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7. Involvement of nicotinic receptors in methamphetamine and MDMA induced neurotoxicity: Pharmacological studies

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Abstract. During the last years, our emphasis has focused in the study of the neurotoxic effects of 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) on central nervous system and their pharmacological prevention. In the process of this research, we have used a semi-purified synaptosomal preparation from striatum of mice or rats as a reliable in vitro model to study reactive oxygen species (ROS) production by these amphetamine derivatives, which is well correlated with their dopaminergic injury in in vivo models. Using this preparation we have demonstrated that blockade of alpha7 nicotinic receptors with methyllycaconitine (MLA) and memantine (MEM) prevents ROS production induced by MDMA and METH.

Studies at molecular level showed that both, MDMA and METH, displaced competitively the binding of radioligands for homomeric alpha7 and heteromeric nAChRs, indicating that they can directly interact with them. In all the cases MDMA displayed higher affinity than METH and it was higher for heteromeric than

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for alpha7 subtype. Preincubation of differentiated PC12 cells with MDMA or METH induces nicotinic acetylcholine receptors (nAChR) up-regulation in a concentration- and time-dependent manner, as many nicotinic ligands do, supporting their functional interaction with nAChRs. Such interaction expands the pharmacological profile of amphetamines and can account for some of their effects.

Introduction

Amphetamine derivatives, such as methamphetamine (METH, speed) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) are widely abused drugs, mainly by young people in recreational settings. Besides their stimulatory effects, these drugs have been shown to be neurotoxic in animal models (for reviews see [1, 2]) in which deleterious effects in dopamine (DA) and serotonin (5-HT) nerve terminals have been reported. In addition, signs derived from neurotoxicity have been described in humans who are heavy users of such drugs [3-6]. The patterns of neurotoxicity of MDMA in mice and rats differ in that mice typically exhibit neurotoxicity to both DA- and 5-HT-containing neurons, whereas rats commonly display selective neurotoxicity to 5-HT-containing neurons [7, 8].

Two theories have arisen to explain this neurotoxicity. Firstly, the neurotoxicity induced by MDMA may at least partially be a consequence of its metabolism [9]. This hypothesis is based on the fact that a direct intracerebral injection of MDMA failed to reproduce the neurotoxicity profile that appears after its peripheral administration [10]. The other main theory involves reactive oxygen species (ROS), although the two theories cannot be considered mutually exclusive. Oxidative stress appears to be one of the main factors involved in the serotonergic and dopaminergic terminal injury induced by MDMA [11, 12].

Although oxidative stress has been proposed as a key neurotoxic mechanism induced by these drugs [13, 14], several aspects surrounding the concrete pathways involved in METH- and MDMA-induced ROS generation remain unresolved. Our research group has recently reported not only that METH and MDMA induce ROS production inside rat and mouse striatal synaptosomes, but also that endogenous DA is needed for this reaction to occur [15-17]. We also determined that methyllycaconitine (MLA), an antagonist of α7 nicotinic acetylcholine receptors (α7 nAChR), prevented in vitro ROS generation and attenuated in vivo neurotoxicity, thus implicating α7 nAChR in the toxicity of amphetamine derivatives.

α7 nAChR are homomeric ligand-gated ion channels whose activation induces calcium influx. Calcium entry could favour the activation of Ca^{2+}-dependent enzymes such as protein kinase C (PKC) and neuronal nitric oxide synthase (nNOS), which have similarly been implicated in the neurotoxicity

of amphetamines [18, 19]. In light of these findings, we believed it necessary to assess whether METH and MDMA have a direct interaction with α7 nAChR.

Certain previous reports have suggested that amphetamines interact with different nicotinic receptors. Liu et al. [20] reported that D-amphetamine acts as an agonist on nicotinic receptors (probably α7) in bovine chromaffin cells, inducing catecholamine release. In addition, Skau and Gerald (1978) [21] had reported that D-amphetamine inhibits α-bungarotoxin binding at the neuromuscular junction in mice, while Klingler et al. [22] recently identified nAChR as one of the physiological targets of MDMA in the neuromuscular junction. Our previous findings [15-17] relate such an interaction to neurotoxicity. Moreover, as it has been extensively reported that chronic treatment with nicotine and nicotinic ligands induces an up-regulation of nicotinic receptors in central nervous system (CNS) [23, 24], the effect of amphetamines on nicotinic receptor populations warrants further study.

We demonstrated, using radioligand binding assays, the interaction of METH and MDMA with homomeric α7 nAChR and heteromeric subtypes of nicotinic receptors, such as α4β2. We previously demonstrated in vitro that Ca^{2+} chelation with EGTA prevented ROS production to a similar extent as nAChR blockade [12, 15-17]. This indicates that calcium influx, probably through α7 nAChR, is a key step in this process. Consequently, one of the objectives of the final work was to use a fluorimetric method to investigate the effect of MDMA on Ca^{2+} levels in cultured PC12 cells and the involvement of different nAChR subtypes and other cell pathways related to Ca^{2+} mobilization. In addition, we investigated the effects of pretreatment with METH and MDMA on nAChR densities.

PC12 cells have been utilized by other scientists to study the neurotoxicity of amphetamines [25-27]. In addition, this cell line expresses nAChRs, including the α7 subtype [28-30], and also provides an in vitro model for the up-regulation of nAChR, which occurs following chronic exposure to nicotine [31, 32]. Moreover, the pathways involved in cytosolic Ca^{2+} increase induced by different selective nicotinic agonists have been characterized in this cell line [33]. For this reason, we chose this model and the isolated synaptosomes as the most appropriate for our purposes.

Herein we present the most important points brought out by our research.

1. DA, PKC and nNOS involvement in METH- and MDMA-induced ROS production

Our study was undertaken with the goal of developing an alternative in vitro model that might be useful for studying the molecular mechanisms of METH-induced DA neurotoxicity. With this purpose we used the fast and

simple method for isolating synaptosomes described by Myhre and Fonnum [34]. Using this model the formation of intrasynaptosomal ROS was measured using the conversion of the non fluorescent 2',7'– dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent compound 2',7'-dichlorfluorescein (DCF).

METH increases DCF fluorescence when added to our preparation, which indicates that it induces ROS production [15]. Incubation of synaptosomes with METH for a few minutes also causes release of DA from presynaptic nerve terminals and inhibits DA uptake, probably by reversion of dopamine transporter (DAT) functionality. When we used synaptosomes from DA-depleted rats (pretreated with reserpine or reserpine plus alpha-methyl-p-tyrosine) the METH-induced ROS production was inhibited, thus corroborating DA as the main source of ROS detected (Fig. 1). Besides, METH, by altering the intracellular pH gradient, prevents vesicular monoamine transporter (VMAT) function and promotes DA release from vesicles to cytosol [35] where it can be oxidized. By this way, *in vitro* incubation of synaptosomes with substances that block VMAT (reserpine) prevents METH oxidative effect.

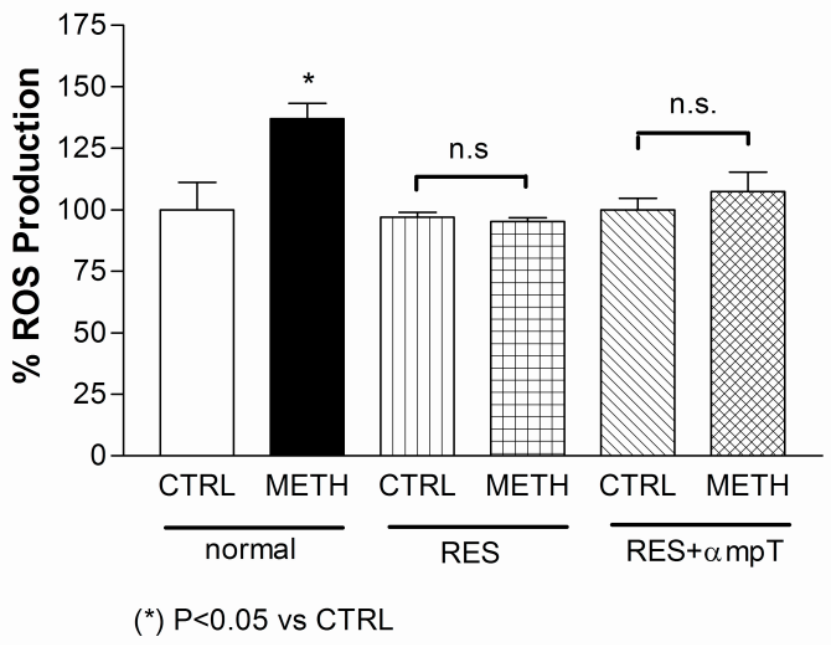

Figure 1. Effect of catecholamine depletion on METH-induced ROS in rat striatal synaptosomes. Rats were pretreated with saline (normal), reserpine (RES), or with reserpine plus alpha-methyl-p-tyrosine (RES + AMPT). Synaptosomes were obtained, and incubated alone (CTRL) or with 2 mM METH. *P<0.05 versus normal control group. Results are mean ± S.E.M. of at least three separate experiments run by triplicate. Differences between groups were compared using one-way analysis of variance (ANOVA, two-tailed). Significant ($p < 0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons where appropriate.

Activation of nNOS produces NO, which reacts with the peroxide radicals which would originate from DA autooxidation, producing the more toxic radical peroxynitrite ($ONOO^-$). In our model, the inhibitor of nNOS, 7-NI, completely abolished METH-induced ROS production, demonstrating a role of the enzyme nNOS in METH oxidative effects.

PKC has been implicated in various aspects of DAT function and its direct phosphorylation [36]. In our model, inhibition of PKC (by NPC 15437, 2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]hexanamide dihydrochloride) fully prevented METH-induced ROS, corroborating a key role of PKC in this process.

Therefore an increase in cytosolic DA and activation of nNOS and PKC (blocking DA transport through DAT) are needed to generate ROS inside the dopaminergic terminal. Both, PKC and nNOS are enzymes that require calcium to be activated. Accordingly, when calcium of the medium was chelated with EGTA, the oxidative effect of METH was prevented.

Also MDMA induces oxidative stress [12]. The MDMA concentration-response curve showed an inverted "U" shape and the maximal oxidative effect appeared at 50-100 μM and showed the same dependences.

2. nAChR involvement in METH and MDMA-induced ROS production

Ionotropic homomeric nicotinic receptors made of five alpha7 subunits are permeant to Na^+ and Ca^{2+}. Although alpha7 neuronal nicotinic receptors are a minority type in the striatum, they are expressed on DA axon terminals [37]. For this reason we tested the involvement of nicotinic alpha7 receptors in our preparation, using the specific antagonist MLA. MLA completely inhibited METH and MDMA-induced ROS production, as well as alpha-bungarotoxin (another prototypic alpha7 antagonist) while dihydro-beta-erythroidine (DBE), an antagonist that blocks receptors containing beta2 subunits was devoid of effect, thus implicating alpha7 receptors [15]. On the basis of these antecedents, we considered the possibility that MDMA might also exert an oxidative effect dependent on nAChR stimulation. Specific beta2-subunit-containing and alpha7 nAChR antagonists fully inhibited the oxidative stress induced by MDMA.

Additionally, MLA inhibited the remaining effect of MDMA in the presence of catalase plus superoxide dismutase (attributed to NO), pointing a role of alpha7 nAChR in the activation of nNOS induced by MDMA.

2a. *In vivo* MLA protects from METH- and MDMA-induced dopaminergic neurotoxicity

Assessment of neurotoxicity markers after an *in vivo* treatment with a neurotoxic schedule of METH or MDMA was used to determine the

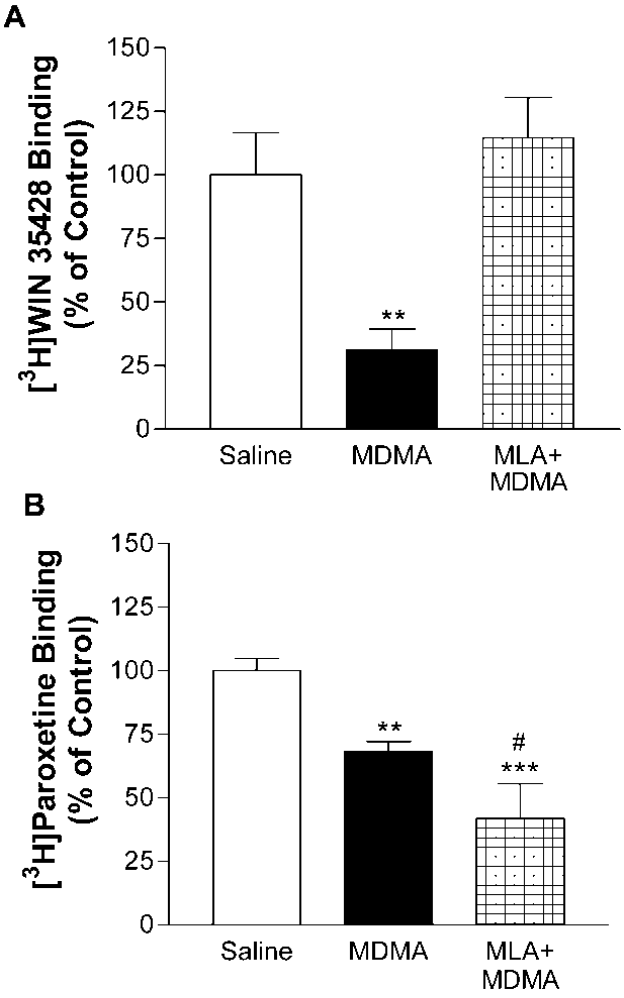

Figure 2. Panel A. Effect of *in vivo* treatment with MDMA (three injections, 25 mg/kg s.c., 3-h intervals) or in combination with MLA (three injections, 6 mg/kg i.p., 20 min before MDMA) on the density of mouse striatal dopamine reuptake sites at 7 days after treatment, and measured as specific binding of [^{3}H]WIN 35428. ** P< 0.01 vs saline-treated group. **Panel B**: Effect of treatment with MDMA alone or in combination with MLA (same dose schedule as above) on the density of mouse striatal serotonin reuptake sites 7 days after treatment, measured as specific binding of [^{3}H] paroxetine. **P<0.01, *** P<0.001 vs saline-treated group; #P< 0.05 vs MDMA-treated group. In both panels values are expressed as means ± S.E.M. of those obtained from 5 - 6 animals in each group. One-way ANOVA and Tukey's post hoc test.

neuroprotective effect of MLA (administered 20 min before each dose of METH or MDMA) in mice [16, 17].

METH induced, at 72 h post-treatment, a significant loss of striatal DA reuptake sites of about 73%, measured as specific binding of [^{3}H]WIN 35428 ((–)-2- β-carbomethoxy-3-β-(4-fluorophenyl)tropane) in mouse striatum membranes. This dopaminergic injury was attenuated in mice pretreated with MLA (from 73% to 43%).

The in vivo neurotoxic model of MDMA used was characterized by a significant loss of DA terminals (69%) and a decrease of tyrosine hydroxylase levels (65%) in striatum from mice sacrificed 7 days post-treatment. This terminal loss was prevented by pretreatment with MLA, pointing also to a neuroprotective effect against this amphetamine derivative.

[^{3}H]Paroxetine binding, which labels SERT, was measured also in the hippocampus of these animals, as a marker of degeneration of serotonin terminals. Conversely, MLA did not prevent the loss in [^{3}H]paroxetine binding sites, indicating that its neuroprotective effect is selective for DA terminals.

2b. Memantine protects from MDMA-induced CNS injury

Memantine (MEM), a non-competitive antagonist of the NMDA receptor, is a drug used to treat moderate-to-severe Alzheimer's disease. It reduces tonic [38] but not synaptic, NMDA receptor activity.

In 2005, the group of Aracava demonstrated that MEM, at clinically relevant concentrations, can block alpha7 nAChR in a non-competitive manner, and more effectively that it does at NMDA receptors [38].

Our *in vitro* neurotoxicity studies showed that MEM had not antioxidant effect versus H_2O_2 but inhibited the ROS production induced by MDMA at all the concentrations tested. The oxidative effect of amphetamine derivative was reinforced by PNU 282987 an alpha7 specific agonist.

In vivo experiments were carried out with Dark Agouti rats, that are more sensitive to low doses of drugs, and we used it as a model of serotonergic neurotoxicity induced by MDMA. A significant decrease in the SERT density was observed in both, the hippocampus and frontal cortex of MDMA-treated Dark Agouti rats sacrificed 1 or 7 days post-treatment. In both cases, MEM significantly prevented the loss of [^{3}H]paroxetine binding sites, suggesting a neuroprotective effect on serotonin terminals [39] (Table 1).

Table 1. Effect of memantine in the abundance of 5-HT (rat hippocampus) transporters (labeled by [^{3}H]paroxetine), and also in the glial activation measured by the [^{3}H]PK11195 binding in those tissues in rats killed 7 days post-treatment.

Treatment	[^{3}H]Paroxetine binding (%)	[^{3}H]PK 11195 binding (%)
Saline	100.00 ± 9.34	100.00 ± 6.35
MDMA	70.02 ± 7.85 *	168.22 ± 20.65 **
MEM	116.40 ± 3.95	94.90 ± 7.05
MDMA + MEM	94.87 ± 4.96	104.41 ± 9.77 #

Results are expressed as mean ± S.E.M. from 6-9 different experiments. * P<0.05 and ** P<0.01 vs Saline; # P<0.05 and ### P<0.001 vs. MDMA group.

Accordingly with our results, MEM could be used not only to treat these addictions or to prevent the effects of these amphetamine derivatives, but it may also have a beneficial effect on the memory impairment that abusers of these drugs usually suffer [40].

3. Nicotinic receptors and the cognitive impairment induced by MDMA

In our laboratory we performed some experiments to demonstrate a specific effect of MDMA treatment on the object recognition memory test and the Morris water maze in Long Evans rats [41]. Animals pre-treated with MEM did not exhibit the lack of memory that appeared in the MDMA-treated animals. Therefore, MEM by preventing MDMA-induced neuronal injury contributes to ameliorate cognitive impairment produced by MDMA, this preventive effect on MDMA-induced impairment suggesting a new therapeutic approach to the treatment of long-term adverse effects of amphetamine derivatives.

4. Amphetamine derivates directly interact with nAChR

METH and MDMA displaced both [^{3}H]epibatidine ([^{3}H]EB) and [^{3}H]MLA binding in PC12 cells and mouse brain, indicating that they can directly interact with nAChR. MDMA displayed higher affinity than METH for both subtypes of nAChR. The resulting K_i values fell in the micromolar range, although some of them are in the low micromolar range and other in the high micromolar range (**Table 2**).

Special attention must be paid in the affinity for heteromeric receptors (K_i about 0.7 μM) which is practically the same that the K_i displayed by MDMA for the serotonin transporter, its main physiological target (0.61 μM) [42]. Therefore an interaction of MDMA on heteromeric nAChR at recreational doses

Table 2. K_i values of METH and MDMA against [^{3}H]MLA and [^{3}H]EB binding in mouse brain.

	[^{3}H]EB		**[^{3}H]MLA**	
Drugs	**K_i (μM)**	**n_H**	**K_i (μM)**	**n_H**
METH	23.90 ± 2.65	1.27 ± 0.28	369.77 ± 95.61	0.29 ± 0.08*
MDMA	0.76 ± 0.11	0.83 ± 0.12	34.21 ± 6.71	0.40 ± 0.27*

The K_i values from competition binding curves were calculated using the Cheng–Prusoff equation.* Significantly different from 1 (one sample t-test). Results are expressed as mean ± S.E.M. from 4-6 different experiments.

is certainly possible. The fact that the lowest K_i values were found against [^{3}H]epibatidine binding indicates that METH and MDMA displayed higher affinity for heteromeric nAChR which are the most abundant in the CNS.

We also performed binding experiments with MDMA enantiomers in order to determine a difference between (*R*)-MDMA and (*S*)-MDMA in their capability of displacing [^{3}H]EB and [^{3}H]MLA from their binding sites. When [^{3}H]EB was used as a radioligand to label α4β2 nAChR, (*R*)-MDMA and (*S*)-MDMA induced a concentration-dependent binding displacement, with IC_{50} values in the micromolar range (see Table 3). The affinity of (*R*)-MDMA for [^{3}H]EB binding sites was higher than (*S*)-MDMA. The Hill coefficients resulting from the analysis of competition data of MDMA *vs* [^{3}H]EB were not significantly different from unity, pointing to a competitive displacement.

When [^{3}H]MLA was used as a radioligand to label α7 nAChR, no differences between (*R*)-MDMA and (*S*)-MDMA were found. The Ki values were similar for both enantiomers.

Table 3. K_i values of (*R*)- and (*S*)-MDMA against [^{3}H]MLA and [^{3}H]EB binding to rat membranes.

Radioligand	Ligand	IC_{50} ± SEM (μM)	Ki ± SEM (μM)	n_H
[^{3}H]EB	(*R*)-MDMA	0.37 ± 0.0	0.6 ± 0.2	1.0 ± 0.0
[^{3}H]EB	(*S*)-MDMA	9.7 ± 1.0	8.2 ± 2.0	0.8 ± 0.1
[^{3}H]MLA	(*R*)-MDMA	149.3 ± 22	71.8 ± 10	0.6 ± 0.1*
[^{3}H]MLA	(*S*)-MDMA	131 ± 27	63.1 ± 17	0.5 ± 0.3*

The K_i values from competition binding curves were calculated using the Cheng–Prusoff equation. .* Significantly different from 1 (one sample t-test). . Results are expressed as mean ± S.E.M. from 3-4 different experiments.

5. METH and MDMA increase the density of nicotinic receptors

A particular feature of nAChR is that chronic exposure to nicotine induces a higher level of either nicotine or epibatidine binding, termed as up-regulation [43]. Accordingly, we tested whether METH and MDMA had any effect on α7 and heteromeric nAChR binding densities and found that both were increased in a time- and concentration- dependent manner (Fig. 3).

The mechanism through which nicotine induces nAChR up-regulation is complex and not fully clarified to date (reviewed by Gaimarri et al. [44]). There are reports indicating that nicotine-induced increases in nAChR are not accompained by changes in mRNA encoding for the different subunits [45, 46]. This led to other hypotheses, such as reduced receptor turnover, promotion of the assembly and migration to the plasma membrane of pre- existing

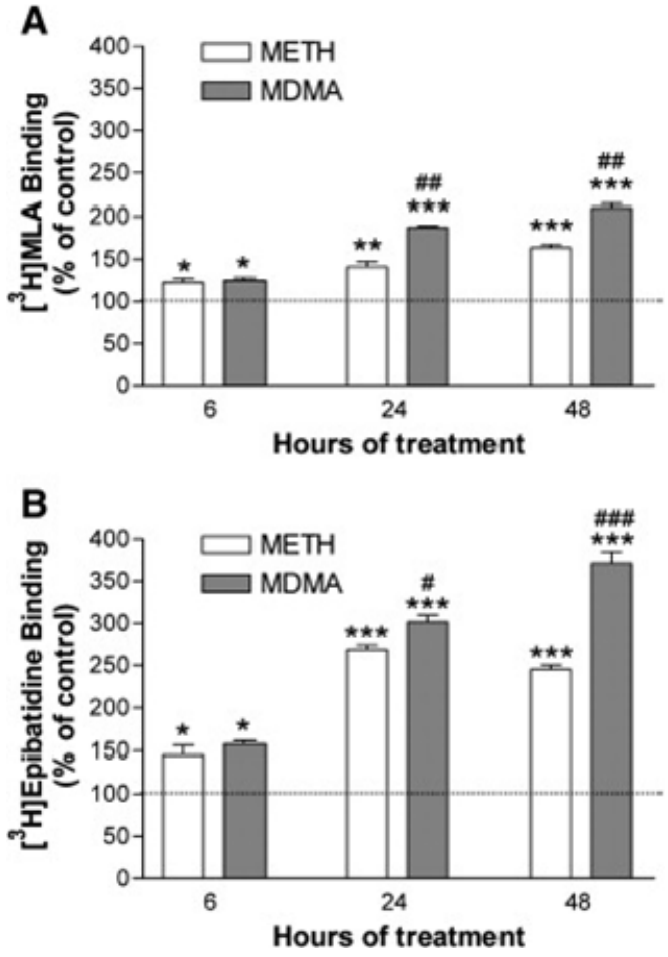

Figure 3. Time-course study of the increases in [^{3}H]MLA (panel A) and [^{3}H]epibatidine (panel B) binding sites induced by METH and MDMA (300 μM). PC12 cells were incubated with amphetamine derivatives over 6, 24 and 48 h. Thereafter radioligand binding was performed on intact cells in culture. Data represent the means ± S.E.M. of three separate experiments carried out in triplicates ($^{*}P < 0.05$, ** < 0.01, $^{***}P < 0.001$ vs. untreated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. METH at the same incubation time).

intracellular subunits [29] or decrease in the rate of receptor turnover [47]. More recently, Sallette et al. [48] demonstrated that nicotine acts as a maturation enhancer of those intracellular nAChR precursors that would otherwise be degraded. However, different authors show controversial results. Vallejo et al. [49] reported that α4β2 up-regulation by nicotine is due to an increase/stabilisation of the proportion of receptors in a high affinity state and not to an enhancement in receptor maduration.

Regardless the underlying mechanism, according to our competition experiments demonstrating the affinity of METH and MDMA for nAChR, it could be hypothesised that the up-regulation of nAChR induced by these drugs would follow a similar mechanism.

6. Role of protein synthesis, cyclophilin A, protein kinase and receptor trafficking

The protein synthesis inhibitor cycloheximide (CHX) inhibited the increase in both [^{3}H]MLA and [^{3}H]epibatidine binding sites, suggesting that such up-regulation require, at least in part, rapid protein synthesis. The chaperone cyclophilin A participates in α7-nAChR maturation [29, 50]. Therefore we tested the cyclophilin A inhibitor cyclosporin A (CsA) on METH/MDMA-induced nAChR up-regulation. CsA inhibited METH/MDMA-induced up-regulation.

To assess whether receptor trafficking to the plasma membrane could be contributing to METH/MDMA-induced nAChR up-regulation we exposed the cells to BFA to inhibit such transport and late steps of maturation. Although BFA induced dramatic decreases in basal binding sites owing to its main mode of action, there was still up-regulation after treatment with METH/MDMA. This indicates that trafficking is not crucial for METH/MDMA-induced nAChR up-regulation and that this takes place previously to surface expression. This result is in agreement with those reported in other studies on the mechanisms involved in nicotine-induced up-regulation of heteromeric receptor [49,51].

PKA- and PKC-dependent pathways have been found to play a role in the up-regulation of α4β2 receptors induced by cholinergic ligands in several cell lines [52, 53]. For this reason we tested the PKA inhibitor H-89 on METH/MDMA-induced up-regulation. We saw that there was an inhibition by H-89, corroborating that PKA plays a role in heteromeric nAChR regulation, but not in that of α7 nAChR [53]. The PKC inhibitor, GF-109203X, did not affect α7 nAChR in our experiments with amphetamine derivatives. This result is in agreement with those reported by Nuutinen *et al.* [53] who found no effect of this compound, at nanomolar concentration, on nicotine-induced α7 nAChR up-regulation.

Protein tyrosine kinases have been reported to play a role in α7 nAChR regulation [54,55]. When genistein, an inhibitor of tyrosine kinases, was assayed for 24 h, it reduced METH/MDMA effects on nAChR densities, indicating that they participate in the up-regulation induced by METH and MDMA.

All these results confirm that METH and MDMA up-regulate nAChR in a complex process but in a similar manner than nicotine. However, the work done to date indicates that up-regulation can occur if the drug has a particular affinity to one or more nAChR subunits; regardless of the agonist/antagonist properties of the drug (i.e., the antagonist DHβE is also able to induce it [52]. In addition, up-regulation is enhanced when the drug crosses the cell membrane to interact with immature forms of the receptor [56]. The affinity of MDMA towards both heteromeric and α7 nAChRs has been demonstrated

[30], and this drug can reach the cytosplasm after transport through the dopamine transporter [57, 58], which is abundant in PC12 cells. Therefore, the interaction of MDMA with immature receptor subunits is feasible.

7. Intracellular Ca^{2+} increase induced by MDMA in PC12 cells

We used a fluorimetric method [33] to investigate the effect of MDMA on Ca^{2+} levels in cultured PC12 cells and the involvement of different nAChR subtypes and other cell pathways related to Ca^{2+} mobilization.

MDMA acutely inhibited the effects of nAChR agonists (ACh, Nic and PNU282987) (Fig. 4a) but, owing to its effects increasing basal calcium levels

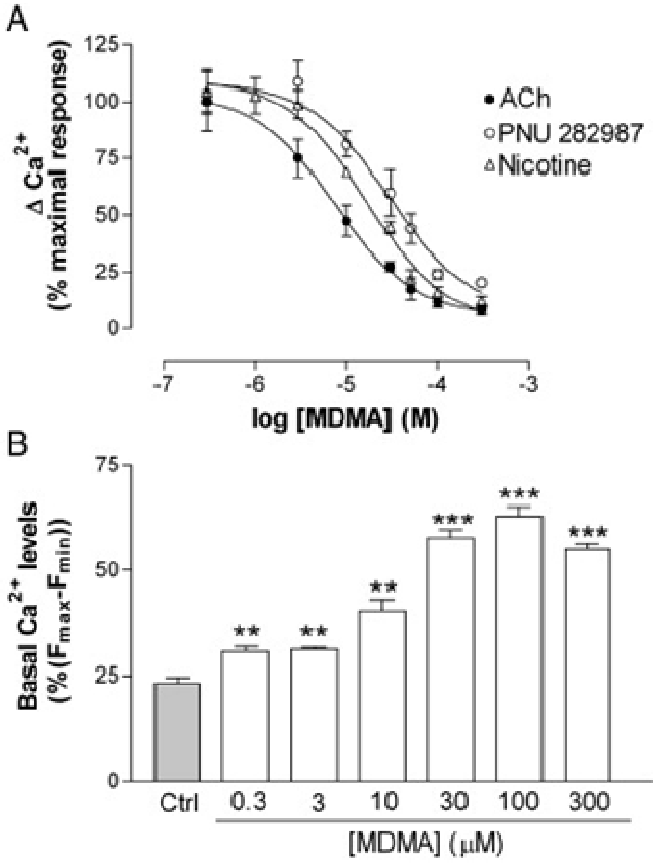

Figure 4. (Panel A) Effect of increasing concentrations of MDMA on the responses to the nicotinic agonists ACh (100 μM), nicotine (100 μM), and PNU 282987 (0.1 μM) in PC12 cells loaded with Fluo-4. MDMA was added to the cells 5 min before the agonist. Basal fluorescence levels were measured for 5 s before the agonist and for a further 30 s after its addition by means of an automated injector. (Panel B) Basal Ca^{2+} levels 5 min after the addition of MDMA and before adding the agonist. All the measurements were performed in the presence of the positive allosteric modulator PNU 120596 (10 μM). The results were normalized as percentage ($F_{max} - F_{min}$) and are the means ± SEM of at least three experiments, carried out in quadruplicates for each condition. ** $P < 0.01$, *** $P < 0.001$ vs. control basal levels.

(Fig. 4b), we explored the possibility of a partial agonist mode of action. We tested the effect of acute application of MDMA and found a concentration-effect relationship in the micromolar concentration range. The effect of MDMA did not reach the maximum values induced by ACh, which indicates a partial agonist mode of action (Fig. 5). The EC_{50} value was in the micromolar range, which is in agreement with previous binding results.

The experiments performed with MDMA enantiomers demonstrated that there are no differences in the calcium increase between the enantiomers and the racemic mixture. This results is in agreement with the similar affinity values obtained of both enantiomers in the binding experiments with [^{3}H]MLA.

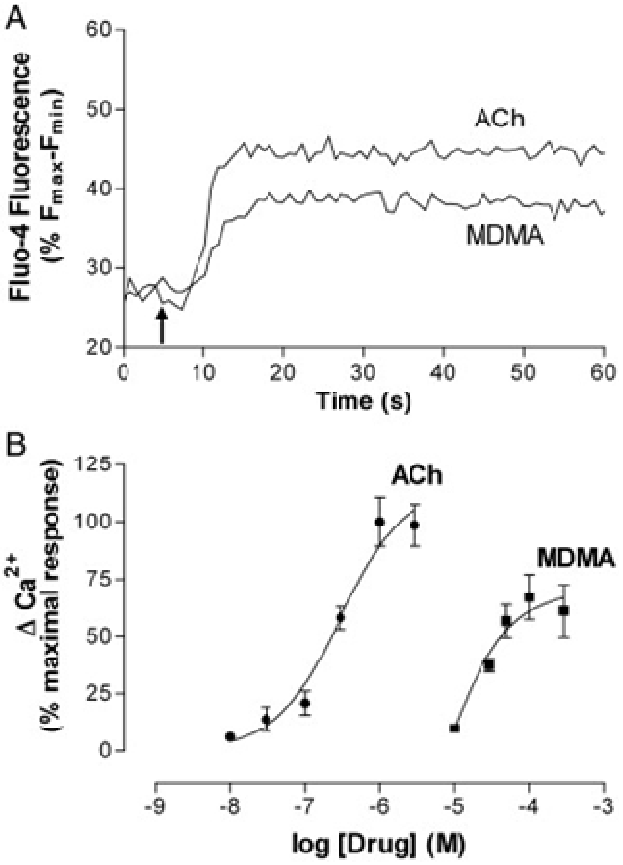

Figure 5. (Panel A) Representative tracings of the increases in Fluo-4 fluorescence in PC12 cells after the addition (arrow) of ACh (1 μM) and MDMA (50 μM). (Panel B) Representative concentration–response curves showing the increase in cytosolic Ca^{2+} induced by MDMA and ACh as a total agonist. Responses were normalized as percentage ($F_{max} - F_{min}$) and represented as a percentage of the maximum response (ACh 100 μM) for both curves. After 5 s of basal recording, ACh and MDMA were added by means of an automated injector to PC12 cells loaded with Fluo-4 and the fluorescence was measured for a further 30 s. Represented data are the mean ± SEM of four replicates for each condition.

8. Pathways involved in MDMA-induced Ca^{2+} increase

The fact that MDMA induced an increase in cytosolic Ca^{2+} led us to study the pathways involved using specific blockers (Fig. 6). According to our previous work, the α7 nAChR blockers MLA and α-bungarotoxine (α-BgTX) abolished the effect of MDMA, which showed that such effects take place mainly through activation of these receptors. However, as we had previously described that MDMA also has affinity for heteromeric receptors [12, 30], we tested the effect of DBE, an antagonist of these receptors, and we found that it had no significant effect on MDMA-induced Ca^{2+} increase, ruling out an antagonistic effect on theses receptors. This finding suggests that MDMA behaves as an antagonist at heteromeric receptors while it is a partial agonist at α7 nAChRs. These receptors are permeant to Na^{+}, which could induce depolarization and open voltage-operated calcium channels (VOCC) [33]. We used nitrendipine, an L-type VOCC inhibitor, and Cd^{2+}, a non selective

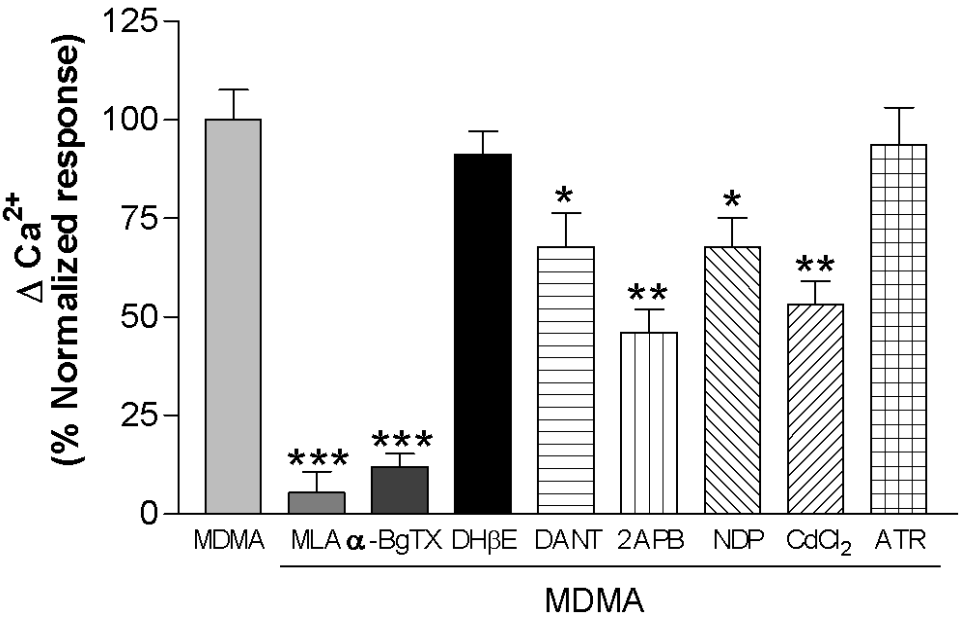

Figure 6. Pharmacological modulation of MDMA-induced increase in cytosolic Ca^{2+}. Drugs were manually added to cultured PC12 cells loaded with Fluo-4, 10 min before the automated addition of MDMA (50 μM). The response was measured for 5 s before and 30 s after MDMA, then normalized as percentage ($F_{max} - F_{min}$) and expressed as a percentage of the response induced by MDMA alone. All the measurements were carried out in the presence of PNU 120596 (10 μM). Abbreviations and concentrations used: MLA (methyllycaconitine, 1 μM), DHβE (dihydro-β-erythroidine, 50 μM), DANT (dantrolene, 20 μM), 2-APB (2-aminoethyl diphenyl borate, 150 μM), NDP (nitrendipine, 30 μM), ATR (atropine, 0.1 μM). Results are mean ± SEM of three experiments carried out in quadruplicates. *$P < 0.05$,** $P < 0.01$,*** $P < 0.001$ vs. MDMA alone.

blocker of VOCC and found that they inhibited MDMA-induced Ca^{2+} suggesting that, in our model, the initial depolarization induced after α7 receptor activation by MDMA, partially couples to L-type channel opening.

Calcium-induced calcium release (CICR) from endoplasmic reticulum (ER) stores has been shown to be coupled to a α7 nAChR activation [33, 59]. CICR could occur after activation of two intracellullar calcium release channels localized in the ER: IP_3 receptors and ryanodine receptors [60, 61]. Accordingly we tested two blockers of these receptors (2-APB and dantrolene, respectively) with MDMA. We found that the MDMA effect was significantly inhibited by these blockers, which demonstrates the participation of CICR. In fact, these blockers had also been effective in preventing MDMA-induced ROS generation in striatal synaptosomes [62].

The MDMA response was dependent on extracellular Ca^{2+}, as suppression of this cation totally inhibited its effect. Extracellular Ca^{2+} could enter through either α7 channels or L-type VOCCs and, as stated above, this Ca^{2+} increase would also induce subsequent CICR.

Although mechanisms other than nAChR activation cannot be totally ruled out in the MDMA-induced increase in cytosolic Ca^{2+}, the practically complete inhibition by MLA and α-BgTX indicates that α7 nAChR activation plays a major role in this process.

9. Effects of MDMA on basal Ca^{2+} levels

Preincubation with MDMA for 24 h induced an increase in basal cytosolic Ca^{2+} levels, as measured after drug washout. Surprisingly, preincubation with nicotine increased basal levels when it was carried out for 1 h, but not after longer preincubation times. This indicates that cells are able to buffer sustained activation by nicotine, but not that induced by MDMA, which suggests increased vulnerability to this drug.

10. Calpain/Caspase-3 activation induced by MDMA

Sustained Ca^{2+} influx after MDMA treatment could favor cytotoxicity through activation of Ca^{2+}-dependent pathways (i.e., calpain). Calpain is a calcium-dependent protease whose activation is a primary mechanism that contributes to several types of neurodegenerative conditions, including the excitatory amino acid-induced neurotoxicity that is associated with traumatic brain injury, ischemia, and hyperthermia [63,64]. Calpain specifically degrades the cytoskeletal membrane protein, spectrin, into 145 and 150 kDa breakdown products [65]. Caspase 3 is another cysteine protease that is

involved in apoptotic pathways. It also degrades spectrin but produces a 120 kDa spectrin fragment [66] and also can produce a 150-kDa fragment [67].

Incubation of PC12 cells with MDMA for 24 h induced a significant increase in α-spectrin breakdown products (SBDP) of 145 and 150 kDa, which indicates calpain activation, and a rise in the 120 kDa band that, together with the increase in the 150 kDa SBDP, points to caspase 3 activation (Fig. 7). In fact, in a previous work using cerebellar granule cells, we demonstrated the proapoptotic role of caspase 3 in the neurotoxic effects of amphetamines [68]. Moreover, the increases in SBDP induced by MDMA were prevented by MLA, indicating that α7 nAChRs play a key role in this process.

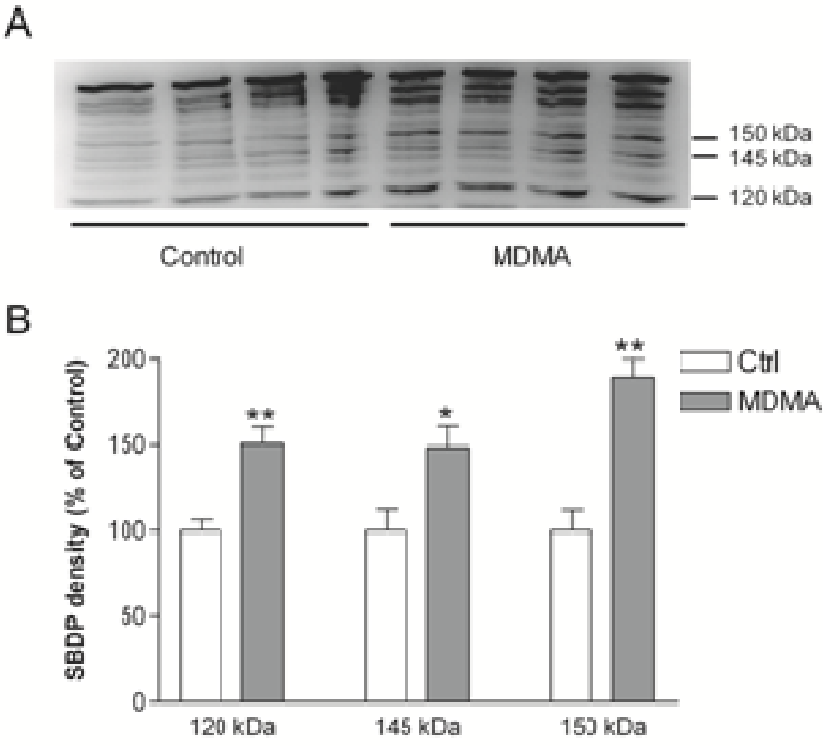

Figure 7. (Panel A) Representative Western blot of α-spectrin breakdown products (SBDP) originated by calpain activation (145 and 150 kDa) and caspase 3 (120 and 150 kDa) after 24-h treatment with culture medium (Ctrl), MDMA (50 μM), MDMA + MLA (10 nM), and MLA alone. The localization of the molecular weight (MW) markers is shown on the left of the picture. (Panel B) Quantification of dot intensity of the SBDPs. Data are the means ± SEM of three different cultures, loaded in duplicates. *P < 0.05 and **P < 0.001 vs. control.

11. Functional up-regulation

After chronic nicotine exposure, some nAChR subtypes undergo radioligand binding up-regulation, changes in stoichiometry, and an increase

in functional state (functional up-regulation) (reviewed in [44]). Such up-regulation occurs at a post-transcriptional level and has been reported in cell cultures for α4β2 nAChRs [56,69] as well as for α7 nAChRs [70]. For this reason, we measured cytosolic Ca^{2+} levels to test whether pretreatment with MDMA induced persistent changes in nAChRs, leading to an increased response to agonists. Our experiments showed that, when incubated for 24 h with MDMA, PC12 cells exhibited increased responses to PNU282987 (*N*-(3*R*)-1-azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide) (α7-selective) and to 5-I-A-85380 (3-[(2*S*)-2-azetidinylmethoxy]-5-iodopyridine dihydrochloride) (selective for β2 subunit-containing receptors), measured after drug washout. This indicates that MDMA also induces functional nAChR up-regulation.

12. Final remarks

Our work demonstrates an additional mode of action for amphetamine derivatives. Their activation of α7 nAChR contributes to the toxicity of these drugs, which points to a new target to reduce damage. Moreover, the effects on different nAChR densities may account for long term effects on neural pathways and addiction processes due the important role of these receptors in CNS functions.

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