Heteromeric nicotinic receptors are involved in the sensitization and addictive properties of MDMA in mice

Andrés Ciudad-Roberts, Jorge Camarasa, David Pubill* and Elena Escubedo

Department of Pharmacology and Therapeutic Chemistry (Pharmacology Section) and Institute of Biomedicine (IBUB), University of Barcelona, Barcelona, Spain

Author for correspondence:

David Pubill

Department of Pharmacology and Therapeutic Chemistry,
Faculty of Pharmacy,
University of Barcelona,
Avda. Joan XXIII s/n
Barcelona 08028, Spain.
Tel: +34-934024531

Fax: +34-934035982

E-mail: d.pubill@ub.edu

Short Title: Nicotinic receptors and MDMA rewarding effects.

Abstract

We have investigated the effect of nicotinic receptor ligands in the behavioral sensitization (hyperlocomotion) and rewarding properties (conditioned place preference paradigm, CPP) of 3,4-methylenedioxy-methamphetamine (MDMA) in mice. Each animal received intraperitoneal pretreatment with either saline, dihydro-β-erythroidine (DHβE, 1 mg/Kg) or varenicline (VAR, 0.3 mg/Kg), 15 min prior to subcutaneous saline or MDMA (5 mg/Kg), for 10 consecutive days. On day 1, both DHβE and VAR inhibited the MDMA-induced hyperlocomotion. After 10 days of treatment, MDMA induced a hyperlocomotion that was not reduced (rather enhanced) in antagonist-pretreated animals. This early hyperlocomotion was accompanied by a significant increase in heteromeric nicotinic receptors in cortex that was not blocked by DHβE or VAR. Behavioral sensitization to MDMA was highest 2 weeks after the discontinuation of MDMA treatment. This additional increase in sensitivity was prevented in animals pretreated with DHβE or VAR. At this time, MDMA-treated mice showed a significant increase in heteromeric receptors in cortex that was prevented by DHβE and VAR. An involvement of α7 nicotinic receptors in this effect is ruled out.

MDMA (10 mg/Kg) induced positive CPP that was abolished by DHβE (2 mg/Kg) and VAR (2 mg/Kg). Moreover, chronic nicotine pretreatment (2 mg/Kg, ip, b.i.d., for 14 days) caused MDMA, administered at a low dose (3 mg/kg), to induce CPP, which would otherwise not occur. Finally, present results point out that heteromeric nicotinic receptors are involved in locomotor sensitization and addictive potential induced by MDMA. Thus, varenicline might be a useful drug to treat both tobacco and MDMA abuse at once.

Key words: MDMA, nicotinic receptors, sensitization, addiction

1. Introduction

MDMA[1] is a synthetic drug that has properties of both stimulants and hallucinogens. Compared to other amphetamine derivatives, MDMA triggers a larger increase in serotonin and a smaller increase in dopamine release (Johnson et al., 1986). The behavioral and neurochemical adaptations related to chronic MDMA treatment are largely unknown. For instance, an increase in the functionality of cortical 5-HT_{2A} and a decrease in striatal D_2 receptors in mice treated with MDMA have been described (Varela et al., 2011). Many drugs of abuse, at low doses, can increase motor behavior producing heightened locomotion and exploration (Wise and Bozarth, 1987) and, after repeated administration, behavioral sensitization can arise from various neuroadaptations in multiple brain nuclei. This is not only the result of distinct molecular targets for the drugs, but may also include a differential involvement of learned associations. It is postulated that the relatively more robust pharmacological capacity of amphetamine derivatives to release dopamine may induce a form of sensitization that is more dependent on adaptations in mesoaccumbens dopamine transmission in comparison to cocaine and morphine sensitization (Vanderschuren and Kalivas, 2000).

There is evidence that acetylcholine plays an important role in the hyperlocomotor activity induced by psychostimulants (Williams and Adinoff, 2008). Dihydro-β-erythroidine (DHβE), a high-affinity competitive antagonist of α4 subunit-containing nAChR (nicotinic acetylcholine receptor) inhibits the induction of locomotor sensitization to d-amphetamine (Karler et al., 1996; Schoffelmeer et al., 2002). Moreover, knockout mice lacking the β2 nAChR subunit do not self-administer nicotine (Picciotto et al., 1999) and show less cocaine-conditioned place preference than wild-type mice (Zachariou et al., 2001). All of these results indicate that heteromeric α4β2 nAChR subtypes appear to play an essential role in nicotine dependence (Govind et al., 2009); in this regard, an activation of α4β2 nAChR is strongly associated with dopamine release in the nucleus accumbens (NAcc) (Champtiaux et al., 2003) and with drug-seeking behavior (Balfour et al., 2000; Picciotto et al., 1999). A particular feature of nAChR is that chronic exposure to nicotine and other nicotinic ligands induces a higher level of epibatidine binding (up-regulation) that can lead to an increase in

[1] *Abbreviations:* AUC, area under the curve; CPP, conditioned place preference; DHβE, dihydro-β-erythroidine; MDMA, 3,4-methylenedioxy-methamphetamine; MLA, methyllycaconitine; NAcc, nucleus accumbens; nAChR, nicotinic acetylcholine receptors; VAR, varenicline; VTA, ventral tegmental area.

receptor function (functional up-regulation) (reviewed by Gaimarri et al., 2007). Therefore, the up-regulation of heteromeric nAChR could, via dopamine release, explain the reinforcing effect of nicotine on the mesolimbic system mediating nicotine addiction (Balfour et al., 2000).

Studies examining the interactions between nAChR and psychostimulant drugs have focused primarily on d-amphetamine and cocaine but it is unclear whether such findings can be extended to other psychostimulants. Previous results from our group (for a review see Pubill et al., 2011) have demonstrated that nAChR are a pharmacological target for both methamphetamine and MDMA and are involved in some actions of these drugs of abuse such as analgesia or locomotor activity (Camarasa et al., 2009), tumor necrosis factor alpha suppression (Camarasa et al., 2010) and neurotoxicity (Chipana et al., 2008b; 2008c; Escubedo et al., 2009). We have described the direct and specific interaction of MDMA with α7 and α4β2 nAChR in mouse brain membranes and cultured PC12 cells (García-Ratés et al., 2007). The interaction with nAChR occurs at low micromolar concentrations that can be reached in the mammalian central nervous system after its administration (Chipana et al., 2008a). Also, similarly to nicotine, MDMA induces nAChR up-regulation in PC12 cells and in rat brain, where it also potentiates the regulatory effects of nicotine (García-Ratés et al., 2007; Pubill et al., 2013).

MDMA's interaction with nAChR might account for some clinical features of this drug such as fasciculation and muscle cramps, which occur especially in MDMA abusers after high-dose intake (Klingler et al., 2005). Moreover, tobacco is one of the most widely consumed drugs and MDMA abusers very often smoke (Scholey et al., 2004); thus, a pharmacodynamic interaction between nicotine and MDMA can be expected and could have several consequences that will be suggested at a later point in this text.

This study was undertaken to determine whether nAChR are involved in the behavioral sensitization and addictive potential of MDMA. DHβE (antagonist) and varenicline (partial α4β2 nAChR agonist and full α7 nAChR agonist; Mihalak et al., 2006; Rollema et al., 2007) were associated with MDMA in order to investigate the involvement of heteromeric nAChRs on its effects. Also, the effect of a chronic pretreatment with nicotine on MDMA addictive effects was investigated. We focused

on the locomotor hyperactivity induced by MDMA as an indicator of its psychostimulant effect and on the conditioned place preference (CPP) paradigm to assess its addictive properties. Also, we investigated the changes in the density of homomeric and heteromeric nAChRs in determined brain areas as a possible consequence of the treatment that could be related with the observed behavioural effects.

2. Material and Methods.

2.1. Animals and treatment groups

Data were collected from adult male Swiss CD-1 mice (Charles River, Barcelona, Spain) weighing 24 to 30 g at the beginning of the experiments (first drug administration). They were housed three per cage under standard laboratory conditions (21 ± 1 ºC room temperature and a 12-h light/dark cycle from 8:00 am to 8:00 pm). Animals had free access to food (standard laboratory diet, PANLAB SL, Barcelona, Spain) and drinking water. All experimental procedures were conducted between 9:00 am and 5:00 pm and were in compliance with the guidelines of the European Community Council (86/609/EEC) and approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia. Efforts were made to minimize suffering and reduce the number of animals used.

In our experiments we administered MDMA at doses closely related to its recreational use in humans rather than at high doses that would lead to neurotoxic effects.

Mice were assigned randomly to one of six treatment groups: Saline (saline i.p. + saline s.c.), MDMA (saline i.p. + MDMA s.c.), DHβE (DHβE i.p. + saline s.c.), DHβE+MDMA (DHβE i.p. + MDMA s.c.), VAR (saline i.p. + varenicline s.c.), VAR+MDMA (varenicline i.p. + MDMA s.c.). Doses and schedule are detailed below.

Prior to experimentation, all of the animals received two habituation sessions (48 and 24 h before testing) that were intended to reduce the novelty and stress associated with handling and injection.

2.2. Drugs

Drugs and reagents were obtained from the following sources: 3,4-methylenedioxymethamphetamine hydrochloride was provided by the National Health Laboratory (Barcelona, Spain). Varenicline was a gift from Pfizer Laboratories (New York, USA). Aprotinin, DHβE, methyllycaconitine (MLA), nicotine bitartrate dihydrate, phenylmethylsulfonyl fluoride and sodium orthovanadate were purchased from Sigma–Aldrich (St. Louis, MO, USA). [^{3}H]epibatidine was from PerkinElmer (Boston, MA, USA), while [^{3}H]MLA came from American Radiolabeled Chemicals (St. Louis, MO, USA). Drugs were dissolved in saline (NaCl 0.9%). All other reagents were of analytical grade

2.3. Locomotor Activity

This test was used to assess the psychostimulant effects of MDMA along the treatment and its modulation by nicotinic drugs.

2.3.1. Drug treatment

According to its treatment group allocation, each animal received pretreatment with either saline (5 ml/Kg), DHβE (1 mg/Kg) or varenicline (0.3 mg/Kg), given intraperitoneally, 15 min prior to saline or MDMA (5 mg/Kg), given subcutaneously, for 10 consecutive days. These doses were chosen based on previous reports (Camarasa et al., 2009; Kim et al., 2011). We administered MDMA at a 5 mg/Kg dose because, although it is relatively low, it induces robust behavioral activation (Ball et al., 2009). Once the 10-day repeated treatment phase was completed, all of the animals remained in their home cages for a 14-day drug-free period (days 11-24). On day 25, all of the mice were accordingly challenged with either a dose of saline or DHβE or varenicline plus saline or MDMA to assess for conditioned hyperactivity. Locomotor activity was measured on days 1, 10 and 25. To evaluate the development of behavioral sensitization we compared data from day 1 *vs* day 10 or day 25 of the same group .

2.3.2. Measurement

On the different testing days and immediately after the i.p. injection (saline or MDMA), the mice were placed in a plexiglas cage. This cage constituted the activity box that was placed inside a frame system of two sets of 16 infrared photocells (LE8811, PANLAB SL, Barcelona, Spain) mounted according to the x, y axis coordinates and 1.5 cm above the wire mesh floor. The registration of horizontal locomotor activity then began. Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, PANLAB SL, Barcelona, Spain). The interruption counts (beam breaks), in a 10-min block, were used as a measure of horizontal locomotor activity. The locomotor activity of each mouse was monitored over 180 min. All experiments were conducted between 9:00 am and 3:00 pm. Results are expressed as cumulative breaks per mouse for 180 min or as AUC (area under the curve), which was measured as the total changes from baseline at each recording interval over the total measuring time.

2.4. Radioligand binding experiments

2.4.1. Tissue Sample Preparation

Six hours after the challenge with MDMA on day 10 or on day 25, 5-6 animals per group were killed by cervical dislocation, then decapitated and the brains rapidly removed from the skull. Cortex, striata and hippocampus were quickly dissected out, frozen on dry ice and stored at -80 ºC until use. When required, tissue samples were thawed and homogenized at 4 ºC in 10 volumes of buffer consisting of 5 mM Tris-HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 mg/ml, 0.1 mM PMSF and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at 15,000 × g for 30 min at 4 °C. The pellets were resuspended in fresh buffer and incubated at 37 °C for 10 min to remove endogenous neurotransmitters. The protein samples were subsequently re-centrifuged and washed two additional times. The final pellets (crude membrane preparations) were resuspended in 50 mM Tris–HCl buffer plus protease inhibitors and stored at −80 °C until later use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent (Bio-Rad Labs. Inc., Hercules, CA, USA), according to the manufacturer's instructions.

2.4.2. [^{3}H]Epibatidine Binding.

[^{3}H]Epibatidine binding was used to label heteromeric nAChR, which in CNS are mainly α4β2. Binding of [^{3}H]epibatidine to brain membranes from cortex and striatum was measured as described previously (Chipana et al., 2008b). Briefly, experiments were carried out in glass tubes containing 1 nM [^{3}H]epibatidine (55.5 Ci/mmol)—at this concentration primarily α4β2 receptors are labeled (Avila et al., 2003)—and incubation was carried out for 3 h at 25 °C. The incubation buffer was 50 mM Tris-HCl plus protease inhibitors. Non-specific binding was determined in the presence of 300 μM nicotine. Binding was terminated by filtration, and data were treated as described below.

2.4.3. [^{3}H]MLA Binding.

[^{3}H]MLA binding was used to quantify homomeric α7 nAChR. Binding of [^{3}H]MLA to brain hippocampal membranes was measured as described by Davies et al. (1999). Briefly, 0.25 ml of membranes (containing 200 μg of brain membranes) was incubated in borosilicate glass tubes with 2 nM [^{3}H]MLA (60 Ci/mmol), in a final volume of 0.5 ml for 2 h at 4 °C. The incubation buffer consisted of 50 mM Tris–HCl, 120 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.1% bovine serum albumin. Non-specific binding was determined from tubes containing 1 μM unlabeled MLA. Incubation was completed by rapid filtration under vacuum through Whatman GF/B glass fiber filters (Whatman Intl. Ltd., Maidstone, U.K.) pre-soaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly 3 times with 4 ml ice-cold 50 mM Tris–HCl and the radioactivity trapped was measured by liquid scintillation spectrometry. Specific binding was calculated as the difference between the radioactivity measured in the absence (total binding) and in the presence (non-specific binding) of the excess of non-labeled ligand, and expressed as the percentage of that obtained from saline-treated mice.

2.5. Conditioned Place Preference (CPP) Paradigm.

The place conditioning protocol used was non-biased (Robledo et al., 2004). The apparatus was composed of three distinct compartments separated by manually operated doors. The central compartment (corridor) measured 27x10x25 cm (w x d x h) and served as a thoroughfare between the two pairing sides. The pairing compartments are 20x20x25 cm (w x d x h). One compartment had black and white checkered walls with

a smooth and shiny floor. The other compartment had white and light blue painted walls and rough flooring. The light intensity within the conditioning chambers was 30 lux. CPP was performed in three phases: preconditioning, conditioning and test. During the pre-conditioning phase (day 1), naive or nicotine pre-treated mice were placed in the middle of the corridor and had free access and roam among the three compartments of the apparatus for 20 min. The time spent in each compartment was recorded by computerized monitoring software (Smart Junior, PANLAB SL, Barcelona, Spain). During the conditioning phase (days 2, 4, 6 and 8), mice were treated with MDMA (3 and 10 mg/kg, s.c.), or saline, 20 min before being confined into one of the two conditioning compartments for 30 min. On days 3, 5, 7 and 9 of the conditioning phase, animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day and treatments were counterbalanced to assure that some animals received MDMA in the black and white compartment while others received MDMA in the white and light blue compartment.

Control animals received saline every day. For conditioning studies with DHβE or varenicline, these drugs or saline were administered intraperitoneally 15 min before MDMA, at doses previously described as effective in antagonizing nicotine-induced CPP (2 mg/Kg) (Biala et al., 2010; Walters et al., 2006). The test phase (day 10) was conducted identically to the preconditioning phase; animals were drug-free and had free access to the three compartments for 20 min.

To investigate whether nicotine (administered in a previous chronic treatment) potentiates MDMA-induced CPP, nicotine was given intraperitoneally at a dose of 2 mg/Kg (Dougherty et al., 2008) b.i.d. for 14 days. The day after, nicotine was withdrawn and preconditioning for CPP was started with MDMA at a dose of 3 mg/Kg as above. A preference score was expressed in seconds and calculated for each animal as the difference between the times spent in the drug-paired compartment in the post-test minus the time spent in the pre-conditioning phase.

2.8. Statistical Analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using two-tailed one-way analysis of variance

(ANOVA). Significant ($p<0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons, where appropriate. AUC values were calculated by nonlinear regression using GraphPAD Prism (GraphPAD software, San Diego, CA, USA). All statistic calculations were performed using Graph Pad Instat (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Effect of nAChR ligands on induction of behavioral sensitization to MDMA

Locomotor activity was used to measure behavioral sensitization to MDMA in the different treatment groups through time. On day 1 an acute challenge of MDMA (5 mg/Kg) produced significantly greater locomotor activity than saline alone (total breaks (TB): 3423 ± 267 saline, 4870 ± 244 MDMA, $p<0.001$). This psychostimulant effect was fully abolished by pretreatment with DHβE or varenicline ($F_{5,89}=6.92$, $p<0.001$, see figure 1, table 1). DHβE and VAR control groups revealed the absence of effect of these drugs alone on locomotor activity.

Similarly, on day 10, one-way ANOVA showed a significant effect of treatment ($F_{5,89}=23.04$, $p<0.001$). Daily exposure to MDMA or DHβE+MDMA or varenicline+MDMA revealed sensitization, expressed as a significant increase in the psychostimulant effect of MDMA. The inhibitory effect of DHβE and varenicline observed in the acute challenge of MDMA on day 1 was not present after 10 consecutive days of treatment. Day10/day1 ratio of total breaks ($F_{2,41}=175.92$, $p<0.001$; 136.32 ± 3.24% MDMA, 169.23 ± 3.10% DHβE+MDMA and 225.29 ± 2.59% VAR+MDMA) revealed that these drugs enhanced rather than attenuated this early sensitization. As on day 1 the animals treated with DHβE/VAR alone denoted the absence of effect of these antagonists on locomotor activity on day 10.

Behavioral sensitization was monitored up to 2 weeks after the discontinuation of MDMA treatment. Analysis of results on day 25 to assess conditioned hyperactivity showed an overall significant difference among treated groups ($F_{5,74}=37.25$, $p<0.001$, see figure 1, table 1). A challenge dose of MDMA induced a stronger behavioral response than that administered on day 10 (day 25: 8075 ± 404; day 10: 6639 ± 332;

p<0.01). DHβE- or varenicline- pretreated mice showed a response on day 25 that did not differ from that on day 10 (see figure 1). These results were assessed when analyzing day25/day10 ratio of total breaks ($F_{2,23}$=7.12, p<0.01: 118.12±2.49% MDMA, 105.81±3.02% DHβE+MDMA p<0.01 vs MDMA and 108.00±2.86% VAR+MDMA p<0.05 vs MDMA). Differences between total breaks on day 25 and total breaks on day 10, confirms the results ($F_{2,23}$=29.15 p<0.001; 1436±163 MDMA, 128±12 DHβE+MDMA, varenicline+MDMA=193±18).

3.2. Effect of nAChR ligands on the density of nicotinic receptor subtypes in different mouse brain areas

Due to the effects observed in locomotor activity experiments, the density of nAChR was measured in several brain areas of the same animals in order to establish a possible relationship between such effects and changes in receptor populations. 5 animals of each treatment group were killed on day 10 after treatment and locomotor activity measurement, while the rest were kept to obtain the results on day 25.

Treatment with MDMA, DHβE or varenicline for 10 days induced a significant increase in [^{3}H]epibatidine binding in cortex, compared with those receiving saline alone ($F_{5,34}$=2.908, p<0.05) . DHβE also induced such an increase in the striatum. In this area, MDMA did not modify [^{3}H]epibatidine binding and did not alter the increase in heteromeric nAChR expression induced by DHβE. Moreover, pretreatment with varenicline significantly reduced [^{3}H]epibatidine binding in mouse striatum; this was not altered by MDMA ($F_{5,29}$=27.231, p<0.001) (Fig. 2B).

After the 14-day drug-free period, the mice treated previously with MDMA (but not those pretreated only with DHβE or varenicline alone), showed a significant increase in heteromeric nAChR density in cortex and striatum. The cortical increase in [^{3}H]epibatidine binding was not present in animals which received pretreatment with DHβE or varenicline ($F_{3,21}$=18.936, p<0.001) (Fig. 3A). Only pretreatment with DHβE prevented the up-regulation induced by MDMA in striatum ($F_{3,23}$=3.376, p<0.05) (Fig 3B).

When analyzing the density of homomeric α7 nAChR in hippocampus, where they are more highly expressed, no differences in receptor densities, measured as [^{3}H]MLA binding, were found in MDMA-treated mice (Fig 4).

3.3. Effect of nAChR ligands on the acquisition of MDMA-induced CPP

The CPP paradigm was used to study the effect of the different treatments on the addictive/rewarding properties of MDMA.

Throughout all experiments, a within-subjects comparison revealed that mice had no bias. Time (in seconds) spent in both compartments during pre-conditioning were 367.58 ± 56.70 and 326.05 ± 35.69, indicating a lack of preference for either side. This did not significantly change in the test session (309.12 ± 35.14 and 276.19 ± 28.73) when saline was paired with both compartments during the conditioning phase.

We first investigated the effect of varenicline and DHβE in the CPP induced by MDMA (10 mg/kg). On the test day (day 10, post-conditioning), one-way ANOVA revealed a significant effect of treatment ($F_{5,36} = 4.56$, $p<0.01$). The ability of MDMA to produce a CPP was assessed while some mice were under the influence of DHβE or varenicline (2 mg/kg) treatment, administered 15 min before the MDMA dose. Both reduced MDMA's ability to produce a CPP, fully blocking MDMA's effects ($p<0.05$ for varenicline and $p<0.01$ for DHβE vs. MDMA-treated mice) (Fig 5B). Neither DHβE nor varenicline alone had any effect on CPP.

During the pre-conditioning phase and test day we measured the distance and speed of travel in each of the two compartments. Results corresponding to the drug-paired compartment are shown in Table 2 and demonstrate that treatment with MDMA during the conditioning phase induces an increase in locomotor activity in the test day that is not present in animals pretreated with varenicline or DHβE. This increase in locomotor activity was not accompanied by an increase in speed and confirms a psychostimulant effect in these animals.

To explore the effect of a chronic nicotine treatment on the addictive behavior caused by a low dose of MDMA (3 mg/Kg) which is not supposed to induce CPP when given alone (Robledo et al., 2004), we pretreated mice with nicotine at a dose of 2 mg/Kg,

given subcutaneously (b.i.d.) for 14 days. This treatment induced a significant increase in α4β2 nAChR density in the striatum (147.98 ± 13.13%, nicotine-treated *vs* 100.00 ± 10.56%, saline-treated, $p<0.05$, Student's *t* test). This nicotine treatment schedule did not induce a significant CPP on its own (Dougherty et al., 2008) and, therefore, at the end of the nicotine treatment, animals did not show preference for either of the two compartments (445.85 ± 69.28 *vs* 551.02 ± 27.82). Repeated nicotine administration during the 14 days prior to pre-conditioning led to a decreased MDMA threshold for CPP. As reflected in Fig. 6, when animals were exposed to chronic nicotine pretreatment, they showed a positive preference score at a dose of MDMA (3 mg/Kg) that proved to be ineffective when administered alone ($F_{2,23} = 5.808$, $p<0.01$).

4. Discussion

This study examines the involvement of heteromeric nAChR in the behavioral sensitization as well as the addictive potential of MDMA in mice. The results indicate that an antagonism or a partial agonism on nAChR reduces the addiction, blocks the acute locomotor effects and changes the development of sensitization induced by MDMA. α4β2 nAChR appear to mediate these effects given that DHβE and varenicline, but not MLA (data not shown), antagonized the acute effects of MDMA. In fact, previous studies (Walters et al., 2006) have demonstrated that MLA at doses of 5 and 10 mg/Kg (s.c), does not inhibit nicotine-induced CPP, ruling out an involvement of the α7 nAChR in this behavior.

The psychomotor stimulant effect of MDMA is considered subsequent to an extracellular increase in DA and 5-HT in the NAcc and VTA (Bankson and Cunningham, 2001). In a previous study we demonstrated the involvement of nicotinic receptor subtypes in the hyperlocomotion induced by methamphetamine (Camarasa et al., 2009). Here we report that the stimulant effects of an acute dose of MDMA are blocked by antagonists acting on α4β2 nAChR. Nicotinic agonists can differentially affect neurotransmitter release in a given brain region and the magnitude of such responses will largely be determined by the subtype selectivity of the agonist (Rao et al., 2003). Nicotine activates nAChR localized in the dopaminergic nerve terminals in the nucleus accumbens and elicits a complex pattern of inhibitory–stimulatory effects on locomotion (Avale et al., 2008).

Although there are subtle differences between MDMA and other commonly abused amphetamines, a clear overlap in the behavioral pharmacology of MDMA and other amphetamine-like compounds can be found, especially in the induction of behavioral excitation. In rodents, this effect, called behavioral sensitization, persists many months after the last administration, thus mimicking long-term sensitivity to drugs observed in human addicts. Expression of this persistent drug-induced behavioral sensitization has been suggested to contribute to craving and high relapse rates in addicts (Robinson and Berridge, 2003). Studies of the neurobiological basis of behavioral sensitization have focused on the increased capacity of these drugs to release dopamine in the midbrain dopamine system (Cadoni et al., 2000) although multiple limbic-associated areas such as the prefrontal cortex provide the excitatory cortical innervation to the NAcc (Kita and Kitai, 1990). This dopaminergic system mediates locomotor stimulation as well as the ability of drugs to elicit craving and lead to abuse.

When MDMA was administered daily for 10 consecutive days, there was an increase in the hyperlocomotion induced by this drug on day 10 respect with that measured on day 1 (early behavioral sensitization). These results are in agreement with those previously described in rats (Kalivas et al., 1998) demonstrating that repeated administration of MDMA over the course of ten days produces sensitization to the behavioral stimulant effects of MDMA. Furthermore, the behavioral sensitization in mice was found to be highest after a 2 week-period following the discontinuation of MDMA treatment, (a challenge dose of MDMA showed a stronger behavioral response than on day 10) demonstrating that the treatment schedule of MDMA used in this study induces not only an early but also a delayed sensitization that can be modulated by drugs acting on α4β2 nAChR.

Neither DHβE nor varenicline blocked but rather enhanced the development of early behavioral sensitization by MDMA, conversely to the inhibitory effect observed in the acute challenge (day 1). When comparing the ratios D10/D1 of the different groups, a potentiation was revealed for those treated with MDMA plus DHβE or varenicline. In other words, the groups receiving MDMA plus the nicotinic ligand showed a day-to-day greater increase in locomotion than the group receiving MDMA alone.

The increased delayed sensitization to MDMA was prevented when it was administered together with either the α4β2 nAChR antagonist (DHβE) or the partial

agonist (varenicline). It is known that nAChR ligands regulate sensitization to stimulant drugs such as d-amphetamine and cocaine. For instance, DHβE, a high-affinity competitive antagonist of α4 subunit-containing nAChR, attenuates the induction of locomotor sensitization to d-amphetamine, cocaine, ephedrine and methylphenidate in mice and rats (Karler et al. 1996; Miller and Segert 2005; Schoffelmeer et al. 2002; Woorters and Bardo, 2009). Additionally, the sensitizing effect of acute nicotine on amphetamine-stimulated behavior and dopamine efflux requires activation of β2 subunit-containing nAChRs (Kim et al., 2011).

Varenicline is an effective aid in smoking cessation. This drug, by acting on α4β2 nAChR, stimulates dopamine release when the basal tone is depressed and simultaneously blocks the effects of a full agonist when simultaneously present. Partial agonists aim to provide a low-to-moderate level of dopamine stimulation to reduce craving and withdrawal symptoms. When varenicline is administered to nicotine-sensitized rats, it reduces the expression of nicotine sensitization (Zaniewska et al., 2008). Similarly, in our experiments, varenicline inhibited the increase in the delayed sensitization observed on day 25.

Due to the described dynamic plasticity of nAChR after treatment with nicotinic ligands, we assessed the density of heteromeric (mainly α4β2) and homomeric α7 receptors through radioligand binding studies. The results showed that early sensitization on day 10 was accompanied by changes in α4β2 nAChR density in certain brain areas. MDMA induced in cortex, but not in the striatum, a significant increase in α4β2 nAChR that was not blocked by DHβE or varenicline. However, the results on day 25 correlate with the *in vivo* effects: although these animals had a 14-day drug-free period, the increased α4β2 nAChR density in cortex and striatum was still present in the MDMA group, but not in the animals co-treated with DHβE. Varenicline appears to do the same in the cortex. From these results it can be deduced that the α4β2 nAChR subtype is involved in the early and delayed sensitization elicited by MDMA. If treatment leads to an increase in α4β2 nAChR subtype population in cortex, the sensitization takes place. By contrast, when this up-regulation is prevented, sensitization is attenuated. The role of the cortex in sensitization is not an exception as it is known that the prefrontal cortex and the hippocampus exhibit converging projections to the NAcc and have functional reciprocal connections via indirect pathways (Day et al., 1991; Goto and Grace, 2008). Medial prefrontal neurons, including those projecting to

the NAcc (McGinty and Grace, 2008), are also excited by conditioned stimuli (Laviolette et al., 2007) and Ball et al (2009) demonstrating that long-lasting locomotor sensitization to MDMA is accompanied by reorganization of synaptic connectivity, not only in NAcc, but also in the medial prefrontal cortex.

Effects derived from changes in α7 nAChR population can be ruled out from present binding studies. The difference between the effects of DHβE and varenicline can be explained by their different pharmacological profile.

Once the correlation between nAChR and behavioral sensitization to MDMA was demonstrated, we examined the effect of α4β2 nAChR ligands as well as that of a nicotine chronic treatment on the CPP score induced by MDMA. In this study we provide evidence that MDMA at a dose of 10 mg/kg, but not 3 mg/Kg, causes positive CPP in mice. These results are in agreement with those of Salzmann et al. (2003) and Robledo et al. (2004). Bilsky et al. (1998) demonstrated that the CPP induced by MDMA was effectively blocked by the dopamine release inhibitor CGS10746B. These results and those of Vidal-Infer et al. (2012) demonstrate that, in mice, the dopaminergic system is involved in the acquisition and expression of MDMA-produced CPP. Moreover, results of the present study provide pharmacological evidence of the involvement of the α4-containing nAChR in the CPP induced by MDMA, as this effect was antagonized by DHβE and varenicline.

Acute nicotine challenge induces behavioral sensitization to amphetamines (Birrell and Balfour, 1998, Jutkiewicz et al., 2008) and consequently can enhance its addictive potential. In this study we used a chronic nicotine treatment in order to increase the density of α4β2 nAChR (Dougherty et al., 2008). It is important to note that nicotine treatment took place previously and this drug was not present during the CPP experiments with MDMA, avoiding any interaction on the test day. Abstinence signs of nicotine are dose-dependent and appear at doses equal to or higher than 6.3–8 mg/kg/day (Isola et al., 1999, Gould et al., 2012) and not at 6 mg/kg/day or lower (Damaj et al., 2003), as in our experiments. These signs last for a maximum of 3-4 days (Zhang et al., 2012) and are supplemented with deficits in contextual learning (Gould et al., 2012). In the present study, sustained exposure to nicotine significantly increased MDMA rewarding in the CPP paradigm. While MDMA at a low dose (3mg/kg) did not

induce CPP on its own, this dose of MDMA showed a very significant preference score in nicotine-pretreated mice.

As in the behavioral sensitization experiments, this increase in the CPP score caused by MDMA runs parallel to an increase in α4β2 nAChR density induced by nicotine, pointing to an up-regulation of these receptors as an additional factor in MDMA's reinforcing effect. The up-regulated nAChR could mediate enhanced synaptic transmission when stimulated by local and brief releases of ACh at synapses. Stimulation of dopamine neurons in the VTA via the α4β2 nAChR leads to an increase of dopamine in the NAcc that plays a crucial role in drug reward as measured by CPP (Di Chiara and Imperato, 1988). Consequently, the modulation of dopamine release by means of α4β2 nAChR activation could result in a modification of the CPP induced by MDMA. Although animals were not under the effect of nicotine when tested in the CPP paradigm, and despite the very low dosage of this stimulant administered during the pretreatment phase, we cannot rule out an influence of nicotine withdrawal in the first days of the conditioning phase.

The influence of chronic nicotine treatment on MDMA effects extends not only to CPP but also to its hyperlocomotion properties. In previous studies (Camarasa et al., 2009) we have described that nicotine, when administered in a chronic low-dose schedule, significantly potentiates the methamphetamine-induced increase in locomotor activity and rearing. These results suggest that up-regulation of nAChR leads to a very significant potentiation of the increase in locomotor activity induced by this drug. Similar results were obtained for MDMA-induced hyperlocomotion using the same nicotine pretreatment than in the study with methamphetamine (a 30% potentiation,, unpublished results).

A great number of MDMA consumers also smoke concomitantly (Scholey et al., 2004). In view of results obtained in the present paper it can be deduced that smoking can increase neuronal sensitization to MDMA and its addictive potential, making MDMA-users more susceptible to addiction. Although further research must be done on this subject, our results suggest that α4β2 nAChRs are a potential target towards treating nicotine and MDMA polyabuse. Although DHβE is a useful pharmacological tool for preclinical studies on nAChR, it is not adequate for clinical use due to its toxicity: it can produce neuromuscular blockade, hypotension and has a vey narrow dosage window

(the i.p. DL50 in mice is 4.5 mg/kg, Megirian et al., 1955). Also DHβE, as a pure antagonist, can precipitate nicotine abstinence syndrome (Malin et al., 1998). Conversely varenicline, as a marketed drug for smoking cessation with a good security profile, should be taken into consideration as a possible candidate drug.

5. Conclusion

In summary, although it is well known that nAChRs are a pharmacological target for understanding the neurotoxic effects of amphetamine derivatives (Chipana et al., 2008c), they are also involved in other behavioral effects of these drugs such as hyperlocomotion and addictive properties. This paper demonstrates the involvement of specific α4-containing nAChR subtypes by using specific modulators of these receptors. Our results point out that effects induced by MDMA such as locomotor sensitization and addictive potential, both related with the release of dopamine, are modulated by DHβE and varenicline. Consequently, varenicline, a commercial drug used to treat tobacco addiction, could also be considered for treating MDMA abuse. Finally, these results may have clinical implications because MDMA abusers are often smokers; in this regard, varenicline would be the first useful drug to simultaneously treat both tobacco and MDMA abuse.

Disclosure Statement

All authors disclose any actual or potential conflict of interest including financial, personal or other relationships with other people or organizations that could inappropriately influence the present work. All authors reviewed the content and approved the final version.

Contributors

JC and EE were responsible for the study concept and design. AC, JC and DP contributed to the acquisition of animal data. JC and DP performed data analysis. EE interpreted findings and provided critical revision of the manuscript.

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Figure captions

Fig 1.

Cumulative breaks after 180 min for the effect of saline, DHβE (1 mg/Kg), or varenicline (VAR) (0.3 mg/Kg) on saline/MDMA (5 mg/Kg)-induced hyperlocomotion. Locomotor activity was measured on day 1 (acute challenge), day 10 (after a daily dose for ten days) and day 25 (acute challenge of saline, DHβE or varenicline plus saline or MDMA after 14-day withdrawal). Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, significantly different from day 1 of the same treated group. $^{\#\#}$p<0.01 significantly different from day 10 of the same treated group. $^{\phi\phi\phi}$ p<0.001 significantly different from saline day 1.

Fig 2.

Effect of treatment with saline, DHβE (1 mg/Kg), or varenicline (VAR) (0.3 mg/Kg) plus saline or MDMA (5 mg/Kg) during 10 consecutive days on α4β2 nAChR density (measured as [^{3}H]epibatidine binding) in mouse cortex (panel A) or striatum (panel B). Data are expressed as mean ± SEM from the values obtained from 5-6 animals per group. *p<0.05 and **p<0.01, significantly different from saline-treated group.

Fig 3.

Effect of a 14 day withdrawal after a 10 consecutive day treatment with saline, DHβE (1 mg/Kg), or varenicline (VAR) (0.3 mg/Kg) plus saline or MDMA (5 mg/Kg) on α4β2 nAChR density (measured as [^{3}H]epibatidine binding) in mouse cortex (panel A) or striatum (panel B). On day 25, mice were killed 6 h after receiving the assigned treatment and their brains were used for this experiment. Data are expressed as mean ± SEM from the values obtained from 5-6 animals per group. *p<0.05 significantly different from saline-treated group.

Fig 4.

Effect of MDMA (5 mg/Kg) alone for 10 consecutive days (day 10) or after a 14 day withdrawal period (day 25) on α7 nAChR density (measured as [^{3}H]MLA binding) in mouse hippocampus. Data are expressed as mean ± SEM from the values obtained from 5-6 animals per group

Fig 5.

Effect of DHβE (2 mg/Kg) and varenicline (VAR) (2 mg/Kg) alone and on MDMA (10 mg/Kg)-induced conditioned place preference. The *x-axis* represents the treatment group and the *y-axis* represents the preference score (test day minus preconditioning day) in seconds. **p<0.01, significantly different from saline-treated group; #p<0.05 and ##p<0.01, significantly different from the corresponding value of MDMA-treated group.

Fig 6.

Effect of a 14 day chronic nicotine pretreatment (2 mg/Kg, b.i.d.) on the conditioned place preference assay on MDMA (3 mg/Kg). Data are expressed as mean ± SEM. **p<0.01, significantly different from saline- or MDMA-treated groups.

Figure 1

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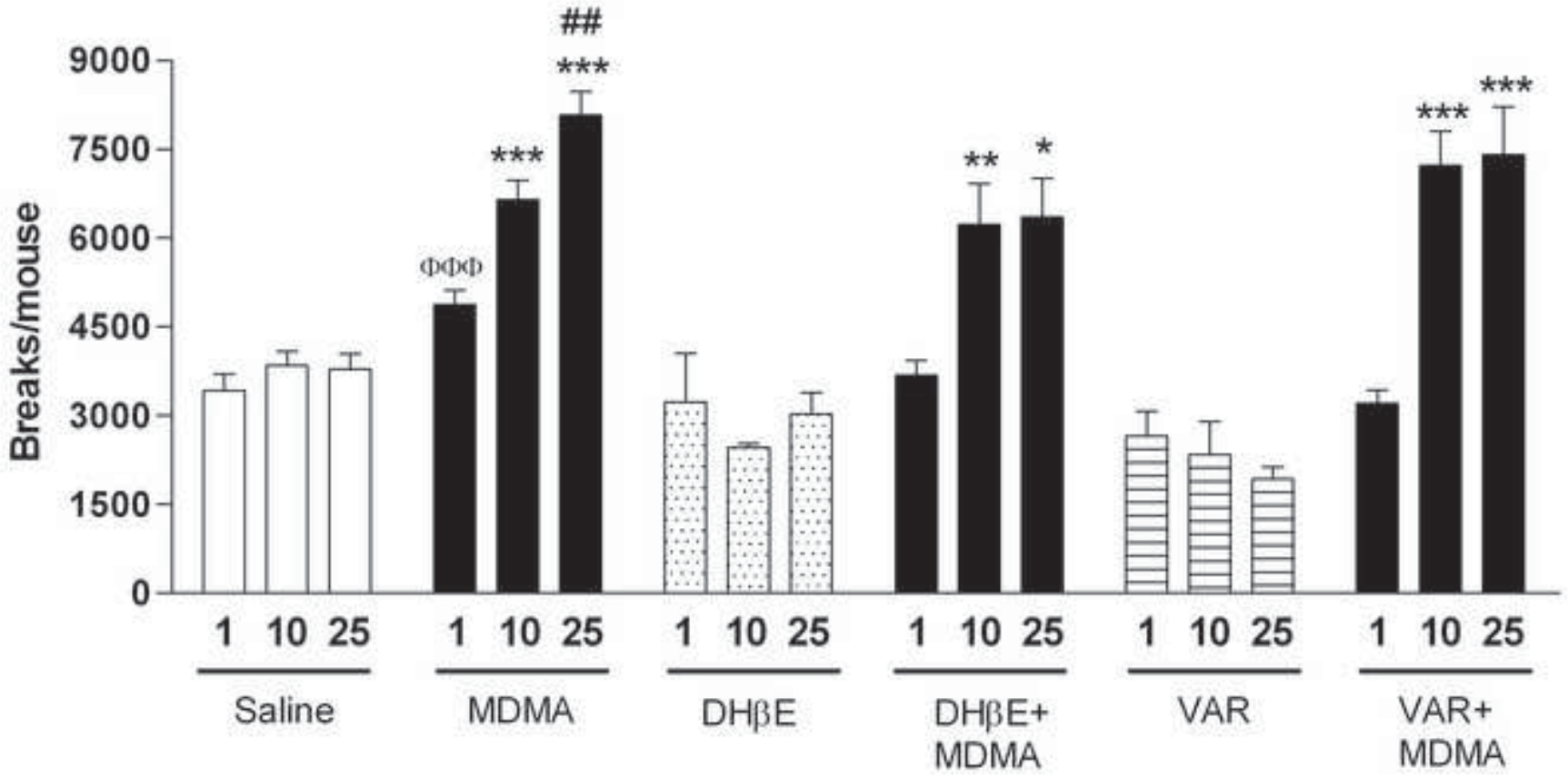

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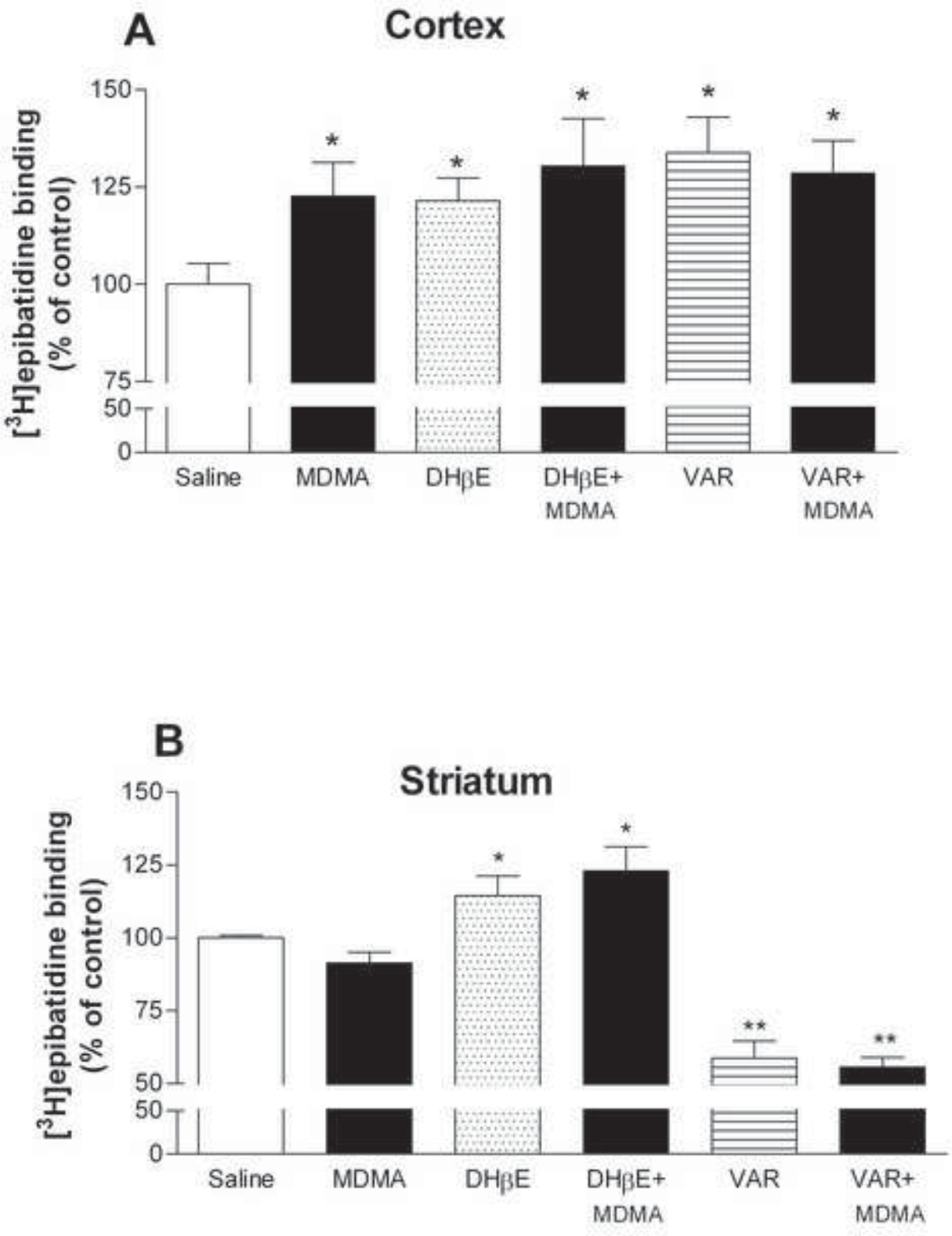

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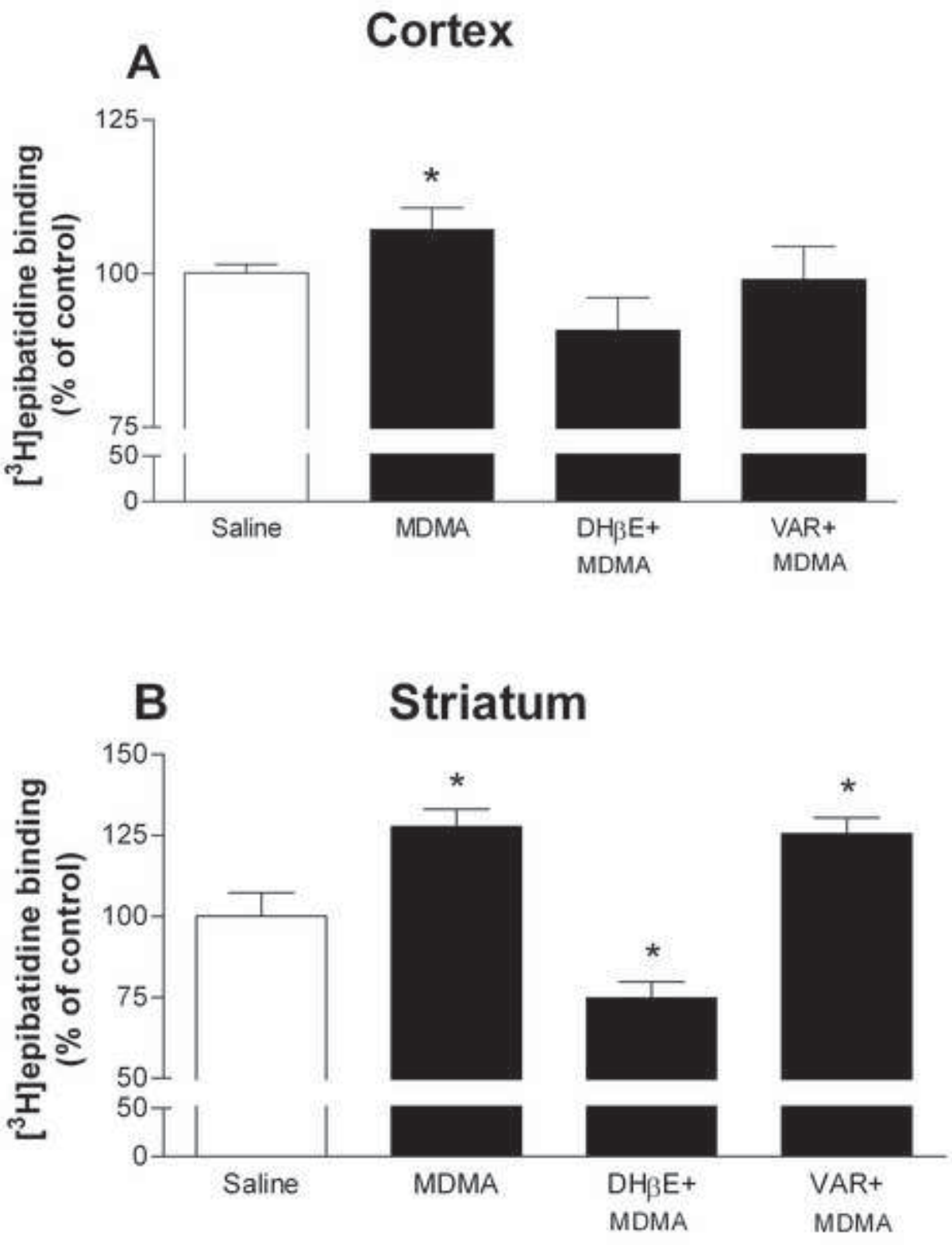

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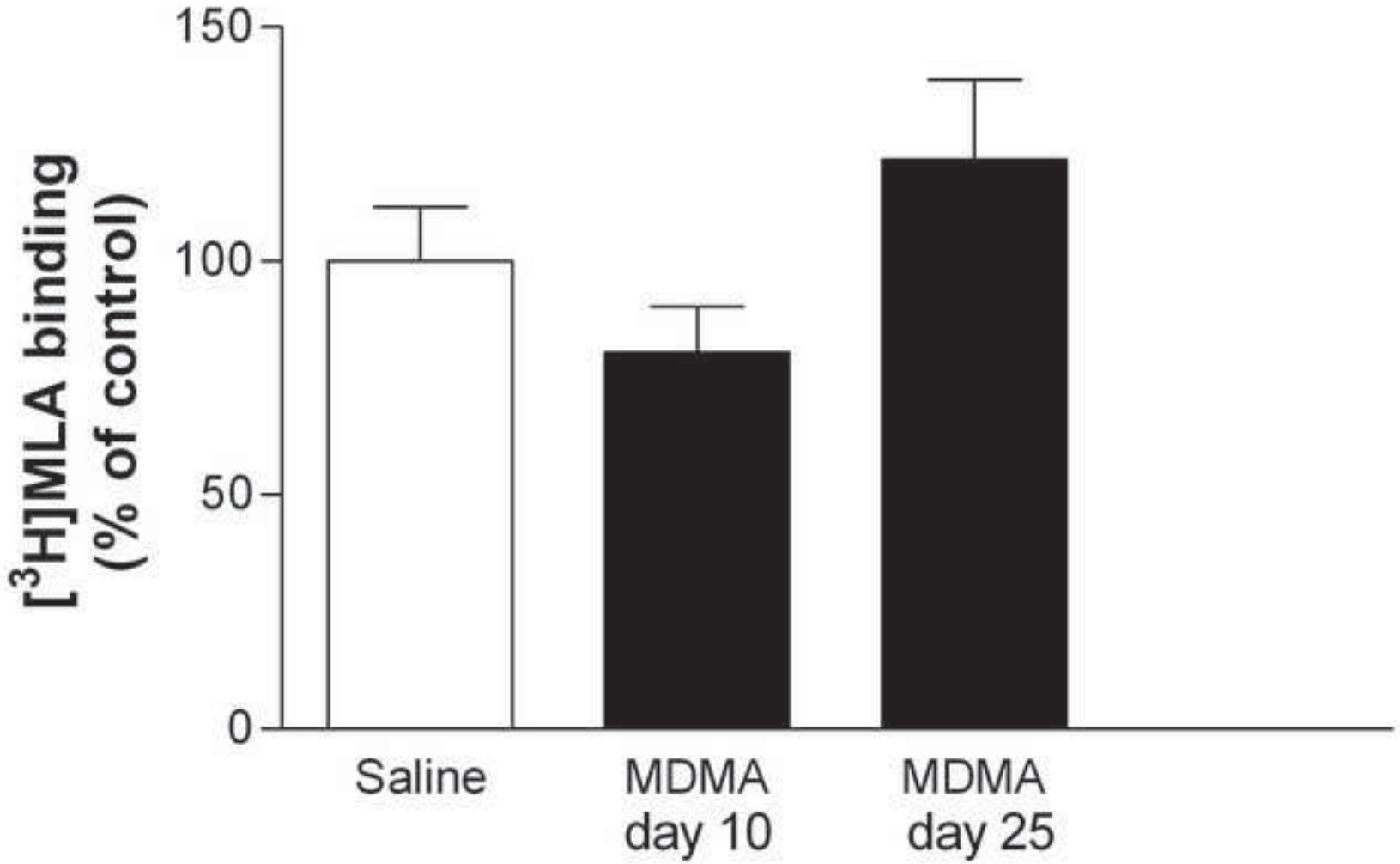

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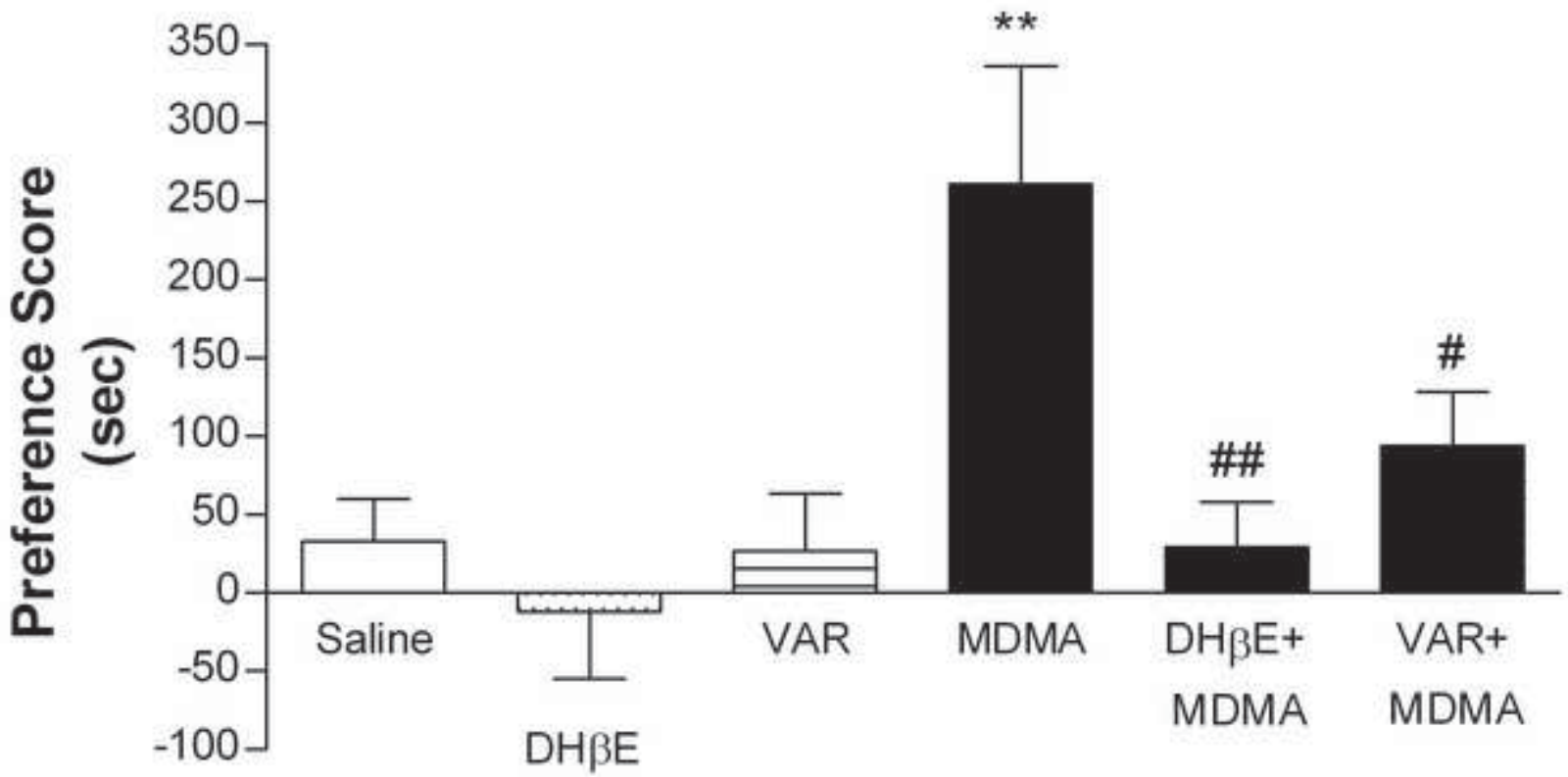

Figure 6

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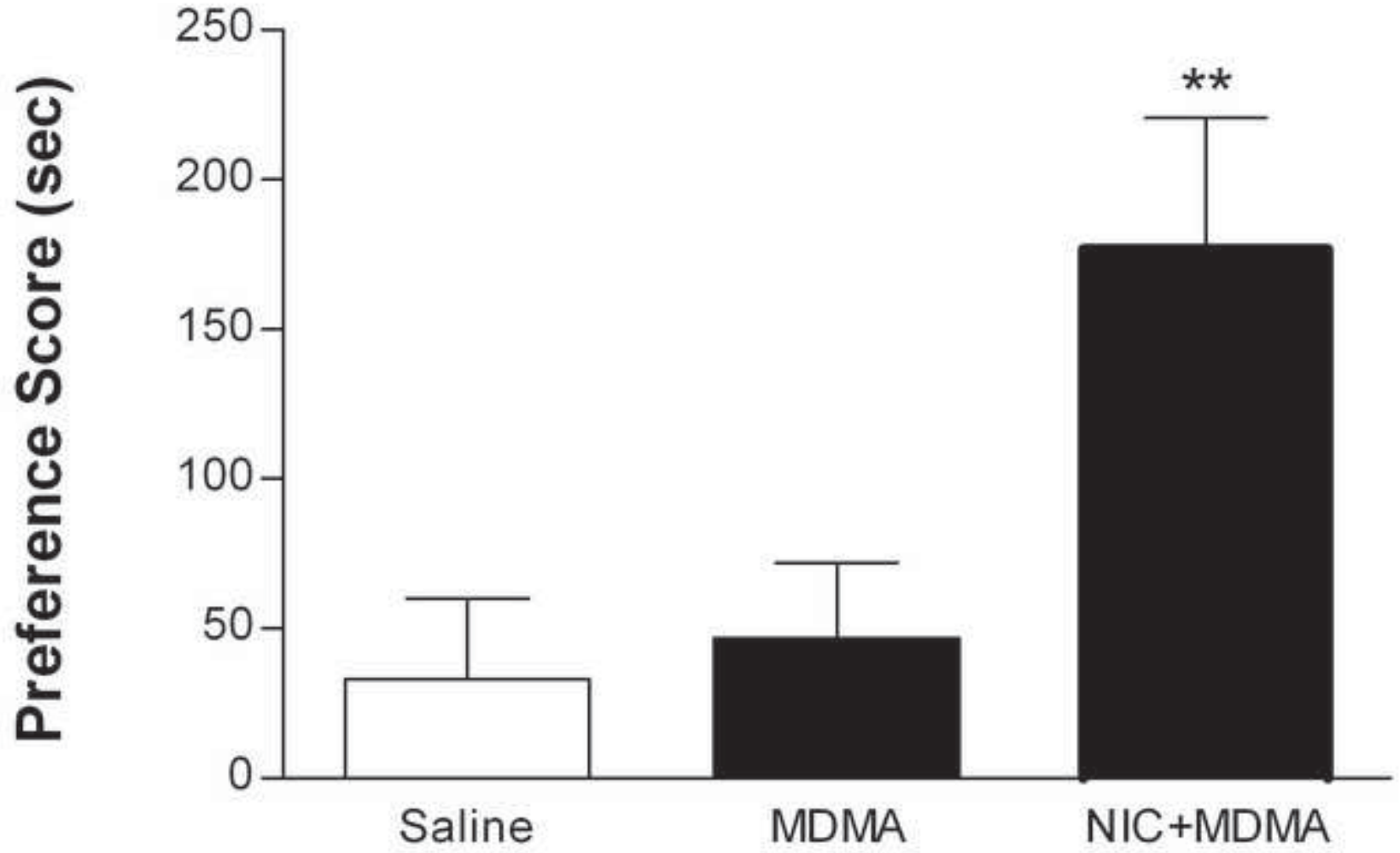

Table 1. Effect of DHβE (1 mg/Kg) and varenicline (VAR) (0.3 mg/Kg) on MDMA (5 mg/Kg)-induced locomotor sensitization in mice. Locomotor activity was measured on day 1 (acute challenge), day 10 (after a daily dose for ten days) and day 25 (acute challenge of saline, DHβE or varenicline plus saline or MDMA after 14-day withdrawal). Results are expressed as mean ± S.E.M. of the total area under the curve (AUC) over a period of 180 minutes (left column) and the time during which a significant hyperlocomotion was present (right column). *p<0.05, **p<0.01 and ***p<0.001 vs saline ##p<0 .01 vs MDMA.

Drug	Locomotor activity	
	Total AUC	Hyperlocomotion for (min)
Day 1		
Saline	71192 ± 6915	60
MDMA	114874 ± 16034*	150
DHβE + MDMA	86100 ± 6782	90
VAR + MDMA	77246 ± 4932	60
DHβE	61718 ± 8959	60
VAR	44405 ± 5329	60
Day 10		
Saline	79914 ± 8790	60
MDMA	161774 ± 22363**	150
DHβE + MDMA	147198 ± 19630**	120
VAR + MDMA	197120 ± 11987***	120
DHβE	47325 ± 1819	30
VAR	47097 ± 6898	60
Day 25		
Saline	78143 ± 8768	60
MDMA	190550 ± 20777***	150
DHβE + MDMA	156582 ± 18953*	90
VAR + MDMA	211860 ± 22595**	90
DHβE	58315 ± 6665	60
VAR	39740 ± 3902	60

Table 2

Table 2.- Distance travelled and the speed in the drug-paired compartment measured in the pre-conditioning day and in the test day (absence of drug treatment). Results are expressed as mean ± standard error of the mean from 8 different animals

Drug treatment	Distance travelled (cm)		Speed (cm/s)	
	Pre-conditioning	Test	Pre-conditioning	Test
Saline	1112.23 ± 176.39	1120.03 ± 143.73	3.30 ± 1.19	2.90 ± 0.97
MDMA	1329.62 ± 51.62	2063.11 ± 55.46**	3.63 ± 0.23	3.04 ± 0.43
VAR+MDMA	1660.90 ± 178.57	1953.87 ± 154.16	4.14 ± 0.91	3.66 ± 0.66
DHBE+MDMA	1664.25 ± 61.64	1872.36 ± 151.60	3.62 ± 0.24	3.71 ± 0.13

**P<0.01 vs. the corresponding value of the preconditioning day (paired Student *t*-test).