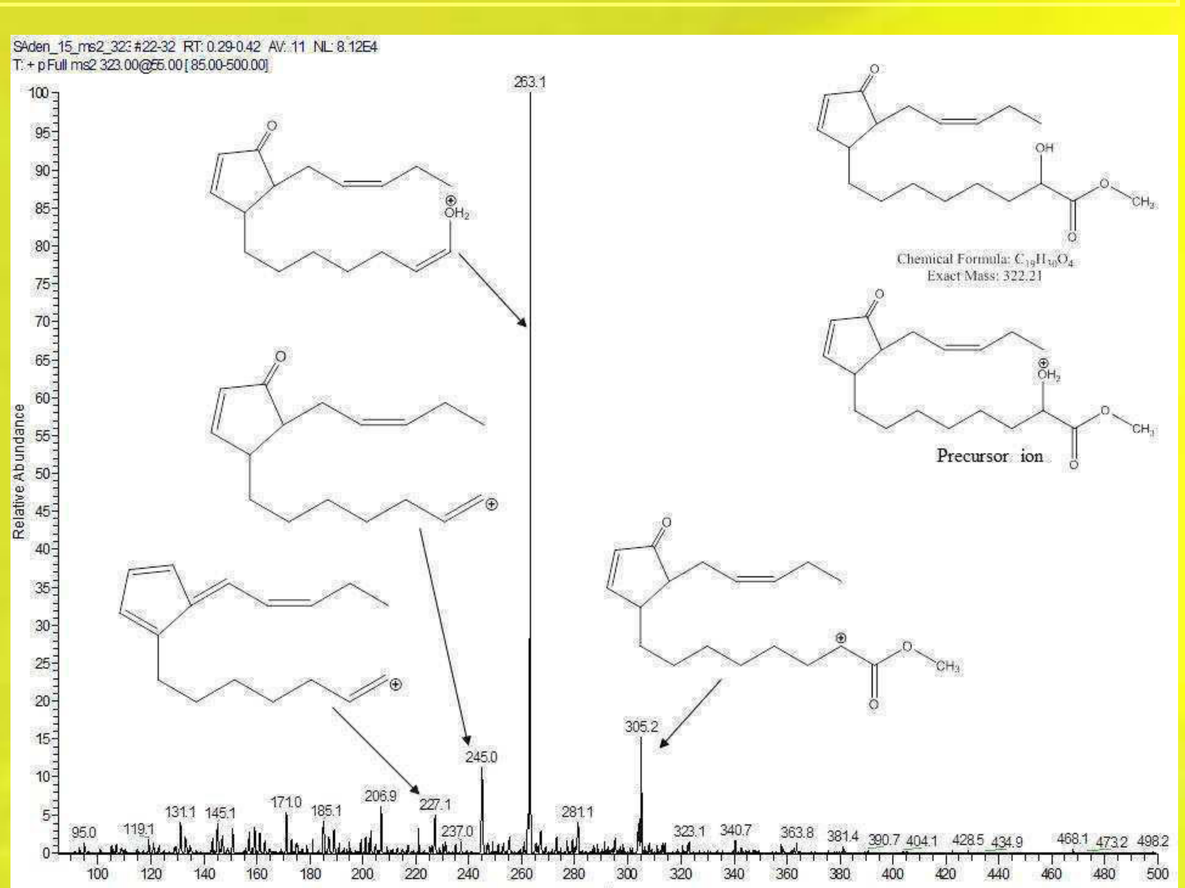


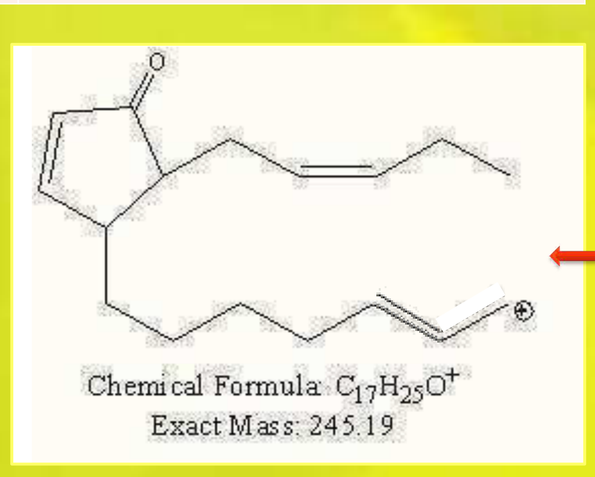


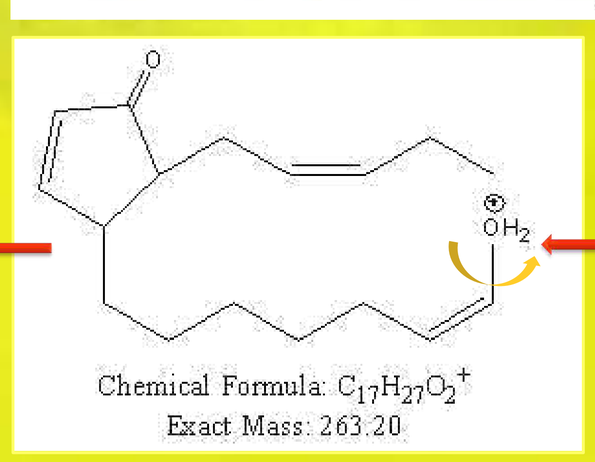
Extraction and isolation. Fresh aerial parts (8.8 kg) were immersed in CH_2Cl_2 for 20s. After filtration, the extraction solvent was removed under reduced pressure giving 60.9 g of exudate material. 36.0 g of exudate were then decerated with *n*-hexane and the residue (25.93 g) was then chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 7:3 as an eluent; analytical TLC control), to give in order of elution five fraction groups: fraction group I (7.38 g), fraction group II (3.02 g), fraction group III (6.48 g), fraction group IV (6.58 g) and fraction group V (1.47 g). The fraction group I was chromatographed on silica gel column (55 x 3.5 cm; analytical TLC control) eluting with mixtures of *n*-hexane-$CHCl_3$ (35:65, 2.4 L; 10:90, 5.0 L; 0:100 2.2 L) and mixtures of $CHCl_3$-CH_3OH (95:5, 10.2L; 90:10, 0.9L; 0:100, 1.6L). Elution with *n*-hexane-$CHCl_3$ 35:65 afforded compound **1** (from 2.1 to 2.25 L) purified by HPLC-MS and MS^2 experiments followed by preparative RP HPLC (solvent A: H_2O with 0.1 % formic acid, solvent B: CH_3OH with 0.05 % formic acid, linear gradient: time 0 min: 40% B, time 25 min 100% B), R_T 22 min, 4.4 mg.
The hexane soluble fraction (11.0g) was chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 7:3 as an eluent; analytical TLC control), to give in order of elution five fraction groups: fraction group I (4.35 g), fraction group II (2.55 g), fraction group III (2.05 g), fraction group IV (0.28 g) and fraction group V (0.09 g). The fraction group I was chromatographed on silica gel column (55 x 3.5 cm; analytical TLC control) eluting with mixtures of *n*-hexane-$CHCl_3$ (35:65, 2.1 L; 0:100 4.0 L) and mixtures of $CHCl_3$-CH_3OH (95:5, 3.7L; 90:10, 3.8L). Elution with $CHCl_3$ afforded compound **2** (from 3.4 to 3.7 L) purified by HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 80:20, R_T 9 min, 6.7 mg. The compounds **1** [7] and **2** had been identified by means of ^1H and ^{13}C-NMR spectra, including TOCSY, COSY, HSQC, HMBC, ROESY experiments, and ESI-TRAP-MS and HR-MS analysis. The spectroscopic data obtained for compounds **1** and **2** relative to the pentenil and cyclopentenil moieties and in particular those concerning the chiral centers in the ring juncture C 9 and C13, were largely consistent with those published in the literature for 12-oxo-trans-10,15-phytodienoic acid (13-epi-12-oxo-PDA) [8, 9, 10] and for its methyl ester [11, 12].

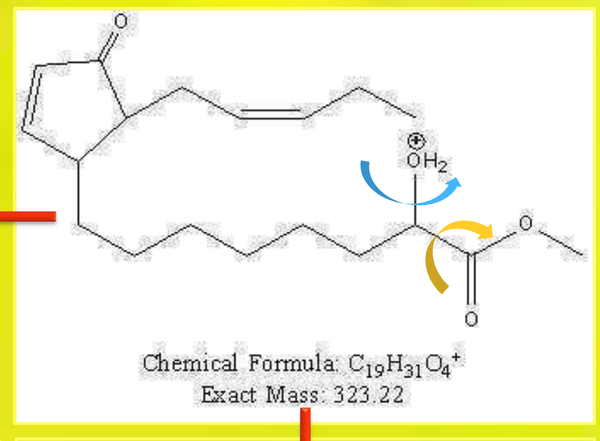
Table 1. NMR spectral data for compound **2** (δ values, CDCl3, 13C-NMR at 150 MHz, 1H-NMR at 600 MHz).

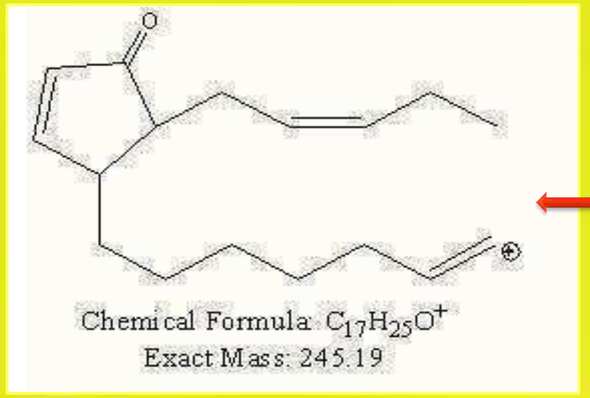
C	^{13}C	^1H (*J* in Hz)	COSY correlations of the H	HMBC correlations of the C
1	177.0	-	-	1.78 - 3.79 - 4.19
2	70.4	4.19 m	1.63 - 1.78	1.78
3	34.3	1.63 m - 1.78 m	1.37 - 1.47 - 4.19	4.19
4	24.7	1.37 m - 1.47 m	1.27 - 1.38 - 1.63 - 1.78	1.38 - 1.63 - 1.78 - 4.19
5	29.5	1.27 m - 1.38 m	1.34 - 1.36 - 1.37 - 1.47	1.63 - 1.78
6	29.1	1.34 m - 1.36 m	1.27 - 1.38 - 1.39 - 1.50	1.27 - 1.38 - 1.39 - 1.42 - 1.50 - 1.51
7	27.3	1.39 m - 1.50 m	1.34 - 1.36 - 1.42 - 1.51	1.51
8	34.3	1.42 m - 1.51 m	1.39 - 1.50 - 2.59	2.02 - 2.59
9	47.2	2.59 m	1.42 - 1.51 - 7.60	1.51 - 2.02 - 2.28 - 2.46 - 6.13 - 7.60
10	167.4	7.60 nd (9.0)	2.59 - 6.13	1.51 - 2.59 - 6.13
11	132.8	6.13 nd (9.5)	7.6	7.6
12	212.0	-	-	2.02 - 2.28 - 2.46 - 6.13
13	51.3	2.02 m	-	1.51 - 2.28 - 2.46 - 6.13 - 7.60
14	28.2	2.28 m - 2.46 m	5.27	2.02 - 2.59 - 5.27
15	125.6	5.27 ddd (10; 7.6; 6.7)	2.28 - 2.46 - 5.45	2.02 - 2.05 - 2.06 - 2.28 - 2.46
16	133.7	5.45 ddd (10; 7.6; 6.7)	2.05 - 2.06 - 5.27	0.97 - 2.05 - 2.06 - 2.28 - 2.46
17	20.5	2.05 m - 2.06 m	0.97 - 5.45	0.97 - 5.27 - 5.45
18	14.2	0.97 t (8.6; 8.6)	2.05 - 2.06	2.05 - 2.06 - 5.45
19	52.5	3.79 s	-	-

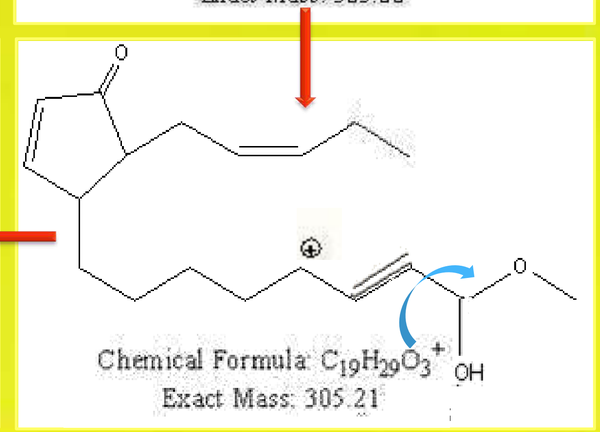












[1].Dayan, F.E., Duke, S.O. *Pesticide Outlook*, **2003**, 8, 175-178; [2].Epling, C. In Fedde F., University of California Press: Berkley, California, **1940**, *Vol.110*; [3].Bisio, A., Fraternale, D., Giacomini, M., Giacomelli, E., Pivetti, S., Russo, E., Caviglioli, G., Romussi, G., Ricci, D., De Tommasi, N. *Crop Protection*, **2010**, 29, 1434-1446; [4].Jahn, U., Galano, J.M., Durand, T., **2008**, *Angewandte Chemie International Edition* 47, 5894-5955; [5] Göbel, C., Feussner, I., **2009**, *Phytochemistry* 70, 1485-1503; [6] Mueller, M.J., Berger, S., **2009**, *Phytochemistry* 70, 1511-1521; [7].Bisio, A., Giacomelli, E., Damonte, G., Salis, A., Fraternale, D., Ricci, D., Romussi, G., Cafaggi, S., De Tommasi, N. *Congresso S.I.F.*, **2011**, Roma 30 giugno-2 luglio; [8] Ainai, T., Matsuumi, M., Kobayashi, Y., **2003**, *The Journal of Organic Chemistry* 68, 7825-7832; [9] Baertschi, S.W., Ingram, C.D., Harris, T.M., Brash, A.R., **1988**, *Biochemistry* 27, 18-24; [10].Kobayashi, Y., Matsuumi, M., **2002**, *Tetrahedron Letters* 43, 4361-4364; [11] Bohlmann, F., Borthakur, N., King, R.M., Robinson, H., **1982**, *Phytochemistry* 21, 125-127; [12] Chu, X.-J., Hong, D., Liu, Z.-Y., **1995**, *Tetrahedron* 51, 173-180.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

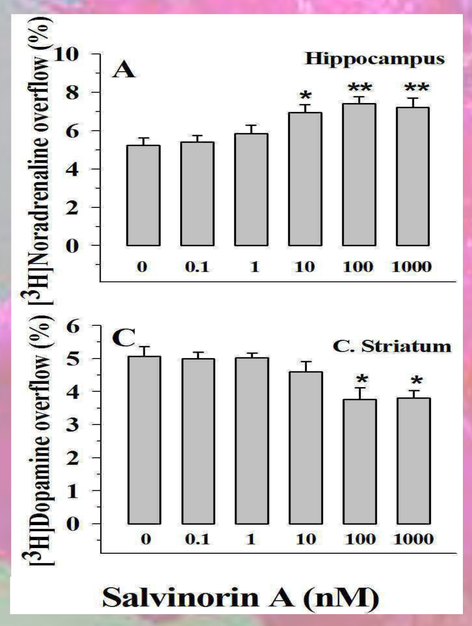
PRODOTTO 26b.5

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Salvinorin A is the main active compound found in Salvia Divinorum; it is a neoclerodane diterpene that acts as an highly selective and potent opioid receptor agonist, representing one of the most potent naturally occurring hallucinogen. Because of its chemical features, this compound could represent the prototype of a new class of drugs useful in therapeutic treatment in amnesia, depression, pain and drug seeking.

In a previous work we have found that Salvinorin A (0.1–1000 nM) failed to affect the basal release of amines, but inhibited the 12 mM K^+-evoked, Ca^{2+}-dependent, exocytotic-like release of [^{3}H]DA from mouse striatal nerve endings. At the same concentration, Salvinorin A facilitated the 12 mM K^+-evoked, Ca^{2+}-dependent, exocytotic-like release of [^{3}H]NA from mouse hippocampal terminals. By using the same experimental approach, the up-down superfusion of a monolayer of synaptosomes, which is considered appropriate to investigate the existence and the functional role of presynaptic receptors, we have now invetsigated the impact of new neocloredane derivatives on central amine release. The new clerodane diterpenes have been isolated from *Salvia jamensis* (the compound SJ217), *Salvia wagneriana* (the compounds SW8 and SW22).



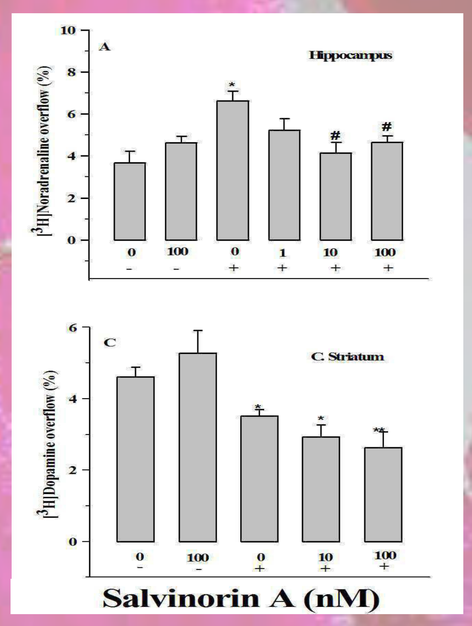


Fig. 2. Effects of pertussis toxin (PTx) on the Salvinorin A induced modification of neurotransmitter overflows evoked by K^+ depolarization from superfused synaptosomes.

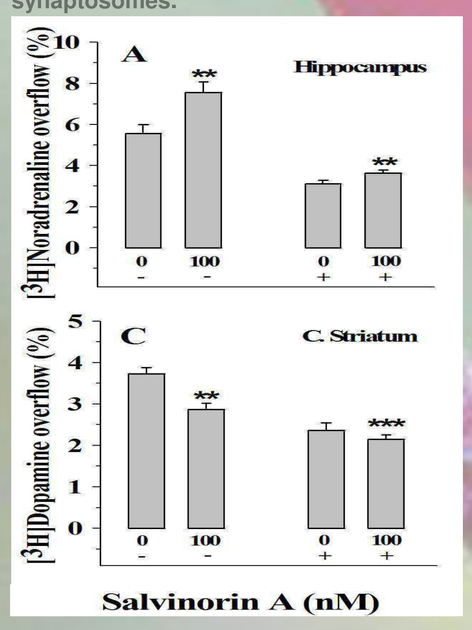


Fig. 5. Concentration-effect relationship of U69593 on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.

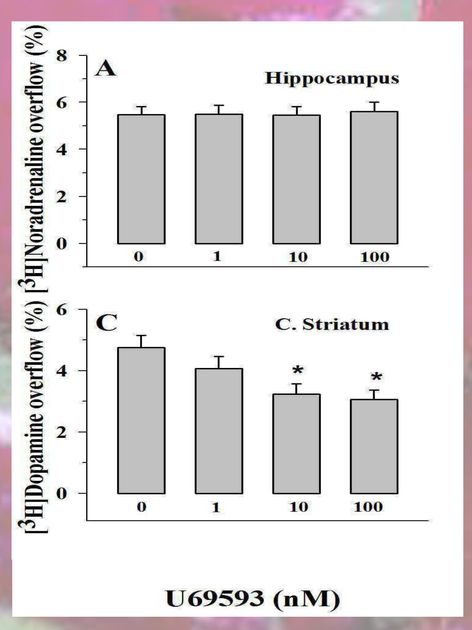
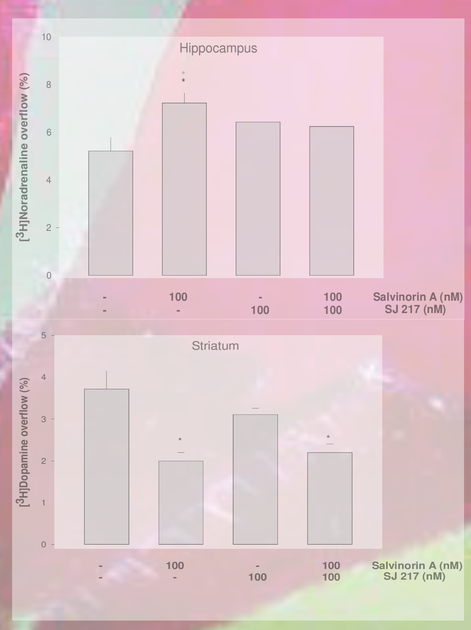


Fig. 7 Effects of SJ 217 on the Salvinorin A induced modification on the 12 mM K^+-evoked release of neurotransmitter from superfused synaptosomes.



CONCLUSIONS

•Salvinorin A facilitates NA exocytosis from hippocampal terminals, while it inhibits the K^+-evoked release of DA from striatal synaptosomesThe presynaptic modulation occurs in the nM range, indicative of the involvement of high-affinity binding sites.

•The opposite effects of Salvinorin A were found to rely on distinct receptor subtypes. In particular, k and d heterodimers presynaptically located on mouse hippocampal noradrenergic terminals may account for the Salvinorine A-induced facilitation of NA release, while inhibition of DA release occurred because of the binding of the diterpene to k homodimers on mouse striatal dopaminergic terminals.

Our recent findings show that the new compounds SW8 and SJ217 mimic Salvinorin A on the 12 mM K^+-evoked release of hippocampal [^{3}H]NA, while SW22 inhibits this release. Furthermore, our results demonstrate that SW8 and SW22, when co-applied with Salvinorin A, could prevent its positive effect on the [^{3}H]NA exocytosis.

•As to the dopamine release is concerned, the three compounds fail to modify the 12 mM K^+-evoked release of dopamine. The compounds were also unable to counteract the inhbitory effect exerted by Salvinorin A on dopamine exocytosis.

•All together these results suggest that the new neocloredane derivatives could pharmacologically discriminate between k homodimers and k/ d heterodimers, tipified by a low affinity towards k/ d homodimers. Future investigations are needed to explore this hypothesis.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.6

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

COMPOSITION OF ESSENTIAL OILS OF

Helichrysum italicum (ROTH) SUBSP. *italicum*

FROM ELBA ISLAND

Luisa Pistelli[a], Guido Flamini[a], Pier Luigi Cioni[a], Bernardo Melai[a],Michele Leonardi[a]

[a]Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno 6, 56100 Pisa, Italy

luipi@farm.unipi.it

Progetto PYRGI
Strategia d'impresa in settori di nicchia per l'economia agroindustriale del Mediterraneo

LA COOPERAZIONE NEL CUORE DEL MEDITERRANEO

INTRODUCTION

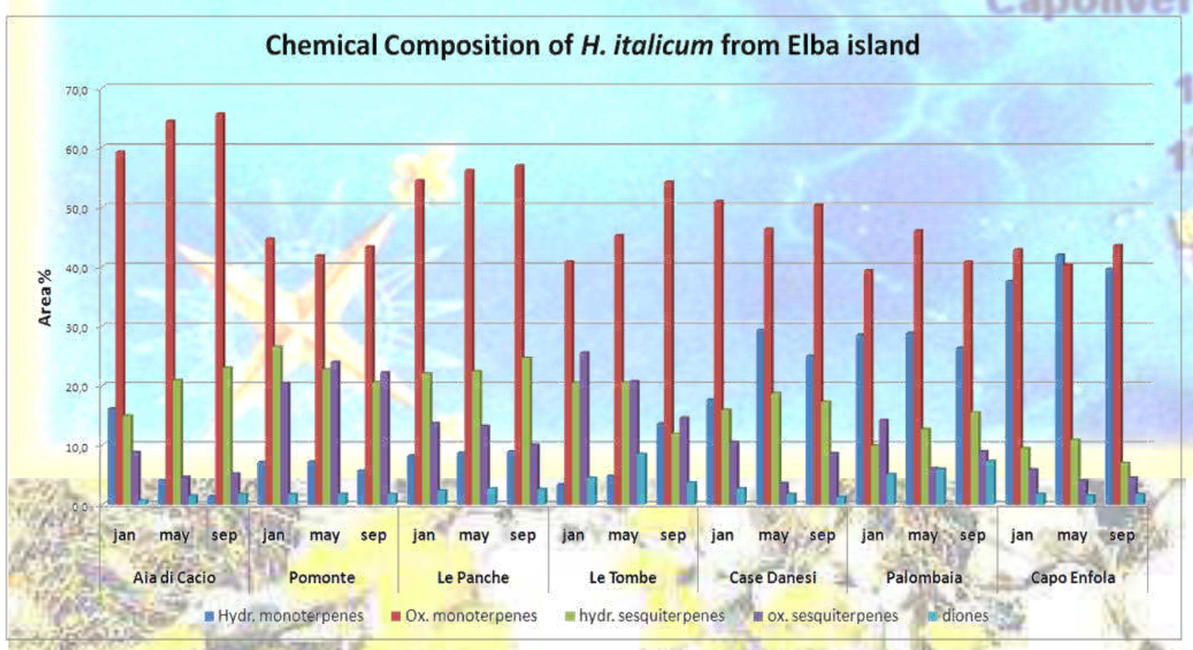
Helichrysum **is a typical aromatic plant of the Asteraceae family. There are about 300 species of** ***Helichrysum*****, six-teen of which are spontaneous in Europe [1]. Among the large number of phytoproducts that can be obtained from Helichrysum are used in cosmetic and pharmaceutical preparations.** ***Helichrysum italicum*** **is a typically Mediterranean species. It consist of three subspecies,** ***Helichrysum italicum*** **subsp.** ***microphyllum*** **(present in Baleares, Sardinia, Corsica);** ***Helichrysum italicum*** **subsp.** ***serotinum*** **(Iberian peninsula) and** ***Helichrysum italicum*** **subsp.** ***italicum*** **(Mediterranean basin) [2]. In a previous paper [3] we describe the essential oil composition of the** ***Helichrysum italicum*** **subspecies** ***italicum*** **(Roth) collected in Corsica, Sardinia, Tuscany and minor islands of Tuscan archipelago (Gorgona, Capraia, Giannutri, Giglio, Montecristo and Pianosa). As part of our on-going work on the characterization of** ***Helichrysum italicum*** **subsp.** ***italicum*** **EOs from various origins, we analyzed eight samples from Elba island in the framework of the European Program Interreg III between Corsica, Tuscany and Sardinia.**

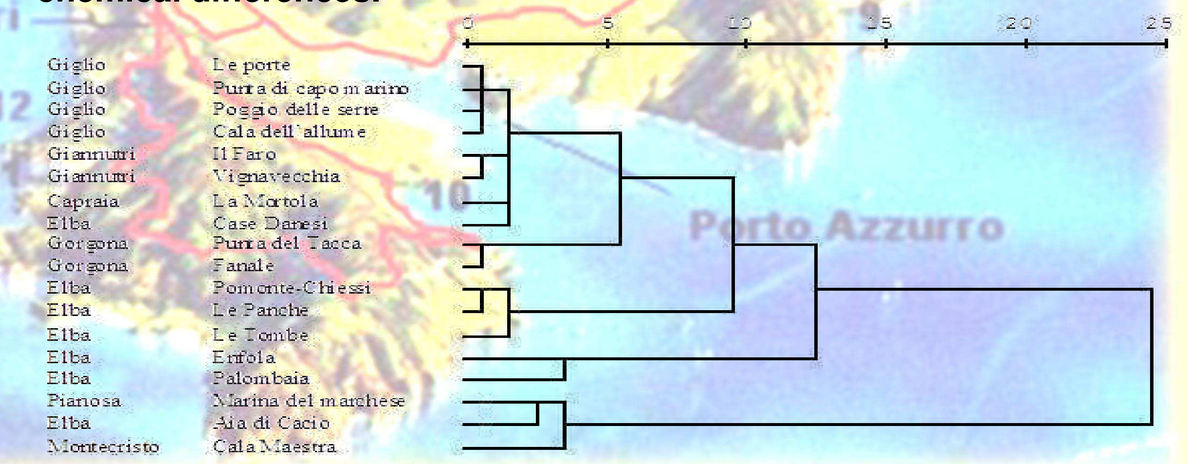
EXPERIMENTAL

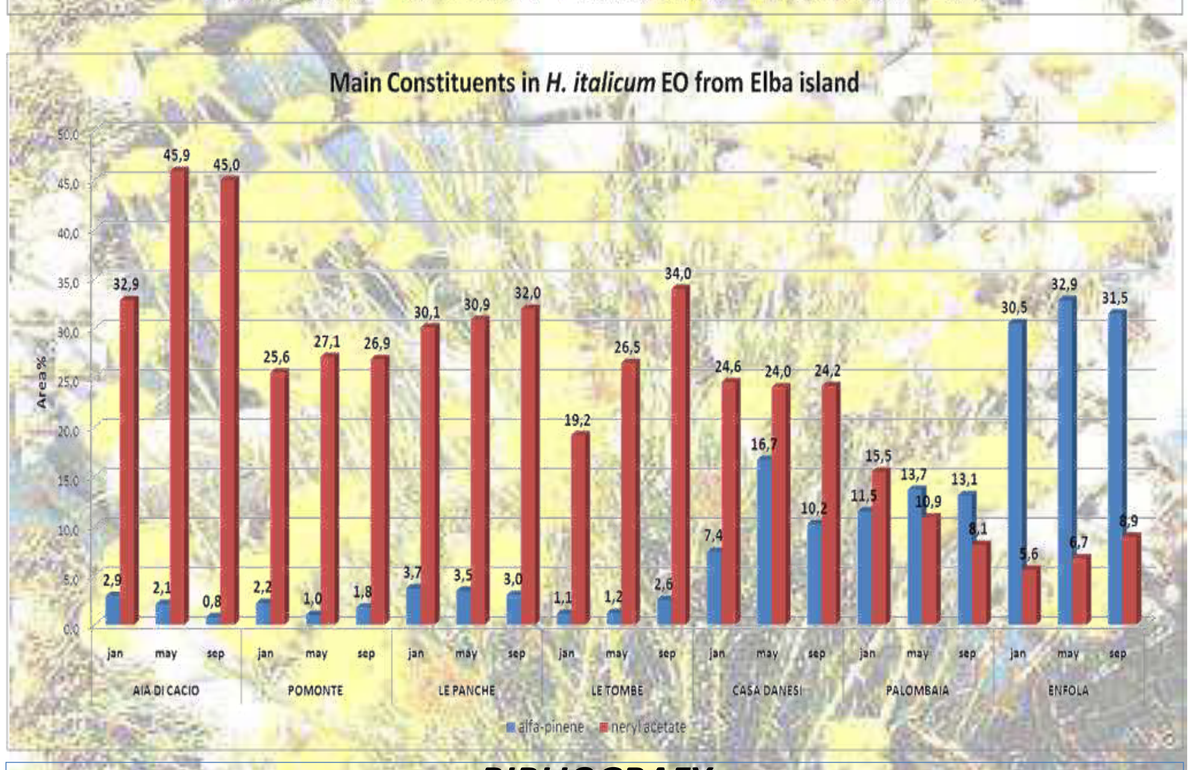
Plant material of Elba island ***H. italicum*** **subsp.** ***Italicum*** **was sampled from seven locations showed in the fig.1. The eight samples of** ***H. italicum*** **subsp.** ***italicum*** **essential oils from eight different place in Elba island collected in three different periods: January, May and October 2010. All the EOs were produced by hydrodistillation of fresh leaves and stems, using a Clevenger-type appratus. The oil yields are: 0,18% Aia di Cacio, 0,22% Capoliveri (Calamita), 0,39% Palombaia, 0,57% Le Tombe, 0,27% Case Danesi, 0,32% Le Panche, 0,34% Enfola, 0,30% Pomonte. All sample were analysed by using HP-5890 Series II instrument equipped with a HP-5 capillary columns (30 X 0.25 mm, 0.25 mm film tickness) working with the following temperature program: 60°C for 10 min, rising at 5 °C/min to 220 °C. The injector and detector temperatures were maintened at 250 °C; carrier gas nitrogen (2 Ml/min); the volume injected was 0.5 mL.**

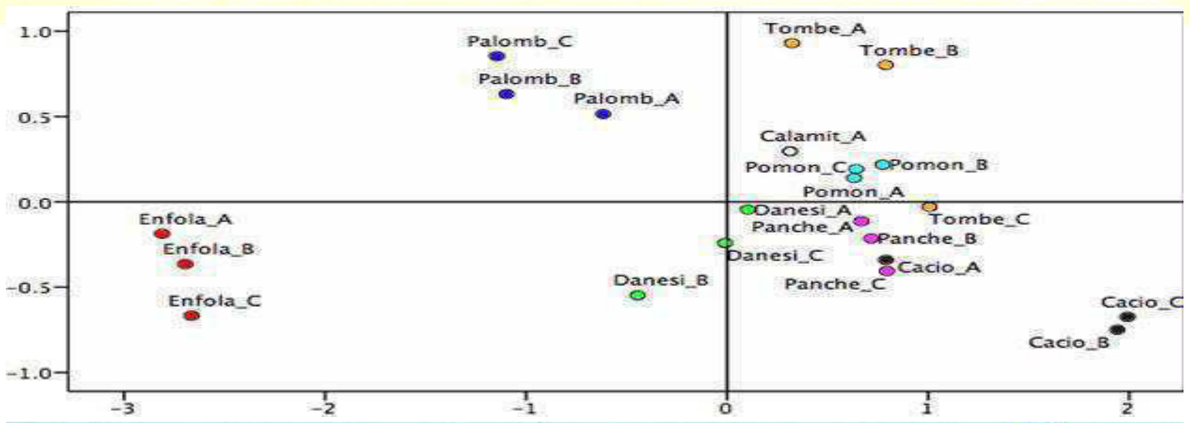
RESULTS AND DISCUSSION

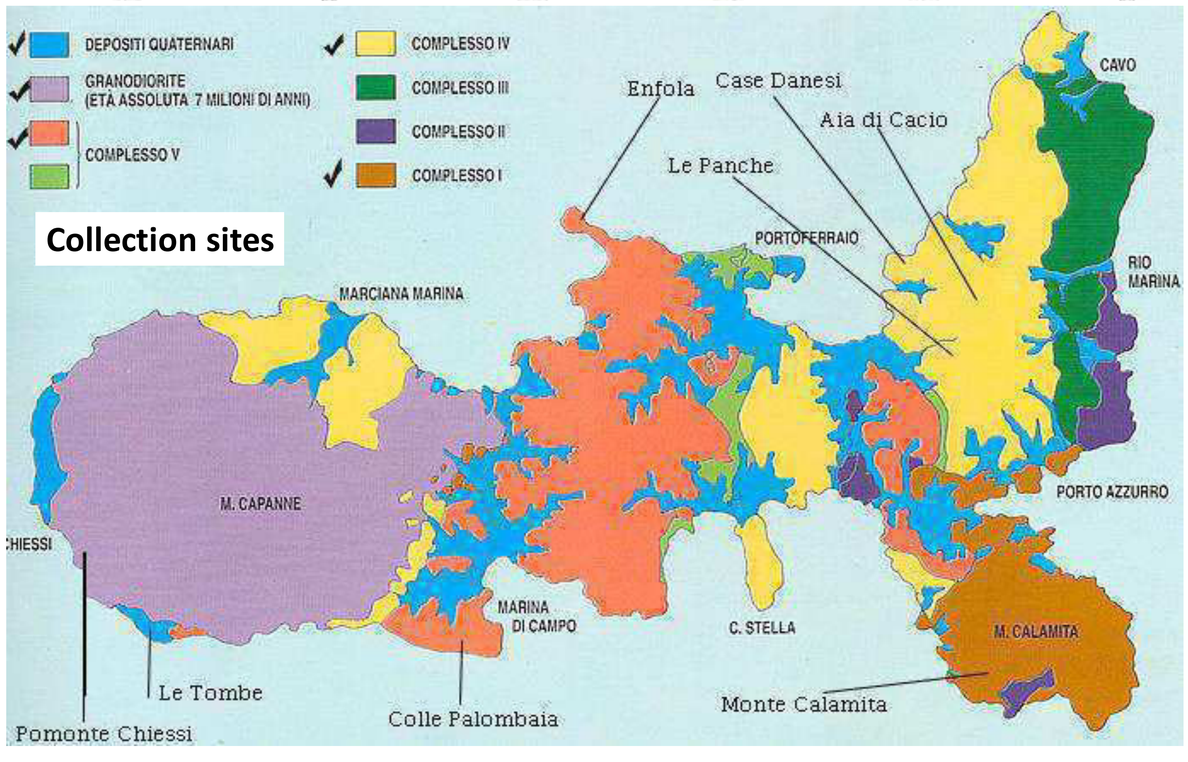
Analysis of the seven samples of ***H. italicum*** **susp.** ***italicum*** **oils led to the identification of one hundred and twenty-six compounds, which represented 91.5-98.3% of the total amount. According to their origin the composition of EO samples of** ***H. italicum*** **subsp.** ***italicum*** **displayed a significant diversity. In Elba island oils except the sample collected in Capo Enfola, the major compound observed was neryl acetate, with a percentage range comprise between 19.2% (Le Tombe) and 45.9% (Aia di Cacio). In the EOs collected in Capo Enfola the major constituents was the a-pinene (30.5-32.9%) while the neryl acetate was present in minor percentages (5.6-8.9%). Elba island oils also exhibited high contents of oxygenated monoterpenes that ranged from 44.2 % (Palombaia) to 83.1% (Aia di Cacio). Only two essential oil samples from plant material collecteted Capo Enfola and Colle Palombaia showed chemical differences.**











BIBLIOGRAFY

[1] J. Gamisans and D. Jeanmonod, *Complements au prodrome de la flore Corse, Asteraceae,* vol. 1; 1998.

[2] J. Paolini, J.M. Desjobert, J. Costa, A.F. Bernardini, C. Buti-Castellini, P.L. Cioni, G. Flamini, L Morelli; *Flavour and Fragrance Journal*, 2006; 21; 805-808.

[3] B. Conti, A. Canale, A. Bertoli, F. Gozzini, L. Pistelli; *Parasitol. Res.* 2010; 107; 1455-1461.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.7

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

MICROPROPAGAZIONE DI SPECIE AROMATICHE DELL'ARCIPELAGO TOSCANO

Laura Pistelli[a*], R. Celata, F. D'Angiolillo[a],, C. Noccioli[b] and Luisa Pistelli[b]

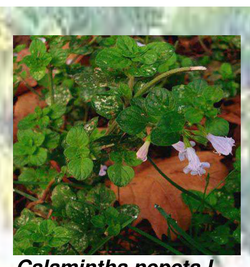
[a] Dipartimento di Biologia, Università di Pisa, via Mariscoglio 34, 56124 Pisa - Italy

[b] Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 33, 56126 Pisa – Italy

Progetto PYRGI
Strategia d'impresa in settori di nicchia per l'economia agroindustriale del Mediterraneo

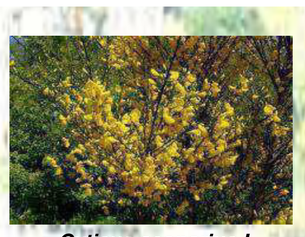
LA COOPERAZIONE NEL CUORE DEL MEDITERRANEO

Il **Parco Nazionale dell'Arcipelago Toscano comprende un'area terrestre di circa diciassettemila ettari e un'area marina di circa cinquantasettemila ettari ed è una delle più grandi aree protette d'Italia, la più estesa fra quelle marine di tutto il Mediterraneo. L'ambiente è caratterizzato da numerose piante aromatiche che contengono metaboliti secondari utilizzati sia a scopo alimentare e cosmetico, sia in aromaterapia e in fitoterapia. Queste specie sono spontanee, ma costituiscono produzioni "di nicchia" per il mercato salutistico; pertanto la loro riscoperta e valorizzazione costituiscono un interessante punto di partenza per nuove strategia d'impresa della popolazione dell'arcipelago. L'obiettivo di questo lavoro è la produzione massiva di piantine, senza depauperare l'ambiente, la conservazione del gerrmoplasma, e la creazione di baby-plants che possono costituite un interessante souvenir per i turisti .**



Calamintha nepeta **L.**

Crithmum maritimum **L.**



Cytisus scoparius **L.**

Lavandula angustifolia. Miller

Myrtus communis **L.**

Nepeta cataria **L.**

Rosmarinus officinalis **L.**

Salvia officinalis L.

Satureja hortensis **L.**

Materiali e metodi

Colture in vitro. Espianti isolati dalle piante selzionate (Fig.2) sono stati sterilizzati come indicato in tabella 1, e successivamente messi in substrati di coltura, utilizzando com substrato comune il Murashige e Skoog (1962) con aggiustamenti come indicato in tabella 2. Le subcolture sono state effettuate ogni 4 settimane.

Gas cromatografia

Le analisi GC sono state effettuate con uno strumento HP 5890 Series II Plus equipaggiato con due colonne capillari HP-Wax e HP-5MS (30 m x 0.25 mm; film di rivestimento 0.25 □m), usando le seguenti condizioni analitiche: temperatura iniettore e detector: 250°C; programma di temperatura del forno da 60°C (10 min) a 220°C a 5°C/min; gas di trasporto: N_2 a 2 ml/min; detector FID; split: ratio 1:30; volume di iniezione: 0.5 □l. L'identificazione dei componenti dello spazio di testa è stata effettuata su entrambe le colonne, confrontando i tempi di ritenzione dei singoli componenti con quelli di campioni puri autentici e dei loro indici di ritenzione relativi alla serie di *n*-alcani.

Gas cromatografia-massa

Le analisi GC-MS sono state effettuate con uno strumento HP 5890 Series II Plus, equipaggiato con una colonna capillare HP-5MS (30 m x 0.25 mm; film di rivestimento 0.25 □m) ed un detector di massa HP 5972, operando nelle seguenti condizioni analitiche: temperatura iniettore e detector: 220°C e 280°C rispettivamente; programma di temperatura del forno da 60°C (10 min) 20°C/min; gas di trasporto: He ad un flusso costante (0.6 ml/min); sorgente: 70eV.

L'identificazione dei componenti degli spazi di testa è avvenuta mediante analisi computerizzata degli spettri di massa, dei tempi di ritenzione ed indici di ritenzione, confrontati con i dati presenti sia nella libreria elaborata dal nostro gruppo di ricerca attraverso campioni di riferimento e olii essenziali noti, sia nelle librerie di spettri di massa NBS-75 e Wiley. Inoltre tutti i dati ottenuti sono stati confrontati anche con letteratura specifica (Adams, 1995; Connolly and Hill, 1991; Swigar and Silverstein, 1981).

Analisi SPME

L'analisi SPME (Solid Phase Micro Extraction) è stata condotta con un dispositivo munito di fibra in polidimetilsilossano (SUPELCO, PDMS, 100 □m) utilizzata per campionare (15 min) la frazione volatile formatisi al di sopra un campione di materiale vegetale fresco chiuso ermeticamente in una beuta e lasciato equilibrare per 30 min. Alla fine del tempo di campionamento, la frazione adsorbita è stata sottoposta ad analisi GC e GC-MS.

Tabella 1: protocolli efficaci di sterilizzazione e vitalità degli espianti post-sterilizzazione in vitro (dopo 2 settimane)

Specie vegetale	Tipo di espianto	sterilizzazione	Vitalità
***Calamintha nepeta* L.**	internodi	Steril A	50%
***Cytisus scoparius* L.**	internodi	Steril B	70%
***Crithmum maritimum* L.**	Porzioni apicali	Steril A	100
***Crithmum maritimum* L.**	Porzioni basali	Steril D	100
***Myrtus communis* L.**	Porzioni apicali	Steril B	100%
***Lavandula angustifolia* Miller**	internodi	Steril B	80%
***Myrtus communis* L.**	internodi	Steril B	100%
***Nepeta cataria* L.**	semi	Steril D	50%
***Rosmarinus officinalis* L.**	internodi	Steril B	0%
***Rosmarinus officinalis* L.**	internodi	Steril E	100
***Rosmarinus officinalis* L.**	Porzioni apicali	Steril E	80
***Salvia officinalis* L.**	internodi	steril D	0%
***Salvia officinalis* L.**	semi	Steril D	8%
***Satureja hortensis* L.**	Porzioni apicali	Steril E	80%
***Satureja hortensis* L.**	semi	Steril D	70

Mezzi di sterilizzazione

Steril A: Tween 20 0.05% 20 minuti , NaOCl 50% 10 min, acqua sterile 5 min ad ogni passaggio

Steril B: Tween 20 0.05% 20 minuti , EtOH al 70%, 1min, ACE commerciale 50% 10 min, acqua sterile 5 min ad ogni passaggio

Steril D: ACE commerciale 20% 10 min, soluzione di Plant preservative mixture (PPM) 2%, $MgSO_4$ 50 mg/L, $Mg(NO_3)$ 50 mg/L, $MgCl_2$ 50 mg/L 18 h 24 °C, acqua sterile 5 min ad ogni passaggio

Steril E: saccarosio20 g/L, ACE commerciale0,01%, 15 g al buio; Tween-20 0.05% 20 minuti, EtOH 70%, 1min, ACE commerciale 50% 10 min, acqua sterile 5 min ad ogni passaggio

Tabella 2: fase di moltiplicazione delle diverse specie aromatiche dell'isola d'Elba. Tasso di moltiplicazione è individuato come numero di germogli per espianto per mese di coltura. Gli espianti utilizzati sono stati incubati su diversi substrati di coltura.

Specie vegetale	Tipo di espianto	mezzo	Tasso di moltiplicazione
Calaminta nepeta L.	internodi	Medium A	4
Cytisus scoparius L.	internodi	Medium MS0	1
Crithmum maritimum L.	porzioni apicali	Medium A	1
Crithmum maritimum L.	porzioni basali	Medium A	2,5
Crithmum maritimum L.	porzioni basali	Medium B	9
***Lavandula angustifolia* Miller**	internodi	Medium B	3
Myrtus communis L.	porzioni apicali	Medium A	3
Myrtus communis L.	internodi	Medium A	2
Nepeta cataria L.	semi	Medium A	5
Rosmarinus officinalis L.	internodi	Medium C	1
Rosmarinus officinalis L.	porzioni apicali	Medium C	1
Salvia officinalis L.	semi	Medium MS0	2
Salvia officinalis L.	semi	Medium A	2
Satureja hortensis L.	porzioni apicali	Medium A	0
Satureja hortensis L.	semi	Medium A	1

Medium MS0: mezzo Murashige e Skoog (1962) completo di vitamine, saccarosio 30%, agar 0,8%, Plant preservative mixture (PPM) 0,05%.

Medium A: MS0 + BA 0,5 g/L

Medium B: : MS0 + BA 0,5 g/L + NAA 0,46 g/L

Medium C: : MS0 + BA 0,5 g/L + IAA 0,017 g/L

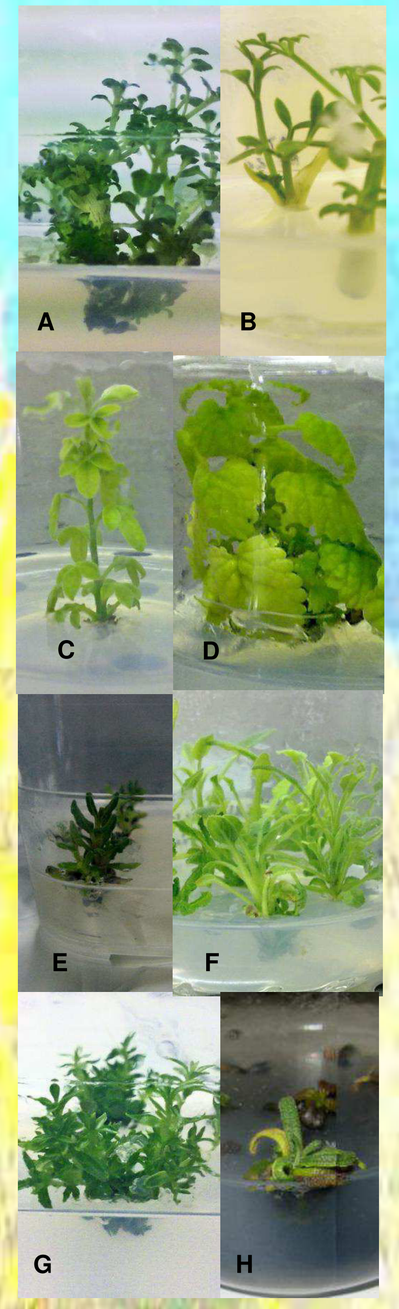


Figura 1: germogli micropropagati di:
A ***Calamintha nepeta* L**;
B ***Crithmum maritimum* L.;**
C ***Cytisus scoparius L.;*** ;
D ***Nepeta cataria* L.;**
E ***Lavandula angustifolia Miller*;**
F ***Salvia officinalis L***;
G ***Myrtus communis* L.**;
H ***Rosmarinus officinalis* L.**

Tabella 3 : costituenti principali identificati nello spazio di testa delle specie esaminate

metabolita	KI	*Cal*	*Cri*	*Lav*	*Myr*	*Nep*	*Ros*	*Sal*	*Sat*
a-pinene	940				65.0		24.7	14.6	
sabinene	978		14.5						
b-pinene	981	42.4						17.0	
myrcene	993						12.0		
p-cymene	1028								15.6
limonene	1032	15.0							
1,8-cineol	1036			11.9					
g-terpinene	1062		31.6						29.0
a-thujone	1109							20.5	
methyl thymol	1235		20.3		7.2				
caryophyllene	1418			19.3	6.8	47.6			20.0
aromadendrene	1445					6.6			
cis muurola 3,5-diene	1448	13.9							
germacrene D	1481			8.5					
b-selinene	1485					8.4			

Cal: *Calamintha nepeta*; *Cri*: *Crithmum maritimum*; ***Lav*: *Lavandula longifolia***; *Myr*: *Myrtus communis*; *Nep*: *Nepeta cataria*; *Ros*: *Rosmarinus officinalis*; *Sal*: *Salvia officinalis*; *Sat*: *Satureja hortensis*.

I risultati ottenuti dimostrano la capacità delle piantine selezionate di produrre una discreta quantità di nuovi germogli tale da rendere questa tecnica molto promettente e tale da dare nuovo impulso allo sviluppo imprenditoriale dell'arcipelago.

Le piantine moltiplicate *in vitro* dimostrano di possedere caratteristici aromi simili a quelli prodotti dalle piante autoctone coltivate in vaso o cresciute spontaneamente

BIBLIOGRAFIA

Adams, R.P., 1995. Identification of essential oil components by gas chromatography-mass spectroscopy. Allured, Carol Stream, Illinois

Connolly, J.D., Hill, R.A., 1991. Dictionary of terpenoids. Chapman & Hall, London;

Landi S. 1989., Flora e ambiente dell'isola d'Elba, Editrice Azzurra, Cavalese (Tn),.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-493.

Swigar, A.A., Silverstein, R.M., 1981. Monoterpenes. Aldrich Chem. Comp., Milwaukee.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.8

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Oxylipin compounds from the aerial part exudate of *Salvia adenophora* Fernald

A. Bisio[1], G. Damonte[2], E. Giacomelli[1], G. Mele[1], D. Fraternale[3], D. Ricci[3], G. Romussi[1], N. De Tommasi[4]

[1] Dipartimento di Farmacia, Università degli Studi di Genova, via Brigata Salerno 13, 16147 Genova;
[2] Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, viale Benedetto XV 7, 16132 Genova;
[3] Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura, Università di Urbino, via Bramante 28, 61029 Urbino;
[4] Dipartimento di Scienze Farmaceutiche, Università di Salerno, via Ponte Don Melillo, Fisciano 84084, Salerno.

Introduction. In course of a research devoted to the identification of natural pesticides in the surface exudates [1] of aromatic plants, we considered *Salvia adenophora* Fernald [2], which previously showed herbicide activity [3]. In this work we focused on the more lipophylic part of the exudate in order to isolate cyclic metabolites of the polyunsaturated fatty acids. As a matter of fact, due to their great structural variety, oxylipins may have various biological roles as antimicrobial, anti-insecticidal and antifungal compounds [4, 5]; these compounds have also shown effects of inhibition of root elongation, alteration of seedling growth and photosynthetic activity [6].

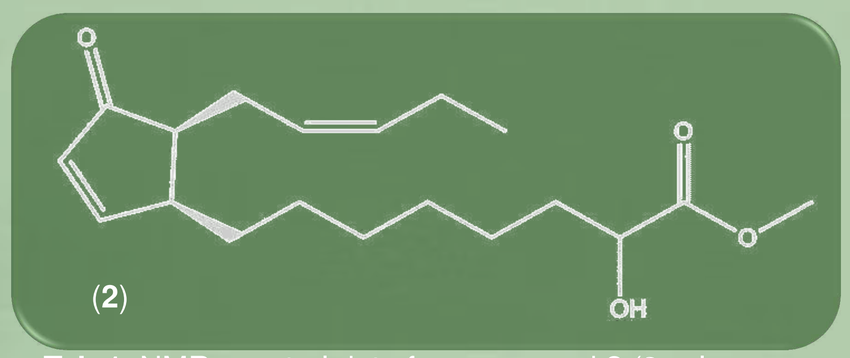
Extraction and isolation. Fresh aerial parts (8.8 kg) of *Salvia adenophora* Fernald were immersed in CH_2Cl_2 for 20s. After filtration, the extraction solvent was removed under reduced pressure giving 60.9 g of exudate material, composed by leaf surface constituents. 36.0 g of exudate were treated with n-hexane giving the hexane-soluble fraction and the hexane-insoluble fraction.

The hexane soluble fraction (11.0 g) was chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 7:3 as an eluent; analytical TLC control), to give in order of elution five fraction groups: fraction group I (4.35 g), fraction group II (2.55 g), fraction group III (2.05 g), fraction group IV (0.28 g) and fraction group V (0.09 g).

The fraction group I was chromatographed on silica gel column (55 x 3.5 cm; analytical TLC control) eluting with mixtures of n-hexane-$CHCl_3$ (35:65, 2.1 L; 0:100 4.0 L) and mixtures of $CHCl_3$-CH_3OH (95:5, 3.7L; 90:10, 3.8L). Elution with $CHCl_3$ afforded compound **1** (from 3.4 to 3.7 L) purified by HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 80:20, R_T 9 min, 6.7 mg. The fraction group II was chromatographed on silica gel column (35 x 1 cm; analytical TLC control) eluting with mixtures of n-hexane-$CHCl_3$ (35:65, 2.1 L; 0:100 4.0 L) $CHCl_3$-CH_3OH (95:5, 3.7L; 90:10, 3.8L). Elution with CHCl3 afforded compounds **2** and **3** (from 3.2 to 3.6. L) purified by HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, (solvent A: H_2O, solvent B: CH_3OH, linear gradient: time 0 min: 40% B, time 15.02 min: 85% B, time 35.73 min: 100% B), R_T 21.50 min, 17.0 mg and R_T 23.35 min, 26.9 mg, respectively.

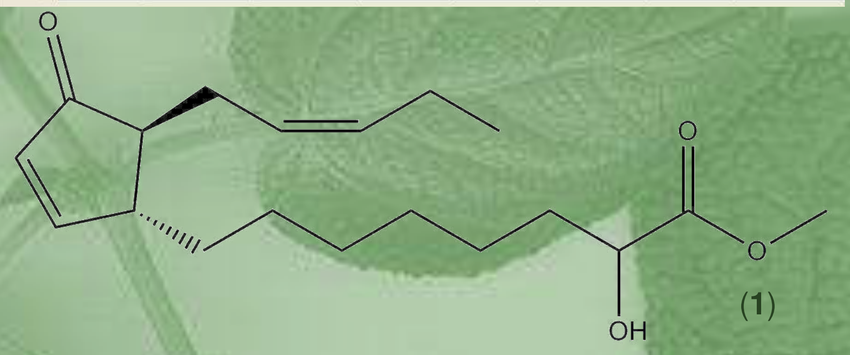
The hexane insoluble fraction (25.93 g) was chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 70:30 as an eluent; analytical TLC control), to give in order of elution five fraction groups: fraction group I (7.38 g) with waxy compounds, fraction group II (3.02 g), fraction group III (6.48 g), fraction group IV (6.58 g) and fraction group V (1.47 g).The fraction group I was chromatographed on silica gel column (55 x 3.5 cm; analytical TLC control) eluting with mixtures of n-hexane-$CHCl_3$ (35:65, 2.4 L; 10:90, 5.0 L; 0:100 2.2 L) and mixtures of $CHCl_3$-CH_3OH (95:5, 10.2L; 90:10, 0.9L; 0:100, 1.6L). Elution with n-hexane-$CHCl_3$ 35:65 afforded compound **4** (from 2.1 to 2.25 L) purified by HPLC-MS and MS^2 experiments followed by preparative RP HPLC (solvent A: H_2O with 0.1 % formic acid, solvent B: CH_3OH with 0.05 % formic acid, linear gradient: time 0 min: 40% B, time 25 min 100% B), R_T 22 min, 4.4 mg.

The compounds **1** [7], **2**, **3** and **4** had been identified by means of ^1H and ^{13}C-NMR spectra, including TOCSY, COSY, HSQC, HMBC, ROESY experiments, and ESI-TRAP-MS and HR-MS analysis.



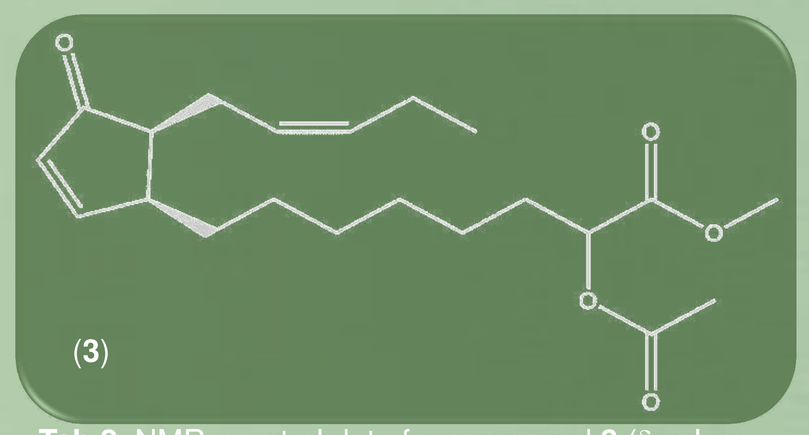
Tab 1. NMR spectral data for compound **2** (δ values, $CDCl_3$, ^{13}C-NMR at 150 MHz, ^1H-NMR at 600 MHz).

C	^{13}C	^1H (J in Hz)	HMBC correlations of the C
1	175.9	-	1.60 - 1.78 - 3.79 - 4.19
2	70.5	4.19 dd (7.55, 4.14)	1.60 - 1.78
3	34.5	1.60 m 1.78 m	4.19
4	24.9	1.39 m 1.44 m	1.32 - 1.78 - 4.19
5	29.3	1.32 m 1.34 m	1.25 - 1.30 - 1.60 - 1.78
6	29.7	1.25 m 1.30 m	1.34 - 1.37 - 1.39 - 1.44
7	27.5	1.32 m 1.37 m	1.14 - 1.34 - 1.73
8	30.9	1.14 m 1.73 m	1.25 - 1.30 - 1.32
9	44.4	2.98 m	1.15 - 2.45 – 2.52 - 6.18 - 7.74
10	167.2	7.74	6.18
11	132.6	6.18 d (6.0)	7.74
12	211.0	-	2.45 - 2.52 - 6.18 - 7.74
13	50.0	2.45 m	2.13 - 2.52 - 6.18 - 7.74
14	24.0	2.13 m 2.52 m	2.45
15	127.1	5.36 m	2.05 - 2.07 - 2.13 - 2.45 – 2.52
16	133.5	5.44 m	0.96 - 2.05 - 2.07 - 2.45
17	20.7	2.05 m 2.07 m	0.96 - 5.36 - 5.44
18	14.0	0.96 m	2.05 - 2.06 - 5.44
19	52.7	3.79 s	-



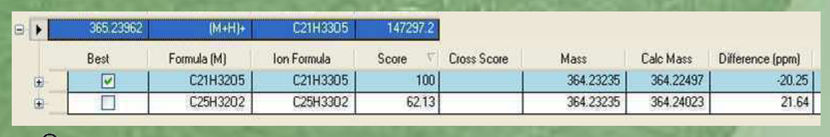
The spectroscopic data obtained for compounds **2** and **3** relative to the pentenil and cyclopentenil moieties and in particular those concerning the chiral centers in the ring juncture C 9 and C13, were largely consistent with those published in the literature for 12-oxo-cis-10,15-phytodienoic acid (13-epi-12-oxo-PDA) [8, 9, 10] and for its methyl ester [11, 12]; similarly the data obtained for 1 and 4 were consistent with those published for 12-oxo trans-10,15-phytodienoic acid and for its methyl-ester.

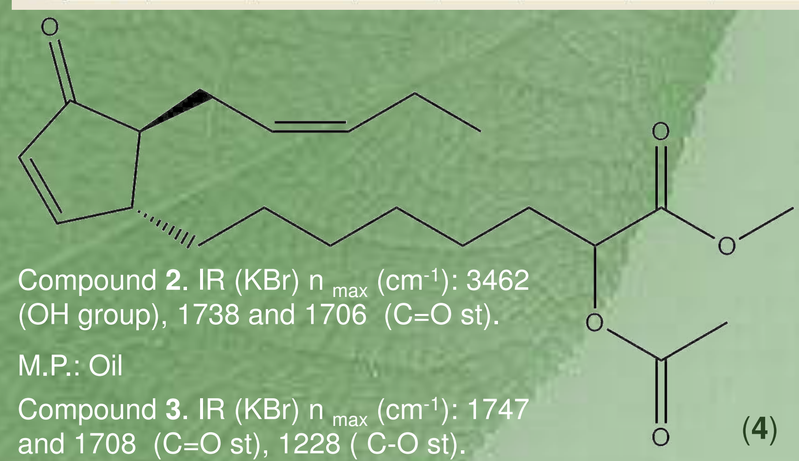
Plant Material. Fresh aerial parts of *S. adenophora* Fernald were obtained from Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).



Tab 2. NMR spectral data for compound **3** (δ values, $CDCl_3$, ^{13}C-NMR at 150 MHz, ^1H-NMR at 600 MHz).

C	^{13}C	^1H (J in Hz)	HMBC correlations of the C
1	170.8	-	1.81 - 1.82 - 3.74 - 4.99
2	72.2	4.99 t (6.4)	1.38 - 1.81 -1.82
3	31.0	1.81 m 1.82 m	1.32 -1.33 -1.38 - 1.39 - 4.99
4	25.0	1.38 m 1.39 m	1.32- 1.81 - 1.82 - 4.99
5	29.0	1.32 m 1.33 m	1.25 - 1.36 -1.38 - 1.39 - 1.40 - 1.81 - 1.82
6	29.5	1.25 m 1.32 m	1.15 - 1.25 - 1.32 - 1.33 - 1.36 - 1.38 - 1.39 - 1.40 - 1.72
7	27.5	1.36 m 1.40 m	1.15 - 1.25 - 1.32 - 1.33 - 1.72
8	30.7	1.15 m 1.72 m	1.25 - 1.32 - 1.36 - 1.40 - 2.44
9	44.2	2.97 m	1.15 - 1.72 - 2.44 - 2.50 - 6.18 - 7.73
10	166.2	7.73 m	1.15 - 1.72 - 2.44 - 6.18
11	132.5	6.18 nd (5.9)	7.73
12	210.7	-	2.44 - 2.16 - 2.50 - 6.18 - 7.73
13	49.8	2.44 m	2.16 - 2.50 - 5.36 - 6.18 - 7.73
14	23.7	2.16 m 2.50 m	2.44 - 5.36 - 5.44
15	126.7	5.36 m	2.04 - 2.12 - 2.16 - 2.44 - 2.50
16	133.0	5.44 m	0.96 - 2.04 - 2.12 - 2.50
17	20.8	2.04 m 2.12 m	0.96 - 5.36 - 5.44
18	14.0	0.96 m	2.04 - 2.12 - 5.44
19	52.2	3.74 s	-
20	170.6	-	2.14
21	20.6	2.14 s	-





Compound **2.** IR (KBr) n_{max} (cm^{-1}): 3462 (OH group), 1738 and 1706 (C=O st).

M.P.: Oil

Compound **3.** IR (KBr) n_{max} (cm^{-1}): 1747 and 1708 (C=O st), 1228 (C-O st).

M.P.: Oil

[**1**].Dayan, F.E., Duke, S.O. *Pesticide Outlook*, **2003**, 8, 175-178; [**2**].Epling, C. In Fedde F., University of California Press: Berkley, California, **1940**, *Vol.110*; [**3**].Bisio, A., Fraternale, D., Giacomini, M., Giacomelli, E., Pivetti, S., Russo, E., Caviglioli, G., Romussi, G., Ricci, D., De Tommasi, N. *Crop Protection*, **2010**, 29, 1434-1446; [**4**].Jahn, U., Galano, J.M., Durand, T., **2008**, *Angewandte Chemie International Edition* 47, 5894-5955; [**5**] Göbel, C., Feussner, I., **2009**, *Phytochemistry* 70, 1485-1503; [**6**] Mueller, M.J., Berger, S., **2009**, *Phytochemistry* 70, 1511-1521; [**7**] Bisio A., Damonte G., Giacomelli E., Mele G., Pozzo E., Cafaggi S., De Tommasi N., Romussi G. **2011**. A New Derivative of 12-oxo-Phytodienoic Acid from *Salvia adenophora* Fernald. XX Congresso Italo-Latino Americano de Etnomedicina "Prof. Dr. Francisco Josè de Abreu Matos. Fortaleza - Cearà, Brasil, 19-22 Setembro 2011.; [**8**] Ainai, T., Matsuumi, M., Kobayashi, Y., **2003**, *The Journal of Organic Chemistry* 68, 7825-7832; [**9**] Baertschi, S.W., Ingram, C.D., Harris, T.M., Brash, A.R., **1988**, *Biochemistry* 27, 18-24; [**10**].Kobayashi, Y., Matsuumi, M., **2002**, *Tetrahedron Letters* 43, 4361-4364; [**11**] Bohlmann, F., Borthakur, N., King, R.M., Robinson, H., **1982**, *Phytochemistry* 21, 125-127; [**12**] Chu, X.-J., Hong, D., Liu, Z.-Y., **1995**, *Tetrahedron* 51, 173-180.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.9

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

A NEW DITERPENOID FROM *SALVIA ADENOPHORA* FERNALD (LAMIACEAE)

Angela Bisio[1], Gianluca Damonte[2], Emanuela Giacomelli[1], Giacomo Mele[1], Aldo Profumo[3], Giovanni Romussi[1], Nunziatina De Tommasi[4]
[1]Department of Pharmacy, University of Genoa, Via Cembrano 4, 16146, Genoa, Italy,
[2]Center of Excellence for Biomedical Research, Viale Benedetto XV 7, 16132, Genoa, Italy,
[3]S.C. Integrated Molecular Pathology- IRCCS AOU San Martino – IST- 16132 Genoa,
[4]Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Via Ponte Don Melillo, 84084 Salerno, Italy.

The secretion product of epidermal structures is involved in plant-plant and plant-environment interactions [1-2], and its phytotoxic chemicals are released into the environment by foliar leaching, volatilization or residue decomposition [3]. In course of a research devoted to the identification of natural pesticides in the surface exudates [4] of aromatic plants, we considered *Salvia adenophora* Fernald [5], a Mexican species whose aerial part exudate showed herbicide activity against *Papaver rhoeas* L. and *Avena sativa* L. in a preliminary test (Total Germination-GT of 0.33±0.6 against *Papaver* in Petri dish experiments at 5 mg/L and 3.67±1.2 in pot experiments at 40 mg/L; germination inhibition against *Avena* 0.33±0.6 in Petri dish experiments at 5 mg/L and 3.00±1.0 in pot experiments at 40 mg/L; LC50 against *Papaver* 1.87±0.25 mg/L in Petri dish experiments and 3.52±0.73 mg/L in pot experiments; LC50 against *Avena* 5.3±1.5 in Petri dish experiments and 3.99±0.31 mg/L in pot experiments) [4].

Plant Material. Fresh aerial parts of *S. adenophora* Fernald were obtained from Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

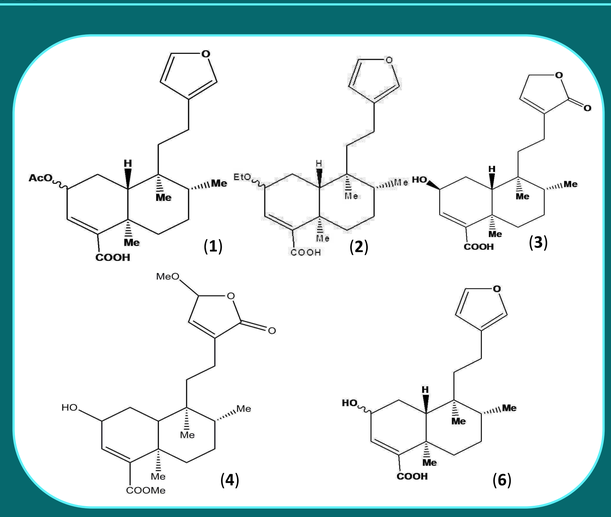
Extraction and isolation. Fresh aerial parts (8.8 kg) of *Salvia adenophora* Fernald were immersed in CH_2Cl_2 for 20s. After filtration, the extraction solvent was removed under reduced pressure giving 60.9 g of exudate material, composed by leaf surface constituents. 36.0 g of exudate were treated with n-hexane giving the hexane-soluble fraction and the hexane-insoluble fraction.

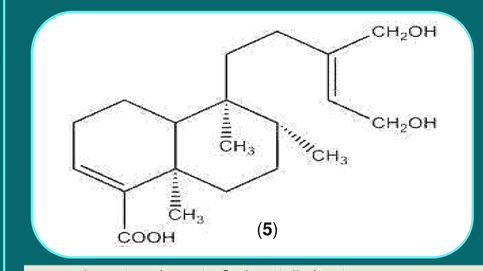
The hexane insoluble fraction (25.93 g) was chromatographed on Sephadex LH-20 (1 g portions; 53 x 2.5 cm; $CHCl_3/CH_3OH$ 70:30 as an eluent; analytical TLC control), to give in order of elution five fraction groups: fraction group I (7.38 g) with waxy compounds, fraction group II (3.02 g), fraction group III (6.48 g), fraction group IV (6.58 g) and fraction group V (1.47 g).

The fraction group IV was chromatographed on silica gel column (55 x 3.5 cm; analytical TLC control) eluiting with mixtures of n-hexane-$CHCl_3$ (25:75, 1.7 L; 20:80, 9.2 L; 10:90, 2.25 L; 0:100 8.0 L) and mixtures of $CHCl_3$-CH_3OH (95:5, 10.2L; 90:10, 0.9L; 0:100, 1.6L). Elution with $CHCl_3$ afforded compounds **1** and **2** (from 4.7 to 8.0 L) purified by HPLC-MS and MS^2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 80:20, RT 8 min, 5.4 mg and RT 11 min, 5.8 mg respectively. Elution with $CHCl_3$-CH_3OH 90:10 afforded **3** (from 0 to 0.4 L) purified by HPLC-MS and MS2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 60:40, RT 22 min, 24.8 mg.
Elution with $CHCl_3$-CH_3OH 95:5 afforded thirteen fraction groups.The eighth fraction group (from 2.8 to 7.4 L) was re-chromatographed on silica gel column (35 x 1 cm; analytical TLC control) eluting with mixtures of n-hexane-$CHCl_3$ (35:65, 3.0 L; 10:90, 0.6 L; 0:100, 0.3 L) and mixtures of $CHCl_3$-CH_3OH (60:40, 0.4 L). Elution with n-hexane-$CHCl_3$ 35:65 afforded compounds **4**, and **5** (from 1.4 to 2.0 L) purified by HPLC-MS and MS2 experiments followed by semi-preparative RP HPLC, CH_3OH/H_2O 70:30, RT 13.5 min, 13.2 mg and CH_3OH/H_2O 50:50, RT 43.5 min, 5.1 mg and RT 68 min, 4.4 mg respectively.

The fraction group V was purified by semi-preparative RP HPLC (CH_3OH/H_2O 70:30) directly, obtaining compound **6** (RT 19 min, 34.1 mg).

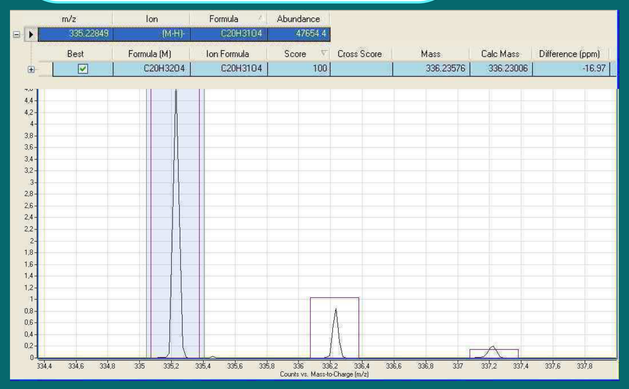
The compounds **1, 4** and **6** were identified as 2-acetoxyhardwickiic acid , 15-methoxy- 16-oxo-15,16H-hardwickiic acid and 2-hydroxyhardwickiic acid, respectively, on the basis of their ^1H- and ^{13}C-NMR data, that were largely consistent with those published in the literature [6-7-8]. However, in these papers, the relative stereochemistry of these compound as α-acetoxy and β-hydroxy, respectively, is described by statements that in our opinion are not sufficient for defining these stereo-structures. On the other hand, we didn't obtain sufficient ROESY data to confirm the relative stereochemistry of the C-2 for both the compounds.
The compounds **2**, **3** [9] and **5** had been identified by means of their UV, IR, ^1H and ^{13}C-NMR spectra, including TOCSY, COSY, HSQC, HMBC, ROESY experiments, and ESI-TRAP-MS and HR-MS analysis.





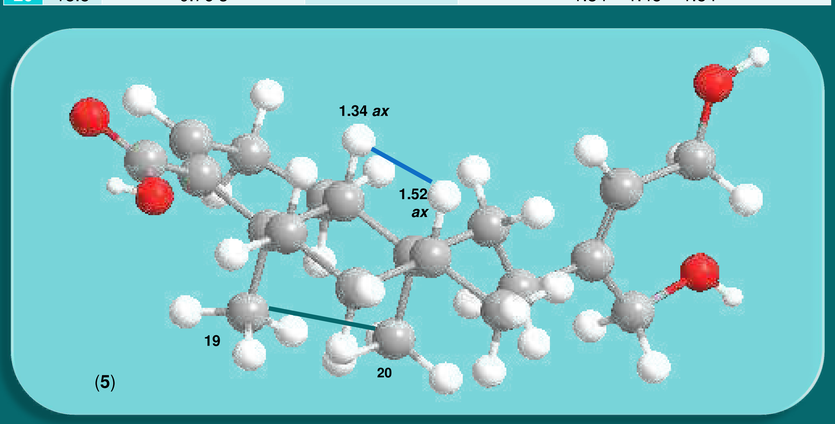
Compound **5**. IR (KBr) n_{max} (cm^{-1}): 3406 (OH group), 1708 (CO carbossilic acid).

M.P.: 112-114°C

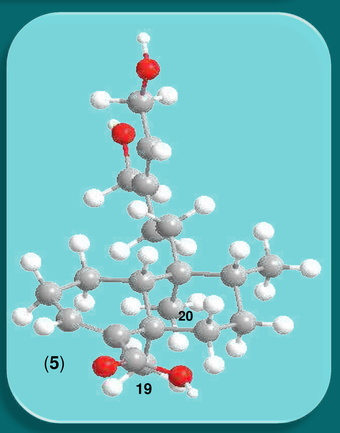


Tab 1. NMR spectral data for compound **5** (δ values, $CDCl_3$, ^{13}C-NMR at 150 MHz, ^1H-NMR at 600 MHz).

C	^{13}C	^1H (J in Hz)	COSY correlations of the H	HMBC correlations of the C
1	17.6	1.47 m 1.67 m	1.34 - 2.22 - 2.32 1.34 - 2.22 - 2.32	1.34 - 2.32
2	27.4	2.22 m 2.32 m	1.47 - 1.67 - 6.81 1.47 - 1.67 - 6.81	1.34 – 1.47 -1.67 – 6.81
3	140.1	6.81 ns	2.22 - 2.32	1.47 - 1.67 - 2.22 – 2.32
4	141.3	-	-	1.15 – 1.25 – 1.34 – 2.32
5	38.9	-	-	1.25 – 1.34 – 1.43 – 1.44 - 1.67
6	35.9	1.15 td (13.5, 7.0, 7.0) 2.40 m	1.43 - 1.44 1.43 - 1.44	1.25 –1.33 - 1.43 – 1.52
7	27.6	1.43 m 1.44 m	1.52 1.15 -1.52 - 2.40	0.82 – 1.52 – 2.40
8	36.4	1.52 m	0.82 - 1.43 - 1.44	0.76 – 0.82 - 1.34 – 1.43 – 1.54 – 2.40
9	39.0	-	-	0.76 – 0.82 – 1.34 – 1.43 – 1.44 – 1.47 - 1.52 –1.54 – 1.67
10	46.8	1.34 m	1.47 - 1.67	0.76 – 1.25 – 1.45 – 1.47 – 1.67 – 2.32
11	37.3	1.45 m 1.54 m	1.89 - 2.02 1.89 - 2.02	0.76 – 1.34 – 1.52 – 1.89 – 2.02
12	29.2	1.89 td (13.6, 13.6, 4.18) 2.02 m	1.45 - 1.54 1.45 - 1.54	1.45 – 1.54 – 4.18 – 5.61
13	144.8	-	-	1.45 – 1.54 – 1.89 – 2.02 – 4.21 – 4.18
14	126.3	5.61 t (7.0)	4.21	1.89 – 2.02 – 4.18 – 4.21
15	58.8	4.21 d (7.0)	5.61	5.61
16	61.3	4.18 s	-	1.89 – 2.02 – 5.61
17	16.1	0.82 d (6.5)	1.52	-
18	170.2	-	-	6.81
19	20.7	1.25 s	-	1.15 – 1.34 – 2.40
20	18.5	0.76 s	-	1.34 – 1.45 – 1.54



ROESY correlations 19-CH_3 /20-CH_3 and 1-H *axial* (δ_H = 1.47) /20-CH_3 showed that these groups are on the same side.
ROESY correlations 10-H/6-H at δ_H = 2.40 and 10-H/8-H *axial* (δ_H = 1.52) showed that these protons are on the same opposite side and that the 10-H proton at δ_H = 1.34 is in the *axial* position.



Tab 3. *Papaver rhoeas* L and *Avena sativa* L. germination indices after ten days exposure to *Salvia adenophora* isolated compounds (**2-3**), in Petri dish experiments compared to Pendimethalin (**P**) and Control (**C**), at various concentrations. Significant differences among the means were evaluated by one-way ANOVA, using the Tukey's honest significant difference test. Percent germination data were arcsin-square root transformed for analysis to meet the requirements of the test. Retransformed data are presented in the results. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Index	Compound	*Papaver rhoeas* L.				*Avena sativa* L.			
		Compound concentration (mg/L)				Compound concentration (mg/L)			
		10	20	40	60	20	40	60	80
Total germination (GT)	2	80.7 b	81.3 b	67.4 b	42.7 b	81.7 b	79.7 b	61.3 b	50.7 b
	3	81.3 b	79.7 b	82.0 c	80.7 c	81.3 b	80.7 b	82.3 c	81.7 c
	P	15.0 a	0.0 a	0.0 a	0.0 a	68.7 a	12.6 a	0.0 a	0.0 a
	C	83.0 b	83.0 b	83.0 c	83.0 c	82.7 b	82.7 b	82.7 c	82.7 c
Speed of Germination (S)	2	12.8 b	12.9 b	9.3 b	6.2 b	24.3 b	23.1 b	15.5 b	10.8 b
	3	12.9 b	12.6 b	13.0 c	12.7 c	23.3 b	22.6 b	22.9 c	22.4 c
	P	1.7 a	0.0 a	0.0 a	0.0 a	12.8 a	2.4 a	0.0 a	0.0 a
	C	12.9 b	12.9 b	12.9 c	12.9 c	24.3 b	24.3 b	24.3 c	24.3 d
Speed of Accumulated Germination (AS)	2	44.8 b	45.2 b	28.3 b	19.7 b	92.5 b	86.7 b	58.8 b	39.9 b
	3	45.3 b	44.4 b	45.9 c	44.5 c	89.0 b	85.5 b	86.6 c	84.4 c
	P	3.6 a	0.0 a	0.0 a	0.0 a	46.7 a	9.5 a	0.0 a	0.0 a
	C	44.7 b	44.7 b	44.7 c	44.7 c	92.6 b	92.6 b	92.6 c	92.6 d
Coefficient of the rate of Germination (CRG)	2	12.5 b	12.5 b	11.9 b	12.1 b	14.7 b	14.7 b	14.2 b	13.6 b
	3	12.5 b	12.5 b	12.5 b	12.5 b	14.6 b	14.5 b	14.5 b	14.5 b
	P	10.8 a	0.0 a	0.0 a	0.0 a	13.2 a	13.2 a	0.0 a	0.0 a
	C	12.5 b	12.5 b	12.5 b	12.5 b	14.6 b	14.6 b	14.6 b	14.6 d

Species	Exudate concentration (mg/L)					LC_{50} (mg/L)
	0	0.5	1	2	5	
Papaver rhoeas L.	100 ±2	50 ±2	30 ±3	13 ±1	0.4 ±0.7	0.47 ±0.06
Avena sativa L.	100 ±6	84 ±1	50 ±1	16 ±3	0.4 ± 0.7	1.1 ±0.4

Tab 2. Effect of *Salvia adenophora* exudate on the Final Germination of *Papaver rhoeas* L. and *Avena sativa* L. The seeds were surface sterilized in 2% sodium hypochlorite under vacuum for 20 min, rinsed 3 times in sterile distilled water, and dipped in distilled sterile water for 24 h for imbibition prior to the germination trial. The seeds were then equidistantly placed in 9 cm and 15 cm diameter Petri dishes for *Papaver* and *Avena* respectively (100 seeds per Petri dish, 3 replicates per treatment) lined with three layers and moistened on Whatman no. 1 sterilized filter paper. The filter paper was treated with 0 (control); 10; 20; 40; 60 µg/mL of exudate dissolved in DMSO plus sterile distilled water. After the addition of tested solution Petri dishes were sealed with parafilm and placed in a growth chamber at 25± 2°C with a 16h/8h light/dark photoperiod under light intensity of 65 µmol m^{-2} s^{-1}. After ten days germinated seeds (radicle emergence ≥1 mm) were counted. Data of Final germination are means (±S.D.) of three replicates related to an untreated control. LC_{50} were calculated by linear interpolation between the two adjacent values of Final Germination comprehending the 50% response of germination (normalized data). LC_{50} is expressed as 95% confidence interval.

Compound	*Papaver rhoeas* L.				*Avena sativa* L.			
	LC_{50} (mg/L)		LC_{90} (mg/L)		LC_{50} (mg/L)		LC_{90} (mg/L)	
2	ND		ND		ND		ND	
3	ND		ND		ND		ND	
P	6.1 ± 0.9		23 ± 8		30 ± 3		50 ± 7	

Tab 4. LC_{50} and LC_{90} for *Papaver* and *Avena* germination inhibition after ten days exposure to *Salvia adenophora* isolated compounds (**2-3**), compared to Pendimethalin (**P**), at various concentrations in Petri dish experiments, calculated by linear interpolation between two adjacent values of Final Germination [y] (from the data of Table 3 normalized to 100% with respect to control). LC_{50} and LC_{90} are expressed as 95% confidence intervals. (ND= not determinable, since out of the range of concentrations used).

References: [1] R.G. Kelsey, G.W. Reynolds, E. Rodriguez, **1984**, In: *Biology and chemistry of plant trichomes,* Rodriguez E., Healey P.L., Metha J., eds. New York: Plenum Press, 187-241; [2] E. Werker, **2000,** In : *Advances in Botanical Research*, Vol. 31. San Diego: Academic Press, 77-111; [3] C.H. Chou, **2006**, In: *Allelopathy – A Physiological Process with Ecological Implications*, Reigosa M.J., et al. Eds., Springer: Dordrecht, The Nederlands, 1-10; [4] Bisio A., Fraternale D., Giacomini M., Giacomelli E., Pivetti S., Russo E., Caviglioli G., Romussi G., Ricci D., De Tommasi N. 2010. Phytotoxicity of *Salvia* spp. exudates. *Crop Protection* 29: 1434-1446; [5] C. Epling, **1940,** In: *Repertorium Specierum Novarum Regni Vegetabilis*, Vol.110. Fedde F., University of California Press: Berkley, California; [6] P. Singh, V. Krishna, R.B. Pareek, **1998**, *Journal of the Chemical Society*, **75**(10-12): 552-558; [7] Vivek Krishna, Pahup Singh*1999. A clerodane derivative from *Grangea maderaspatana, Phytochemistry 52 (1999) 1341-1343*; [8] M.J. Simirgiotis, L.S. Favier, P.C. Rossomando, O.S. Giordano, C.E. Tonn, J.I. Padron, J.T. Vazquez, **2000**, *Phytochemistry,* **55**(7): 721-726; [9] Bisio A., Damonte G., Fraternale D., Giacomelli E., Salis A., Russo E., Cafaggi S., Ricci D., Romussi G., De Tommasi N. 2010. New clerodane diterpenoids from *Salvia adenophora* Fernald (Lamiaceae). Abstracts of the 58th International Congress and Annual Meeting of The Society for Medicinal Plant and Natural Product Research. 29th August - 2nd September 20, 2010, Berlin, Germany, **76**: 1274.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.10

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Culletività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

PHYTOTOXIC ACTIVITY AND A NEW CLERODANE DITERPENOID FROM THE EXUDATE OF THE AERIAL PARTS OF *SALVIA BUCHANANII* HEDGE

A. BISIO[1*], E. GIACOMELLI[1], G. MELE[1], G. DAMONTE[2], D. FRATENALE[3], G. ROMUSSI[1], D. RICCI[3], N. DE TOMMASI[4]

[1] Dipartimento di Farmacia, Università di Genova, Viale Cembrano 4, 16147 Genova, bisio@dictfa.unige.it;
[2] Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, Viale Benedetto XV 7, 16132 Genova;
[3] Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura, Università di Urbino, Via Bramante 28, 61029 Urbino;
[4] Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Salerno.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

107° Congresso SBI Onlus
Benevento 18/22 settembre 2012

In the course of our study on *Salvia* species with potential interest for phytotoxic activity we considered *Salvia buchananii* [1] whose exudate showed activity against *Papaver rhoeas* L. and *Avena sativa* L. in a preliminary test [2] (Fig. 1).

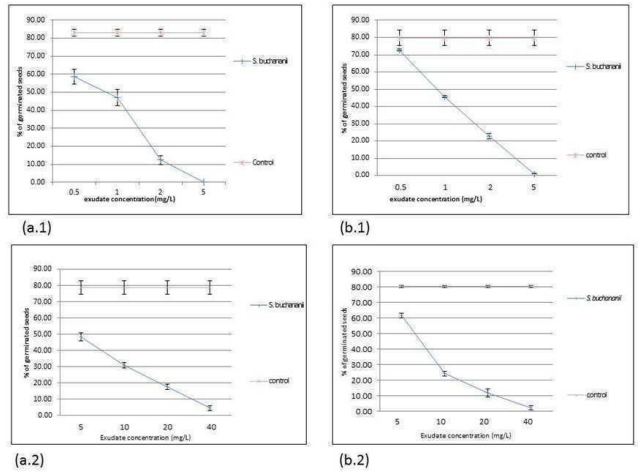


Fig 1. *Papaver rhoeas* L. (a) and *Avena sativa* L. (b) Total germination in Petri dish (1) and pot (2) experiments at various exudate concentrations.

Plant Material. Fresh aerial parts of *Salvia buchananii* Hedge were obtained from the Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and the Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). The species was identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K). Commercial seeds of *Papaver rhoeas* L. (La Semeria-www.Lasemeria.it- Italy) and *Avena sativa* L. (Il Monastero-Sementi-Italy), chosen as model species of dicotyledonous and monocotyledonous plants [3], were used.

Table 1. Effect of the various fractions obtained from the first chromatographic separation of *Salvia buchananii* exudate on the Final germination (maximum average percentage of seeds that germinated during the experiment) and early growth of *Papaver rhoeas* L. (a) and *Avena sativa* L. (b) at various concentrations. Data are expressed as mean (± S.D.) of three triplicates.

Fractions	2µg/mL	5 µg/mL	10 µg/mL	20 µg/mL
2	11.6±1.2	3.2±0.3	0.0±0.0	0.0±0.0
3	80.4±8.5	80.4±8.5	80.4±8.5	80.4±8.5
4	14.3±1.4	5.8±0.6	0.0±0.0	0.0±0.0
5	80.4±8.5	80.4±8.5	80.4±8.5	80.4±8.5

(a)

Fractions	2µg/mL	5 µg/mL	10 µg/mL	20 µg/mL
2	20.2±2.2	10.3±0.9	0.0±0.0	0.0±0.0
3	75.0±7.8	75.0±7.8	75.0±7.8	75.0±7.8
4	24.6±2.7	8.8±0.9	0.0±0.0	0.0±0.0
5	75.0±7.8	75.0±7.8	75.0±7.8	75.0±7.8

(b)

Extraction and Isolation. For the isolation of leaf surface constituents, fresh aerial parts (5.5 Kg) were immersed in CH_2Cl_2 for 20 s. After filtration, the extraction solvent was removed under reduced pressure. The exudate (16.6 g) was chromatographed on a Sephadex LH-20, to give five fraction groups in order of elution: fraction group I (from 0 to 170 ml; 0.6 g) with waxy compounds, fraction group II (from 170 to 190 ml; 1.6 g), fraction group III (from 190 to 210 ml; 7.6 g), fraction group IV (from 210 ml to 230 ml; 2.1 g); and fraction group V (from 230 to 390 ml; 1.1 g).
Fraction group II was fractionated by flash chromatography (elution with an increasing polarity gradient of n-hexane/$CHCl_3$ from 50:50 to 0:100, then $CHCl_3$/CH_3OH from 100:0 to 0:100) obtaining nine fractions. Other fraction groups, i.e. fraction groups III, IV and V, were fractionated following the same scheme, obtaining respectively six, six and seven fractions. The fractions that resulted similar in composition by TLC analytical control were then gathered together, obtaining five final fractions. Fraction I contained waxy compounds. Fractions II, III, IV and V, which showed the presence of ursolic and oleanolic acids by TLC control with these previously isolated compounds, were then treated with CH_3OH/H_2O 80:20, heating, in order to separate the triterpenoids that were then separated by filtration. Four fractions were so obtained, fraction I, II, III and IV.
Fraction I was then fractionated by flash chromatography (elution with an increasing polarity gradient of n-hexane/$CHCl_3$ from 35:65 to 0:100, then $CHCl_3$/CH_3OH from 100:0 to 0:100) obtaining fourteen fractions: fraction number 2 was fractionated by flash chromatography following the same procedure above, obtaining nineteen fractions. Fraction number 8 was then purified by RP-HPLC separation with a gradient of H_2O/CH_3OH 0:5 to 0:100 to afford pure compound **1** (12.3 mg). Compound **1** was identified by IR and NMR analysis, including TOCSY, COSY, HSQC and HMBC experiments, and HR-MS analysis.

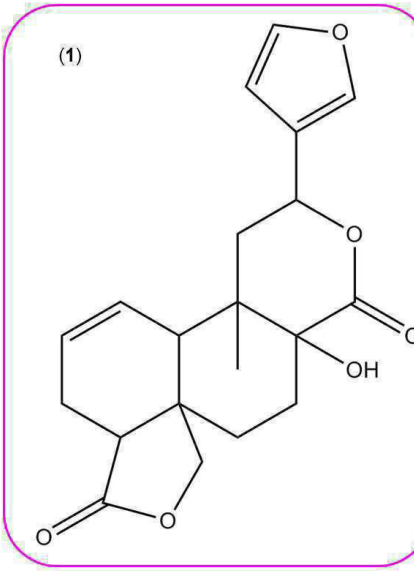
Table 2. NMR spectral data for compound **1** (δ values, CDCl3, 13C-NMR at 150 MHz, 1H-NMR at 600 MHz).

C	^{13}C	^{1}H (J in Hz)	HMBC correlations of the C
1	128.6	5.91 m	1.14, 2.41, 2.45, 5.79
2	128.0	5.79 m	5.91
3	21.2	2.25 m, 2.41 m	2.50, 5.79
4	46.2	2.50 m	2.41, 2.45, 4.04, 4,80
5	41.3	-	1.70, 1.82, 2.23, 2.25, 2.41, 2.45, 2.50, 4.80, 5.79
6	20.6	1.70 m	1.82, 2.23, 4.04, 4.80
7	29.6	1.82 m, 2.23 m	1.70
8	73.3	-	1.14, 1.70, 1.82, 2.09, 2.23, 2.36
9	40.5	-	1.14, 2.09, 2.36, 5.58
10	51.3	2.45 m	1.14, 1.70, 2.09, 2.36, 2.50, 4.04, 5.79, 5.91
11	40.1	2.36 m, 2.09 m	5.58
12	72.3	5.58 dd (9.6, 6.1)	6.49
13	125.6	-	2.09, 2.36, 5.58, 6.49, 7.43, 7.49
14	108.6	6.49 n s	5.58, 7.49
15	144.1	7.43 n s	6.49, 7.49
16	140.0	7.49 n s	5.58, 6.49, 7.43
17	175.1	-	1.82, 2.23, 5.58
18	175.9	-	2.25, 2.50, 4.80
19	78.7	4.04 d (8.4), 4.80 d (8.4)	1.70, 2.50
20	27.4	1.14 s	2.09, 2.36

IR absorption bands at 3414 (OH group), 1758, 1717 (CO groups), and 3139, 1509, 873 (β-substituted furan ring) cm^{-1} and 20 carbon resonances in the ^{13}C NMR could suggest an oxygenated clerodane diterpenoid structure.

By inspection of ESI-MS, HR ESI-MS (*m/z*: 358.14239 $[M+H]^+$), COSY, HSQC and HMBC spectra it was possible to assign all the protons and carbons as belonging to the planar structure **1** that is similar to that of Tehuanine D [4], thus differing for the absence of the 1(10),2-diene system of Tehuanine D (δ_H 6.11 H-1; 6.19, H-2; and 5.633, H-3): only one double bond was present in **1** (δ_H 5.91 H-1; 5.79, H-2; 2.25 and 2.41, H-3; 2.45, H-10), on the basis of HMBC correlations. Compound **1** is also similar to 8-hydroxysalviarin [5], thus differing for the position of the double bond in C-2.

(1)



References.
[1] Hedge I.C. , 1963. Tab.430. *Salvia buchananii- Labiatae*. In: *Curti's Botanical Magazine*. *Volume CLXXIV*. Taylor G. Ed., The Royal Horticultural Society, London; [2] Bisio A., Fraternale D., Giacomini M., Giacomelli E., Pivetti S., Russo E., Caviglioli G., Romussi G., Ricci D., De Tommasi N., 2010. Phytotoxicity of *Salvia* spp. exudates. *Crop Protection*, 29: 1434-1446; [3] Allaie R.R., Reshi Z., Rashid I., Wafai B.A., 2006. Effect of aqueous leaf leachate of *Anthemis cotula* e an alien invasive species on germination behaviour of some field crops. *Journal of Agronomy and Crop Science* 192, 186e191; [4] Bautista E., Maldonado E., Ortega A., 2012. neo-Clerodane Diterpenes from *Salvia herbacea*. *Journal of Natural Products*, 75, 951−958; [5] Nieto M., Gallardo O.V., Rossomando P.C., Tonn C.E., 1996. 8-Hydroxysalviarin and 7,8-Didehydrorhyacophiline, Two New Diterpenes from *Salvia reflexa*. *Journal of Natural Products*, 59, 880-882.

Acknowledgements. We thank the project PO Italia-Francia MARITTIMO: "Pyrgi" for the financial support.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.11

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

Chemical Constituents of roots of *Salvia* x *jamensis* J. Compton grown in aeroponic culture

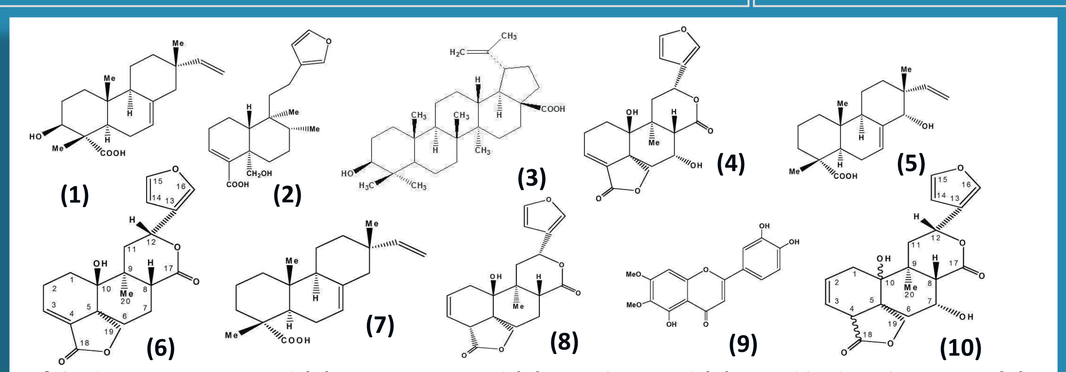
Bisio A.[1], *Damonte G.*[2], *Fraternale D.*[3], *Giacomelli E.*[1], *Mele G.*[1], *Romussi G.*[1], *Ricci D.*[3], *De Tommasi N.*[4]

1 Dipartimento di Farmacia, Università di Genova; 2 Centro di Eccellenza per la Ricerca Biomedica, Università di Genova; 3 Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura, Università di Urbino; 4 Dipartimento di Scienze Farmaceutiche, Università di Salerno

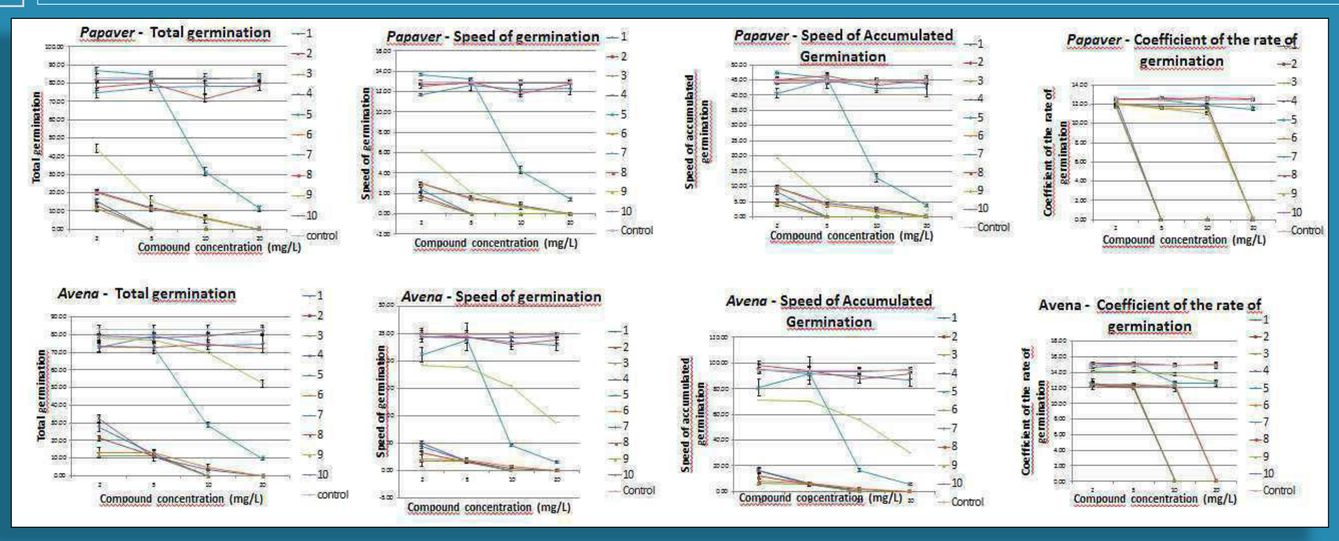
In the course of our search for allelochemicals from Salvia species we have previously described the phytotoxic activity of compounds extracted from *Salvia x jamensis* against Papaver rhoeas L. and Avena sativa L. [1, 2]. In the present work we have investigated the production of these bioactive compounds by the root system, because of its ability to exude a vast array of compounds, including diterpenes [3], into the rhizosphere with the potential to affect the inter-relationships between plants [4]. An aeroponic system has been chosen as this soilless culture allows to access roots with minimal disturbance, decreased plant water stress, enhanced plant growth rates, and optimal aeration of the root zone [5, 6].

Plant Material: Plotted plants of *Salvia x jamensis* J. Compton were obtained from Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

Dose–response studies: The seeds were surface sterilized in 2% sodium hypochlorite under vacuum for 20', rinsed 3 times in sterile distilled water, and dipped for 24 h in sterile distilled water for imbibition prior to the germination trial. The seeds were then equidistantly placed in 9 cm and 15 cm diameter Petri dishes for *Papaver* and *Avena* respectively (50 seeds per Petri dish, 3 replicates per treatment) lined with three layers of no. 1 Whatman sterilized filter paper. Standard solutions (2; 5; 10 and 20 µg/mL) of each compound were obtained by dissolving each weighed compound in DMSO and diluting with the appropriated quantity of sterile distilled water. Treatment with distilled water, with the same DMSO concentration, in a similar manner served as a control. The filter paper was treated with the solutions, and the Petri dishes were sealed with parafilm and placed in a growth chamber at 25±2°C under a 16h/8h light/dark photoperiod and light intensity of 65 µmol m^{-2} s^{-1}. Emergence of the radicle (≥1 mm) was used as an index of germination and it was recorded daily in each replicate. Germination counts were performed for a period of 10 days. Seed germinability was assessed by the total seed germination at the end of the test; germination progress was followed by the calculation of other indices, i.e. speed of germination, speed of accumulated germination and coefficient of the rate of germination [1].

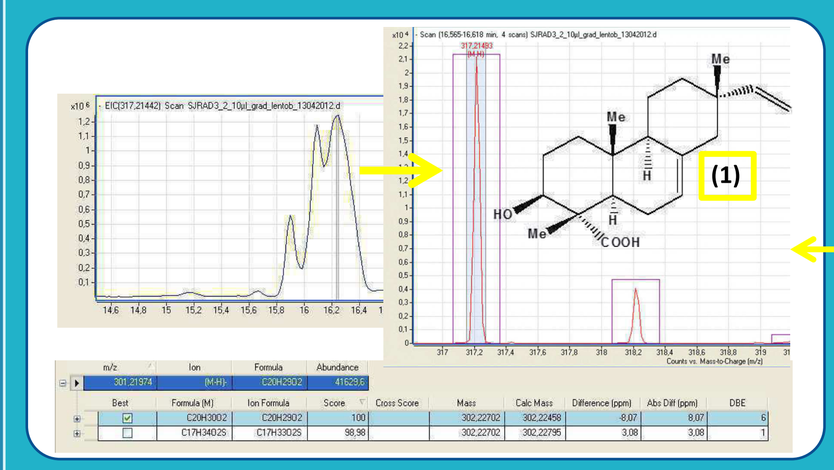


3-β-hydroxysopimaric acid **(1)**; Hautriwaic acid **(2)**; Betulinic acid **(3)**; 7,8-dihydrosalviacoccin **(4)**; Isopimaric acid **(5)**; 15,16-epoxy-cleroda-3,13(16),14-trien-10β-hydroxy-12,17;19,18-diolide **(6)**; 14-α-hydroxy-isopimaric acid **(7)**; 15,16-epoxy-cleroda-2,13(16),14-trien-7a,10β-dihydroxy-12,17;19,18-diolid **(8)**; cirsiliol **(9)**; 15,16-epoxy-cleroda-2,13(16),14-trien-7α,10-dihydroxy-12,17;19,18-diolide **(10)**.



Aeroponic culture: Rooting of cuttings with three nodes of *Salvia x jamensis* J. Compton, was induced in distilled water containing 5.0 µM IBA. Cuttings with three or more roots were transferred in the aeroponic culture experiment system in order to obtain the growth of the root apparatus. The aeroponic culture was performed at 25±2°C under 16 h photoperiod with fluorescent tubes at a light intensity of 60 µmol m^{-2} s^{-1}. The nutrient solution was supplied as nebulized solution for 15 min every 60 min only on the roots zone at a flow rate of 1dm^3/min and a pressure of 0.2 MPa. The nutrient solution (pH 5.8) was replaced biweekly and solution temperature was kept at 17-20°C. After 4 months of aeroponic culture, the roots were removed from plants, dried and extracted.

Extraction: The biomass obtained from aeroponic culture (8.65 g) was extracted with CH_3OH. After filtration the extraction solvent was removed under reduced pressure. The crude extract (0.79 g) was fractionated by RP-C18 flash chromatography (elution with a gradient of n-exane/$CHCl_3$ 1:0 to 0:1, then $CHCl_3$, then $CHCl_3$/CH_3OH from 1:0 to 0:1) (flow: 10 mL/min), obtaining six fractions. The fraction 2 was subsequently purified by RP SPE (Strata Si-1 Silica, 55um, 70A, 200mg/3mL SPE tubes, Phenomenex). Columns were preconditioned with methanol (2 × 3 mL) and eluted sequentially with CH_3OH/H_2O from 1:1 to 0:1, CH_2Cl_2/CH_3OH (1:1), and CH_2Cl_2, yielding 5 fractions. Fractions 2,3 and 4 were monitored by reverse phase high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC--MS). The HPLC-MS experiments were performed using an Agilent (Santa Clara, CA) 6210 time of flight instrument equipped whit an electrospray ion source (ESI-TOF); the HPLC was an Agilent 1200 system and eluents were A: water with 0.1% formic acid and B: methanol with 0.05% formic acid. Starting from an A:B composition of 60:40, the gradient reached 80% of B concentration in 8 min and 100% of B concentration in 35 min, at a flow rate of 0.25 ml/min. The column used was a Symmetry C18 (150 x 2.1 mm ID, 3.5 µm particle size). The HR-ESI-TOF analysis was performed in reflectron negative mode with a capillary voltage of 3000V (drying gas 5.0 l/min). The following voltages were applied: fragmentor, 250 V; skimmer, 60 V; OCT RF, 250 V. The exact mass calculations were performed using an utility integrated in the instrument software and the calculated elemental formulas were unambiguous for all the compounds.



References: [1] Bisio A.; Fraternale D.; Damonte G.; Millo E.; Lanteri A.P.; Russo E.; Romussi G.; Parodi B.; Ricci D.; De Tommasi N. Natural Product Communications, 2009, 4, 1621-1630. [2] Bisio A.; Fraternale D.; Giacomini M.; Giacomelli E.; Pivetti S.; Russo E.; Caviglioli G.; Romussi G.; Ricci D.; De Tommasi N. Crop Protection, 2010, 29, 1434-1446. [3] Toyomasu T.; Kagahara T.; Okada K.; Koga J.; Hasegawa M.; Mitsuhashi W.; Sassa T.; Yamane H. Bioscience, Biotechnology and Biochemistry, 2008, 72, 562–567. [4] Flores H.E.; Vivanco J.M.; Loyola-Varga V.M. Trends in plant science, 1999, 4, 220-226. [5] Zobel R.W; Del Tredici P.; Torrey J.G. Plant Physiology, 1976, 57, 344-346. [6] Martin-Laurent F.; Lee S.K.;•Tham F.G.; J. He J.; Diem H.G.; Durand P. Biology and Fertility of Soils, 1997, 25, 7–12. [7] Ohara S.; Ohira T. Journal of Wood Science, 2003, 49, 59-64. [8]. Gonzalez-Coloma A.; Lopez-Balboa C.; Santana O.; Reina M.; Fraga B.M. Phytochem Rev, 2011, 10, 245–26.

MARITTIMO - IT FR - MARITIME
TOSCANA - LIGURIA - SARDEGNA - CORSE

La Cooperazione al cuore del Mediterraneo

La Coopération au coeur de la Méditerranée

PRODOTTO 26b.12

REGIONE AUTÒNOMA DE SARDIGNA
REGIONE AUTONOMA DELLA SARDEGNA
REGIONE LIGURIA
REGIONE TOSCANA
Collectivité Territoriale de CORSE
Cullettività Territoriale di CORSICA

Programma cofinanziato con il Fondo Europeo per lo Sviluppo Regionale
Programme cofinancé par le Fonds Européen de Développement Régional

PROGETTO PYRGI: STRATEGIA D'IMPRESA IN SETTORI DI NICCHIA PER L'ECONOMIA AGRO-INDUSTRIALE DEL MEDITERRANEO

VALORIZZAZIONE DI SPECIE SPONTANEE PER LA PRODUZIONE DI COLORANTI VEGETALI PER PRODUZIONI INNOVATIVE DI NICCHIA E LO SVILUPPO LOCALE SOSTENIBILE

Silvia Tavarini[1], Barbara Pieve[1], Luisa Pistelli[2], Luciana Gabriella Angelini[1]

[1] *Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali, Università di Pisa*
angelini@unipi.it

[2] *Dipartimento di Farmacia, Università di Pisa* luipi@farm.unipi.it

INTRODUZIONE

Negli ultimi anni si sta assistendo ad una crescente rivalutazione dei prodotti naturali, sia nel loro utilizzo diretto, sia quale elemento di produzione di prodotti di trasformazione. Nel campo alimentare, farmaceutico, cosmetico, edilizio, architettonico, vi è la tendenza allo sfruttamento di risorse naturali, che comportino un minor impatto ambientale, una maggiore salvaguardia della salute degli esseri viventi e della terra, in generale. Anche nel campo tessile, l'utilizzo di materie naturali, ed in particolare di coloranti di origine vegetale comporta una serie di importanti vantaggi sia per il consumatore che per il settore produttivo, con nuove opportunità di mercato per le imprese, un minor impatto ambientale dei nuovi processi di tintura, con conseguente risparmio sulle misure anti-inquinamento, il recupero di zone eventualmente degradate o abbandonate. Alcune zone geografiche sono particolarmente ricche di specie vegetali spontanee, che possono e potrebbero essere utilizzate per il reperimento delle sostanze naturali da impiegare nei vari settori. In quest'ottica, di particolare interesse è senza dubbio l'area del Mediterraneo che, con il suo clima temperato, la sua configurazione morfologica, la sua posizione strategica, la sua storia, presenta una varietà di piante che possono essere protagoniste di questi nuovi orizzonti. Il progetto Pyrgi, nato dalla collaborazione tra Italia e Francia, e che interessa in particolare i territori della Liguria, Sardegna, della fascia costiera della Toscana, e della Corsica, ha tra le proprie finalità proprio quella di incrementare la valorizzazione dei prodotti ottenuti da specie vegetali attraverso l'attivazione di filiere per lo sfruttamento agro-industriale e commerciale delle loro caratteristiche di pregio e la commercializzazione finale, così da realizzare lo sfruttamento di specie vegetali comunemente coltivate nell'areale mediterraneo quali fonte di sostanze naturali potenzialmente utilizzabili a scopo farmaceutico o per la riduzione dell'impatto ambientale in sostituzione di tradizionali composti (agrofarmaci, farmaci) di natura chimica", nonché la "promozione dello sviluppo economico e dell'occupazione attraverso lo sfruttamento e la rivalutazione di prodotti ad oggi poco valorizzati".

SCOPO DELLA RICERCA

Lo scopo di tale ricerca è stato quello di valorizzare alcune specie spontanee del territorio dell'Isola d'Elba per la produzione di coloranti e di ausiliari di tintura (mordenti) al fine di ottenere produzioni innovative di nicchia, attraverso il recupero del patrimonio culturale, la conservazione e il mantenimento della biodiversità per la promozione di uno sviluppo locale sostenibile.

MATERIALI E METODI

Nel presente lavoro, sono state utilizzate piante da flavonoidi: *Solidago canadensis* L., *Solidago virgaurea* L., *Punica granatum* e piante da tannini *Punica granatum* L., *Cotinus coggygria* Scop., *Arbutus unedo* L., *Ficus carica* L. (**Tabella 1**). Dopo essiccamento in stufa, le parti utili delle piante da cui ricavare i principi coloranti sono state separate, finemente macinate e conservate in contenitori di plastica sigillati fino al momento delle analisi.

La preparazione degli estratti ha previsto un'estrazione per decozione della droga in acqua distillata con rapporto 1:100. Sono stati messi a confronto 2 procedimenti: macerazione in acqua della droga per 24h a T ambiente prima dell'estrazione (ammollo) e estrazione senza macerazione (non ammollo). Dopo filtrazione, l'estratto ottenuto è stato utilizzato per la tintura e per le successive analisi (contenuto in fenoli totali, flavonoidi totali, tannini totali e capacità antiossidante).

Tintura: le tinture sono state condotte utilizzando un rapporto di 1:50 tra filato e bagno di tintura La lana è stata precedentemente mordenzata con allume e cremor tartaro in accordo a Tavarini et al. (2011)
Fenoli totali: metodo colorimetrico del Folin-Ciocalteu
Flavonoidi totali: metodo spettrofotometrico proposto da Barros et al. (2010)
Tannini totali: in accordo con il metodo proposto da Rasineni et al. (2008)
Attività anti-radicalica: saggio del DPPH (2,2-difenil-1-picrilidrazile), in accordo con quanto proposto da Tadhani et al. (2007)

Tabella 1: Piante tintorie spontanee del territorio dell'Isola d'Elba, selezionate per le loro proprietà tintorie.

Famiglia e nome scientifico	Nome comune	Parte utilizzata	Colore	Principio attivo
Anacardiaceae Cotinus coggygria Scop.	Scotano, sommacco selvatico	Foglie e giovani rami	Giallo brillante	Flavonoidi: fustina, quercetina e canferolo. Tannini condensati.
Asteraceae Solidago canadensis L.	Verga d'oro del Canada	Infiorescenze	Giallo brillante-	Flavonoidi: quercetina, canferolo,rutina, astragalina.
Asteraceae Solidago virgaurea L.	Verga d'oro	Infiorescenze	Giallo-	Flavonoidi: quercetina,canferolo,rutina Antociani:cianidolo
Ericaceae Arbutus unedo L.	Corbezzolo	Foglie	Giallo scuro-grigio marrone	Flavonoidi: arbutoflavonoli. Tannini condensati.
Moraceae Ficus carica L.	Fico	Foglie	Giallo pallido-verde chiaro	Tannini.
Punicaceae Punica granatum L.	Melograno	Pericarpo del frutto	Giallo pastello-marrone	Flavogallolo. Ellagitannini: punicalina, punicalagina.

Tabella 2: Contenuto in fenoli totali

Specie selezionate	Fenoli totali (mg GAE/gPS)		
	Ammollo	Non ammollo	Media specie
Solidago canadensis L.	110,96 ± 10,08 d	91,54 ± 4,81 de	**101,25** ± 13,73 C
Solidago virgaurea L.	70,70 ± 10,78 e	73,72 ± 8,30 e	**72,21** ± 2,14 D
Punica granatum L.	155,45 ± 10,77 c	178,26 ± 20,37 b	**166,86** ± 16,13 B
Cotinus coggygria Scop.	170,16 ± 16,98 bc	161,10 ± 26,96 bc	**165,63** ± 6,41 B
Arbutus unedo L.	268,00 ± 3,00 a	139,40 ± 16,60 c	**203,70** ± 90,93 A
Ficus carica L.	72,02 ± 4,98 e	76,55 ± 2,44 e	**74,29** ± 3,20 D
Media trattamento	**141,22** ± 74,55 A	**120,10** ± 45,39 B	

Tabella 3: Contenuto in flavonoidi totali

Specie selezionate	Flavonoidi totali (mg rutina/gPS)		
	Ammollo	Non ammollo	Media specie
Solidago canadensis L.	73,88 ± 3,85 c	135,43 ± 4,32 a	**104,66** ± 43,52 A
Solidago virgaurea L.	63,98 ± 7,90 d	108,86 ± 2,49 b	**86,42** ± 31,73 B
Punica granatum L.	12,82 ± 3,37 e	9,77 ± 2,93 e	**11,30** ± 2,16 C
Media trattamento	**50,23** ± 32,77 B	**84,69** ± 66,23 A	

Tabella 4: Contenuto in tannini totali

Specie selezionate	Tannini totali (mg acido tannico/gPS)		
	Ammollo	Non ammollo	Media specie
Punica granatum L.	128,80 ± 3,75 a	121,62 ± 0,24 a	**125,21** ± 5,08 B
Cotinus coggygria Scop.	140,93 ± 5,43 a	174,91 ± 7,89 a	**157,92** ± 24,03 A
Arbutus unedo L.	160,80 ± 34,71 a	173,00 ± 40,80 a	**166,90** ± 8,63 A
Ficus carica L.	39,39 ± 1,36 a	28,93 ± 0,59 a	**34,18** ± 7,37 C
Media trattamento	**117,48** ± 53,71 A	**124,62** ± 68,38 A	

I risultati relativi al contenuto dei principali composti responsabili del colore (fenoli, flavonoidi e tannini) e all'attività anti-radicalica delle differenti specie sono riportati nelle **Tabella 2, 3, 4 e 5**. Valori medi (± deviazione standard) seguiti da lettere uguali in ciascuna colonna non sono significativamente differenti per P = 0,05 in base al test della Differenza Minima Significativa (DMS). Differenze statisticamente significative sono state osservate, tra le diverse specie, per ciascun parametro analizzato, mostrando un ampio range di variabilità per i diversi costituenti. *Arbutus unedo* ha mostrato un elevato contenuto in fenoli totali e tannini, congiuntamente ad un'elevata attività anti-radicalica. Tra le specie da flavonoidi particolarmente interessante si è dimostrata *Solidago canadensis*.

Tabella 5: Attività anti-radicalica

Specie selezionate	Attività Anti-radicalica (% Inibizione)		
	Ammollo	Non ammollo	Media specie
Solidago canadensis L.	81,77 ± 0,31 b	81,44 ± 0,33 b	**81,61** ± 0,23 B
Solidago virgaurea L.	80,70 ± 0,62 b	82,90 ± 0,41 b	**81,80** ± 1,56 B
Punica granatum L.	90,88 ± 0,16 a	92,09 ± 0,31 a	**91,45** ± 0,86 A
Cotinus coggygria Scop.	90,27 ± 0,12 a	91,18 ± 1,40 a	**90,73** ± 0,64 A
Arbutus unedo L.	90,41 ± 0,06 a	89,99 ± 0,27 a	**90,20** ± 0,30 A
Ficus carica L.	62,46 ± 8,01 c	33,51 ± 1,40 d	**47,99** ± 20,47 C
Media trattamento	**82,75** ± 10,94 A	**78,50** ± 22,50 B	

Il presente studio suggerisce come l'azione antiossidante che caratterizza le differenti specie vegetali sia dovuta ad un'azione sinergica e/o competitiva tra i differenti composti bioattivi in esse presenti. Tuttavia, si rendono necessari ulteriori studi al fine di identificare la natura dei composti predominanti, soprattutto in relazione ai meccanismi d'azione (riduzione e *radical-scavenging*) e alle possibili sinergie. Per quanto riguarda i test di tintura su lana, le prove realizzate hanno dimostrato come le specie spontanee selezionate abbiano permesso di ottenere buoni risultati in termini di proprietà tintorie fornendo tinture molto intense e brillanti.






CONCLUSIONI

I risultati ottenuti hanno evidenziato come alcune delle specie spontanee del territorio dell'Isola d'Elba, possiedano interessanti caratteristiche tintorie, e a una buona attività antiossidante. Tra tutte, ***Arbutus unedo***, si è dimostrata particolarmente interessante per l'alto contenuto di metaboliti secondari di interesse e per l'elevata attività anti-radicalica. Le interessanti proprietà tintorie e fitochimiche messe in evidenza per la prima volta in alcune di queste specie, potrebbero consentire l'applicazione degli estratti vegetali, nel settore tessile, cosmetico ed, in alcuni casi, fitoterapico.

Riferimenti Bibliografici

- Barros N., Heleno S.A., Carvalho A.M., Ferreira I.C.F.R., 2010. *Lamiaceae often used in Portuguese folk mnedicine as a source of powerful antioxidants: vitamins and phenolics*. LWT – Food Science and Technology 43: 544-550.
- Rasineni G.K., Siddavattam D., Reddy A.R., 2008 *Free radical quenching activity and polyphenols in three species of Coleus*. Journal of Medicinal Plant Research 2: 285-291.
- Tavarini S., Zublena S., Angelini L. 2011. *Relationship between coloring power and antioxidant properties of different weld (Reseda luteola L.) extracts*. Proceedings ISEND 2011 Europe. 25-30 April 2011, La Rochelle, France