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RESEARCH PAPER

# The anandamide transport inhibitor AM404 reduces the rewarding effects of nicotine and nicotine-induced dopamine elevations in the nucleus accumbens shell in rats

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## BACKGROUND AND PURPOSE

The fatty acid amide hydrolase inhibitor URB597 can reverse the abuse-related behavioural and neurochemical effects of nicotine in rats. Fatty acid amide hydrolase inhibitors block the degradation (and thereby magnify and prolong the actions) of the endocannabinoid anandamide (AEA), and also the non-cannabinoid fatty acid ethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). OEA and PEA are endogenous ligands for peroxisome proliferator-activated receptors alpha (PPAR-α). Since recent evidence indicates that PPAR-α can modulate nicotine reward, it is unclear whether AEA plays a role in the effects of URB597 on nicotine reward.

## EXPERIMENTAL APPROACH

A way to selectively increase endogenous levels of AEA without altering OEA or PEA levels is to inhibit AEA uptake into cells by administering the AEA transport inhibitor N-(4-hydroxyphenyl)-arachidonamide (AM404). To clarify AEA's role in nicotine reward, we investigated the effect of AM404 on conditioned place preference (CPP), reinstatement of abolished CPP, locomotor suppression and anxiolysis in an open field, and dopamine elevations in the nucleus accumbens shell induced by nicotine in Sprague-Dawley rats.

## KEY RESULTS

AM404 prevented the development of nicotine-induced CPP and impeded nicotine-induced reinstatement of the abolished CPP. Furthermore, AM404 reduced nicotine-induced increases in dopamine levels in the nucleus accumbens shell, the terminal area of the brain's mesolimbic reward system. AM404 did not alter the locomotor suppressive or anxiolytic effect of nicotine.

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## CONCLUSIONS AND IMPLICATIONS

These findings suggest that AEA transport inhibition can counteract the addictive effects of nicotine and that AEA transport may serve as a new target for development of medications for treatment of tobacco dependence.

## LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

### Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, anandamide; AM404, N-(4-hydroxyphenyl)-arachidonamide; CPP, conditioned place preference; FAAH, fatty acid amide hydrolase; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PPAR, peroxisome proliferator-activated receptor; THC, $\Delta^9$-tetrahydrocannabinol; TRPV, transient receptor potential vanilloid; URB597, cyclohexyl carbamic acid 3′-carbamoyl-3-yl ester; VTA, ventral tegmental area

## Introduction

Emerging evidence suggests that rewarding and other abuse-related effects of nicotine related to tobacco dependence are modulated by the endocannabinoid system of the brain. For example, pharmacological blockade or genetic deletion of cannabinoid $CB_1$ receptors can reduce or eliminate many abuse-related behavioural and neurochemical effects of nicotine (Scherma *et al.*, 2008a). These and other similar studies have used systemically-administered CB1-receptor inverse agonists/antagonists and gene deletion techniques, which affect cannabinoid $CB_1$ receptors throughout the brain. A more functionally selective way to alter endocannabinoid activity is to inhibit fatty acid amide hydrolase (FAAH), the main enzyme responsible for degradation of the endocannabinoid anandamide (AEA), when and where it is synthesized and released. We recently reported that the FAAH inhibitor cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597) can counteract abuse-related effects of nicotine in several animal models (Melis *et al.*, 2008; Scherma *et al.*, 2008b; Forget *et al.*, 2009). In rats, FAAH inhibition suppresses the development of nicotine-induced conditioned place preference (CPP) and intravenous (i.v.) nicotine self-administration, two widely used animal models of nicotine's habit-forming rewarding effects (Scherma *et al.*, 2008b). Inhibition of FAAH also suppresses reinstatement of nicotine seeking, an animal model of relapse (Scherma *et al.*, 2008b; Forget *et al.*, 2009). In addition to these behavioural effects, FAAH inhibition reduces nicotine-induced excitation of dopamine neurons in the ventral tegmental area (VTA), the brain area where nicotine appears to trigger its rewarding effects (Melis *et al.*, 2008), and reduces nicotine-induced elevation of dopamine levels in the shell of the nucleus accumbens, the terminal area of the brain's mesolimbic reward system (Scherma *et al.*, 2008b).

Although research with FAAH inhibitors has generally focused on enhancement of cannabinoid signalling mediated by prolongation of AEA's effects, FAAH inhibition also increases brain levels and magnifies and prolongs effects of the non-cannabinoid fatty acid ethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), which are endogenous ligands for the peroxisome proliferator-activated receptors alpha (PPAR-α) (Fegley *et al.*, 2005; Astarita *et al.*, 2006). Mascia *et al.* (2011) showed that the selective PPAR-α agonists WY14643 and methyloleoylethanolamide (methOEA; a long-lasting form of OEA) dose-dependently counteract the rewarding effects of nicotine in rats and monkeys. These findings converge to suggest that URB597 modulates the rewarding effects of nicotine by elevating levels of the endogenous PPAR-α ligands OEA and PEA; further studies are needed to delineate the role of AEA.

A way to selectively increase endogenous levels of AEA without altering levels of OEA or PEA is to inhibit uptake of AEA into cells where it is degraded by FAAH, by administering the N-(4-hydroxyphenyl)-arachidonamide (AM404), the first synthetic inhibitor of endocannabinoid transport that has been shown to increase endogenous brain levels of AEA, without significantly affecting brain levels of PEA or OEA (Fegley *et al.*, 2004; Bortolato *et al.*, 2006). AM404 potentiates many effects elicited by AEA *in vitro* and *in vivo* (Beltramo *et al.*, 1997; Calignano *et al.*, 1997) but does not closely mimic the spectrum of pharmacological responses produced by direct cannabinoid agonists, like $\Delta^9$-tetrahydrocannabinol (THC), because it does not elicit catalepsy or hypothermia (Beltramo *et al.*, 1997; 2000) and does not produce THC-like discriminative effects or alter dopamine levels in the shell of nucleus accumbens in rats (Solinas *et al.*, 2007). These differences have been attributed to the ability of AM404 to increase AEA levels in the brain by inhibition of AEA transport into cells without directly activating cannabinoid receptors (Beltramo *et al.*, 1997; 2000; Bortolato *et al.*, 2006).

The aim of this study was to investigate the effect of AM404 on the development of nicotine-induced CPP in rats, a frequently used animal model of nicotine's rewarding effects (Liu *et al.*, 2008; Le Foll and Goldberg, 2009; Panlilio *et al.*, 2010), in order to clarify the role played by AEA in nicotine reward. We also evaluated the effects of the cannabinoid $CB_1/CB_2$ receptor agonist THC on the development of nicotine-induced CPP in rats, to compare the effects of a directly acting cannabinoid receptor agonist with those of indirectly acting cannabinoid receptor agonists like AM404 and URB597. In addition, we evaluated the effects of different doses of AM404 and THC alone to determine whether or not either of these drugs induced development of CPP under our test conditions. Based on the effect of AM404 on the development of nicotine-induced CPP, we then evaluated the effects of AM404 on nicotine-induced reinstatement of extinguished CPP, an animal model of relapse to tobacco use in humans (Fattore *et al.*, 2007; 2009; Scherma *et al.*, 2008b). Finally, as an elevation of dopamine levels in the nucleus

accumbens shell is an effect considered central for the reinforcing effects of many abused drugs, including nicotine (Pontieri *et al.*, 1996), we evaluated the effects of AM404 on nicotine-induced increases in extracellular dopamine levels in the shell of the nucleus accumbens. We found that AM404 prevented the development of nicotine-induced CPP, blocked nicotine-induced reinstatement of extinguished place-preference and reduced nicotine-induced elevation of dopamine levels in the nucleus accumbens shell.

## Methods

### Animals

Subjects were male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA, or Harlan-Nossan, Milan, Italy) weighing 250–275 g at the beginning of experiments, and housed in temperature- and humidity-controlled rooms on a 12 h light-dark cycle with *ad libitum* access to food and water. Experiments were conducted during the light phase. All animal care and experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse (NIDA), NIH and European Commission regulations for animal use in research (86/609/EEC), and were approved by the Animal Ethics Committee of the University of Cagliari. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

### CPP

Apparatus and procedure were as described previously (Le Foll and Goldberg, 2004).

The general procedure consisted of three consecutive phases:

*Pretest.* Rats were placed at the intersection of two compartments, with the guillotine door separating the two compartments raised to allow exploration of both sides for 15 min. Time spent by the animal in each of the two compartments was recorded to monitor any initial preference for one side versus the other side. Animals showing a pronounced unconditioned preference for one compartment (more than 600 s spent in one compartment) were excluded from the subsequent (conditioning) phase of the experiment.

*Conditioning.* Conditioning sessions were conducted over 3 consecutive days, with two sessions per day. In the morning all rats received an injection of saline before being placed in one of the two compartments for 20 min, with the door separating the two compartments closed. Four hours later, the rats received an injection of drug or vehicle and were placed in the opposite compartment for 20 min. In the preliminary experiment, AM404 and THC at different doses (1.25 to 10 $mg \cdot kg^{-1}$, i.p. and 0.1 to 3 $mg \cdot kg^{-1}$, respectively) or vehicle were injected 30 min before being placed in the compartment. To study the development of nicotine-induced CPP, nicotine (0.05 or 0.4 $mg \cdot kg^{-1}$, s.c.) or saline were injected immediately before being placed in the compartment. A dose of 0.4 $mg \cdot kg^{-1}$ nicotine was chosen for conditioning because this dosage is known to produce reliable CPP in rats in our experimental conditions (Le Foll and Goldberg, 2004, 2005; Scherma *et al.*, 2008b). To evaluate the effects of AM404 and THC on the development of nicotine-induced CPP, AM404 (5 $mg \cdot kg^{-1}$, i.p.) or THC (1 $mg \cdot kg^{-1}$) or vehicles were injected in the home cage, 30 min before saline or nicotine injection. The 5 $mg \cdot kg^{-1}$ dose of AM404 was selected for testing in combination with nicotine based on our results that this dose of AM404 was the maximal dose of AM404 that did not induce CPP under our conditions. The 1 $mg \cdot kg^{-1}$ dose of THC was selected from the range of THC doses studied for development of CPP (0.1 to 3 $mg \cdot kg^{-1}$, i.p.) because we have previously found that 1 $mg \cdot kg^{-1}$ THC produces reliable discriminative stimulus effects in rats without the depressant effects on behaviour that may occur with the higher 3 $mg \cdot kg^{-1}$ dose (Solinas *et al.*, 2007).

*Test.* The day after the last conditioning session, a test session was conducted in which the animals did not receive any drug or vehicle treatment. During the test session, the door separating the two compartments was raised and the animals were placed at the intersection of the two compartments, with access to both compartments. Time spent by the animal in each of the compartments during a 15 min period was recorded.

### Nicotine-induced reinstatement of extinguished CPP

After development of CPP with a nicotine dose of 0.4 $mg \cdot kg^{-1}$, s.c., with the same procedure as described above, 20 min extinction sessions were conducted twice a day for 8 days, using the same procedure as in conditioning sessions, except that saline was administered instead of nicotine. An extinction test was then conducted in the same manner as the earlier test session. Animals that did not meet the extinction criterion (preference for compartment previously associated with nicotine reduced by 80% or more), were removed from the study. One day after the extinction test, a 15 min reinstatement test was then conducted. Rats were randomly divided into groups and were given a priming injection of either, nicotine (0.1 $mg \cdot kg^{-1}$, s.c., immediately before the session), AM404 (5 $mg \cdot kg^{-1}$, i.p., 30 min before the session) or AM404 plus nicotine (30 min and immediately before the session respectively) and were then allowed to explore both compartments; time spent in each compartment was recorded.

### Locomotor activity

Eight rectangular (41 × 41 × 32 cm), transparent Plexiglas chambers, each with a 16 × 16 array of photocells, were enclosed in sound attenuated cubicles. Rats were acclimatized to the experimental room for 1 h before the start of the experiment. Rats were then pretreated with vehicle, THC (1 or 3 $mg \cdot kg^{-1}$, i.p.), or AM404 (5 or 10 $mg \cdot kg^{-1}$, i.p.), and 30 min later injected with saline or nicotine (0.05 or 0.4 $mg \cdot kg^{-1}$, s.c.). Immediately after the last injection each rat was placed in the centre of the apparatus, and activity was measured for 20 min. After each session, the apparatus was cleaned with water, and clean bedding was placed on the

floor. The software (Med Associates, East Fairfield, VT, USA) provided measures of distance travelled and time spent in the centre zone, defined as the central area occupying 43% of the apparatus floor.

## In vivo *microdialysis*

Apparatus and procedure were the same as described previously (Fadda *et al.*, 2003). Sprague-Dawley rats were surgically implanted with a concentric dialysis probe aimed at the shell of the nucleus accumbens [anterior +2.0 and lateral 1.1 from bregma, vertical –7.9 from dura, according to the atlas by Paxinos and Watson (1998)] and dialysate samples were collected every 20 min and immediately analysed by an HPLC system coupled to electrochemical detection. Rats were treated only after dopamine values (<10% variability) were stable for at least three consecutive samples. AM404 (5 mg·kg$^{-1}$, i.p.) or its vehicle were injected 40 min before saline or nicotine (0.4 mg·kg$^{-1}$, s.c.). Only rats with correct probe placement were included in the study.

## *Statistical analysis*

All results are presented as group means (±SEM). Place-conditioning data were analysed by one-way ANOVA. For the locomotor activity experiment, distance travelled and centre zone data were analysed in separate one-way ANOVAs. The percentage of total session time spent in the centre zone, which was recorded as a measure of anxiety, was arcsine-root transformed for analysis. One rat was excluded from the analyses of locomotor activity as an outlier. Microdialysis data were analysed using two-way ANOVA. *Post hoc* comparisons, when appropriate, were performed by Student–Newman–Keuls test for CPP and microdialysis data and Dunnett's *post hoc* test (comparing each condition to vehicle control) for locomotor activity data. In all cases, differences with a $P < 0.05$ were considered significant.

## *Drugs*

Nicotine [(-)-nicotine hydrogen tartrate salt] (Sigma Chemical Company, St. Louis, MO, USA or Sigma, Italy) was diluted in saline (adjusted to pH 7.0) and injected s.c. AM404 (synthesized in the laboratory of Dr Alex Makriyannis at the Center for Drug Discovery, Northeastern University, Boston, MA, USA) and THC (NIDA Research Resources Drug Supply Program, Bethesda, MD, USA) were dissolved in a vehicle containing Tween 80 2%, ethanol 2%, and saline and injected i.p. All drugs were injected in a volume of 1.0 mL·kg$^{-1}$.

# Results

## *Effects of AM404 and THC on development of CPP*

When different doses of AM404 (1.25 to 10 mg·kg$^{-1}$, i.p.) were examined in the CPP test, only the high dose of 10 mg·kg$^{-1}$ was able to induce a significant CPP (one-way ANOVA $F_{9,96}$ = 3.985, $P = 0.0002$; Newman–Keuls Multiple Comparison Test $P < 0.05$) (Figure 1A). In contrast, administration of different doses of THC (0.01 to 3 mg·kg$^{-1}$, i.p.) did not produce any significant CPP (one-way ANOVA $F_{9,90}$ = 0.3378, $P$ = ns) (Figure 1B).

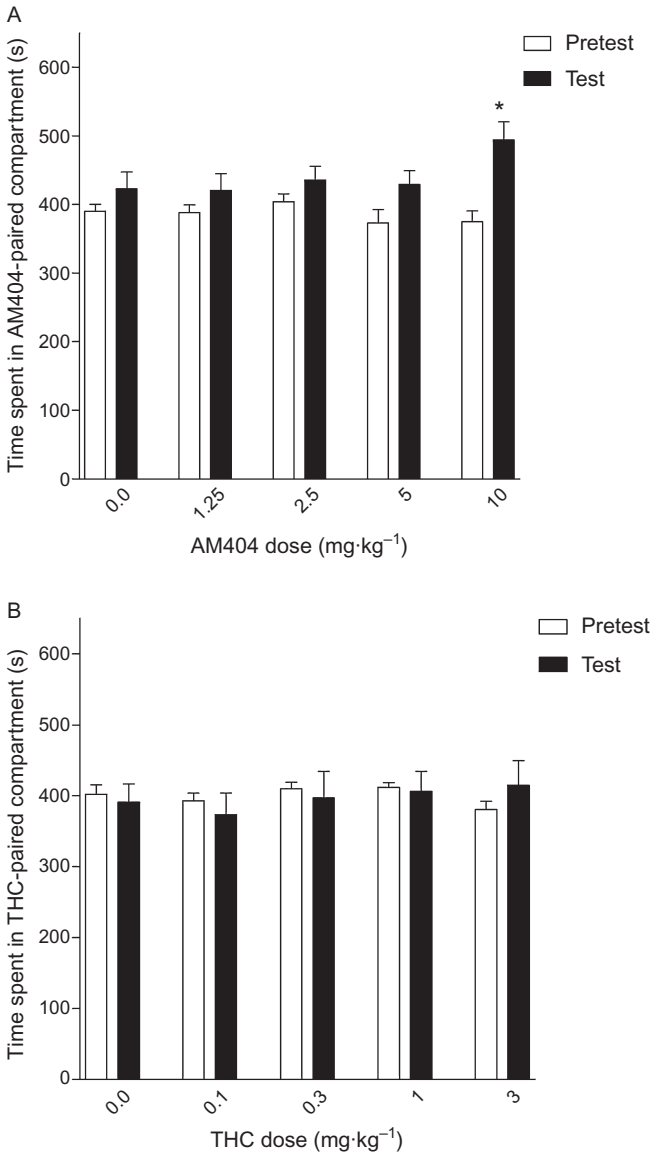
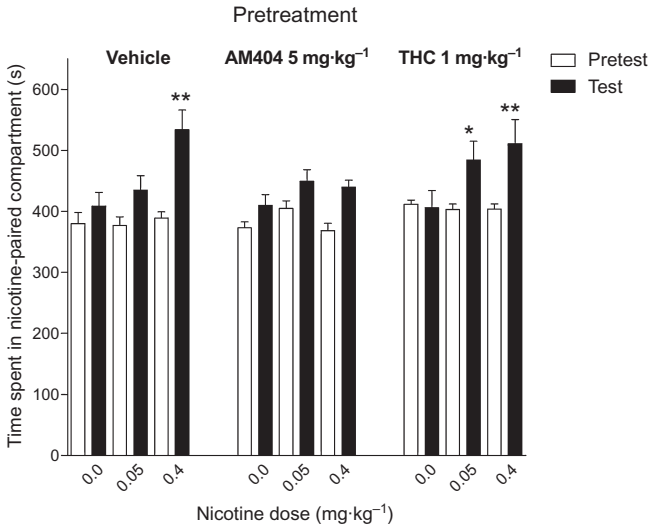



**Figure 1**

Effects of AM404 and THC on CPP. (A) When different doses of AM404 (1.25 to 10 mg·kg$^{-1}$, i.p.) were examined in the CPP test, only the higher dose of 10 mg·kg$^{-1}$ was able to induce a significant CPP ($P < 0.05$ vs. corresponding pretest). (B) THC alone (0.1 to 3 mg·kg$^{-1}$) produced no significant CPP or aversion compared with the vehicle group ($P$ = ns). All data are expressed as time spent in s (mean ± SEM, $n$ = 9–10) in the nicotine-paired compartment during a15 min period. Pretest sessions and 15 min Test sessions and were analysed by one-way ANOVA between subjects.

## *Effects of AM404 and THC on development of nicotine-induced CPP*

Consistent with our previous findings using this place-conditioning paradigm (Le Foll and Goldberg, 2005; Scherma




## Figure 2

Effects of AM404 and THC on development of nicotine-induced CPP. A nicotine dose of 0.4 mg·kg$^{-1}$ produced significant CPP (**$P$ < 0.001 vs. corresponding pretest; $n$ = 9–10). When rats were pretreated with AM404 (5 mg·kg$^{-1}$) before each nicotine conditioning session, CPP did not develop ($P$ = ns; $n$ = 9–10) and AM404 did not potentiate effects of a low 0.05 mg·kg$^{-1}$ ineffective threshold dose of nicotine. When rats were pretreated with THC (1 mg·kg$^{-1}$) before each nicotine conditioning session, nicotine-induced CPP was not reduced (**$P$ < 0.01 vs. corresponding pretest; $n$ = 9–10) and THC potentiated the effects of a low 0.05 mg·kg$^{-1}$ dose of nicotine (*$P$ < 0.05 vs. corresponding pretest; $n$ = 9–10). All data are expressed as time spent in s (mean ± SEM) in the nicotine-paired compartment during a 15-min period. Pretest session and 15 min Test session and were analysed by one-way ANOVA between subjects.

*et al.*, 2008b), we found that a nicotine dose of 0.4 mg·kg$^{-1}$ (s.c.) induced a significant CPP (one-way ANOVA $F_{5,50}$ = 7.619, $P$ < 0.0001; Newman–Keuls Multiple Comparison Test $P$ < 0.001) in Sprague-Dawley rats (Figure 2), while THC did not induce a significant CPP (Le Foll *et al.*, 2006). However, when rats were pretreated with a 5 mg·kg$^{-1}$ (i.p.) dose of AM404 before each nicotine conditioning session, nicotine-induced CPP did not develop (one-way ANOVA $F_{5,50}$ = 1.705, $P$ = ns) (Figure 2). Importantly, AM404 did not potentiate the effects of a subthreshold dose of nicotine (0.05 mg·kg$^{-1}$), which alone did not induce CPP. In contrast to results with AM404, when rats were pretreated with a 1 mg·kg$^{-1}$ (i.p.) dose of THC before each nicotine conditioning session, nicotine-induced CPP was not affected. In addition, THC potentiated the effects of a subthreshold dose of nicotine (0.05 mg·kg$^{-1}$), which failed to induce a significant CPP when tested alone but induced a significant CPP after pretreatment with THC (one-way ANOVA $F_{5,48}$ = 3.837, $P$ = 0.0053; Newman–Keuls Multiple Comparison Test $P$ < 0.01 and $P$ < 0.05) (Figure 2).

### *Effects of AM404 on nicotine-induced reinstatement of extinguished CPP*

After CPP had been abolished by substituting saline for nicotine, a priming injection of nicotine (0.1 mg·kg$^{-1}$, s.c.) completely reinstated the abolished CPP (one-way ANOVA $F_{3,32}$ = 21.51, $P$ < 0.0001; Newman–Keuls Multiple Comparison Test $P$ < 0.001) (Figure 3A). Pretreatment with AM404 (5 mg·kg$^{-1}$, i.p.) prevented this nicotine-induced reinstatement (one-way ANOVA $F_{3,36}$ = 5.225, $P$ = 0.0043; Newman–Keuls Multiple Comparison Test $P$ > 0.05) (Figure 3B). In addition, AM404 (5 mg·kg$^{-1}$) by itself did not reinstate the attenuated CPP (one-way ANOVA $F_{3,24}$ = 12.33, $P$ < 0.0001; Newman–Keuls Multiple Comparison Test $P$ > 0.05) (Figure 3C).

### *Effects of nicotine, AM404 and THC on locomotor activity*

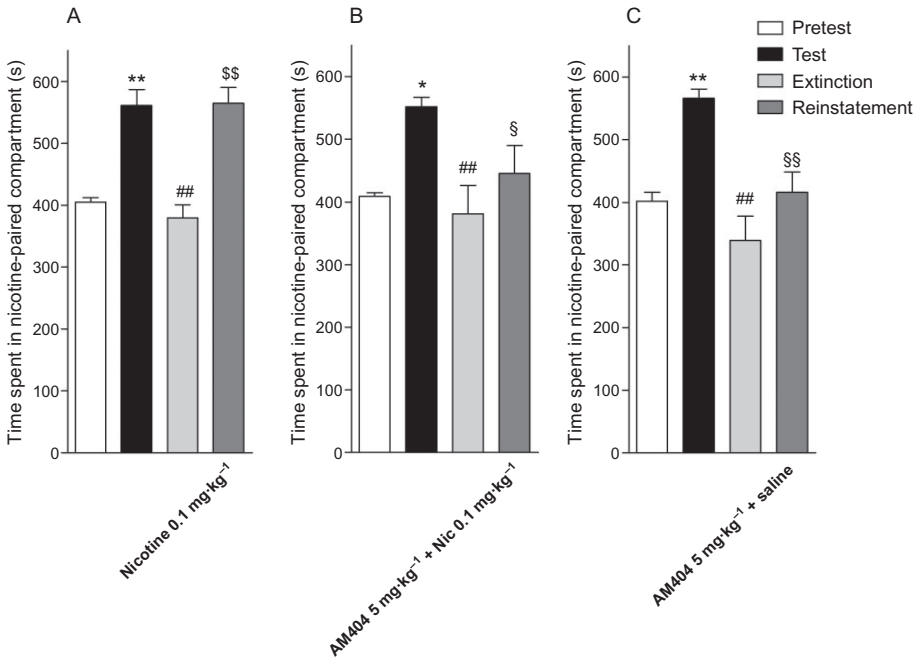
Groups that received 0.4 mg·kg$^{-1}$ nicotine moved less than the groups injected with saline (one-way ANOVA $F_{10,76}$ = 3.8479, $P$ < 0.01; Dunnett's *post hoc* vs. Veh-Sal: Veh-Nic 0.4, $P$ < 0.05; AM404 5-Nic 0.4, $P$ < 0.01; THC 1-Nic 0.4, $P$ < 0.01) (Figure 4A) and also spent more time in the centre zone (one-way ANOVA $F_{10,77}$ = 4.327, $P$ < 0.01; Dunnett's *post hoc* test Veh-Nic 0.4, $P$ < 0.05; AM404 5-Nic 0.4, $P$ < 0.01; THC 1-Nic 0.4, $P$ < 0.05) (Figure 4B), suggesting an axiolytic effect. Administration of AM404 or THC alone did not significantly influence either measure of locomotor activity. AM404 and THC did not modify the locomotor suppressant or anxiolytic effects of 0.4 nicotine, but the combination of the low dose of THC (1 mg·kg$^{-1}$) with the low dose of nicotine (0.05 mg·kg$^{-1}$) significantly decreased distance travelled (Dunnett's *post hoc* vs. Veh-Sal: $P$ < 0.01).

### *Effects of AM404 on nicotine-induced elevations in dopamine levels in the nucleus accumbens shell*

*In vivo* microdialysis experiments showed that nicotine (0.4 mg·kg$^{-1}$, s.c.) increased extracellular levels of dopamine in the nucleus accumbens shell by about 80%, compared with basal levels (one-way ANOVA $F_{8,36}$ = 7.626, $P$ < 0.0001). Administration of AM404 (5 mg·kg$^{-1}$) reduced nicotine-induced elevations in dopamine levels. Two-way ANOVA showed a significant effect of AM404 treatment ($F_{1,88}$ = 10.26, $P$ = 0.0019). Importantly, AM404 (5 mg·kg$^{-1}$) alone did not alter dopamine levels in the nucleus accumbens shell (Figure 5).

## Discussion and conclusions

The present study shows that the AEA transport inhibitor AM404 prevented development of nicotine-induced CPP and blocked nicotine-induced reinstatement of attenuated place-preference. Furthermore, AM404 reduced nicotine-induced increases in extracellular dopamine levels in the nucleus accumbens shell, the terminal area of the brain's mesolimbic reward system. These alterations in nicotine's reward-related effects by AM404 were not associated with changes in nicotine's locomotor or anxiety-related effects in the open field. Our findings with AM404 are similar to our earlier findings with the FAAH inhibitor URB597 (Scherma *et al.*, 2008b). However, URB597 appears to modulate the rewarding effects of nicotine primarily by elevating levels of OEA and PEA (Melis *et al.*, 2008; Mascia *et al.*, 2011), which are structurally similar to AEA but do not bind to or activate cannabinoid receptors (Fegley *et al.*, 2005) and, instead, are endogenous ligands for PPAR-α (Fu *et al.*, 2003; Astarita *et al.*, 2006). AM404 adminis-




## Figure 3

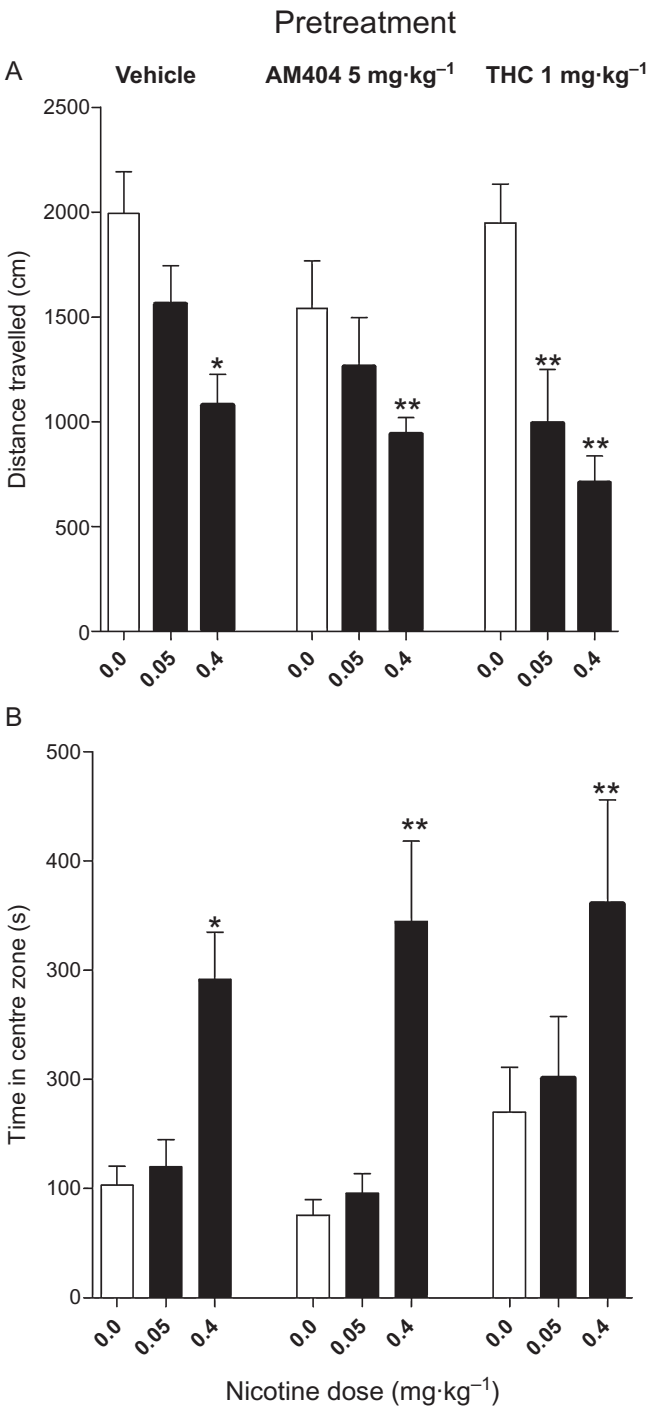
Effects of AM404 on nicotine-induced reinstatement of abolished CPP. (A) A priming injection of nicotine (0.1 mg·kg$^{-1}$) completely reinstated the attenuated CPP (extinction) developed by a nicotine dose of 0.4 mg·kg$^{-1}$ (**$P$ < 0.001 Test vs. Pretest; ##$P$ < 0.001 Extinction vs. Test; $$$P$ < 0.001 Reinstatement vs. Extinction; $P$ > 0.05 Reinstatement vs. Test; $n$ = 9). (B) AM404 pretreatment prevented nicotine-induced reinstatement of the CPP (*$P$ < 0.05 Test vs. Pretest; ##$P$ < 0.01 Extinction vs. Test; $P$ > 0.05 Reinstatement vs. Extinction; §$P$ < 0.05 Reinstatement vs. Test; $n$ = 10). (C) AM404 alone failed to reinstate the attenuated CPP (**$P$ < 0.001 Test vs. Pretest; ##$P$ < 0.001 Extinction vs. Test; $P$ > 0.05 Reinstatement vs. Extinction; §§$P$ < 0.001 Reinstatement vs. Test; $n$ = 7). All data are expressed as time spent in s (mean ± SEM) in the nicotine-paired compartment during a 15 min period. Pretest sessions, 15 min Test sessions, 15 min Extinction sessions and 15 min Reinstatement sessions and were analysed by one-way ANOVA between subjects.

tration increases brain levels of AEA, but it does not significantly affect brain levels of OEA or PEA (Fegley *et al.*, 2004; Bortolato *et al.*, 2006). Thus, the inhibitory effects of AM404 on nicotine reward are probably due to its ability to increase AEA levels, rather than OEA or PEA levels, in the brain.

Although AM404 is generally considered an AEA uptake inhibitor, several reports show that it can also act as an inhibitor of 2-arachidonoylglycerol (2-AG), the other main endocannabinoid in the brain (Bisogno *et al.*, 2001; Hajos *et al.*, 2004; Di *et al.*, 2005). Evidence suggests that AEA and 2-AG share the same membrane transport mechanism, to the extent that several authors define the process as endocannabinoid uptake (or transport) rather than simply AEA uptake. Thus, 2-AG may have contributed to the effects we observed with AM404. Also, AEA binds to cannabinoid $CB_1$ receptors with high affinity, but also acts on $CB_2$ receptors and may have non-cannabinoid-mediated effects, notably through the transient receptor potential vanilloid type 1 (TRPV1; Zygmunt *et al.*, 2000). The present experiments do not provide information on which of these mechanisms mediate AM404's effects on nicotine-induced development of CPP and its reinstatement. Activation at $CB_1$, $CB_2$ and TRPV1 are all possible mechanisms and must be examined in future experiments through the use of selective antagonists. Finally, AEA has nonspecific effects on calcium signalling and cell proliferation (Chen *et al.*, 2001; Jonsson *et al.*, 2003; Kelley and Thayer, 2004).

Blockade of the development of nicotine-induced place preference and of its reinstatement after extinction by AM404 was not due to a motor depressant effect of AM404 and does not appear related to an AM404-induced decrease in general motivational state, as AM404 was not effective in reducing activity in the open field and did not alter time spent in the centre of the open field, a measure of anxiety. Also, AM404 produced a selective blockade of reward-related effects of nicotine, as it was not effective in altering the effects of nicotine in the open field. Interestingly, Cippitelli *et al.* (2007) found that AM404 reduced alcohol self-administration in rats but did not affect the reinstatement of alcohol seeking, suggesting that the role AEA uptake inhibitors play on drug reward may be specific to nicotine.

In contrast to the effects of AM404, the directly acting cannabinoid $CB_1/CB_2$ receptor agonist THC potentiated rather than reduced the rewarding effects of nicotine in rats under the present CPP procedure and potentiated the locomotor suppressive effects of nicotine in the open field. These data are consistent with a previous finding by Valjent *et al.* (2002), who showed that co-administration of subthreshold doses of THC and nicotine (i.e. doses that were ineffective when administered alone) induced significant CPP when administered together. In addition, we have recently shown that the synthetic $CB_1/CB_2$ receptor agonist WIN55,212-2 increases responding for nicotine under a progressive-ratio schedule of i.v. nicotine self-administration in rats and



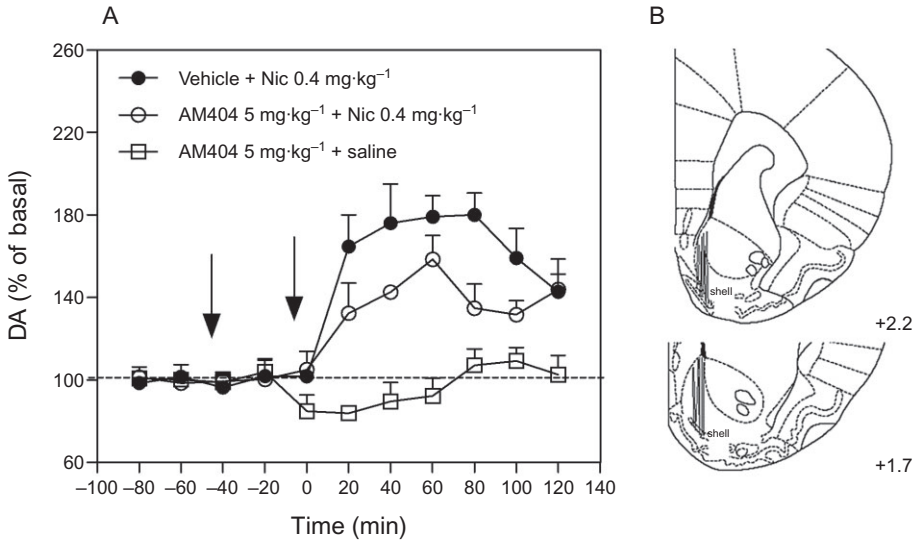

## Figure 4

Effects of nicotine, AM404 and THC on locomotor activity. (A) Distance travelled, expressed in cm. (B) Amount of time spent in the centre zone during the 20 min session, as a measure of anxiety. (*$P < 0.05$; **$P < 0.01$; all comparisons vs. Vehicle-saline condition). Nicotine (0.4 mg·kg$^{-1}$) decreased general activity (distance travelled) and increased time spent in the centre zone, which may indicate an anxiolytic effect. Pretreatment with AM404 or THC did not alter the effects of 0.4 mg·kg$^{-1}$ nicotine, but general activity was decreased by the combination of 1 mg·kg$^{-1}$ THC with 0.05 mg·kg$^{-1}$ nicotine.

enhances nicotine-seeking (Gamaleddin *et al.*, 2011b). Systemically administered cannabinoid $CB_1/CB_2$ receptor agonists like THC and WIN55,212-2 affect signalling at cannabinoid receptors globally, wherever cannabinoid receptors occur in the brain. In contrast, inhibition of AEA transport by AM404 is a more selective way to alter activity of the endocannabinoid system. AEA is synthesized on demand and only affects cannabinoid receptors located in activated synapses (Marsicano *et al.*, 2003; Pertwee, 2005; Di Marzo and Petrosino, 2007). Thus, unlike directly acting cannabinoid agonists, the effects of AEA transport inhibition are functionally selective, increasing the actions of AEA only when and where there is a demand for it (Piomelli, 2003; Pertwee, 2005; Solinas *et al.*, 2008). The selectivity of AM404 versus cannabinoid agonists like THC and WIN55,212-2 may also be due to the fact that it enhances only one of the endocannabinoid signals, the AEA signal, resulting in a functional selectivity, which in turn might result in regional differences, with certain regions of the brain accumulating more AEA than others (Bortolato *et al.*, 2006).

Although the mechanism through which AM404 exerts its effects on nicotine reward was not fully addressed in our study, previous experiments by Gonzalez *et al.* (2002) showed that chronic treatment with nicotine increases AEA, but not 2-AG, levels in the limbic forebrain, a key brain region for reward. Thus AEA, when protected from internalization by AM404, could activate cannabinoid receptors on presynaptic nerve endings of glutamatergic neurons in the VTA (Melis *et al.*, 2004), reducing glutamate release from these neurons, and thereby reducing activation of dopaminergic neurons in the VTA and the release of dopamine from their terminals in the nucleus accumbens shell. This hypothesis is supported by the present finding that AM404 is able to reduce nicotine-induced dopamine elevations in the shell of the nucleus accumbens. An elevation of dopamine levels in the nucleus accumbens is considered central for the reinforcing effects of many abused drugs, including nicotine (Pontieri *et al.*, 1996; Koob, 2000; Di Chiara, 2002; Wise, 2004; Everitt and Robbins, 2005). An alternative mechanism is that AEA transport is bidirectional and is involved in both the release and re-uptake of endocannabinoids (Ligresti *et al.*, 2004). Previous work by Ronesi *et al.* (2004) and others (Ligresti *et al.*, 2004; Straiker and Mackie, 2005; Pillolla *et al.*, 2007) shows that the acute actions of AEA transport inhibitors can actually result in a blockade of endocannabinoid-mediated effects, in a manner similar to that of cannabinoid receptor inverse agonists/antagonists, possibly because of the disruption of endocannabinoid release. It is well known that cannabinoid $CB_1$ inverse agonists/antagonists, such as rimonabant, can prevent both the acquisition and the expression of nicotine-induced CPP in rats (Le Foll and Goldberg, 2004; Forget *et al.*, 2005) and can block nicotine-induced elevations in dopamine levels in the nucleus accumbens shell in rats (Cohen *et al.*, 2002).

The present findings are limited by the fact that only one AEA uptake inhibitor was tested. However, the fact that the present findings with AM404 are similar to recent findings with the structurally related AEA uptake inhibitor VDM11 (which lacks TRPV activity) on maintenance and reinstatement of i.v. nicotine self-administration behaviour in rats (Gamaleddin *et al.*, 2011a) provides some general validity to




## Figure 5

Effects of AM404 on nicotine-induced elevations in dopamine levels in the nucleus accumbens shell. (A) Pretreatment with AM404 (5 mg·kg$^{-1}$), but not its vehicle, given before nicotine (0.4 mg·kg$^{-1}$), significantly reduced the increase in extracellular dopamine levels produced by nicotine (two-way ANOVA $P = 0.0019$, $n = 5$). AM404 alone did not alter dopamine levels. Arrows represent time of injection of each drug. Results are means, with vertical bars representing SEM, of dopamine levels in 20 min dialysate samples, expressed as a percentage of basal values. (B) Verification of microdialysis probe location by histology. Vertical lines indicate the placement of microdialysis probes within brain sections (as modified from Paxinos and Watson, 1998) in the shell of the nucleus accumbens. Numbers beside each plate represent distance from bregma.

our findings and suggests that elevating endocannabinoid levels could be a novel therapeutic strategy for preventing relapse to nicotine use. It has also been reported that AM404 is a FAAH substrate and can reduce the metabolism of AEA, presumably as a result of substrate competition, if not direct FAAH inhibitor properties (Jarrahian *et al.*, 2000; Vandevoorde and Fowler, 2005). This property is shared by VDM11, but other AEA uptake inhibitors such as UCM707 and OMDM-11 are reportedly weaker FAAH inhibitors. Further studies evaluating the ability of other AEA uptake inhibitors, such as UCM707 and OMDM-11 are needed.

In conclusion, our results suggest that AEA transport inhibitors, such as AM404, can counteract the addictive effects of nicotine. This therapeutic behavioural effect of AM404 may be because of an indirect blockade of nicotine-induced increases in dopaminergic transmission in the nucleus accumbens shell, the terminal area of the brain's mesolimbic dopamine system, which is intimately involved in mediation of the rewarding effects of abused drugs. Thus, AEA transport serves as a new target for development of medications for treatment of tobacco dependence.

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## Conflicts of interest

None.

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