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Salvinorin A exerts opposite presynaptic controls on neurotransmitter exocytosis from mouse brain nerve terminals

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ABSTRACT

We investigated the effects of salvinorin A on the basal and the 12 mM K^+-evoked release of preloaded [^{3}H]noradenaline ([^{3}H]NA) and [^{3}H]serotonin ([^{3}H]5-HT) from mouse hippocampal nerve terminals (synaptosomes), as well as on the basal and 12 mM K^+-evoked release of preloaded [^{3}H]dopamine ([^{3}H]DA) from mouse striatal and prefrontal cortex (PFc) synaptosomes. 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]5-HT and [^{3}H]DA. At the same concentration, salvinorin A facilitated the 12 mM K^+-evoked, Ca^{2+}-dependent, exocytotic-like release of [^{3}H]NA. These effects could not be observed in pertussis toxin (PTx) entrapped synaptosomes. The broad spectrum κ-opioid receptor (KOR) antagonist norbinaltorphimine (norBNI, 1–100 nM) antagonized the inhibition of [^{3}H]5-HT and [^{3}H]DA exocytosis as well as the facilitation of [^{3}H]NA overflow induced by 100 nM salvinorin A. The KOR agonist U69593 (1–100 nM) mimicked salvinorin A in inhibiting [^{3}H]5-HT and of [^{3}H]DA exocytosis, its effect being prevented by norBNI, but leaving unchanged the K^+-evoked release of [^{3}H]NA. The effects of Salvinorin A on neurotransmitter exocytosis were not prevented by the selective μ opioid (MOR) receptor antagonist CTAP (10–100 nM), whereas facilitation of [^{3}H]NA exocytosis, but not inhibition of [^{3}H]5-HT and [^{3}H]DA K^+-evoked release, was counteracted by the δ opioid receptor (DOR) antagonist naltrindole (1–100 nM). We conclude that salvinorin A presynaptically modulates central NA, 5-HT, and DA exocytosis evoked by a mild depolarizing stimulus by acting at presynaptic opioid receptors having different pharmacological profiles.

1. Introduction

Salvia divinorum, a plant of the Lamiaceae used in traditional and ethno-pharmacological practices to produce mystical experiences, and its main active compound, the neoclerodane diterpene Salvinorin A, have recently become popular drugs of abuse (Sheffler and Roth, 2003). In vivo Salvinorin A induces an intense and short-lived hallucinogenic experience distinct from that caused by other hallucinogens that usually associates to sedation and anti-nociception (Valdes, 1994; Wang et al., 2005). Since, in ligand-binding studies, Salvinorin A acts as a selective *k*-opioid receptor (KOR) full agonist (Roth et al., 2002; Sheffler and Roth, 2003; Prisinzano and Rothman, 2008), its central effects were proposed to depend on activation of these receptors. This is in part due to the fact that the natural compound has negligible affinity for any of a large number of receptors, ion channels and transporters, including 5-HT_2 receptors, which represent the primary targets for classic hallucinogens (Roth et al., 2002).

KORs are widely distributed in the central nervous system (CNS). Similarly to what already described for other opioid receptor subtypes (namely MORs and DORs), KORs couple to Pertussis toxin (PTx)-sensitive G-proteins, whose activation preferentially transduces inhibitory signaling linked to inhibition of adenylyl cyclase (AC) activity or to activation of neuronal inwardly rectifying K^+-channels (Connor and Christie, 1999 and references cited therein). Interestingly, however, excitatory events, such as activation of phospholipase C and mobilization of intracellular calcium, were also reported to be triggered by this receptor subtype (Connor and Christie, 1999). KORs also exist at the presynaptic level, where they modulate the release of several neurotransmitters including

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noradrenaline (NA), dopamine (DA), glutamate, γ-aminobutyric acid and serotonin (5-HT; Jackisch et al., 1986; Mulder et al., 1987; Schoffelmeer et al., 1997; Shippenberg and Rea, 1997; Rawls et al., 1999; Hjelmstad and Fields, 2003; Berger et al., 2006; Carlezon et al., 2006).

The aim of the present study was to investigate whether Salvinorin A can presynaptically control the release of central amines from nerve terminals isolated from different brain areas and if the modulatory effects observed involve KORs. Thus, we studied the effects of Salvinorin A on the basal and the K^+-evoked release of preloaded [^{3}H]noradrenaline ([^{3}H]NA), [^{3}H]5-HT, and [^{3}H]DA from synaptosomes isolated from mouse hippocampus, striatum, and prefrontal cortex (PFc). Our results are consistent with the idea that Salvinorin A exerts opposite effects on neurotransmitter release. Indeed, we were able to demonstrate that the natural compound inhibits the K^+-evoked release of 5-HT and DA, while it facilitates the K^+-induced [^{3}H]NA exocytosis. The different sensitivity to opioid receptor antagonists suggests that Salvinorin A-induced effects could rely on activation of native presynaptic norBNI-sensitive opioid receptor subtypes having different pharmacological profile that are located on axonal terminals of distinct neuronal types.

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 (7.00 a.m.–7.00 p.m.). Food and water were freely available. The animals were killed by decapitation and the hippocampus, the striatum, and the frontal cortex 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 vol of 0.32 M sucrose, buffered at pH 7.4 with phosphate (final concentration 0.01 M, Raiteri and Raiteri, 2000). The homogenate was first centrifuged at 1000 g for 5 min to remove nuclei and cellular debris, while synaptosomes were isolated by centrifugation at 13 000 g for 20 min. In a set of experiments, the tissue was homogenized in buffered sucrose containing 5 nM PTx, in order to entrap these agents into subsequently isolated synaptosomes (Raiteri et al., 2000). 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, see Raiteri and Raiteri, 2000) is considered an approach of choice to investigate the existence and the functional role of presynaptic receptors. 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 labeling 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 (Raiteri and Raiteri, 2000).

Hippocampal synaptosomes were labeled with [^{3}H]NA (final concentration 30 nM), or with [^{3}H]5-HT (final concentration 60 nM) in the presence of 0.1 μM 6-nitroquipazine, a selective 5-HT uptake inhibitor, or desipramine, a selective NA uptake inhibitor, respectively, to avoid false labeling of serotonergic or noradrenergic terminals. Striatal and PFc synaptosomes were labeled with [^{3}H]DA (final concentrations 50 and 100 nM, respectively) in the presence of 0.1 μM 6-nitroquipazine and 0.1 μM desipramine (to avoid false labeling of serotonergic and noradrenergic terminals). Pargyline (final concentration 0.1 μM) was added to minimize enzymatic amine metabolism. Incubation was performed at 37 °C, for 15 min in a rotary water bath. After the labeling 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) maintained at 37 °C and synaptosomes were then superfused at 0.5 ml/min with standard a physiological solution as above.

When studying the effect of Salvinorin A on the basal release of amines, synaptosomes were first equilibrated during 36 min of superfusion and then six consecutive 1-min fractions were collected. Salvinorin A was introduced at the end of the first fraction collected ($t = 39$ min) and maintained until the end of the superfusion period.

When studying the effect of Salvinorin A on the release of neurotransmitters 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). In a set of experiments carried out to evaluate the Ca^{2+}-dependency of the 12 mM K^+-induced release of tritiated neurotransmitters, the superfusion medium was replaced, starting at $t = 20$ min, with a medium containing 0.1 mM Ca^{2+} and 500 μM ethylene glycol-bis(2-aminomethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). 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). In these experiments, synaptosomes were exposed to Salvinorin A or U69593 concomitantly with the depolarizing stimulus. Antagonists were added 8 min before agonists and maintained throughout the superfusion.

Fractions collected and superfused synaptosomes were counted for radioactivity. The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). The release of neurotransmitter in absence of depolarizing stimulus (basal release) was expressed as percent basal release and was calculated as the sum of the percentage of tritium content in the six 1-min fractions collected. The K^+-induced tritium overflow was expressed as percent induced overflow, and was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release) from that in the 6 min-fraction collected during and after the depolarization pulse (evoked release).

2.3. Statistical analysis

Analysis of variance was performed by one-way analysis of variance ANOVA followed by Dunnett's test or Newman Keuls multiple-comparisons test, as appropriate (software Graph Pad Prism version 4.03). Direct comparisons were performed with Student's *t*-test. 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); 5[1,2-^{3}H(N)]-hydroxytryptamine creatinine sulfate ([^{3}H]5-HT, specific activity 38 Ci/mmol) from NEN, DuPont products (Boston, MA). Pertussis toxin (PTx) were obtained from Calbiochem (La Jolla, CA), 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*-naphto[2,1-c]pyran-7-carboxylic acid methyl ester, purity >99% was purchased from Ascent Scientific (Weston Super-Mare, UK). (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]*dec*-8-yl]-benzeneacetamide (U69593), CTAP, naltrindole–HCl and norbinaltorphimine (norBNI) were purchased from Sigma Chemical Co (St. Louis, MO). Salvinorin A was solubilized in DMSO (final concentration 10 mM), subsequently diluted with standard physiological medium to 100 μM.

3. Results

3.1. Effects of Salvinorin A on the spontaneous and the 12 mM K^+-evoked release of [^{3}H]NA, [^{3}H]5-HT and [^{3}H]DA from synaptosomes

First, we investigated the effects of Salvinorin A on the basal release of NA and 5-HT from mouse hippocampal nerve terminals as well as on the basal release of DA from striatal and PFc synaptosomes. Mouse hippocampal terminals preloaded with [^{3}H]NA or with [^{3}H]5-HT and striatal or PFc synaptosomes labeled with [^{3}H]DA were exposed in superfusion to Salvinorin A: the natural compound added at 100 nM failed to affect, on its own, the basal release of these neurotransmitters (Table 1).

The effects of Salvinorin A on the release of tritiated neurotransmitters caused by a mild depolarizing stimulus, (i.e., 12 mM KCl) were then evaluated. When synaptosomes isolated from the different mouse brain areas were transiently exposed in superfusion to 12 mM K^+, a significant increase of neurotransmitter release was observed. Omission of external Ca^{2+} caused a drastic reduction in the K^+-evoked release of [^{3}H]NA and [^{3}H]5-HT from hippocampal synaptosomes as well as in preloaded [^{3}H]DA from striatal and PFc synaptosomes (Table 2). This is consistent with the

Table 1
Effects of Salvinorin A on the basal release of neurotransmitters from superfused synaptosomes.

Neurotransmitter brain area	Control	100 nM Salvinorin A
[^{3}H]NA Hippocampus	3.88 ± 0.21	4.02 ± 0.37$^{n.s.}$
[^{3}H]5-HT Hippocampus	5.14 ± 0.42	4.99 ± 0.51$^{n.s.}$
[^{3}H]DA C. striatum	4.78 ± 0.06	4.51 ± 0.12$^{n.s.}$
[^{3}H]DA PFc	4.59 ± 0.03	4.44 ± 0.09$^{n.s.}$

Synaptosomes isolated from the different brain areas were exposed to Salvinorin A starting at $t = 39$ min till the end of the superfusion. Results are expressed as % basal release; data are as mean ± SEM of three experiments run in triplicate (three superfusion chambers for each experimental condition). Statistical analysis was performed by Student's *t*-test. *n.s.*, not significant.

idea that the K^+-evoked release mainly occurred in a Ca^{2+}-dependent, exocytotic-like manner.

Salvinorin A (0.1–1000 nM) facilitated the 12 mM K^+-induced release of [^{3}H]NA from mouse hippocampal synaptosomes (Fig. 1A, $F_{5,25} = 5.89$, $p < 0.05$ and $p < 0.01$, one-way ANOVA and Dunnett's test), causing the maximum potentiation (~42%, expressed as percent of control) when added at 100 nM. On the contrary, Salvinorin A inhibited the K^+-evoked release of [^{3}H]5-HT from mouse hippocampal synaptosomes (Fig. 1B, $F_{5,20} = 4.74$, $p < 0.05$ and $p < 0.01$, one-way ANOVA and Dunnett's test) as well as that of [^{3}H]DA from striatal (Fig. 1C, $F_{4,15} = 4.13$, $p < 0.05$, one-way ANOVA and Dunnett's test) and PFc (Fig. 1D, $F_{5,17} = 5.37$, $p < 0.05$, one-way ANOVA and Dunnett's test) mouse synaptosomes. 5-HT and striatal DA K^+-induced overflows were maximally inhibited (~34 and ~26, respectively, expressed as percent of control) by 100 nM Salvinorin A, while lower concentration of Salvinorin A (10 nM) was sufficient to cause the most notable reduction (~25, expressed as percent of control) of PFc DA exocytosis.

3.2. Effects of Salvinorin A on the K^+-induced exocytosis of neurotransmitters are mediated by a Pertussis toxin-sensitive event

KORs are known to couple to PTx-sensitive GPCR (Connor and Christie, 1999). To evaluate whether these receptors could play a role in the presynaptic modulation exerted by Salvinorin A on neurotransmitter exocytosis, we evaluated if intoxication of synaptosomes with PTx can modify the events so far described. To avoid excessively long incubations with PTx, synaptosomes were enriched with the toxin by using an experimental approach (i.e. entrapping technique, Raiteri et al., 2000), previously shown to successfully entrap compounds into subsequently isolated synaptosomes.

Entrapped PTx halved the K^+-evoked [^{3}H]NA exocytosis elicited by 12 mM K^+ from hippocampal synaptosomes (Fig. 2A, $F_{3,10} = 23.77$, $p < 0.01$, one-way ANOVA and Newman Keuls test).

Table 2
Ca^{2+}-dependency of the 12 mM KCl-evoked release of neurotransmitters from superfused synaptosomes.

Neurotransmitter brain area	12 mM KCl 1.2 mM $CaCl_2$ (% induced overflow)	12 mM KCl 0.0 mM $CaCl_2$ (% induced overflow)
[^{3}H]NA Hippocampus	4.43 ± 0.39	0.23 ± 0.08**
[^{3}H]5-HT Hippocampus	3.58 ± 0.11	0.63 ± 0.21**
[^{3}H]DA C. Striatum	3.72 ± 0.27	0.39 ± 0.17**
[^{3}H]DA PFc	3.95 ± 0.41	0.91 ± 0.13*

Synaptosomes isolated from the different brain areas were exposed starting at $t = 20$ min till the end of the superfusion to a medium where Ca^{2+} were omitted and 500 μM EGTA was added. Results are expressed as neurotransmitter % induced overflow, data are mean ± SEM of four experiments run in triplicate. Statistical analysis was performed by Student's *t*-test. *$p < 0.01$; **$p < 0.001$ vs. respective control.

Facilitation of the K^+-evoked [^{3}H]NA release induced by 100 nM Salvinorin A could not be further observed (Fig. 2A).

A significant reduction of the release of [^{3}H]5-HT induced by high K^+(Fig. 2B, $F_{3,12} = 5.34$, $p < 0.05$, one-way ANOVA and Newman Keuls test) was also observed in PTx-enriched hippocampal synaptosomes. Once again, 100 nM Salvinorin A failed to affect the indolamine exocytosis from PTx-entrapped synaptosome (Fig. 2B).

Finally, entrapped PTx significantly reduced the 12 mM K^+-evoked release of [^{3}H]DA from striatal (Fig. 2C, $F_{3,12} = 12.15$, $p < 0.001$, one-way ANOVA and Newman Keuls test), but not that from PFc synaptosomes (Fig. 2D). In both cases, Salvinorin A (100 nM) did not modify the K^+-evoked [^{3}H]DA exocytosis from PTx-entrapped synaptosomal preparations.

The efficacy of PTx in inhibiting the K^+-evoked release of all the neurotransmitters, with the exception of DA exocytosis from PFc synaptosomes, could be suggestive of the existence of presynaptic PTx-sensitive G-protein-coupled receptors (GPCRs) constitutively activated on nerve terminals (Wang and Sihra, 2004). Further studies are needed to test this hypothesis.

3.3. Effects of opioid receptor antagonists on the presynaptic modulation by Salvinorin A of the K^+-induced exocytosis of neurotransmitters

The effect of norBNI, a selective KOR antagonist, of CTAP, a selective MOR antagonist and of naltrindole, a selective DOR antagonist on Salvinorin A-induced modifications to neurotransmitter exocytosis was then investigated. In opioid receptor binding assays, norBNI possesses over a 150-fold selectivity for KOR over MOR and DOR sites (Takemori and Portoghese, 1992), CTAP shows DOR activity at very high concentrations and no KOR affinity (Kramer et al., 1989), while naltrindole has high affinity for DOR but low activity at MORs and KORs (Alexander et al., 2008).

Fig. 3 (A–D) shows that NorBNI (100 nM) did not modify the 12 mM K^+-evoked release of [^{3}H]NA and of [^{3}H]5-HT from hippocampal nerve endings as well as that of [^{3}H]DA from PFc synaptosomes. When the antagonist was added at 100 nM, it inhibited the 12 mM K^+-evoked release of DA from striatal terminals (Fig. 3C, $F_{9,70} = 2.82$, $p < 0.05$, one-way ANOVA and Newman Keuls test). Lower concentrations of norBNI (1–10 nM) were ineffective (Fig. 3C).

Facilitation of [^{3}H]NA exocytosis as well as inhibition of the 12 mM K^+-evoked release of [^{3}H]5-HT and [^{3}H]DA caused by 100 nM Salvinorin A were concentration-dependently prevented by norBNI. An almost maximal reversal of the 100 mM Salvinorin A-induced inhibition of [^{3}H]5-HT and [^{3}H]DA release was observed when the antagonist was added at 1–10 nM (Fig. 3B, $F_{5,18} = 11.86$; Fig. 3C, $F_{9,70} = 2.82$; Fig. 3D, $F_{5,21} = 3.90$, $p < 0.05$, one-way ANOVA and Newman Keuls test). A higher concentration (100 nM) was needed to observe a complete reversal of Salvinorin A-induced facilitation of [^{3}H]NA exocytosis (Fig. 3A, $F_{5,19} = 7.50$, $p < 0.01$, one-way ANOVA and Newman Keuls test).

CTAP slightly affected, although not significantly, the K^+-evoked release of [^{3}H]NA and [^{3}H]5-HT from hippocampal synaptosomes as well as the K^+-induced overflow of [^{3}H]DA from striatal and PFc nerve terminals. Facilitation of [^{3}H]NA exocytosis or inhibition of K^+-evoked [^{3}H]5-HT and [^{3}H]DA release caused by 100 nM Salvinorin A was not prevented by CTAP. When added up to 100 nM, however, the MOR antagonist further increased the inhibitory effect exerted by 100 nM Salvinorin A on the K^+-evoked release of [^{3}H]DA from striatal synaptosomes (Table 3).

The release of tritiated neurotransmitters evoked by high K^+ was not modified by the presence of Naltrindole (Fig. 4A–D). The DOR antagonist also failed to prevent the inhibitory effects exerted by 100 nM Salvinorin A on the K^+-evoked release of [^{3}H]5-HT and

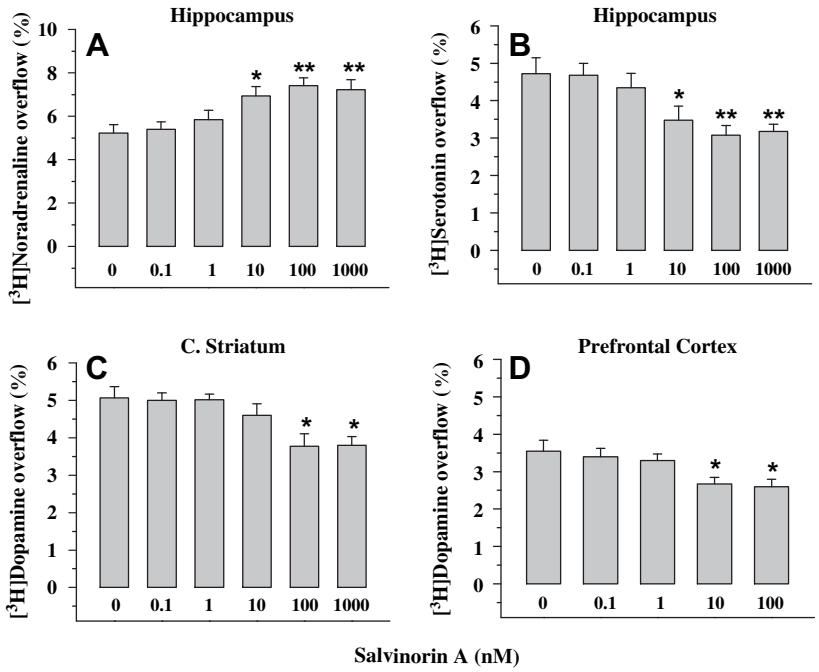

Fig. 1. Concentration-effect relationship of Salvinorin A on the 12 mM K^+-evoked release of neurotransmitters from superfused synaptosomes. Effects of Salvinorin A on the 12 mM K^+-evoked release of [^{3}H]NA (A) and [^{3}H]5-HT (B) from hippocampal synaptosomes and on the 12 mM K^+-evoked release of [^{3}H]DA from striatal (C) and prefrontal cortex (D) synaptosomes. Results are expressed as % induced overflow; data are mean ± SEM of at least three experiments run in triplicate (three superfusion chambers for each experimental condition). $^*p < 0.05$; $^{**}p < 0.01$ vs. respective K^+-evoked overflow.

[^{3}H]DA release (Fig. 4B–D). On the contrary, naltrindole was highly effective in preventing the Salvinorin A-induced facilitation of 12 mM K^+-evoked [^{3}H]NA release from mouse hippocampal synaptosomes (Fig. 4A, $F_{5,17} = 5.01$, $p < 0.05$, one-way ANOVA and Newman Keuls test).

3.4. Effects of U69593 on the K^+-induced exocytosis of neurotransmitters

The effects elicited by Salvinorin A on amine exocytosis were compared with those induced by U69593, a selective KOR agonist

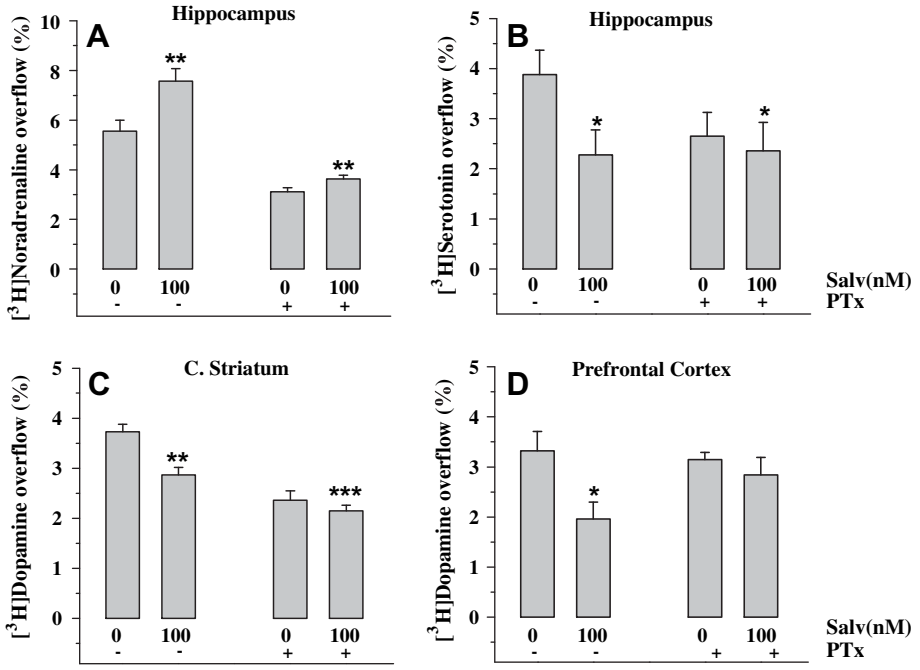

Fig. 2. Effects of pertussis toxin (PTx) on the Salvinorin A(Salv)-induced modification of neurotransmitter overflows evoked by K^+ depolarization from superfused synaptosomes. (A) release of [^{3}H]NA from hippocampal synaptosomes. (B) release of [^{3}H]5-HT from hippocampal synaptosomes. (C) release of [^{3}H]DA from striatal synaptosomes. (D) release of [^{3}H]DA from prefrontal cortex synaptosomes. Results are expressed as % induced overflow; data are mean ± SEM of at least three experiments run in triplicate. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. respective K^+-evoked overflow.

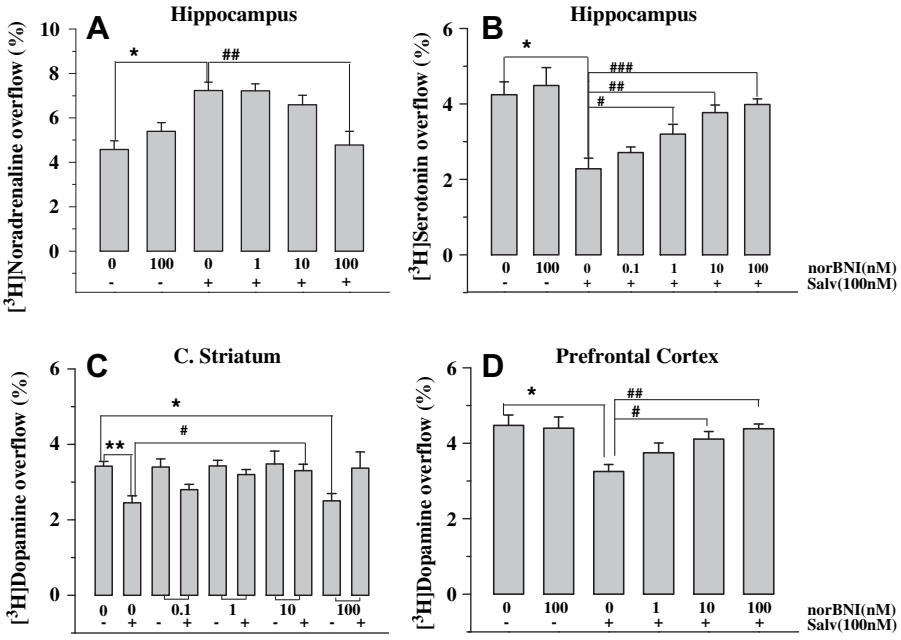

Fig. 3. Effects of norBNI on the Salvinorin A(Salv)-induced modification of neurotransmitter overflows evoked K^+ depolarization from superfused synaptosomes. (A) release of [^{3}H]NA from hippocampal synaptosomes. (B) release of [^{3}H]5-HT from hippocampal synaptosomes. (C) release of [^{3}H]DA from striatal synaptosomes. (D) release of [^{3}H]DA from PFc synaptosomes. Results are expressed as % induced overflow; data are mean ± SEM of at least three experiments run in triplicate. $^*p < 0.05$; $^{**}p < 0.01$ vs. respective K^+-evoked overflow. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ vs. respective (K^+/100 nM Salvinorin A)-evoked overflow.

(Alexander et al., 2008). Fig. 5A shows that U69593 (1–100 nM) did not modify the release of [^{3}H]NA caused by 12 mM K^+, but that it significantly inhibited the K^+-evoked release of [^{3}H]5-HT from hippocampal synaptosomes (Fig. 5B, $F_{3,12} = 9.88$, $p < 0.05$ and $p < 0.01$, one-way ANOVA and Dunnett's test). The inhibitory effect was totally prevented by co-application of the KOR antagonist norBNI (100 nM; Table 4, $F_{2,8} = 26.0$, $p < 0.001$, one-way ANOVA and Newman Keuls test).

U69593 (1–100 nM) also inhibited the K^+-evoked exocytosis of [^{3}H]DA from either striatal (Fig. 5C, $F_{3,16} = 4.75$, $p < 0.05$, one-way ANOVA and Dunnett's test) or PFc synaptosomes (Fig. 5D, $F_{3,16} = 3.77$, $p < 0.05$, one-way ANOVA and Dunnett's test). Once again, the KOR antagonist norBNI (100 nM) completely reverted the inhibitory effects elicited by the KOR agonist (Table 4, striatum: $F_{2,8} = 5.77$, $p < 0.05$; PFc: $F_{2,8} = 6.09$, $p < 0.05$, one-way ANOVA and Newman Keuls test).

Table 3
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)
[^{3}H]NA hippocampus	0	4.10 ± 0.52	6.34 ± 0.51*
	10	4.15 ± 0.0.61	6.83 ± 0.94#
	100	5.13 ± 0.21	7.10 ± 0.16§
[^{3}H]5-HT hippocampus	0	4.31 ± 0.11	2.72 ± 0.11**
	10	3.58 ± 0.22	2.70 ± 0.26#
	100	4.04 ± 0.20	2.90 ± 0.26 §§
[^{3}H]DA C. striatum	0	4.74 ± 0.10	3.68 ± 0.15**
	10	4.26 ± 0.39	3.06 ± 0.12##
	100	5.04 ± 0.34	2.48 ± 0.18§§§; †
[^{3}H]DA PFc	0	2.87 ± 0.21	2.08 ± 0.20*
	10	2.65 ± 0.11	1.65 ± 0.25#
	100	2.85 ± 0.25	1.61 ± 0.08§§

Synaptosomes were exposed in superfusion to high K^+ in presence or in absence of 100 nM Salvinorin A. CTAP (concentration as indicated) was added 8 min before agonist till the end of the superfusion period. Results are expressed as percent induced overflow (%); data are mean ± SEM of three to four experiments run in triplicate. Analysis of variance was performed by ANOVA followed by Newman Keuls multiple-comparisons test, $^*p < 0.05$; $^{**}p < 0.01$ vs. respective 12 mM K^+-evoked neurotransmitter release; #$p < 0.05$; ##$p < 0.01$ vs. respective (12 mM K^+/10 nM CTAP)-evoked neurotransmitter release; §$p < 0.05$; §§$p < 0.01$; §§§$p < 0.001$ vs. respective (12 mM K^+/100 nM CTAP)-evoked neurotransmitter release; †$p < 0.01$ vs (12 mM K^+/100 nM Salvinorin A)-evoked striatal [^{3}H]DA release.

4. Discussion

This study provides the functional evidence of the role of Salvinorin A as presynaptic modulator of neurotransmitter release from nerve terminals isolated from distinct mouse brain regions. The present results show that Salvinorin A controls the Ca^{2+}-dependent exocytosis of central biogenic amines (namely NA, 5-HT and DA) that was evoked by a mild depolarizing stimulus (12 mM K^+), leaving unmodified their basal release.

The main finding of this investigation is that 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.

Salvinorin A-induced central effects were proposed to preferentially involve the binding to PTx-sensitive KORs (Roth et al., 2002; Sheffler and Roth, 2003), although recently PTx-sensitive MOR also has been suggested as low affinity binding site (Rothman et al., 2007). This hypothesis relies on the observation that *in vivo* Salvinorin A-induced effects were i) prevented by the KOR antagonist norBNI (Zhang et al., 2005; Gehrke et al., 2008), ii) mimicked by specific KOR agonists, including U69593 (Butelman et al., 2004; Chavkin et al., 2004) and iii) abolished in KOR1 knockout mice (Ansonoff et al., 2006).

Here we show that 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.

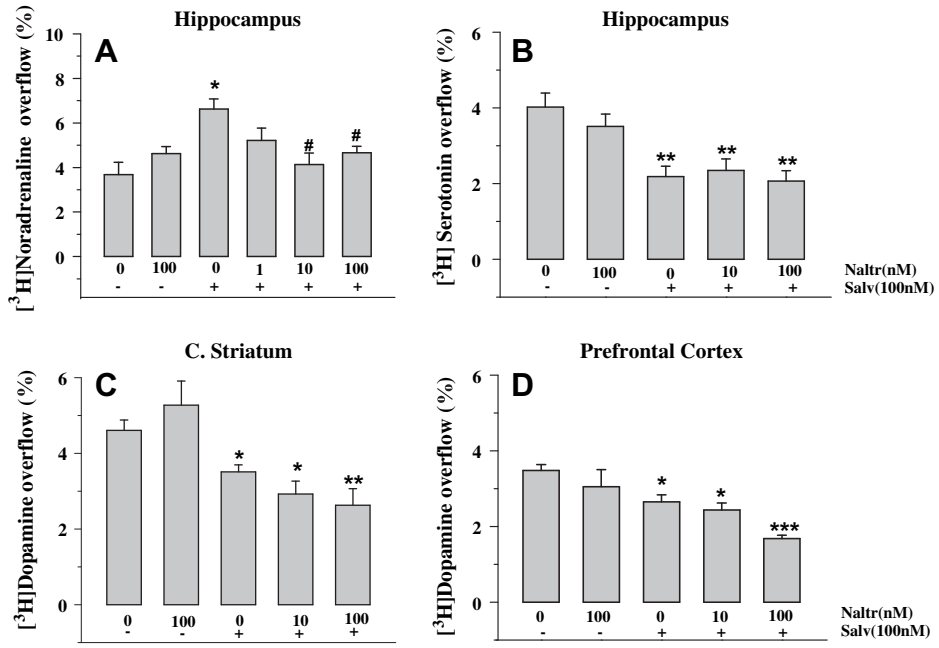

Fig. 4. Effects of naltrindole (Naltr) on the Salvinorin A(Salv)-induced modification of neurotransmitter overflows evoked by K^+ depolarization from superfused synaptosomes. (A) release of [^{3}H]NA from hippocampal synaptosomes. (B) release of [^{3}H]5-HT from hippocampal synaptosomes. (C) release of [^{3}H]DA from striatal synaptosomes. (D) release of [^{3}H]DA from PFc synaptosomes. Results are expressed as % induced overflow; data are mean ± SEM of at least three experiments run in triplicate. $^*p < 0.05$ vs. respective K^+-evoked overflow. $\#p < 0.05$ vs. respective (K^+/100 nM Salvinorin A)-evoked overflow.

Furthermore, experiments aimed to define the pharmacological profile of the GPCR involved showed that norBNI, but not CTAP, prevented (although with different potencies) the Salvinorin A-induced changes to neurotransmitter overflows. These observations support the view that KORs could play a major role in the Salvinorin A-induced control of neurotransmitter release, but also suggest that MORs probably were not involved. Opposite results were obtained when investigating the effects of the selective DOR antagonist naltrindole and of the KOR selective agonist U69593. Naltrindole failed to revert the inhibition of 5-HT and DA overflows, but was effective in preventing the Salvinorin A-evoked facilitation of NA release. On the contrary, U69593 mimicked Salvinorin A in inhibiting 5-HT and DA exocytosis, but failed to modify the K^+-evoked release of NA.

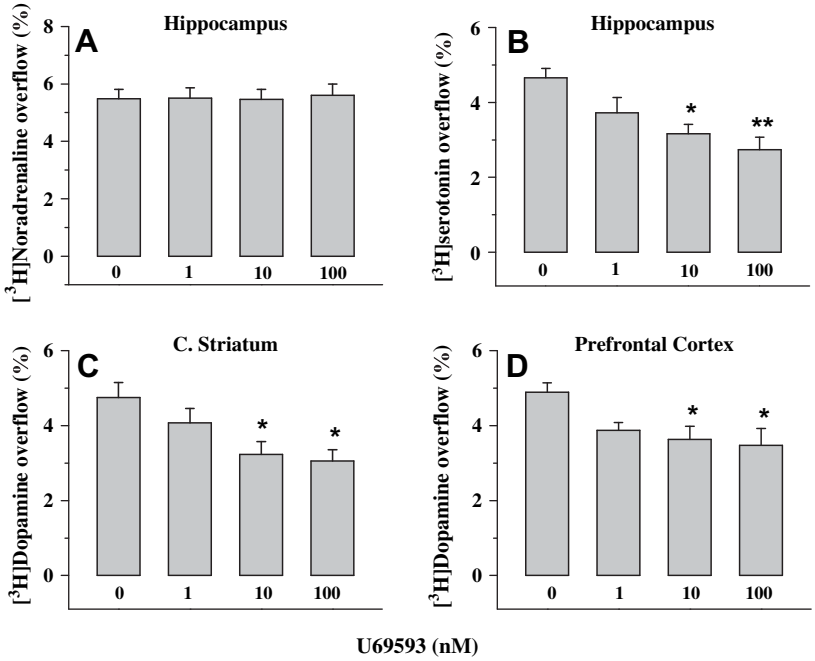

Fig. 5. Concentration-effect relationship of U69593 on the 12 mM K^+-evoked release of neurotransmitters from superfused synaptosomes. Effects of U69593 on the 12 mM K^+-evoked release of [^{3}H]NA (A) and [^{3}H]5-HT (B) from hippocampal synaptosomes and on the 12 mM K^+-evoked release of [^{3}H]DA from striatal (C) and PFc (D) synaptosomes. Results are expressed as % induced overflow; data are mean ± SEM of at least three experiments run in triplicate. $^*p < 0.05$; $^{**}p < 0.01$ vs. respective K^+-evoked overflow.

Table 4
U69593-induced inhibition of the 12 mM K^+-evoked neurotransmitter release from superfused synaptosomes: effects of nBNI.

Brain area	Neurotransmitter	12 mM KCl (% induced overflow)	12 mM KCl 100 nM U69593 (% induced overflow)	12 mM KCl 100 nM U69593 100 nM nBNI (% induced overflow)
Hippocampus	[^{3}H]5-HT	4.02 ± 0.18	$2.21 \pm 0.23^{***}$	3.99 ± 0.21††
C. Striatum	[^{3}H]DA	4.45 ± 0.14	$3.06 \pm 0.30^{*}$	4.17 ± 0.52†
PFc	[^{3}H]DA	4.69 ± 0.25	$3.44 \pm 0.33^{**}$	4.39 ± 0.19†

Synaptosomes were exposed to U69593 contemporarily to the depolarizing stimulus. nBNI was added 8 min before the agonist. Results are expressed as percent induced neurotransmitter overflows. Data are expressed as mean ± SEM of at least three experiments run in triplicate. Analysis of variance was performed by ANOVA followed by Newman Keuls multiple-comparisons test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. K^+-evoked neurotransmitter overflow; †$p < 0.05$; ††$p < 0.001$ vs. (K^+/U69593)-evoked neurotransmitter overflow.

All together, these observations suggested the involvement of different receptor subtypes, also questioning however a possible role for DORs in the positive modulation exerted by Salvinorin A on NA exocytosis.

It is well accepted that, in the CNS, KORs exist as different receptor subtypes having distinct location (Unterwald et al., 1991) and different functional roles (Schoffelmeer et al., 1997). Evidence has been provided showing presynaptic KOR subtypes are also located on distinct subpopulations of axon terminals, whose activation modifies the release of distinct neurotransmitters (Kim and Cox, 1993; Schoffelmeer et al., 1997). More specifically, based on ligand receptor binding studies, it was hypothesized the existence of at least three different KOR subtypes, namely KOR1, 2 and 3 subtypes. KORs typified by a high affinity and selectivity for the selective KOR agonist U69593 and for norBNI were proposed to belong to the KOR1 subtype (Zukin et al., 1988; Clark et al., 1989; Portoghese et al., 2001), while U69593-insensitive receptors having a low affinity for norBNI were classified as receptor belonging to the KOR2 subtype.

However, the lack of genetic evidence of gene coding for all the different KOR subtypes, and the fact that KORs can exist as homo and heterodimers in the CNS (Jordan and Devi, 1999), led us to reconsider this receptor classification. The observation that κ–δ heterodimers show altered pharmacological profiles when compared to δ and κ oligomers provided an alternative explanation to the existence of KOR subtypes. Whereas κ oligomers were found to be U69593-sensitive receptors typified by low nanomolar affinity for norBNI and high nanomolar affinity for naltrindole, κ–δ heterodimers were classified as U69593-insensitive receptors with reduced affinities for the respective selective antagonists (namely norBNI and naltrindole, Jordan and Devi, 1999).

Based on these observations and considering the results obtained in the present investigation, one may speculate that 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 κ 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, although probably present on noradrenergic synaptic membranes, activation of κ 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 δ oligomers on mouse hippocampal terminals and with their involvement in the effect observed. The hypothesis is attractive but is in overt contrast with the reported inconsistent activity of Salvinorin A at DORs (Wang et al., 2005). In alternative to the involvement of opioid receptors homodimers, a more conservative hypothesis considers that δ and κ heterodimers could be presynaptically located on mouse hippocampal noradrenergic terminals, whose activation may account for the Salvinorin A-induced facilitation of NA release. In this respect, it is important to stress that receptor heterodimerization can drive the coupling to transducing pathways, allowing the switch from inhibitory to excitatory signaling. Thus, further investigation is needed to test this hypothesis.

Modulation of the mesolimbic dopaminergic system is known to contribute to hedonic effects caused by addictive drugs (Nestler and Carlezon, 2006). Impairment of this pathway, due to the activation of presynaptic KORs, was proposed to be at the origin of dysphoria caused by Salvinorin A (Gehrke et al., 2008). Presynaptic inhibitory KORs were shown to be located striatal dopaminergic terminals (Schoffelmeer et al., 1997; Shippenberg and Rea, 1997), whose activation was considered to underline the reduction of in-vivo striatal DA observed following local administration of Salvinorin A (Zhang et al., 2005; Chartoff et al., 2008; Gehrke et al., 2008). Our results showing that Salvinorin A inhibits striatal DA exocytosis by acting at presynaptic KORs located on dopaminergic terminals confirm this hypothesis, adding new insights on the KOR subtype involved.

An interesting finding of the present investigation is that presynaptic KORs also located on PFc dopaminergic terminals, where their activation hampers DA exocytosis. The pharmacological characterization of these receptors again indicates the involvement of U69593 and norBNI-sensitive receptors, also suggesting that the presynaptic KORs located on PFc and striatal dopaminergic terminals may belong to the same KOR subtype. The observation could be of particular relevance to the comprehension of the effects caused by systemic Salvinorin A, particularly when considering that, following its systemic administration, the highest concentration of Salvinorin A occurs in the cerebellum and in the cortex (Hooker et al., 2008).

As the effect of Salvinorin A on 5-HT is concerned, systemic administration of this compound (as well as of U69593) was reported to cause behavioral effects opposite to those elicited by 5-HT uptake blockers (Carlezon et al., 2006). This observation was considered predictive of a modulatory effect exerted by Salvinorin A, *via* KORs, on 5-HT pathways in CNS. In line with this hypothesis, we demonstrate that Salvinorin A inhibits hippocampal 5-HT exocytosis by acting at U69593 and norBNI-sensitive, naltrindole-insensitive KORs.

Considering that a decreased 5-HT bioavailability in CNS could be at the basis of mood disorders, the present finding may be relevant to the comprehension of the sedative and the depressive-like effects Salvinorin A exerts in mammalians (Ansonoff et al., 2006; Carlezon et al., 2006).

5-HT, however, does not represent the sole neurotransmitter involved in mood disorders. According to the classic aminergic theory of depression, also NA plays a major role. We speculate that the presynaptic facilitation of NA exocytosis caused by Salvinorin

A could be at the origin of an anomalous Salvinorin A-induced decrease of NA e 5-HT bioavailability in CNS, a condition considered at the basis of depression. NA nerve terminals possess α_2 autoreceptors (Langer, 1981; Starke, 1981) whose activation controls NA exocytosis. Similarly, serotonergic terminals carry inhibitory α_2 heteroreceptors controlling the 5-HT exocytosis (Göthert and Huth, 1980). Thus, Salvinorin A, by augmenting the extracellular concentration of NA, may indirectly cause the activation of inhibitory α_2 auto and heteroreceptors, ultimately reducing amine bioavailability in CNS (see Pittaluga et al., 2007).

To conclude, 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.

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