British Journal of Pharmacology (2009), 157, 844–853

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Potential anxiolytic- and antidepressant-like effects of salvinorin A, the main active ingredient of *Salvia divinorum*, in rodents

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Background and purpose: Drugs targeting brain κ-opioid receptors produce profound alterations in mood. In the present study we investigated the possible anxiolytic- and antidepressant-like effects of the κ-opioid receptor agonist salvinorin A, the main active ingredient of *Salvia divinorum*, in rats and mice.
Experimental approach: Experiments were performed on male Sprague-Dawley rats or male Albino Swiss mice. The anxiolytic-like effects were tested by using the elevated plus maze, in rats. The antidepressant-like effect was estimated through the forced swim (rats) and the tail suspension (mice) test. κ-Opioid receptor involvement was investigated pretreating animals with the κ-opioid receptor antagonist, nor-binaltorphimine (1 or 10 $mg \cdot kg^{-1}$), while direct or indirect activity at CB_1 cannabinoid receptors was evaluated with the CB_1 cannabinoid receptor antagonist, N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, 0.5 or 3 $mg \cdot kg^{-1}$), binding to striatal membranes of naïve rats and assay of fatty acid amide hydrolase in prefrontal cortex, hippocampus and amygdala.
Key results: Salvinorin A, given s.c. (0.001–1000 $\mu g \cdot kg^{-1}$), exhibited both anxiolytic- and antidepressant-like effects that were prevented by nor-binaltorphimine or AM251 (0.5 or 3 $mg \cdot kg^{-1}$). Salvinorin A reduced fatty acid amide hydrolase activity in amygdala but had very weak affinity for cannabinoid CB_1 receptors.
Conclusions and implications: The anxiolytic- and antidepressant-like effects of Salvinorin A are mediated by both κ-opioid and endocannabinoid systems and may partly explain the subjective symptoms reported by recreational users of *S. divinorum*.
British Journal of Pharmacology (2009) **157,** 844–853; doi:10.1111/j.1476-5381.2009.00230.x; published online 5 May 2009

Keywords: κ-opioid receptor; endocannabinoid system; emotional response; binding; tricyclic antidepressant; benzodiazepine; *Salvia divinorum*

Abbreviations: AM251, N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CP-55,940, (–)-cis-3-[2-hydroxy-4-(1,1-dimethyl heptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan-1-ol; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; EDTA, 2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid; EGTA, glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; FAAH, fatty acid amide hydrolase; HEPES, 4-2-hydroxyethyl-1-piperazine ethanesulfonic acid; nor-BNI, nor-binaltorphimine; pNPP, para-nitrophenylphosphate; U 50,488H, (trans)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene-acetamide methane sulphonate; URB 597, [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate

Introduction

Recreational use of salvinorin A, the main active ingredient of *Salvia divinorum* has greatly increased in recent years for the modified state of awareness it can elicit (Valdes *et al.*, 1983; Sheffler and Roth, 2003). It is a highly selective κ-opioid receptor agonist with hallucinogenic properties (Siebert, 1994; Roth *et al.*, 2002; Butelman *et al.*, 2004; Chavkin *et al.*, 2004).

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Received 14 November 2008; revised 19 December 2008; 1 February 2009

Salvinorin A has a low toxicity (Mowry *et al.*, 2003) and induces analgesic effects (McCurdy *et al.*, 2006), discriminative stimulus effects (Butelman *et al.*, 2004), conditioned place aversion (Zhang *et al.*, 2005) and inhibition of intestinal motility in the guinea pig ileum (Capasso *et al.*, 2006; 2008), through the activation of κ-opioid receptors. Recently, salvinorin A has been reported to show rewarding effects both in zebrafish (Braida *et al.*, 2007) and rats (Braida *et al.*, 2008) through the activation of both κ-opioid and cannabinoid CB_1 receptors, suggesting a potential for abuse in humans.

Drugs targeting brain κ-opioid receptors produce profound mood alterations in humans (Pfeiffer *et al.*, 1986; Roth *et al.*, 2002). In animals, κ-opioid agonists induce an increased immobility in the forced swim test (McLaughlin *et al.*, 2003) and signs of anhedonia in reward models (Todtenkopf *et al.*, 2004). However, the κ-opioid receptor agonist, U 50,488H [(trans)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene-acetamide methane sulphonate], attenuated the escape failure induced by pre-exposure to shock in the learned helplessness model of depression in mice (Ukai *et al.*, 2002). Recently, dynorphin has been found to modulate emotional control, and mice lacking prodynorphin display marked anxiety (Wittmann *et al.*, 2008). Also big dynorphin (a precursor peptide consisting of dynorphin A and B) was reported to exhibit anxiolytic properties (Kuzmin *et al.*, 2006). In contrast, κ-opioid receptor antagonists showed anxiolytic- (Knoll *et al.*, 2007) and antidepressant-like (Mague *et al.*, 2003) effects. There are discrepancies in the reports of the central actions of salvinorin A. It has pro-depressant-like effects in the forced swim test (Carlezon *et al.*, 2006), but antidepressant effects have been reported (Hanes, 2001), albeit in a single human case.

The aim of the present work was to explore the effect of salvinorin A in rodent models of emotional behaviour. We evaluated the ability of salvinorin A to produce changes in anxiety-like behaviour, at doses devoid of motor impairment (Braida *et al.*, 2008). The elevated plus maze paradigm, the forced swim and the tail suspension tests were used. To elucidate the mechanism, the involvement of the κ-opioid system was investigated by pretreating animals with the κ-opioid antagonist, nor-binaltorphimine (nor-BNI). As 20% of the users of *S. divinorum* reported its effects to be comparable to those elicited by marijuana smoking (González *et al.*, 2006) and because reinforcing effects of salvinorin A were mediated through CB_1 cannabinoid receptors (Braida *et al.*, 2008), any interaction with these receptors was also evaluated by using the CB_1 cannabinoid receptor antagonist, N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251). We also measured the binding of salvinorin A to CB_1 receptors, using competition with [^{3}H]CP-55,940 [(–)-cis-3-[2-hydroxy-4-(1,1-dimethyl heptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan-1-ol] in striatal membranes from naïve rats. In parallel, spiradoline, a κ-opioid agonist and CP-55,940 were also investigated in the same binding conditions. Furthermore, indirect modulation of the endocannabinoid system by salvinorin A was assessed by its effects on fatty acid amide hydrolase (FAAH), the major inactivating enzyme of anandamide, using extracts from specified brain areas.

Methods

Animals and housing

All animal care and these experimental procedures followed the guidelines established by the Italian Council on Animal Care. The procedures were approved by the Italian Government decree No. 35/2007. All efforts were made to minimize the number of animals used and their suffering. Adult male Sprague-Dawley rats (150–175 g upon arrival) and Swiss mice (22–24 g upon arrival) (Charles-River, Calco, Italy) were housed in cages (10 per cage) in a climatically controlled colony room under a 12 h light–dark cycle (lights on at 0800 h). When submitted to the experiments, rat body weight was about 200 g, which corresponds to a very young adult age. Food and water were continuously available, and each animal was handled daily during the 7 days preceding the experiment. The day before the experiments, animals were individually housed (for technical convenience) and randomly assigned to each experimental group (10 naïve animals per group). All testing took place during the first half of the light period (between 0900 and 1300 h). Animals were used only once and each animal only for one test.

Elevated plus maze

The elevated plus maze test was carried out as described previously (Braida *et al.*, 2008). The apparatus consisted of two opposite open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) extended from a common central platform (10 × 10 cm) based on a design validated by Lister (1987). A total of 120 animals were used. Upon completion of injections made in the colony room, animals were moved to the plus maze laboratory to facilitate adaptation to novel surroundings for 20 min. Then, rats were placed individually onto the centre of the apparatus facing an open arm, and the time spent on and entries onto each arm were noted for 5 min by a trained observer who remained unaware of the treatments. The maze was wiped clean with water and dried after each trial. An arm entry was recorded when all four paws of the rat were in the arm. The number of open- and closed-arm entries and the time spent in open arms were recorded and expressed as percentage (open entries/total entries × 100; open time/total time × 100). The percentage of time spent in the open arms and the percentage of open-arm entries were used as measures of anxiety (Hogg, 1996). Total closed-arm entries were analysed as measures of non-specific changes in locomotor activity.

Forced swim test

Forced swim test (Porsolt *et al.*, 1977) is a 2 day procedure in which rats swim under conditions where escape is not possible. A total of 150 rats were used. On the first day, rats were forced to swim for 15 min in a 50 cm tall, 30 cm diameter glass cylinder filled to 25 cm with 24–26°C water. They initially struggled to escape from water, but later they adopted a posture of immobility in which they only made the movements necessary to keep their head above water. After the 15 min forced swim session, rats were removed from the

water, wiped with towels and placed under a warming lamp until dry. The cylinder was emptied and cleaned between rats. Twenty-four hours later, rats were re-tested for 5 min in the same conditions, and immobility was increased if the animals showed a depressive-like behaviour. *Immobility* was defined as the time spent by rats making only movements necessary to keep their head above water; *swimming* was defined as making active swimming movements to the centre of the cylinder; *climbing* if they were making forceful thrashing movements with their forelimbs against the walls of the cylinder. The time rats spent in each of these behaviours was measured by a trained observer who remained unaware of the treatments.

Spontaneous motor activity in mice

A total of 40 mice were used. Spontaneous motor activity was evaluated as previously described (Braida and Sala, 2000) in an activity cage (43 × 43 × 32 cm) (Ugo Basile, Varese, Italy), placed in a sound-attenuating room. The cage was fitted with two parallel horizontal infrared beams located 0 cm from the floor. Cumulative horizontal movement counts were recorded for 15 min, 15 min after treatment with salvinorin A. This brief recording time (15 min) in the motor activity experiment has been reported sufficient for mice to evaluate gross abnormalities in locomotion (Crawley, 2000).

Tail suspension test

This test is based on the observation that a mouse suspended by the tail alternates periods of immobility and agitation. The test was conducted according to the procedure of Steru *et al.* (1985). A total of 120 mice were used. Mice were moved from the colony room to the testing area in their home cages and allowed to adapt to the new environment for at least 1 h before testing. They were then suspended individually on a paper adhesive tape, 35 cm above the table top. The tape was placed approximately 1 cm from the tip of the tail. Animals were suspended for 6 min, and the duration of immobility was measured by a trained observer who remained unaware of the treatments. Mice were considered immobile only when they hung passively and completely motionless. Approximately 10% of mice climbed their tails during these tests, and these mice were excluded from data analysis.

Drugs and treatments

Salvinorin A (Tocris Bioscience, Cookson Bristol, UK) (0.001–1000 $\mu g \cdot kg^{-1}$) was dissolved in ethanol, Tween 80 and saline (1:1:8) and given s.c. 20 min before each test. AM251 (0.5 or 3 $mg \cdot kg^{-1}$) (Sigma-Aldrich MO, St. Louis, MO, USA) was dissolved in a mixture of saline (90%) and dimethyl sulphoxide (DMSO) (10%) and given i.p. 40 min before testing, while nor-BNI (Tocris Bioscience, Cookson Bristol, UK) (1 or 10 $mg \cdot kg^{-1}$) was dissolved in saline and administered 2 h before testing. Control groups received different combinations of the appropriate vehicles. Diazepam (1 $mg \cdot kg^{-1}$) and imipramine (15 $mg \cdot kg^{-1}$ for mice and 40 $mg \cdot kg^{-1}$ for rats) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and given i.p. 30 min before the test. The volume administered was 1 $mL \cdot kg^{-1}$ for s.c. and 5 $mL \cdot kg^{-1}$ for i.p. route in rats and 1 $mL \cdot kg^{-1}$ for mice. The range of doses of salvinorin A was similar to that previously used to investigate its reinforcing properties (Braida *et al.*, 2008). The dose of nor-BNI (10 $mg \cdot kg^{-1}$) was chosen on the basis of its ability to reverse salvinorin A-induced aversion and to decrease extracellular levels of dopamine in the caudate putamen (Zhang *et al.*, 2005). The dose of AM251 (1 $mg \cdot kg^{-1}$) was chosen within a range (0.3–3 $mg \cdot kg^{-1}$) shown to selectively block CB_1 receptors (Haller *et al.*, 2004). The anxiolytic dose of diazepam (1 $mg \cdot kg^{-1}$) and the antidepressant dose of imipramine (40 $mg \cdot kg^{-1}$) were selected from previous reports (Pellow *et al.*, 1985; Belozertseva *et al.*, 2007)

The drug/molecular target nomenclature in this paper conforms to the *BJP*'s Guide to receptors and channels (Alexander *et al.*, 2008).

Binding studies

Binding studies were conducted on membranes obtained from striata of naïve animals. Briefly rat striata were suspended in buffer A [320 $mmol \cdot L^{-1}$ sucrose, 50 $mmol \cdot L^{-1}$ Tris-HCl pH 7.4, 2 $mmol \cdot L^{-1}$ EDTA (2-[2-(Bis(carboxymethyl) amino)ethyl-(carboxymethyl)amino]acetic acid), 5 $mmol \cdot L^{-1}$ $MgCl_2$] and homogenized by using a Teflon pestle and glass homogenizer. Tissue homogenates were then centrifuged (1000× *g*, 10 min, 4°C) and the resulting supernatant collected. The pellet was resuspended in buffer A and centrifuged twice as before. The combination of the three supernatants was centrifuged (40 000× *g*, 30 min, 4°C), and the final pellet was resuspended in buffer B (50 $mmol \cdot L^{-1}$ Tris-HCl, 2 $mmol \cdot L^{-1}$ EDTA, 3 $mmol \cdot L^{-1}$ $MgCl_2$) and assayed for protein content (Bradford, 1976). The aliquots were stored at –80°C until used. For the competition binding experiments, membrane aliquots (50 µg protein) were incubated in triplicate to equilibrium (1 h at 37°C) with a fixed concentration of 1 $nmol \cdot L^{-1}$ [3H]CP-55,940 and increasing concentrations of salvinorin A (from 3 $pmol \cdot L^{-1}$ to 1 $mmol \cdot L^{-1}$), spiradoline (from 100 $pmol \cdot L^{-1}$ to 300 $\mu mol \cdot L^{-1}$) or CP-55,940 (from 1 $pmol \cdot L^{-1}$ to 1 $\mu mol \cdot L^{-1}$). Specific binding was determined by using 1 $\mu mol \cdot L^{-1}$ of CP-55,940. Assays were terminated by rapid filtration through GF/B glass fibre filters (Whatman, Maidstone, UK) pre-soaked in buffer D (50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.4, 1 $mg \cdot mL^{-1}$ bovine serum albumin). Filters were then washed twice with 5 mL of ice-cold buffer D, and the remaining membrane-bound radioactivity was determined by liquid scintillation counting (Beckman LS 6000IC, CA, USA).

Preparation of nuclear extracts

Each brain region from controls or salvinorin A-treated (10 $\mu g \cdot kg^{-1}$ s.c. 30 min before killing) animals was homogenized in an appropriate volume of ice-cold buffer A [10 $mmol \cdot L^{-1}$ HEPES (4-2-hydroxyethyl-1-piperazine ethanesulfonic acid) pH 7.5, 1.5 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ KCl, 2 $mmol \cdot L^{-1}$ dithiothreitol (DTT), 1 $mmol \cdot L^{-1}$ phenylmethanesulphonyl fluoride, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ EGTA (glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid), 2 $mmol \cdot L^{-1}$ sodium orthovanadate, 50 $mmol \cdot L^{-1}$ NaF, 10 $mmol \cdot L^{-1}$ sodium pyrophosphate, 0.5% Triton, 5 $mg \cdot mL^{-1}$ aprotinin and 5 $mg \cdot mL^{-1}$ leupeptin] and centrifuged at

12 000× *g* at 4°C for 3 min. The pellet was resuspended in an appropriate volume of ice-cold buffer C [20 mmol·L^{-1} HEPES pH 7.5, 400 mmol·L^{-1} NaCl, 1.5 mmol·L^{-1} $MgCl_2$, 10 mmol·L^{-1} NaF, 10 mmol·L^{-1} Na_2MoO_4, 0.1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} sodium orthovanadate, 10 mmol·L^{-1} pNPP (para-nitrophenylphosphate), 10 mmol·L^{-1} b-glycerophosphate, 20% glycerol, 2 mmol·L^{-1} DTT and protease inhibitors as above] and homogenized. After 30 min incubation on ice with gentle rocking, samples were centrifuged at 12 000× *g* at 4°C for 10 min, and the pellet was used for nuclear extracts. Protein concentrations in the respective fractions were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL, USA).

Assay of FAAH activity

Tissues were thawed, weighed and homogenized in 50 mmol·L^{-1} Tris-HCL buffer pH 7.6 containing 1 mmol·L^{-1} EDTA and 3 mmol·L^{-1} $MgCl_2$, in a volume of 5 mL·g^{-1} wet weight and then briefly sonicated. After determination of protein concentration, the homogenates were stored in aliquots of 250 µL at –80°C prior to assay of FAAH activity. FAAH activity was measured, as previously reported (Holt *et al.*, 2005) at 37°C for 4 min in 100 µL of assay buffer (116 mmol·L^{-1} NaCl; 5.4 mmol·L^{-1} KCl; 1.8 mmol·L^{-1} $CaCl_2$; 25 mmol·L^{-1} HEPES, pH 7; 1 mmol·L^{-1} NaH_2PO_4; 0.8 mmol·L^{-1} $MgSO_4$) containing fatty acid-free bovine serum albumin (0.1%), (10 µg), 0.5 mmol·L^{-1} anandamide and anandamide [ethanolamine-^{3}H] (10 000 cpm, specific activity 60 Ci·mmol^{-1}; American Radiolabeled Chemicals, St. Louis, MO, USA). The reactions were stopped with charcoal/HCl (1:4, 100 µL), and radioactivity was measured in the aqueous layers by liquid scintillation counting.

Statistical analysis

Data were expressed as mean ± SEM and analysed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's or Dunnett's post hoc comparisons. Binding competition curves were fitted to one- or two-site binding models, to determine whether the data were best fitted by a one- or two-site model, by using the non-linear least-squares curve-fitting. K_i values were calculated by applying the Cheng–Prusoff equation to the IC_{50} values for the displacement of the bound radioligand by increasing concentrations of salvinorin A. FAAH findings were analysed by using unpaired *t*-test. The accepted level of significance was $P < 0.05$. All statistical analyses were done by using software Prism, version 5 (GraphPad Software Inc, San Diego, CA, USA).

Results

Elevated plus maze

The ability of salvinorin A, given 20 min before the elevated plus maze test, to modulate anxiety responses was examined. Rats treated with doses of salvinorin A, which previously were reported not to affect locomotor activity (Braida *et al.*, 2008), made more open-arm entries and spent a longer time in the open arms than did vehicle-injected rats (Figure 1) [$F_{(7,62)}$ = 5.68, $P < 0.0001$ and $F_{(7,62)} = 6.47$, $P < 0.0001$ for open-arm entries and time respectively]. Post hoc analysis revealed significant differences between salvinorin A-treated and the vehicle groups in a range of doses between 0.1 and 160 µg·kg^{-1}. However, no progressive dose-related increase of anxiolytic effect was shown. The clinically used anxiolytic diazepam (1 mg·kg^{-1}, i.p.) produced a similar effect to salvinorin A on open-arm entries, but it appeared more effective in increasing the time spent in the open arms. Salvinorin A and diazepam treatment had no significant effect on the number of closed-arm entries (Figure 1).

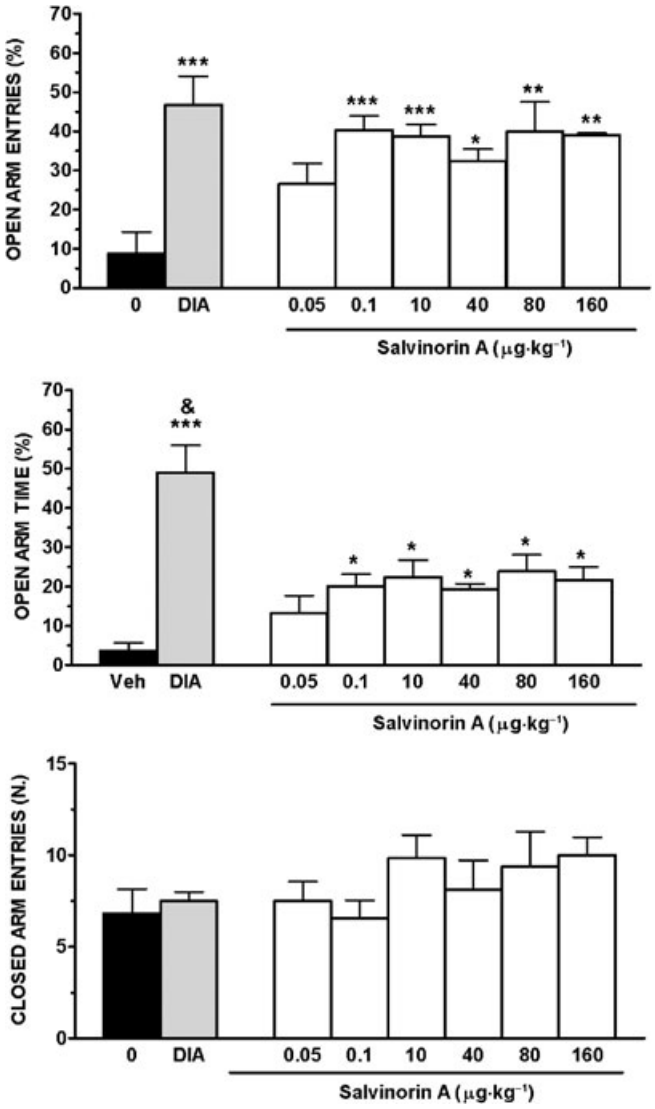

Figure 1 Effect of salvinorin A or diazepam (DIA, 1 mg·kg^{-1} i.p., 30 min before testing) on behaviour in the elevated plus maze in terms of percentage of entries and time spent in open arms and number of closed-arm entries. Sprague-Dawley rats were injected with vehicle (Veh) or salvinorin A (0.05–160 µg·kg^{-1} s.c.), 20 min before testing for 5 min. Each column represents the mean ± SEM of 10 animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle group; $^{\&}P < 0.05$ compared with salvinorin A (all the tested doses); one-way ANOVA followed by Tukey's test.

The effect of treatment with nor-BNI and AM251, alone or in combination with the lowest effective dose of salvinorin A

Table 1 Effect of nor-BNI and AM251 on salvinorin A-induced anxiolytic effects in the elevated plus maze with Sprague-Dawley rats

Pretreatment	*Dose (mg·kg^{-1})*	*Treatment*	*Dose (μg·kg^{-1})*	*Open-arm entries (%)*	*Open-arm time (%)*	*Closed-arm entries (N)*
Veh	–	Veh	–	14.43 ± 4.89	5.71 ± 1.90	7.60 ± 1.02
Nor-BNI	10	Veh	–	10.67 ± 5.81	10.33 ± 4.09	7.25 ± 1.43
AM251	3	Veh	–	10.10 ± 3.16	5.33 ± 5.33	6.00 ± 1.64
Veh	–	Salv-A	0.1	44.44 ± 3.16***	23.96 ± 2.23***	8.84 ± 1.32
Nor-BNI	10	Salv-A	0.1	16.00 ± 1.60###	8.50 ± 3.26###	6.00 ± 0.57
AM251	3	Salv-A	0.1	23.65 ± 2.10#	8.80 ± 1.87##	6.50 ± 1.84

Values represent mean ± SEM, $n = 10$ for each group. Salvinorin A was injected s.c. 20 min before the test; AM251 and nor-BNI were injected i.p. 40 or 120 min before salvinorin A.
AM251, N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; nor-BNI, nor-binaltorphimine; Salv-A, Salvinorin A; Veh, vehicle (different combinations of corresponding vehicles).
***$P < 0.001$ versus respective vehicle group, nor-BNI and AM251 groups; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus salvinorin A alone.

(0.1 μg·kg^{-1}), is reported in Table 1. One-way ANOVA showed a significant effect of drug treatment on the percentage of open-arm entries [$F_{(8,81)} = 8.32$, $P < 0.0001$ and time [$F_{(8,81)} = 4.73$, $P < 0.0001$]. Tukey's test revealed that the two antagonists per se did not change emotional reactivity, but when given in combination with salvinorin A they completely blocked its anxiolytic effect in terms of open-arm entries and time. No significant difference between vehicle and treated groups in the number of closed-arm entries was shown.

Forced swim test

The effects of acute administration of salvinorin A in comparison with imipramine in the forced swim test are shown in Figure 2. The treatments induced significant changes in the mean time spent immobile [$F_{(10,99)} = 11.76$, $P < 0.0001$], in the duration of swimming [$F_{(10,99)} = 8.59$, $P < 0.0001$] and climbing behaviour [$F_{(10,99)} = 25.51$, $P < 0.0001$]. Post hoc analysis revealed that imipramine, a clinically used antidepressant (Belozertseva *et al*., 2007), significantly induced a reduction in the mean time of immobility and an increase in climbing. Salvinorin A specifically, at doses from 0.001 to 10 μg·kg^{-1}, dose-dependently decreased the occurrence of immobility ($R^2 = 0.864$) and increased swimming ($R^2 = 0.908$). Doses between 40 and 1000 μg·kg^{-1} did not produce any further effect. Imipramine, as expected, increased the mean time spent in climbing behaviour while salvinorin A did not.

The effect of pretreatment with nor-BNI and AM251, alone or in combination with the lowest effective dose of salvinorin A (10 μg·kg^{-1}), is reported in Figure 3. Pretreatment with the different antagonists significantly blocked the effect of salvinorin A on the time spent immobile [$F_{(5,54)} = 7.73$, $P < 0.0003$] and on swimming activity [$F_{(5,54)} = 5.95$, $P < 0.001$]. No changes were observed in climbing activity in all groups.

Spontaneous motor activity in mice

Figure 4 shows the effect of salvinorin A on spontaneous motor activity in mice. One-way ANOVA revealed no significant treatment effect.

Tail suspension test

The influence of salvinorin A on the tail suspension test was also examined in mice (Figure 5). Statistical analysis revealed

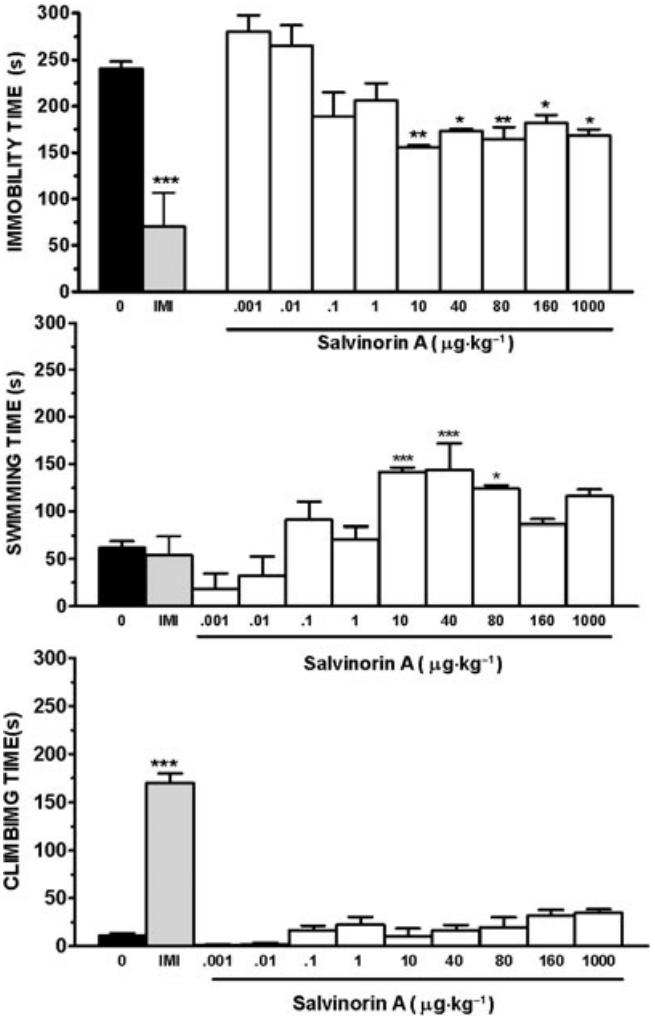

Figure 2 Effect of acute treatment with salvinorin A and imipramine (IMI, 40 mg·kg^{-1} i.p., 30 min before testing) on behaviour in the forced swim test. Male adult Sprague-Dawley rats were injected with salvinorin A (0.001–1000 μg·kg^{-1} s.c.), 20 min before testing. The time spent immobile, the duration of swimming and the time spent trying to climb out were evaluated. Each column represents the mean ± SEM of 10 animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle group; one-way ANOVA followed by Tukey's test.

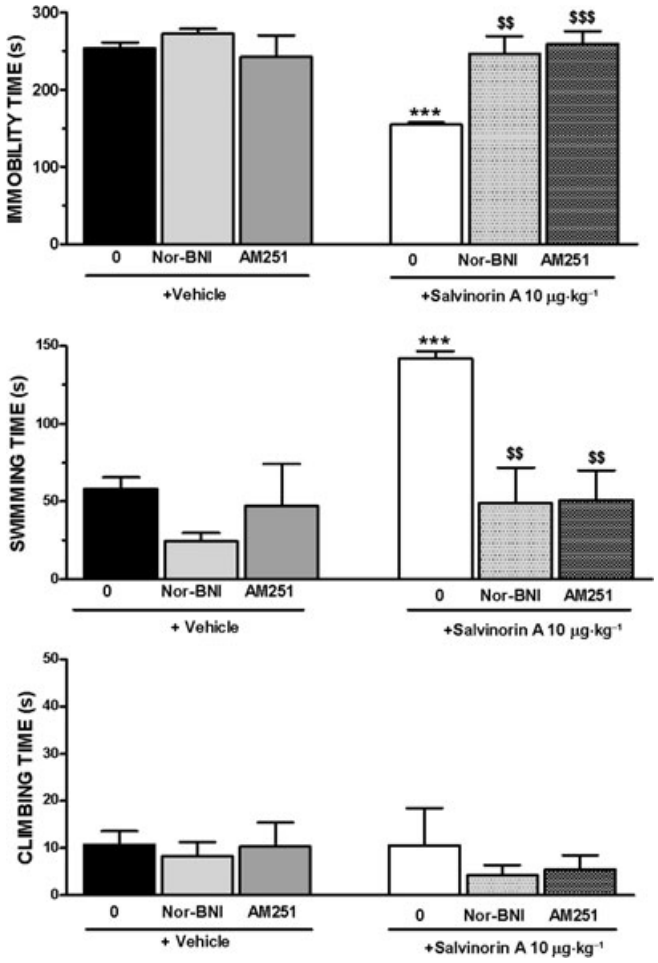

Figure 3 Effect of nor-binaltorphimine (nor-BNI) and AM251 (N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) on salvinorin A-induced antidepressant effects in the forced swim test in terms of time spent immobile, duration of swimming and time spent trying to climb out. Nor-BNI (10 mg·kg^{-1} i.p.) was given 120 and AM251 (3 mg·kg^{-1} i.p.) 40 min before the test. Salvinorin A (10 μg·kg^{-1} s.c.) was given 20 min before the test. Each column represents the mean ± SEM of 10 animals. ***P < 0.001 compared with vehicle group; $$$P$ < 0.01, $$$$P$ < 0.001 compared with salvinorin A; one-way ANOVA followed by Tukey's test.

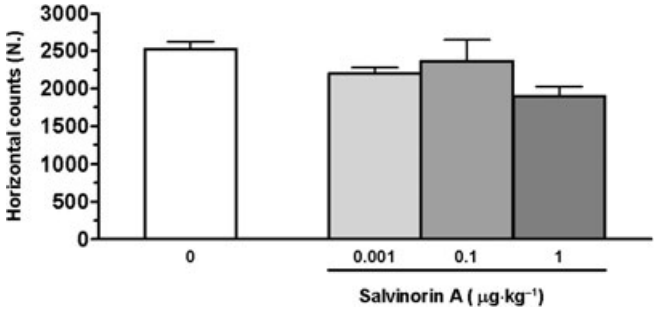

Figure 4 Effect of increasing doses of salvinorin A on spontaneous motor activity in mice. The drug was given 15 min before the test. Bars represent the mean ± SEM of the number of horizontal counts evaluated for 15 min. N = 10 for each group.

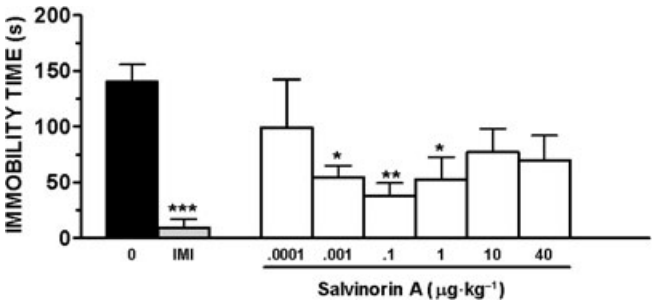

Figure 5 Effect of acute administration of salvinorin A and imipramine (IMI, 15 mg·kg^{-1} i.p., 30 min before testing) on immobility in the tail suspension test in mice. Animals were injected with salvinorin A (0.0001–40 μg·kg^{-1} s.c.) 20 min before testing. The duration of time spent immobile was measured for 6 min. Each column represents the mean ± SEM of 10 animals. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle group; one-way ANOVA followed by Tukey's test.

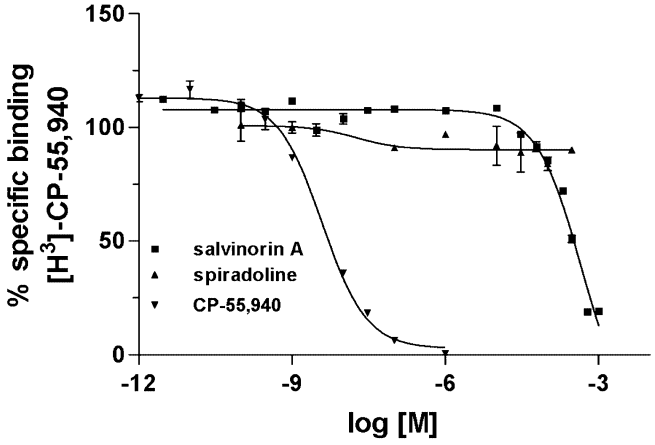

Figure 6 Effect of increasing concentrations of salvinorin A, spiradoline and CP-55,940 [(–)-cis-3-[2-hydroxy-4-(1,1-dimethyl heptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexan-1-ol] on CB_1 receptor binding in rat striatal membranes. Each point represents the mean ± SEM of membranes from at least six animals.

a significant effect of treatment between different groups [$F_{(7,72)}$ = 4.24, P < 0.002]. As comparison, imipramine produced a typical antidepressant-induced decrease in immobility time. Post hoc comparisons showed that salvinorin A produced a significant decrease of the time spent in immobility in a range of doses between 0.001 and 1 μg·kg^{-1}.

The effect of pretreatment with nor-BNI and AM251, alone or in combination with the maximal effective dose of salvinorin A (0.1 μg·kg^{-1}), is reported in Table 2. Both the antagonists completely blocked the effect of salvinorin A on the time spent immobile [$F_{(5,54)}$ = 3.88, P < 0.005].

CB_1 cannabinoid receptor binding

The binding affinity of salvinorin A for the CB_1 receptor was measured as its ability to displace radio-labelled CP-55,940 (1 nmol·L^{-1}) from CB_1 receptors of rat striata (Figure 6). As expected, unlabelled CP-55,940 was able to compete with the labelled compound for CB_1 receptors. In contrast, spiradoline did not alter [^{3}H]CP-55,940 binding even when at high molar

Table 2 The effect of nor-BNI and AM251 on salvinorin A-induced antidepressant effect in the tail suspension test, with mice

Pretreatment	*Dose (mg·kg^{-1})*	*Treatment*	*Dose (μg·kg^{-1})*	*Immobility time (s)*
Veh	–	Veh	–	145 ± 17
Nor-BNI	1	Veh	–	142 ± 26
AM251	0.5	Veh	–	130 ± 36
Veh	–	Salvinorin A	0.1	38 ± 11*
Nor-BNI	1	Salvinorin A	0.1	167 ± 25††
AM251	0.5	Salvinorin A	0.1	114 ± 10††

Salvinorin A was injected s.c. 20 min before the test. AM251 and nor-BNI were injected i.p. 40 or 120 min before salvinorin A. Values represent mean ± SEM, $n = 10$ for each group.
AM251, N-(piperidin-1-yl) -5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; nor-BNI, nor-binaltorphimine; Veh, vehicle (different combinations of corresponding vehicles).
*$P < 0.05$ versus respective vehicle group, nor-BNI and AM251 groups; ††$P < 0.01$, compared with salvinorin A alone.

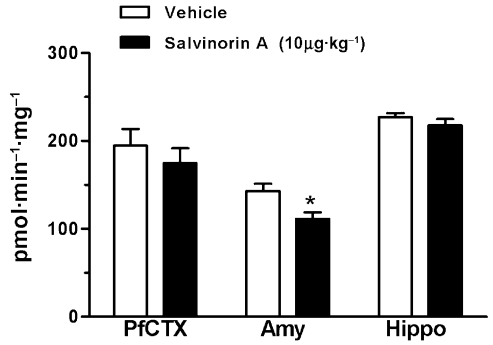

Figure 7 Effect of salvinorin A pretreatment (30 min), on fatty acid amide hydrolase activity (pmol·min^{-1}·mg^{-1}) in nuclear extracts of prefrontal cortex (PfCtx), amygdala (Amy) and hippocampus (Hippo). Mean ± SEM of at least four animals. *$P < 0.05$ versus vehicle group (Student's *t*-test).

excess. Salvinorin A did not alter the binding of the labelled compound within the range 3 pmol·L^{-1}–10 μmol·L^{-1}. However, at higher concentrations (30 μmol·L^{-1}–1 mmol·L^{-1}), salvinorin A displaced [^{3}H]CP-55,940 binding with a curve that fitted a one-site binding model. The EC_{50} value was 3.89 nmol·L^{-1} for CP-55,940 and 457 nmol·L^{-1} for salvinorin A with a K_i of 1.72 and 202 nmol·L^{-1}.

FAAH activity

To test whether, *in vivo*, treatment with salvinorin A (10 μg·kg^{-1}) could indirectly modulate the endocannabinoid system, its ability to alter the activity of FAAH in different brain areas was assessed *ex vivo* (Figure 7). Pretreatment (30 min) with salvinorin A decreased FAAH activity only in extracts of amygdala with no effects on activity in prefrontal cortex or hippocampus.

Discussion

Most findings of the present work provide evidence of anxiolytic- and antidepressant-like effects induced by salvinorin A, which were prevented by κ-opioid and CB_1 cannabinoid receptor antagonists.

The anxiolytic effect of salvinorin A was shown by the progressive dose-dependent increase of both the percentage of entries and time spent in the open arms of the elevated plus maze. We have no adequate explanation for the lack of dose dependence in these assays. Further studies to investigate the effect of salvinorin A at different intervals of time might disclose a dose-dependent effect. The maximal anxiolytic effect was obtained with a dose as 0.1 μg·kg^{-1} and was maintained even by increasing the dosage. The increase in time spent in open arms after salvinorin A treatment was half that seen after treatment with the classical anxiolytic compound, diazepam, but the number of open-arm entries was similar for both drugs, suggesting that salvinorin A possesses a slight anxiolytic effect.

On the other hand, we found a more consistent antidepressant-like activity. Salvinorin A, starting from 10 μg·kg^{-1}, reduced immobility time and simultaneously enhanced swimming behaviour in rats by using the forced swimming test, whereas in mice it was effective in a range between 0.001 and 1 μg·kg^{-1} in the tail suspension test. Reduction of immobility in rats was comparable to that observed after the acute administration of the reference antidepressant drug imipramine. In contrast to the original protocol (Porsolt *et al.*, 1978) that involved triple administration of the compound prior to the forced swim test, in our experiments, a single injection of imipramine (30 mg·kg^{-1}) was sufficient, to decrease immobility (Belozertseva *et al.*, 2007). The observation of detailed behaviours, such as swimming and climbing, in addition to the traditional immobility measure, showed that imipramine increased, as expected, climbing activity suggesting a mechanism that involves noradrenergic neurotransmission (Detke *et al.*, 1995). In contrast, salvinorin A increased swimming behaviour without affecting climbing, suggesting an involvement of 5-hydroxytryptaminergic neurotransmission in this antidepressant-like activity. Our results agree with Bambico *et al.* (2007) who found that the cannabinoid agonist WIN 55,212-2 exhibits antidepressant-like properties through modulation of 5-hydroxytryptaminergic neuronal activity.

Our findings of an antidepressant-like effect of salvinorin A appear to contradict those of Carlezon *et al.* (2006) who found that salvinorin A, given i.p., in triple administration to rats, at doses much greater than those used in our study (0.25–2 mg·kg^{-1}), increased immobility behaviour in the forced swim test, suggesting a pro-depressant-like effect. This effect was accompanied by a decrease of extracellular concentration of dopamine in the nucleus accumbens as measured by *in vivo*

microdialysis, indicating that salvinorin A, through the selective action of κ-opioid receptors, affects the function of the dopaminergic system. However, it must be noted that, in *in vivo* microdialysis studies, an acute injection of a dose of 40 $\mu g \cdot kg^{-1}$, which in our experiments induced antidepressant-like effects, produced an elevation of extracellular dopamine in the shell of nucleus accumbens accompanied by rewarding effects (Braida *et al.*, 2008). Given the growing evidence for a role of the ventral tegmental area–nucleus accumbens pathway in the pathophysiology and symptomatology of depression (Nestler and Carlezon, 2006) and given the extensive connections of the nucleus accumbens with limbic brain areas involved in emotion (Heimer *et al.*, 1991), a salvinorin A-induced increase of dopamine in the nucleus accumbens might be involved in the modulation of affective and motivational properties. Indeed, decreased dopaminergic function in the nucleus accumbens produced depressive-like behaviours such as anhedonia (Wise and Bozarth, 1982). Thus, it appears that salvinorin A, given acutely, at very low doses, produced antidepressant-like effects whereas, when repeatedly administered at high doses, it is pro-depressant. However, it remains to be elucidated how long, after multiple injections, the antidepressant-like effects of salvinorin A last, before suggesting that this compound is antidepressant. Preliminary findings (data not shown) indicate that salvinorin A, at the highest dose ($1 mg \cdot kg^{-1}$), given in a triple administration, produced pro-depressant-like effects, as shown by Carlezon *et al.* (2006).

The mechanisms that mediate the putative anxiolytic/antidepressant-like activity of salvinorin A are still unclear. However, the fact that both the selective κ-antagonist nor-BNI and the selective CB_1 receptor antagonist AM251 prevented these effects led to the idea that salvinorin A acted through both κ-opioid and CB_1 cannabinoid receptors. The possibility that non-specific effects could be responsible for the observed antagonism might be ruled out, because the two antagonists per se had no effect in our behavioural assays on emotional reactivity. However, the possibility that AM251 was acting as a κ-opioid antagonist cannot be ruled out. Our results with the cannabinoid antagonist agree with those of Capasso *et al.* (2008) who found, in a model of croton oil-induced ileitis, that inhibition of intestinal motility induced by salvinorin A was prevented by both nor-BNI and another CB_1 cannabinoid receptor antagonist, rimonabant. Consistent with our results, salvinorin A has been shown to exert some other effects through the activation of κ-opioid receptors (Butelman *et al.*, 2004; Zhang *et al.*, 2005; Capasso *et al.*, 2006; 2008; McCurdy *et al.*, 2006).

Concerning the involvement of the endocannabinoid system, which has a functional role in the expression of emotional behaviour (Patel and Hillard, 2006), our binding data seem to exclude the direct action of salvinorin A at CB_1 cannabinoid receptors. In fact, its K_i is quite high ($202 \mu mol \cdot L^{-1}$), ruling out a specific interaction. However, the dose-related displacement only at high concentrations might suggest the existence of a binding site with very low affinity, which could not account for the *in vivo* effect obtained at very low doses. These findings agree with those of Capasso *et al.* (2008) who found that salvinorin A, added to membranes from HEK cells transfected with human CB_1 or CB_2 receptors, had no affinity for CB_1 and only a weak affinity for CB_2 receptors. More consistently, salvinorin A induced a significant inhibition of FAAH activity in the amygdala, suggesting that this compound could induce elevations in the brain levels of anandamide and other fatty acid ethanolamides that are substrates for FAAH, as reported for the selective FAAH inhibitor, [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB 597) (Kathuria *et al.*, 2003). Decreased FAAH activity after salvinorin A, was only observed in amygdala, which is a brain region linked with the effects of cannabinoids on emotionally relevant behaviours (Katona *et al.*, 2001) and where anatomical studies have reported the presence of κ-opioid receptors (Mansour *et al.*, 1994).

It could be argued that the observed decrease of FAAH activity (22%) is too small to be relevant. However, a similar decrease (30%) was able to induce an anxiolytic effect, evaluated in the elevated plus maze, when URB 597 ($0.01 \mu g \cdot rat^{-1}$) was injected into the prefrontal cortex (Rubino *et al.*, 2008) or given i.p. ($0.3 mg \cdot kg^{-1}$) (Kathuria *et al.*, 2003; Gobbi *et al.*, 2005). Capasso *et al.* (2008) found that salvinorin A exhibited less than 20% inhibition of FAAH activity, concluding that it could have no effect on anandamide inactivation. However, these authors measured FAAH activity in membranes prepared from whole brain whereas we used membranes from specific brain regions and found inhibition in only one of the three regions examined.

How salvinorin A alters FAAH activity is unclear as is the connection between decreased FAAH activity and κ-opioid receptor-mediated responses. However, in a very recent paper (Haller *et al.*, 2008), anandamide, if protected from degradation, acted via the CB_1 cannabinoid receptor to interact with the κ-opioid receptor system mediating opioid analgesia in mice.

In conclusion, the present study provides evidence for the first time of an anxiolytic/antidepressant effect of salvinorin A in rats, which probably could explain some of the subjective symptoms as laughter, happiness, well-being reported by González *et al.* (2006) in young recreational users of *S. divinorum*. Besides the well-known interaction of salvinorin A with κ-opioid receptors, the involvement of the endocannabinoid system in emotional reactivity may contribute to an understanding of the underlying mechanisms of the complex effects of *S. divinorum* or salvinorin A, observed in humans. Further studies are needed to elucidate the biochemical nature of this interaction.

Acknowledgement

This work was supported by PRIN (2006) and Regione Lombardia.

Conflict of interest

The authors state no conflict of interest.

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