3,4-Methylenedioxy-methamphetamine induces *in vivo* regional up-regulation of central nicotinic receptors in rats and potentiates the regulatory effects of nicotine on these receptors

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Abstract

Nicotine (NIC), the main psychostimulant compound of smoked tobacco, exerts its effects through activation of central nicotinic acetylcholine receptors (nAChR), which become up-regulated after chronic administration. Recent work has demonstrated that the recreational drug 3,4-methylenedioxy-methamphetamine (MDMA) has affinity for nAChR and also induces up-regulation of nAChR in PC 12 cells. Tobacco and MDMA are often consumed together. In the present work we studied the *in vivo* effect of a classic chronic dosing schedule of MDMA in rats, alone or combined with a chronic schedule of NIC, on the density of nAChR and on serotonin reuptake transporters. MDMA induced significant decreases in [^{3}H]paroxetine binding in the cortex and hippocampus measured 24 h after the last dose and these decreases were not modified by the association with NIC. In the prefrontal cortex, NIC and MDMA each induced significant increases in [^{3}H]epibatidine binding (29.5 and 34.6%, respectively) with respect to saline-treated rats, and these increases were significantly potentiated (up to 72.1%) when the two drugs were associated. Also in this area, [^{3}H]methyllycaconitine binding was increased a 42.1% with NIC+MDMA but not when they were given alone. In the hippocampus, MDMA potentiated the α7 regulatory effects of NIC (raising a 25.5% increase to 52.5%) but alone was devoid of effect. MDMA had no effect on heteromeric nAChR in striatum and a coronal section of the midbrain containing superior colliculli, geniculate nuclei, substantia nigra and ventral tegmental area. Specific immunoprecipitation of solubilised receptors suggests that the up-regulated heteromeric nAChRs contain α4 and β2 subunits. Western blots with specific α4 and α7 antibodies showed no significant differences between the groups, indicating that, as reported for nicotine, up-regulation caused by MDMA is due to post-translational events rather than increased receptor synthesis.

Keywords: MDMA; ecstasy; up-regulation; nicotinic; nicotine; epibatidine

1. Introduction

3,4-Methylenedioxy-methamphetamine (MDMA, ecstasy) is an amphetamine derivative used illicitly in developed countries for recreational purposes, usually by young people in night clubs and at extended dance parties (known as raves).

A number of fatalities have been reported after acute consumption of this drug but there also exists experimental evidence that chronic MDMA can induce serotonergic and, to a lesser extent, dopaminergic neurotoxicity in rats and primates (see Capela et al. (2009) for a review). Also, serotonergic (Erritzoe et al., 2011; Reneman et al., 2002) and cognitive (Adamaszek et al., 2010; Nulsen et al., 2010; Parrott et al., 1998; Quednow et al., 2006) deficits have been reported in human chronic MDMA users, which could be due to neurotoxicity or to drug-induced long-lasting regulatory changes (Biezonski and Meyer., 2011).

The neurotoxicity of amphetamine derivatives can be a consequence of coordinated oxidative stress, metabolic compromise and inflammation (see Capela et al., 2009 and Yamamoto and Raudensky, 2008 as reviews), and we have recently reported that neuronal acetylcholine nicotinic receptors (nAChR), mainly the homomeric α7 subtype, also play a key role in MDMA-induced neurotoxicity as the blockade of these receptors by the antagonists methyllycaconitine (MLA) or memantine prevents *in vitro* and *in vivo* MDMA-induced neurotoxicity (Chipana et al., 2006, 2008a, 2008b, 2008c) as well as cognitive impairment in rats (Camarasa et al., 2008). Also, using radioligand binding experiments, we have demonstrated that MDMA has affinity for both homomeric and heteromeric nAChRs and behaves as a partial agonist at α7 nAChR (Chipana et al., 2008b, 2008c; Garcia-Rates et al., 2007, 2010).

NAChR are a family of ligand-gated cation channels widely distributed in the brain and the peripheral nervous system, whose subunit composition and signalling effects depend on subtype and localisation (Albuquerque et al., 2009; Gotti et al., 2007). They exert a number of effects on brain functions, involving fast synaptic transmission, cognitive enhancement, memory or reinforcement, and they are the main target of smoked nicotine. In the brain, nAChRs are

pentameric structures formed by the association of α and β subunits and can be either homomeric or heteromeric. The homomeric family is made up of the α7-α10 subunits and is sensitive to α-bungarotoxin (αBgTx), while the heteromeric receptors consist of combinations of α2- α6 and β2-β4 subunits, and are insensitive to αBgTx. Of these combinations, the most abundant are homomeric α7 and heteromeric $(\alpha4)_2(\beta2)_3$ receptors. A particular feature of some nAChR subtypes is that, after chronic nicotine exposure, they undergo radioligand binding up-regulation, changes in stoichiometry and increase in their functional state (functional up-regulation) (reviewed by Gaimarri et al., 2007). Such up-regulation occurs at a post-translational level and several mechanisms have been proposed to explain it, including a chaperone-like maturation enhancing effect of nicotine (Lester et al., 2009; Kuryatov et al., 2005; Sallette et al., 2005; Srinivasan et al., 2011;) and stabilisation of the high-affinity state of the receptors (Vallejo et al., 2005). Moreover, nAChR play a key role in addiction to nicotine (Govind et al., 2009), so up-regulation could enhance addiction to nicotine by increasing the pleasant effects of the drug.

In a previous study on PC12 cells, we demonstrated that MDMA pretreatment induces *in vitro* up-regulation of both homomeric and heteromeric receptors (Garcia-Rates et al., 2007) through a mechanism that seemed to mimic that of nicotine. Then it was of interest to assess whether MDMA induces nAChR up-regulation in *vivo* as well, as changes in these receptors could have a role in drug addiction and explain some psychiatric effects of this drug, such as memory impairment and psychoses, among others in which nAChRs have been found to play a role (Levin et al., 2002; Martin et al., 2004; Ripoll et al., 2004).

Consequently, the aim of this study was to determine whether treatment with MDMA induces *in vivo* nAChR up-regulation and, moreover, to investigate whether it affects or potentiates the up-regulatory effects of nicotine, as MDMA and tobacco are very often associated (Scholey et al., 2004) and this could have implications on the addiction induced by both drugs.

2. Material and Methods

2.1 Drugs and radioligands

MDMA hydrochloride, obtained from the National Health Laboratory (Barcelona, Spain), was dissolved in saline (0.9% NaCl). Nicotine bitartrate dihydrate, purchased from Sigma-Aldrich (St. Louis, MO, USA), was also dissolved in saline. [^{3}H]MLA came from American Radiolabeled Chemicals (St. Louis, MO, USA), while [^{3}H]paroxetine, and [^{3}H]epibatidine came from Perkin-Elmer (Boston, MA, USA). All buffer reagents were of analytical grade and purchased from several commercial sources.

2.2 Animals and treatment

The experimental protocols for the use of animals in this study follow the guidelines set out by the European Communities Council (86/609/EEC) and were supervised by the ethics committee of the University of Barcelona. Male Sprague-Dawley rats weighing 200-230 g (Harlan Ibérica, Barcelona, Spain) were used. They were housed at 21ºC ± 1ºC under a 12 h light/dark cycle with free access to food and drinking water.

At the beginning of the treatment they were housed one per cage and a combined nicotine and MDMA dosing schedule was carried out for 10 days as follows. Six animals were used in each treatment group. The control (Ctrl) group received saline (1 ml/kg s.c.) twice daily (7-h interval) for the 10 days; the nicotine (NIC) group received 2 mg/kg nicotine bitartrate dihydrate (s.c.) twice daily (7-h interval) for 10 days (Flores et al., 1992); the MDMA group was given saline (s.c.) twice a day from days 1 to 6, and 20 mg/kg MDMA (s.c., b.i.d., 7-h interval) from days 7 to 10 (Battaglia et al., 1987). The MDMA+NIC group received nicotine bitartrate for the 10 days as stated for the NIC group, and MDMA (same dosing as above) was also injected during the last 4 days, 15 min. after nicotine and at a different puncture site. The rats were weighed at days 1, 4, 6 and 11 and the percentage increase calculated throughout the treatment.

The rats were killed by decapitation under isoflurane anaesthesia on day 11. The brains were rapidly removed from the skull and dissected on a refrigerated surface. Prefrontal and parietal cortex, striatum, hippocampus, and a coronal block delimited by the thickness of superior colliculi, after removal of cortex and hippocampus (contains the colliculi, the geniculate nuclei, the substantia nigra and the ventral tegmental area, VTA), were excised, frozen on dry ice and stored at -80ºC until use.

These areas were selected on the basis of their abundance in the different types of nAChR, ease to be dissected and the amount of protein to perform binding assays in homogenates. Thus heteromeric nAChR were measured in cortex, striatum and the section containing the colliculi, as they express high levels of these receptors. As for α7 nAChR, they were assessed in the hippocampus (where they are more abundant and there are low levels of α4β2) and in the cortex as well (Tribollet et al., 2004).

2.3 Tissue processing

When required, tissue samples were thawed and homogenised at 4ºC in 10 volumes of buffer consisting of 5 mM Tris-HCl, 320 mM sucrose, and protease inhibitors (aprotinin 4.5 µg/µl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogeniser. The homogenates were centrifuged at 15,000 x g for 30 min at 4ºC. The resulting pellets were resuspended in fresh buffer, incubated 5 min at 37ºC to degrade remaining endogenous ligands and recentrifuged twice. The final pellets of membrane homogenates were resuspended in 50 mM Tris-HCl buffer (plus protease inhibitors) and stored at -80ºC until use in radioligand binding assays or receptor solubilisation for Western blotting or immunoprecipitation. 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 [^{3}H]Paroxetine binding

The density of serotonin transporters (SERT) in each rat's cortex and hippocampus was determined to assess the serotonergic changes/neurotoxicity induced by MDMA (Pubill et al., 2003). This was accomplished by measuring the specific binding of 0.05 nM [^{3}H]paroxetine after incubation with 150 µg protein at 25ºC for 2 h in a Tris-HCl buffer (50 mM, pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 µM) was used to determine non-specific binding.

2.5 [^{3}H]MLA binding

Binding of 2 nM [^{3}H]MLA to label α7 nAChRs was performed in duplicates for each rat and brain area as described by Davies et al.(1999). Membrane homogenates (250 µl containing 200 µg protein) were incubated with the radioligand in glass tubes in a final volume of 0.5 ml for 2 h at 4ºC. 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 of each animal/area was determined from tubes containing 1 µM unlabelled MLA to be subtracted from total binding values. Incubation was terminated by rapid filtration and bound radioactivity counted as described below.

2.6 [^{3}H]Epibatidine binding

[^{3}H]Epibatidine binding was used to label heteromeric nAChRs. Binding was measured for each rat and brain area in glass tubes containing 1 nM [^{3}H]epibatidine and 200 µg of membrane homogenates in buffer (50 mM Tris-HCl plus protease inhibitors) to a final volume of 0.5 ml. Incubation was carried out for 2 h at 25ºC. Non-specific binding was determined in the presence of 300 µM nicotine. Binding was terminated by filtration and data were treated as described below.

2.7 Bound radioligand separation and counting

For all radioligand binding experiments, incubation was finished by rapid filtration under vacuum through GF-B glass fibre filters (Whatman, Maidstone, UK) pre-soaked in 0.5% polyethyleneimine. Tubes and filters were rapidly washed four times with 4 ml of ice-cold buffer, and the radioactivity trapped was measured by liquid scintillation spectrometry. Specific binding was calculated as the difference between the radioactivities measured in the absence (total binding) and in the presence (non-specific binding) of the excess of non-labelled ligand.

2.8 Receptor solubilisation and radioimmunoprecipitation

Aliquots of tissue homogenates were centrifuged at 15,000 x g for 30 min at 4ºC. The supernatants were discarded and the pellets were resuspended in an appropriate volume of ice-cold solubilisation buffer consisting of 20 mM Tris HCl pH 8, 137 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 4.5 µg/µl aprotinin and 0.1 mM phenylmethylsulfonyl fluoride. The receptors were solubilised by incubation for 2 h at 4ºC under gentle rotation. Thereafter, the samples were centrifuged at 15,000 x g for 30 min at 4ºC and the supernatants containing solubilised receptors were stored at -80ºC after determination of protein content using the Bio-Rad Protein Reagent and bovine serum albumin standards prepared in the same dilution of solubilisation buffer, in order to compensate for the reaction with the buffer detergent.

Immunoprecipitation of receptors containing α4 and β2 subunits was performed as described by Turner and Kellar (2005) with some modifications. Rabbit polyclonal antibody anti-nAChR α4 subunit was purchased from Abcam (Cambridge, UK) and rat monoclonal anti-nAChR β2 subunit, clone mAb290 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Aliquots of solubilised receptors containing 300 µg protein were added to sample tubes containing 1.5 nM [^{3}H]epibatidine and 1 µg of either one of the subunit-specific antibodies or the same volume of normal rabbit serum (supplied by the animal facilities

service of the Faculty of Pharmacy, University of Barcelona) in the case of α4 or rat normal IgG (Invitrogen Corp., Carlsbad, CA, USA) in the case of β2, in order to determine non-specific immunoprecipitation. The optimal antibody concentration (1 µg) was obtained from pilot experiments, and 1.5 nM [^{3}H]epibatidine was chosen to ensure the occupation of nearly all the heteromeric receptors. The final volume of each test tube was 180 µl. The samples were incubated overnight at 4ºC under gentle rotation and then 25 µl of a slurry of either Protein A-agarose or Protein G Plus-Agarose (Santa Cruz Biotechnology, Inc.) was added to each tube for α4 or β2 antibody precipitation, respectively. The rotation of the samples was continued for an additional hour. The samples were then centrifuged at 7,000 x g for 5 min. and the supernatants carefully removed. The pellets were washed with 0.75 ml of cold 50 mM Tris-HCl buffer pH 7.4 and recentrifuged. The supernatants were discarded and the immunoprecipitate pellets were dissolved in 100 µl of 1 N NaOH, transferred to scintillation vials and the radioactivity counted in a liquid scintillation counter after addition of liquid scintillation fluid (Ultima Gold MV, Perkin Elmer, Boston, MA, USA). The counts precipitated in tubes containing normal rabbit serum or rat IgG, which were used as control for non-specific precipitation, were subtracted from the counts obtained in the presence of the specific antibody, in order to calculate specific immunoprecipitation.

Total epibatidine binding was measured in parallel samples, incubated under the same conditions but without antibody and agarose beads. After overnight incubation they were filtered through Whatman GF-B glass fibre filters that had been pre-wet with 0.5% polyethyleneimine, using a cell harvester (Perkin Elmer filter mate), followed by four 1 ml washes. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry as above. Non-specific binding was determined from tubes containing 300 µM nicotine.

2.9 Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine α4 and α7 subunit levels in the protein extracts. For each sample, 40

µg of protein was mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue, final concentrations), boiled for 10 min, and loaded onto a 10% polyacrylamide gel. Proteins were separated by electrophoresis until the elution of the migration front and transferred from gels to polyvinylidene fluoride sheets (Immobilon-P; Millipore, Billerica, MA, USA). These sheets were then blocked for 1 h at room temperature with 5% defatted milk in Tris-buffered saline buffer plus 0.05% Tween 20 (TBS-T buffer) and incubated overnight at 4ºC with either rabbit polyclonal antibody against α7 subunit (ab23832) or rabbit polyclonal anti-α4 subunit (ab41172), both purchased from Abcam (Cambridge, UK) and used at a 1:1000 dilution in TBS-T buffer plus 5% defatted milk. Thereafter, membranes were washed with TBS-T buffer and incubated for 45 min with peroxidase-conjugated secondary antibody (donkey anti-rabbit IgG, 1:20,000 dilution; GE Healthcare, Buckinghamshire, UK,).

Immunoreactive protein was visualised using a chemoluminescence-based detection kit following the manufacturer's protocol (Immobilon Western, Millipore, Billerica, MA, USA) and a BioRad ChemiDoc XRS gel documentation system (BioRad Labs., Hercules, CA, USA). Apparent molecular weight bands corresponding to the target proteins were 56 kDa for α7 subunit and 70 kDa for α4 subunit. Scanned blots were analysed using BioRad Quantity One software. Immunodetection of β-actin (mouse monoclonal anti β-actin antibody, Sigma, St. Louis, USA; dil.1:2500) served as a control of load uniformity for each lane and was used to normalise differences due to protein content. The β-actin band appeared at a molecular weight of approximately 42 kDa. The α7 and α4 levels are expressed as a percentage of those obtained from saline-treated animals.

2.10 Statistical Analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) of the values obtained for each treatment group. Two-way analysis of variance (ANOVA) for repeated measures was used to analyse the effect of the treatment in the temporal evolution of body weight gain. The rest of statistical comparisons were made using one-way ANOVA (two-tailed). Significant

(P<0.05) differences were then analysed by Tukey's post-hoc test for multiple means comparisons, where appropriate. All statistic calculations were performed using PASW Statistics v-18 (SPSS software, IBM, New York, USA).

3. Results

3.1 Effect of treatment on body weight gain

Figure 1 depicts the evolution of body weight in the different treatment groups. As can be seen, the main differences among the groups start at the point that MDMA was introduced. The two-way ANOVA analysis for repeated measures reported significant differences in the effects of the treatment ($F_{3,21}$= 5.77, P<0.01) and during the days of treatment ($F_{3,21}$= 18.93, P<0.001). At day 11, the MDMA group significantly had gained less weight than saline (P<0.01) and the MDMA+NIC group had gained even less weight than the MDMA group (P<0.001 *vs.* saline), although differences between these two groups did not reach statistical significance. No significant differences were found between NIC- and saline-treated animals.

3.2 Effects of treatment on serotonin transporter density

Binding of [^{3}H]paroxetine was performed in the parietal cortex and hippocampus in order to assess the possible deleterious effect of drug treatment on serotonergic terminals. Results are presented in table 1. In the cortex, a significant decrease (around 70%) in [^{3}H]paroxetine binding was found in MDMA-treated animals (P<0.001 *vs*. saline), while NIC did not modify these levels. In the hippocampus, the decrease in binding was more modest (around 20%) and it was not modified by nicotine either.

Table 1
Binding of [^{3}H]paroxetine to membranes from parietal cortex (pCTX) and hippocampus of rats treated with saline (control), MDMA, NIC or the combination of NIC+MDMA, as stated in 2. Results are expressed as the percentage of the specific binding obtained in control rats and they are the mean ± S.E.M. of the values obtained from 5-6 animals per group.

	Control	MDMA	NIC	NIC + MDMA
pCTX	100.0 ± 3.6	33.7 ± 3.1 ***	91.0 ± 4.1	26.94 ± 13.7 ***
HC	100.0 ± 2.9	83.9 ± 5.5 *	114.0 ± 7.2	77.1 ± 5.6 **

* P<0.05; ** P<0.01; *** P<0.001 vs. control group. One-way ANOVA and Tukey's post-hoc test.

3.3 Effects on [^{3}H]epibatidine binding

The levels of heteromeric nAChRs were measured through [^{3}H]epibatidine binding assays and are shown in Fig. 2. In the parietal cortex, NIC and MDMA separately induced significant increases in binding of 18.0 ± 3.6% and 16.5 ± 5.8% respectively, compared with saline-treated rats. When NIC and MDMA were associated, such effects were significantly potentiated, rising to a 29.1 ± 5.7% increase. Similar but more pronounced effects were found in the frontal cortex , where NIC and MDMA separately induced increases of 29.5 ± 10.7% and 34.6 ± 9.2%, respectively, that rose to 72.1 ± 17.5% when both drugs were associated. By contrast, in the striatum and in the coronal section delimited by the superior colliculi, NIC alone induced an increase in binding of 41.3 ± 5.5% and 47.4 ± 15.4%, respectively, but not MDMA, which did not modify NIC-induced up-regulation. In the cerebellum, no significant increases were found in any of the treatment groups (data not shown).

3.4 Effects on [^{3}H]MLA binding

The levels of homomeric nAChRs (mainly α7) were measured using [^{3}H]MLA binding. The results are shown in Fig. 3. In the prefrontal cortex, MDMA and NIC failed to induce significant up-regulation separately, but the association of the two drugs led to a significant increase of 42.1 ± 20% in [^{3}H]MLA binding. In

the parietal cortex, MDMA induced a slight up-regulation (15.3 ± 5.2% increase) that was not modified by its association with NIC, which alone did not induce any significant effect.

By contrast, in the hippocampus (Fig. 3C), MDMA alone did not induce any change in [^{3}H]MLA binding but potentiated the regulatory effects of NIC, which rose from a 25.5 ± 7.6% to a 52.5 ± 11.3% increase when both drugs were associated.

In the striatum, none of the treatments induced significant changes in [^{3}H]MLA binding (data not shown).

3.5 α4 and α7 subunit expression

Western blot analysis using specific antibodies against α4 and α7 nAChR subunits was performed in the areas where the most marked increases had been found, that is, in the prefrontal cortex for α4 and the hippocampus for α7. No significant changes in protein expression were observed among the different treatment groups (Fig. 4).

3.6 α4 and β2 subunit immunoprecipitation

To assess which subunits of heteromeric receptors were up-regulated, we performed immunoprecipitation of α4 and β2-containing receptors labelled with [^{3}H]epibatidine using prefrontal cortex extracts, where the most marked effects on radioligand binding were found. The results are shown in Fig. 5. Overall, the increases in binding in the different treatment groups paralleled those observed in binding to membranes. The potentiation of the up-regulation was only seen in the α4 immunoprecipitate. Immunoprecipitation with anti-β2 antibody trapped 100% of specifically-bound radioligand, indicating that in this area, all receptors labelled with [^{3}H]epibatidine contained this subunit. Immunoprecipitation with anti-α4 trapped around 80% of total binding.

4. Discussion

Nicotinic receptors play a key role in addiction to nicotine (Govind et al., 2009). It has been described that the addictive effects of nicotine are produced through its interaction with nAChR in the mesolimbic pathway, especially those in the nucleus accumbens, leading to dopamine release that activates the reward circuitry. In fact, mice with deletion of the β2 gene do not self-administer nicotine after previous administration and do not show increased release of dopamine in the ventral tegmental area (Picciotto et al., 1999). Although the mechanisms involved in the establishment of addiction are complex and still being investigated, up-regulation of nAChR increasing the pleasant effects of the drug is an event that could feasibly play a role. It is thought that up-regulation of nAChR is a homeostatic response to the rapid desensitisation of the receptors induced after prolonged exposure to an agonist (Fenster et al., 1999) in order to re-establish the nicotinic pathways. Several mechanisms have been proposed to explain such up-regulation and they are mentioned and cited in the Introduction section.

According to our previous study (Garcia-Rates et al., 2007, 2010) demonstrating that MDMA had affinity for and induced nAChR up-regulation in PC12 cells, it was of interest to assess whether the regulatory effects of MDMA on nAChR could take place *in vivo* after repeated administration of MDMA as well. Also, because MDMA and nicotine (smoked tobacco) are often associated (Scholey et al., 2004), we tested the effect of such an association on nAChR up-regulation. One important issue was the selection of the dosing schedule. Studies involving nicotine have used either a repeated dosing schedule or the implantation of sustained-release osmotic minipumps (Even et al., 2008; Nguyen et al., 2003) or constant infusion (Marks et al., 2011; Pauly et al., 1996) to achieve constant plasmatic levels, thus reaching the highest levels of nAChR up-regulation. The use of these drug delivery methods was rejected for MDMA because of possible organ failure after prolonged and sustained plasmatic levels and because this drug is not consumed as continuously as nicotine. For this reason we chose the established MDMA chronic dosing schedule (20 mg/kg b.i.d. for 4 days) and combined it with one of the schedules reported in

the literature for nicotine, involving two injections per day for 10 days (Flores et al., 1992). The relatively high dose of MDMA was chosen based on the fact that, according to radioligand binding experiments (Garcia-Rates et al., 2007), low micromolar concentrations had to be reached in the target area and mantained enough time to induce the up-regulation and also because this is a generally accepted schedule of chronic MDMA (Battaglia et al., 1987).

An inconvenience of such a dosing schedule is the possibility of serotonergic neurotoxicity from MDMA. MDMA-induced hyperthermia can potentiate its neurotoxic events, although it is not mandatory for the long–term neurotoxicity that follows MDMA administration (Capela et al., 2009). For this reason, the treatment was carried out at 20ºC-21ºC in order not to exacerbate the neurotoxic effects by facilitating the hyperthermia (Gordon et al., 1991; Green et al., 2005). At ambient temperatures between 20ºC-24ºC, no changes in serotonin and 5-hydroxyindolacetic acid were reported following MDMA administration (Malberg et al., 1998), although decreases in [^{3}H]paroxetine have been reported at these temperatures (O'Shea et al., 2006). In fact the changes in [^{3}H]paroxetine binding have been claimed to be a more reliable marker of MDMA-induced neurotoxicity, rather than the loss of serotonin and its metabolites (O'Shea et al., 2006). However, we must point out that in our treatment the rats were killed 24 h after the last dose, while most studies (i.e. (Biezonski and Meyer, 2010; Broening et al., 1995; Malberg et al., 1998; O'Shea et al., 1998; O'Shea et al., 2006; Pubill et al., 2003) make the measurement after leaving a time of at least one week to allow the neurotoxic process to occur. Interestingly, we found a robust decrease (around 70%) in [^{3}H]paroxetine binding in cortex from MDMA-treated rats as early as 24 h after the last dose, a time at which the development of axonal and terminal degeneration is unlikely. Also we must point out that measuring [^{3}H]paroxetine binding at only one radioligand concentration (0.05 nM) does not inform us on whether the observed decrease is due to a drop in total number of transporters (B_{max}) or to a decrease in the affinity (increase in K_D). Battaglia et al. (1987) used the same MDMA schedule than us but measured binding two weeks after treatment. They performed saturation binding assays and demonstrated that the

reduction in [^{3}H]paroxetine binding after MDMA treatment was due to a decrease in B_{max} without significant changes in K_D.

Recent studies, however, have raised the question whether serotonergic marker depletion caused by MDMA is reflective of neurodegeneration or rather is an effect of biochemical down-regulation in the absence of tissue damage (Biezonski and Meyer, 2011). In fact, a significant reduction in SERT gene expression, which could explain a reduction in SERT protein irrespective of altered terminal integrity, has been reported after treatment with MDMA (Biezonski and Meyer, 2010). Both terminal destruction and SERT down-regulation would produce reductions in B_{max}. On the other hand, a decrease in K_D could be caused by the presence of MDMA in the binding medium (which is unlikely in our case seeing as the preparation is washed several times before binding) or by acute modifications (i.e. phosphorilation, nitrosilation) in the transporter as has been documented for the dopamine transporter (Hansen et al., 2002). In fact, in a previous study from our group (Escubedo et al., 2011), we demonstrated that incubation of rat brain synaptosomes with MDMA for 1 h induced a decrease in [^{3}H]5-HT uptake measured after drug removal, which indicates that a rapid change in SERT leading to an impaired function was produced. This change could also involve a decreased affinity for paroxetine. Due to the fact that our rats were killed 24 h after treatment, such an effect decreasing affinity for the radioligand cannot be definitely ruled out.

Regardless of the fact that neurotoxicity could develop after this treatment, it can be assumed that changes in nAChR density after moderate-high doses of MDMA take place before the neurodegenerative process begins.

The temporal evolution of rats' body weight was studied to ascertain an easily measurable effect of MDMA and to study any possible interaction with nicotine. The rats treated with MDMA gained less weight than controls due to the anorectic effect of the drug. Serotonin (5-HT) 5-HT_4 receptors in the nucleus accumbens are specifically involved in the appetite-suppressant effects of this drug inducing 5-HT release (Francis et al., 2011). Although nicotine had no significant effects on body weight gain, the graph of the association with MDMA

suggests a tendence, although not statistically significant, of impaired weight gain in this treatment group. It is known that nicotine relieves anxiety and people who give up smoking increase food intake (Schnoll et al., 2012). In this context, nicotine could enhance the lack of appetite induced by MDMA leading to less weight gain although, as mentioned, this did not reach statistic significance.

After this treatment, MDMA induced up-regulation of heteromeric and α7 nAChR in several rat brain areas. Moreover, a synergistic effect was observed in the cortex for heteromeric nAChR and in the hippocampus for the α7 type. Accordingly, these two areas contain a high density of serotonergic innervation in addition to nAChR, and are main targets of MDMA. In striatum and the section containing superior colliculi, lateral geniculate nuclei, substantia nigra and VTA, only the effect of NIC was detected. With the data to hand, any explanation for such a difference can only be a matter of speculation. Striatum, geniculate nuclei, substantia nigra and especially superior colliculi exhibit higher heteromeric nAchR density than cortex and hippocampus (Tribollet et al., 2004) and are highly sensitive to up-regulation by nicotine (Nguyen et al., 2003). It might be that MDMA-induced up-regulation in striatum and colliculi was so modest in these tissues with high receptor density that the increases do not reach statistical significance. In fact, an upward trend can be seen in the striatum in the MDMA group.

The effect of NIC on α7 nAChR was less marked than that on heteromeric receptors. In fact, the affinity of NIC for α7 nAChR is in the micromolar range while the K_D for heteromeric nAChR is nanomolar (Marks et al., 1986), so higher concentrations of NIC had to be achieved in a given brain area to induce such up-regulation. Also it must be pointed out that the intermittent NIC dosing schedule used could not be as potent at inducing α7 nAChR up-regulation as continuous administration would be. As far as MDMA is concerned, it has higher affinity for heteromeric than for α7 nAChR (Garcia-Ratés et al., 2007), thus a more marked effect on the heteromeric receptors was expected and confirmed by the experimental results. It has been reported that exposure to α7 nAChR partial agonists increases the expression of these receptors in rodents

(Werkheiser et al., 2011) and that MDMA acts as a α7 partial agonist in PC12 cells (Garcia-Rates et al., 2010). This would account for its effect on the density of α7 nAChR in the parietal cortex and the synergy with NIC seen in the cortex and hippocampus.

When solubilised receptors were immunodetected with Western blotting, no significant changes were observed in levels of protein density, in agreement with the general assertion that up-regulation of nAChR takes place at a post-translational level (reviewed by Gaimarri et al., 2007), promoting the assembly of nAChR subunits and their migration from the endoplasmic reticulum (ER) to the plasma membrane. Since our extracts were obtained from whole-tissue homogenates, the antibodies raised to a specific nAchR subunit did not distinguish between assembled plasma membrane receptors and intracellular ER-associated subunits at different stages of maturation. Conversely, radioligands preferentially labelled assembled/mature receptors. These results suggest that MDMA acts on nAChR similarly to nicotine, possibly even exerting a synergistic effect.

Nicotine mainly induces up-regulation of α4β2 nAChR, which are the most abundant in mammals' CNS. As [^{3}H]epibatidine labels nearly all heteromeric nAChR, we performed the immunoprecipitation of receptors containing α4 and β2 subunits and carried out radioligand binding, in order to ascertain the participation of these subunits in the up-regulation process. The binding of the total solubilised extract paralleled that performed in crude membranes, indicating that up-regulation levels persist after receptor solubilisation. Up-regulation of α4- and β2-containing receptors was found separately in the immunoprecipitates, but the synergy of the nicotine + MDMA association was found only in the α4 immunoprecipitate. This indicates that, similarly to what happens with nicotine, α4β2 nAChR are the main subtype that is up-regulated after treatment with MDMA *in vivo*. However, other associations containing the β2 subunit would be resistant to up-regulation because in the β2 immunoprecipitate the increased binding levels were more modest and there was no synergy in the nicotine + MDMA association. In fact, several studies have demonstrated that not all nAChR subtypes undergo up-regulation and not

all show it to the same extent and under the same experimental conditions. For example, the α4β2α5 combination is resistant to up-regulation (Mao et al., 2008), the α6-containing nAChR only undergo up-regulation at high but transient nicotine concentrations, while α4β2 nAChR require a lower concentration but a more prolonged exposure (Walsh et al., 2008); finally, α3β2 and α3β4 subtypes undergo much lower up-regulation than α4β2 (Nguyen et al., 2003). Also the access of each ligand to a certain brain area and the nAChR subtype predominant in it could modify the up-regulation process.

Both nicotinic agonists and antagonists are able to induce nAChR up-regulation due to their affinity for the receptors or their immature forms (Peng et al., 1994; Gopalakrishnan et al., 1997). In fact, we have previously demonstrated that MDMA behaves as a partial agonist on α7 nAChR and as an antagonist on α4β2, inducing significant up-regulation of both receptor types in PC 12 cells at a concentration of 1 μM (Garcia-Ratés et al., 2007, 2010), which has been reported to be reached *in vivo* (Johnson et al., 2004).

Due to the complexity of brain synapses and regulation, an additional unknown mechanism involved in nAChR up-regulation after MDMA cannot definitely be ruled out. However, the previously reported results using cultured PC 12 cells demonstrate that the simple interaction of MDMA with nAChR is sufficient to induce the up-regulation.

All this evidence makes the study of MDMA's effects on nAChR levels as complex as the studies carried out on nicotine for more than 20 years by many research groups. What is warranted is the use of autoradiography to produce a closer mapping of more defined brain areas that show up-regulation after treatment with MDMA. Also, another challenge to face is to find a different dosing schedule that uses lower and potentially less neurotoxic MDMA doses, while increasing the duration of treatment.

In conclusion, this is the first study to date that demonstrates an *in vivo* up-regulation of nAChR after treatment with MDMA, as well as a synergistic effect when MDMA is associated with nicotine. Given that these two drugs are often

associated, the development of neuroadaptive processes in which nAChR play a role could be enhanced. Were specific areas to be affected, such as the ventral tegmental area and nucleus accumbens, there could be an increase in addiction and drug vulnerability. The fact of having taken one of these drugs could later make the subject more prone to the addictive effects of the other. Also, as α7 nicotinic receptors are involved in MDMA-induced neurotoxicity (cited above), an enhanced risk of toxicity in certain brain areas (i.e. the hippocampus) leading to cognitive impairment could be feasible.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1: Evolution of body weight gain throughout the drug treatment. Nicotine was started at day 1 and MDMA at day 7. Drug administration was finished at day 10 and the rats were killed at day 11. The values are the means ± SEM of the body weight of six animals per group. **P<0.01, *** P<0.001 *vs*. saline.

Figure 2: Binding of [^{3}H]epibatidine to heteromeric nAChR in membranes from prefrontal cortex (A), parietal cortex (B), striatum (C) and the coronal section containing superior colliculi and substantia nigra (D) of rats belonging to the different treatment groups. Data are means ± SEM from 6 animals per group. Control (Ctrl) animals received saline. *P<0.05, **P<0.01; ***P<0.001 *vs.* Ctrl.; #P<0.01 *vs.* NIC; $P<0.05 *vs.* MDMA.

Figure 3: Binding of [^{3}H]methyllycaconitine ([^{3}H]MLA) to homomeric α7 nAChR in membranes from prefrontal cortex (A), parietal cortex (B) and hippocampus (C) of rats belonging to the different treatment groups. Data are means ± SEM from 6 animals per group. Control (Ctrl) animals received saline. *P<0.05, **P<0.01 *vs.* Ctrl., #P<0.05 *vs.* NIC.

Figure 4: Western blot analysis of nAChR subunits α4 (panel A) and α7 (panel B) in extracts of prefrontal cortex and hippocampus, respectively, from rats belonging to the different treatment groups. Bar graphs show overall quantification of the blots (mean ± SEM), while a representative autoradiography of each determination is shown above. β-actin levels were used to ensure gel loading uniformity and to normalise the protein values.

Figure 5: Levels of [^{3}H]epibatidine binging after immunoprecipitation with anti-α4 (panel A) and anti-β2 (panel B) specific antibodies in cortex extracts of rats from the different treatment groups. Also, total binding to these extracts was measured in parallel samples (panel C) in order to calculate the percentage of immunoprecipitated binding. Data are the means ± SEM of values from 5-6 rats per group. *P<0.05, **P<0.01; ***P<0.001 *vs.* Ctrl.; #P<0.05, ##P<0.01 *vs.* NIC.

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