Methyllycaconitine Prevents Methamphetamine-Induced Effects in Mouse Striatum: Involvement of α7 Nicotinic Receptors

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ABSTRACT

In a previous study, we demonstrated that in rat striatal synaptosomes, methamphetamine (METH)-induced reactive oxygen species (ROS) production was prevented by methyllycaconitine (MLA), a specific antagonist of α7 neuronal nicotinic acetylcholine receptors (α7 nAChR). The aim of this study was to test the influence of MLA on acute METH effects and neurotoxicity in mice, using both in vivo and in vitro models. MLA inhibited METH-induced climbing behavior by 50%. Acute effects after 30-min preincubation with 1 μM METH also included a decrease in striatal synaptosome dopamine (DA) uptake, which was prevented by MLA. METH-induced neurotoxicity was assessed in vivo in terms of loss of striatal dopaminergic terminals (73%) and of tyrosine hydroxylase levels (by 90%) at 72 h post-treatment, which was significantly attenuated by MLA. Microglial activation [measured as 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinolinecarboxamide binding] was also present at 24 h post-treatment and was fully prevented by MLA, tending to confirm its neuroprotective activity. MLA had no effect on METH-induced hyperthermia. Additionally, flow cytometry assays showed that METH-induced ROS generation occurs inside synaptosomes from mouse striatum. This effect implied release of vesicular DA and was calcium-, neuronal nitric-oxide synthase-, and protein kinase C-dependent. MLA and α-bungarotoxin, but not dihydro-β-erythroidine (an antagonist that blocks nAChR-containing β2 subunits), fully prevented METH-induced ROS production without affecting vesicular DA uptake. The importance of this study lies not only in the neuroprotective effect elicited by the blockade of the α7 nico- tinic receptors by MLA but also in that it proposes a new mechanism with which to study METH-induced acute and long-term effects.

Amphetamine derivatives such as methamphetamine (METH) and 3,4-methylenedioxymethamphetamine are drugs that are widely abused in the United States and Europe, where they are taken in a recreational context. The growing incidence of the use of these drugs has led to concern because of the extensive evidence that they are neurotoxic in animal models (for review, see Davidson et al., 2001). It is still uncertain what neurotoxic consequences the acute or long-term use of these substances in humans could have, and a great deal of research is being done on the subject.

METH exerts its powerful acute psychostimulant effects by promoting the release of monoamine neurotransmitters (carrier-mediated efflux) and by inhibiting their uptake, thus increasing the extracellular dopamine (DA) concentration. There is compelling evidence that these amphetamine effects are due to the reversion of the operational direction of the high-affinity transport sites present in dopaminergic terminals (DAT) (Fleckenstein et al., 1997).

ABBREVIATIONS: METH, methamphetamine; DA, dopamine; DAT, dopamine transporter; ROS, reactive oxygen species; VMAT, vesicular monoamine transporter; nAChR, neuronal nicotinic acetylcholine receptor(s); 7-NI, 7-nitroindazole; nNOS, neuronal nitric oxide synthase; PBR, peripheral-type benzodiazepine receptors; PKC, protein kinase C; CTRL, control; MLA, methyllycaconitine; WIN 35428, (−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-napthalenedisulfonate; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinolinecarboxamide; TH, tyrosine hydroxylase; PVDF, polyvinylidene fluoride; HBSS, HEPES-buffered saline solution; DCFF-DA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; ANOVA, analysis of variance; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzof[1]quinoxaline-7-sulfonamide; NPC 15437, (S)-2,6-diamino-N-[1-(1-oxotridecyl)-2-piperidinyl][methyl]hexanamide dihydrochloride; PCP, phencyclidine; DBE, dihydro-β-erythroidine.
Neurotoxic effects after high doses of METH in rodents and other species include long-lasting depletion in the striatal content of DA and its metabolites (Ricaurte et al., 1982), decrease in tyrosine hydroxylase activity (Ellison et al., 1978), and loss of DA transporters (Escuedo et al., 1998). However, the mechanisms underlying METH-induced striatal neurotoxicity are complex and still being investigated (Pubill et al., 2003, 2005). It has also been reported that, at higher concentrations and inside the synaptic terminals, METH displaces vesicular DA, increasing cytosolic DA concentration. Free DA can be oxidized to reactive oxygen species (ROS) (Graham, 1978; Hastings, 1995). In fact, oxidative stress appears to be one of the main factors involved in METH-induced dopamine terminal degeneration in the striatum (Sonsalla et al., 1989; Yamamoto and Zhu, 1998; Imam et al., 1999).

Larsen et al. (2002), using vesicular monoamine transporter (VMAT)-knockout mice, proposed that METH-induced injury is due to a redistribution of DA from the vesicular storage pool to the cytoplasm and its subsequent transformation in ROS, suggesting that the enhanced extracellular DA levels after METH were not the main source of the ROS through which METH induces neurotoxicity. Additional mechanisms have been implicated in METH-induced neurotoxicity, including glutamate-mediated neurotoxicity (Sonsalla et al., 1991) and mitochondrial toxicity (Davidson et al., 2001).

In a recent study (Pubill et al., 2005), using a synaptosomal preparation from rat striatum to study the mechanisms involved in METH-induced ROS generation in vitro, we demonstrated that METH induces ROS production inside the synaptosomes. These results explain the selective neurotoxicity of this amphetamine derivative better because intracellular ROS are more likely to induce damage in the synaptic terminal and not in the surrounding unaffected neurons. We also demonstrated that ROS generation induced by METH is concentration- and dopamine-dependent. Methyllycaconitine is a specific antagonist of α7 nicotinic receptors (nAChR), although at concentrations of 40 nM and higher, it can interact also with α4β2 and α6β2 nAChR (Mogg et al., 2002). In rat synaptosomes, methyllycaconitine (MLA), but not dihydro-β-erythroidine (an antagonist that blocks nAChR containing β2 subunits), completely inhibited METH-induced ROS production, thus implicating α7 receptors in METH effect in rats. Among the neuronal acetylcholine receptors, homomeric α7 nAChRs have the highest fractional Ca2+ current. In fact, Liu et al. (2003) found that α7-amphetamine can activate α7 receptors in bovine chromaffin cells. They concluded that amphetamine enhances calcium entry via α7 nicotinic receptor activation and dose-dependently suppressed [3H]nicotine binding.

In view of these results, it was necessary to assess whether MLA had neuroprotective effects in vivo or interfered with METH-induced acute behavioral effects. Thus, the aim of this work was to test the effect of MLA on acute METH effects and neurotoxicity in mice, using both in vivo and in vitro models.

To evaluate the action of MLA on the acute effects of METH, climbing behavior and locomotor activity in mice were measured in vivo, and [3H]HIDA uptake was evaluated in vitro. Assessment of neurotoxicity markers after an in vivo treatment with a neurotoxic schedule of METH and in vivo METH-induced ROS production was used to determine the neuroprotective effect of MLA.

### Materials and Methods

#### Animals

The experimental protocols concerning the use of animals in this work were approved by the Animal Ethics Committee of the University of Barcelona under supervision of the Autonomous Government of Catalonia, following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used.

Adult male Swiss CD-1 mice (Charles River, Barcelona, Spain) were used in all experiments. They were housed at 22 ± 1°C under a 12-h light/dark cycle with free access to food and drinking water.

#### Climbing Behavior

Climbing behavior was measured using Gerhardt’s method (Gerhardt et al., 1985) as modified by us. Briefly, mice of 20 to 26 g were intraperitoneally administered saline (5 ml kg−1) or MLA (6 mg kg−1) at the beginning of the test. Twenty minutes later, the animals received a single dose of saline or METH (1 mg kg−1) subcutaneously and were placed individually, for habituation, into the experimental chamber consisting of a cylindrical cage (diameter, 20 cm; height, 25 cm) with the wall made of plastic bars (0.1-cm diameter; separated by 0.2-cm gaps) and covered with a lid. After a 20-min period of exploratory activity, stereotypy measurement was performed for a period of 30 min. Climbing behavior was scored by an observer who was blind to the drug treatment, and the time spent on climbing the wall (time during which almost two limbs were off the floor) was measured, registered by an electronic device (CompuLet; Letica, Barcelona, Spain), and expressed as the percentage of the total time (30 min).

#### Spontaneous Locomotor Activity

Prior to experimentation, all 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. During these sessions, each mouse was given a subcutaneous injection of saline (5 ml kg−1) and was placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets of 16 infrared photocells (LE8811; Letica) mounted according to the x,y-axis coordinates and 1.5 cm above the wire mesh floor. Occlusions of the photo beams were recorded and sent to a computerized system (Sedac-Com32; Letica). The interruption counts, in a 10-min block, were used as a measure of horizontal locomotor activity. The locomotor activity of each mouse was monitored over 360 min. All experiments were conducted between 9:00 AM and 3:00 PM. In the testing day, the animals received drug treatment and were immediately placed in the activity box and registration of horizontal locomotor activity began. The first 30 min of registered counts were discarded. Results are expressed either as breaks at each 10-min block or as the cumulative count of breaks in 120 min.

#### In Vivo Treatments for Neurotoxicity Assessment

Mice weighing 28 to 32 g were used. The day before the treatment animals were fasted, and drinking water was supplemented with glucose (5%). Methamphetamine was administered to the METH group (7.5 mg kg−1 s.c.) every 2 h, for a total of four doses (equivalent to a chronic schedule). The MLA+METH group received four doses of MLA (6 mg kg−1 i.p.) administered 20 min before each dose of METH. There were also two control groups: one was injected with saline, and the other received MLA alone, following the same injection schedule. The appropriate dose of MLA was determined from pilot experiments according to its pharmacokinetics and affinity for the α7 nicotinic receptor (Turek et al., 1995; Damaj et al., 2003).

All substances were administered at a constant volume of 5 ml kg−1. During the experiment, animals were maintained in an environmental temperature of 26 ± 2°C and were kept under these conditions until 1 h after the last dose. Body temperature was measured at 1 h after the second dose of METH using a lubricated, flexible rectal probe inserted 1.5 cm into the rectum (for 40 s) and attached to a digital thermometer (0331; Panlab, Barcelona, Spain).
When rectal temperature rose above 40°C, animals were placed on ice for 5 min. Body weight was registered at the beginning of the experiment and 24 h after the last dose of METH. Animals were killed by cervical dislocation 3 days after treatment for [3H]WIN 35428 binding and tyrosine hydroxylase studies, and 24 h after the last dose for [3H]PK 11195 binding studies.

**Tissue Sample Preparation.** Immediately after sacrifice, mice were decapitated, and the brains were rapidly removed from the skull. Striata were quickly dissected out, frozen on dry ice, and stored at −80°C until use. When required, tissue samples were thawed and homogenized at 4°C in 10 volumes of buffer consisting of 5 mM Tris-HCl, 320 mM sucrose, and protease inhibitors (aprotinin 4.5 μg ml⁻¹, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at 1000g for 15 min at 4°C. Aliquots of the resulting supernatants were taken, and after the protein concentration was determined, they were frozen and kept for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000g for 30 min at 4°C. The pellets were resuspended in buffer and incubated at 37°C for 10 min to remove endogenous neurotransmitters. Then the protein samples were recentrifuged and washed two more times. The final pellets (crude membrane preparation) were resuspended in the appropriate buffer and stored at −80°C until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent (Bio-Rad Labs. Inc., Hercules, CA), according to the manufacturer’s specifications.

**Western Blotting and Immunodetection.** A general Western blotting and immunodetection protocol was used to determine tyrosine hydroxylase (TH) levels. For each sample, 30 μg of protein was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% w/v) 1:2500 dilution in phosphate-buffered 0.32 M sucrose (final radioligand concentration, 5 nM) and 50 μl of membranes. Incubation was done for 2 h at 4°C. Nonspecific binding was determined in the presence of 30 μM bupropion.

All incubations were finished by rapid filtration through GF-G glass fiber filters (Schleicher and Schuell, Dassel, Germany). Tubes and filters were washed rapidly three times with 4 ml of ice-cold buffer, and the radioactivity in the filters was measured using a liquid scintillation counter. Specific binding was defined as the difference between the radioactivities measured in the absence (total binding) and in the presence (nonspecific binding) of an excess of nonlabeled ligand.

**Preparation of Striatal Synaptosomes.** Striatal synaptosomes were obtained as described elsewhere (Pubill et al., 2005) with minor modifications. Briefly, on the morning of each day of the experiment, seven mice were decapitated, and their striata were homogenized and centrifuged at 1000g at 4°C for 10 min. The supernatant was recovered, and sucrose buffer was added to a final sucrose concentration of 0.8 M. Samples were then centrifuged at 13,000g for 30 min at 4°C. The supernatant was discarded, and the synaptosome layer was separated from mitochondria by carefully adding 1 ml of ice-cold 320 mM sucrose buffer and gently shaking. Finally, the synaptosome fraction was diluted in HEPES-buffered solution (HBSS; 140 mM NaCl, 5.37 mM KCl, 1.26 mM CaCl₂, 0.44 mM KH₂PO₄, 0.49 mM MgCl₂·6H₂O, 0.41 mM MgSO₄·7H₂O, 4.17 mM NaHCO₃, 0.34 mM Na₃HPO₄·7H₂O, 5.5 mM glucose, and 20 mM HEPES sodium), to a final protein concentration of about 0.1 mg ml⁻¹. Protein concentration was determined as cited above. The final synaptosome suspension was distributed in 1-ml aliquots in centrifuge tubes for the experiments. For reserpine-pretreated mice (to deplete vesicular DA), reserpine was prepared as a microsuspension in an aqueous vehicle consisting of 0.5% carboxymethylcellulose sodium salt and 0.1% Tween 80 and administered at a dose of 5 mg kg⁻¹ in a volume of 5 ml kg⁻¹ (s.c.) 20 h before sacrifice.

**Measurement of Methamphetamine-Induced ROS.** The formation of intrasynaptosomal ROS was measured using fluorochrome 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which passively diffuses through membranes and, after being deacetylated by esterases, is accumulated inside the synaptosomes in the form of 2′,7′-dichlorofluorescein, which is not fluorescent. This compound reacts quantitatively with oxygen species to produce the fluorescent dye 2′,7′-dichlorofluorescein (DCF), whose intensity can be measured to provide an index of oxidative stress.

Fifty micromolar DCFH-DA was added to each tube, together with the drugs at the appropriate concentrations. The synaptosomes were incubated for 15 min in a shaking bath at 37°C in the dark, and methamphetamine was then added at the desired concentration. Incubation was continued in the dark for 2 h and was finally stopped by centrifugation at 13,000g for 30 min at 4°C. The pellets were resuspended in 1 ml ice-cold Tris-sucrose buffer (320 mM) and re-centrifuged. The final pellets were resuspended in 0.2 ml of ice-cold HBSS, and the tubes were kept on ice in the dark until fluorescence measurements were performed, within the hour. Fluorescence measurements were performed on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an argon laser. The excitation wavelength was 488 nm, and the emission was detected at 525 nm. Sample was diluted in HBSS to obtain a flow rate of 500 to 900 synaptosomes per second, and each sample was measured for 1 min. Fluorescence data were analyzed using Elite software (Beckman Coulter). Mean fluorescence values were taken to compare the degree of ROS production in each treatment group. Values were taken from triplicates of each experimental condition, and individual experiments were performed at least three times. Mean fluorescence values of each experimental condition are expressed as a percentage of control (100%). When the test compound significantly reduced basal ROS levels, the effect of METH in the presence of this compound was compared with its respective control (compound + vehicle METH). Results are mean ± S.E.M. of at least three separate experiments run on triplicates.

To test the possibility that the presence of test compounds could...
alter the ability of synaptosomes to accumulate the dye, parallel experiments were performed with the same synaptosomal preparations in which some samples were preloaded with the dye, then washed, and incubated with METH, whereas in others, DCFH-DA remained in the medium during the incubation with blocker compound and METH. Although the arbitrary fluorescence values were slightly higher in nonwashed preparations, the percentage of increase in fluorescence was the same in both cases. Thus, all the experiments were carried out on nonwashed preparations to avoid an additional centrifugation step.

Plasmalemmal and Vesicular [3H]Dopamine Uptake. For measuring [3H]DA uptake via plasmalemmal transporters, synaptosomes were obtained as described above and preincubated in a shaking water bath at 37°C with METH for 30 min (see Results for particular conditions). Specific compounds such as MLA or EGTA were added when appropriate 10 min before METH. After preincubation, synaptosomes were centrifuged at 13,000g for 20 min, resuspended and lysed in ice-cold distilled deionized water. Osmoticers (VMAT), the method described by Hansen et al. (2002) was used.

In preliminary studies (data not shown), METH given subcutaneously induced dose-response (ranging from 0.2 to 5 mg kg\(^{-1}\)) climbing behavior; 1 mg kg\(^{-1}\) appeared to be an optimal and submaximal (37%) dose for inducing climbing behavior in mice, so this dose of METH was used in this experiment. MLA (6 mg kg\(^{-1}\) given alone intraperitoneally did not cause climbing behavior when compared with the saline group. Pretreatment with MLA significantly inhibited METH-induced climbing behavior, by about 50% (see Fig. 1A). DBE (3 mg kg\(^{-1}\) i.p.) did not inhibit but potentiated METH-induced climbing behavior (35.70 ± 4.70% METH, n = 11 versus 85.43 ± 5.30% DBE + METH, n = 4; P < 0.001).

Locomotor Activity

Preliminary experiments (data not shown) demonstrated that subcutaneous administration of METH elicited a dose-dependent (doses from 0.2 to 5 mg kg\(^{-1}\)) locomotor response, and a dose of 0.4 mg kg\(^{-1}\) was chosen for further studies. Horizontal locomotor activity profile was registered for 360 min after administration of saline (5 ml kg\(^{-1}\) s.c.), MLA (6 mg kg\(^{-1}\) i.p.), METH (0.4 mg kg\(^{-1}\) s.c.), or MLA + METH (MLA was administered 20 min prior to METH).

The significant increase of locomotor activity induced by METH remained constant for 120 min. MLA did not modify either basal locomotor activity or METH-induced hyperlocomotion. At this time, the comparative results of total counts obtained in the different treatment groups are displayed in Fig. 1B.

[3H]DA Uptake in Mouse Striatal Synaptosomes

Preincubation of synaptosomes with METH (1 μM) for 30 min significantly reduced [3H]DA uptake (80%). MLA (0.1 μM) did not affect basal uptake values and prevented the inhibition of [3H]DA uptake induced by METH. In experiments carried out with the calcium chelator EGTA (5 mM), the basal [3H]DA uptake was not modified, and the inhibitory effect of METH was partially abolished (Fig. 2). Unexpectedly, DBE alone, at a concentration of 0.1 μM, abolished plasmalemmal dopamine uptake (from 100 to 2.17%). Therefore, its association with METH was not tested.
In Vivo Treatment

Radioligand Binding Experiments. Methamphetamine induced, at 72 h post-treatment, a significant loss of striatal dopamine reuptake sites of about 73% (123 ± 10 fmol mg⁻¹, n = 6 compared with control values: 458 ± 83 fmol mg⁻¹, n = 5, p < 0.01), measured as specific binding of [³H]WIN 35428 in mouse striatum membranes. The METH-induced depletion of dopamine neuron terminals was attenuated in mice pretreated with MLA (250 ± 43 fmol mg⁻¹, n = 7) (see Fig. 3A).

The peripheral-type benzodiazepine receptor density in striata of the different treatment groups sacrificed at 24 h post-treatment was measured as [³H]PK 11195 specific binding. In this area, METH-treated animals showed a significant increase of 53% in peripheral-type benzodiazepine receptor density compared with control animals (from 260 ± 11 fmol mg⁻¹, n = 5 to 397 ± 29 fmol mg⁻¹, n = 5, p < 0.05), indicating microglial activation (Fig. 3B). When MLA was administered 30 min before each METH dose, such an increase was fully prevented (286 ± 36 fmol mg⁻¹, n = 5), pointing to the protective effect of this nicotinic receptor antagonist against METH neurotoxicity in mouse striatum.

**Fig. 1.** A, effects of saline (5 ml kg⁻¹ s.c.), METH (1 mg kg⁻¹ s.c.), MLA (6 mg kg⁻¹ i.p.) and MLA+METH on climbing behavior registered for 30 min. Data are represented as mean ± S.E.M. (n = 7–11 per group) of total time spent in climbing (as percentage). ***, p < 0.001 versus saline-treated group; ##, p < 0.01 versus METH group. One-way ANOVA was used for statistical analysis. Significant differences were then analyzed by Tukey’s post hoc test for multiple means comparisons. B, comparative effects presented as total breaks per animal over a 120-min period beginning 30 min after the administration of different compounds. Bars denote mean ± S.E.M., from n = 9 per group. ***, p < 0.001 versus saline-treated group. One-way ANOVA was used for statistical analysis. Significant differences were then analyzed by Tukey’s post hoc test for multiple means comparisons.

**Fig. 2.** Effects of preincubation with METH (1 μM), MLA (0.1 μM), and EGTA (5 mM) on [³H]DA uptake in mouse striatal synaptosomes. After preincubation, synaptosomes were washed and [³H]DA uptake performed (5 min). Data are presented as mean ± S.E.M. percentage of control [³H]DA uptake from at least three separate experiments run in duplicates. Nonspecific [³H]DA uptake was determined at 4°C in parallel samples containing 100 μM cocaine and was about 7% of total [³H]DA uptake. *, p < 0.05; and **, p < 0.01 versus control [³H]DA uptake; Tukey’s post hoc test.

**Fig. 3.** A, effect of treatment with METH alone (four injections, 7.5 mg kg⁻¹ s.c., 2-h intervals) or in combination with MLA (four injections, 6 mg kg⁻¹ i.p., 20 min before METH) on the density of mouse striatal dopamine reuptake sites 72 h after treatment, measured as specific binding of [³H]WIN 35428. #, p < 0.05 versus METH group. B, effect of treatment with METH alone or in combination with MLA (same dose schedule as above) on the density of mouse striatal peripheral-type benzodiazepine receptors 24 h post-treatment, measured as specific binding of [³H]PK 11195. Increase in binding indicates microglial activation. *, p < 0.05 versus saline-treated group; #, p < 0.05 versus METH group. In both panels values are expressed as means ± S.E.M. of those obtained from five to six animals in each group; Tukey’s post hoc test.
Furthermore, MLA administered alone did not induce any change in dopamine terminal density or in microglia activation.

Effects on Core Temperature and Body Weight. METH induced a slight hyperthermia in mice (36.5 ± 0.6°C, n = 5, saline group versus 36.5 ± 0.3°C, n = 6, METH group, measured 1 h after the second dose of saline or METH, respectively, p < 0.05). A direct effect of MLA on body temperature is ruled out because this compound did not affect basal body temperature (37.0 ± 0.5°C, n = 5) or reduce the METH-induced hyperthermia (38.2 ± 0.4°C, n = 6, MLA + METH group, n.s. versus METH group).

METH-treated animals showed a significant loss in body weight (−9.0 ± 1.8% METH group, n = 6 versus 0.05 ± 0.23% saline group n = 5, p < 0.01) measured 24 h after the last dose of the treatment. MLA administered alone did not induce any change in body weight (−0.9 ± 0.6%, n = 5), but pretreatment with this compound was not able to prevent the loss of body weight induced by METH (−6.4 ± 1.5% MLA + METH group, n = 6, n.s. versus METH group).

Effect of Antioxidants. Vitamin E, an antioxidant compound, antagonized the oxidative effect of METH on striatal synaptosome preparations and fully abolished the METH-induced ROS production at a concentration of 100 μM. At this concentration, vitamin E alone also significantly reduced basal ROS production (56.9 ± 1.9% vitamin E versus 100 ± 5.2% CTRL, P < 0.001). Incubation of striatal mouse synaptosomes with 2 mM METH in the presence of vitamin E (100 μM) did not induce a significant increase in ROS production (56.9 ± 1.9% vitamin E versus 69.1 ± 1.4% vitamin E + METH).

Effect of Specific Enzyme Inhibitors. To investigate whether an activation of neuronal nitric oxide synthase or protein kinase C may participate in the ROS production induced by METH, we determined the effect of specific inhibitors. 7-Nitroindazole (7-NI) (100 μM), an nNOS inhibitor, significantly prevented the effect of METH, although it did not modify the basal fluorescence (Fig. 6). On the other hand, NPC 15437 (100 μM), the PKC inhibitor, significantly reduced the basal fluorescence but also significantly blocked the ROS production induced by 2 mM METH (Fig. 6).

Role of Calcium and Dopamine Transporter. When striatal synaptosomes were incubated in a medium containing EGTA (5 mM), the basal level of ROS was not modified (109.9 ± 1.6% EGTA versus 100 ± 5.2% CTRL), but the oxidative effect of 2 mM METH was significantly abolished (189.7 ± 8.7% METH versus 135.4 ± 0.2% EGTA + METH, p < 0.001). Cocaine (100 μM), a dopamine transport blocker, did not affect basal values (100 ± 2.7% CTRL versus 105.3 ± 5.4% Cocaine) or METH-induced ROS production (144.4 ± 5.3% METH versus 143.1 ± 10% Cocaine + METH, n.s.).

Effect of Reserpine in Vitro and in Vivo. To evaluate the role of vesicular DA in the oxidative effect of METH, experiments with reserpine were carried out. In the first series, mouse striatal synaptosomes were incubated with 10 μM reserpine to avoid the METH effect on vesicular transport. The presence of reserpine alone in the incubation medium significantly reduced basal ROS production, but the incubation of synaptosomes with METH in the presence of reserpine did not induce any increase in ROS production (see Fig. 7A).

In the second series, mice were previously depleted of vesicular dopamine with a subcutaneous dose of reserpine (5 mg kg⁻¹, 20 h before). Synaptosomes from the animals previously depleted had no oxidative response after incubation with METH (either 2 or 5 mM; Fig. 7B).

Effect of Nicotinic Antagonists. Methyllycaconitine (MLA) is an antagonist of homomeric α7 nicotinic receptors. MLA (10 and 50 μM) prevented the oxidative effect of 2 mM METH (Fig. 8). At the concentrations used in the present study (1, 10, and 50 μM), MLA had no effect on hydrogen peroxide-induced ROS (data not shown), ruling out a nonspecific antioxidant effect of this compound.

Because MLA, at the concentrations used, could also block the nicotinic receptors containing α4β2 or α6β2 subunits, we tested DBE, an antagonist that blocks nAChRs containing β2
subunits. DBE (10 or 50 μM) failed to block METH-induced ROS production (see Fig. 8) without modifying basal values [104.3 ± 1.7% DBE (50 μM), n.s. versus CTRL].

To confirm that MLA effect was due to a specific blockade of α7 nicotinic receptors, α-bungarotoxin, a prototypic α7 antagonist, was used. When striatal synaptosomes were incubated in a medium containing α-bungarotoxin (40 or 500 nM) the basal level of ROS was not modified, but the oxidative effect of METH (2 mM) was significantly abolished [174.2 ± 8.1% METH versus 137.4 ± 6.5% α-bungarotoxin (40 nM) + METH; 103.7 ± 1.3% α-bungarotoxin (500 nM) + METH, p < 0.01 and p < 0.001, respectively].

**Effect of Glutamate Ionotropic Receptors Antagonists.** To study the implication of glutamate ionotropic receptors, the effect of NBQX (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic/kainate receptor antagonist; 10 μM) and PCP (NMDA-associated channel blocker; 1 μM) was evaluated. PCP did not affect basal values or METH-induced ROS production. However, NBQX had a significant effect on METH-induced increase in ROS production, reversing partially the METH effect (204.3 ± 5.6% METH versus 146.8 ± 3.2% NBQX + METH, p < 0.001).

**[3H]DA Vesicular Uptake**

Vesicular uptake of DA was assayed in the presence of two different METH concentrations (the concentration used in synaptosomal DA uptake and a concentration that induces ROS in synaptosomal preparation) and in the presence of MLA from 5 nM to 50 μM, to rule out an effect of this specific α7 nAChR antagonist on VMAT. Incubation with 10 μM...
Discussion

Neurotoxicological studies have established that certain dose regimens of METH administration evoke the degeneration of striatal dopaminergic fibers in the brains of many animal species. Our group has characterized this lesion in the rat (Escubedo et al., 1998; Pubill et al., 2002, 2003). Oxidative stress appears to be one of the main factors involved in this METH degenerative effect. In a recent study (Pubill et al., 2005), we used a synaptosomal preparation from rat striatum to study the mechanisms involved in METH-induced ROS generation. This preparation not only allowed us to determine the most important mechanisms involved in the METH oxidative effect in rat but also brought the first data indicating a preventive effect of MLA, an α7 nAChR antagonist. Thus we initiated the present study to determine whether MLA had neuroprotective effects in vivo or interfered with METH-induced acute behavioral effects in mice, while also performing an in vitro test that could corroborate the results.

nAChRs are ligand-gated ion channels formed by the association of five subunits, leading to heteromeric and homomeric structures (for review, see Hogg et al., 2003). Among the homomeric type, only α7 receptors are widely distributed in the mammalian central nervous system. Matsubayashi et al. (2004) revealed that mRNA for α7 nicotinic receptor subunit and TH were detected in the same single neuron in substantia nigra, suggesting that activation of postsynaptic α7 (and also α4-β2) nAChR in this area results in the excitation of dopaminergic neurons.

MLA binds potently (Kᵢ around 2 nM; Davies et al., 1999) to α-bungarotoxin-binding sites (α7 subunits). Moreover, MLA has been classified as a competitive antagonist of α7 nicotinic receptors (Ward et al., 1990) and, at concentrations of 40 nM and higher, can interact with α4β2 and α6β2 nAChR (Mogg et al., 2002).

Amphetamines, at low doses, can block DA uptake and elicit a nonexocytotic transporter-mediated DA release. Thus, acute administration of METH at low doses such as those used in the behavioral tests performed induced both weak stereotypies (measured as climbing behavior), thought to reflect an increased DA transmission in the neostriatum, and increased locomotion, thought to reflect an increased dopamine transmission in the nucleus accumbens (Ljungberg and Ungerstedt, 1985).

MLA, but not DBE, inhibited METH-induced climbing behavior. However, MLA did not modify either basal locomotor activity or the METH-induced hyperlocomotive profile. Thus, it seems that in neostriatum activation of α7 nAChRs is required to permit METH-induced DA release through reverse transport.

Incubation of striatal synaptosomes with METH induces a decrease in DA uptake that persists even after drug washout (Sandoval et al., 2001). In our preparation, preincubation with MLA prevented the inhibition of DAT induced by METH, without affecting basal uptake values. Because METH inhibition of DA uptake was attenuated in the presence of EGTA, it can be established that extracellular calcium could modulate such inhibition, permitting us to speculate that acute effects after METH administration implicate activation of striatal α7 nAChRs, which consequently in-

reserpine, as expected, fully abolished [³H]DA vesicular uptake (100 ± 7% CTRL versus 0 ± 4.6% reserpine, p < 0.001). METH (500 μM, but not 1 μM) prevented [³H]DA vesicular uptake [0.6 ± 6.0% METH (500 μM), p < 0.001 versus CTRL]. MLA had no effect on this transport [92 ± 12.4% MLA (50 μM), n.s. versus CTRL].

![Fig. 7. A, effect of reserpine added in vitro on METH-induced ROS production. Mouse striatal synaptosomes were incubated alone (CTRL), with METH (2 mM), with reserpine (RES; 10 μM), or with RES plus METH. *** p < 0.001 versus CTRL; ns, non significant. Results are expressed as means ± S.E.M. of three separate experiments run in triplicates. Tukey's post hoc test. B, effect of in vivo catecholamine depletion on METH-induced ROS production in mouse striatal synaptosomes. Animals were pretreated as described under Materials and Methods with saline or reserpine. Synaptosomes were obtained from pretreated animals and incubated alone (CTRL) or with METH (2 or 5 mM). *** p < 0.001 versus CTRL group. Tukey's post hoc test.](image)

![Fig. 8. Effect of different concentrations of MLA (1, 10, and 50 μM) and DBE (10 and 50 μM) on METH (2 mM)-induced ROS production in mouse striatal synaptosomes. ROS production was measured in the absence of drugs (CTRL) or in the presence of METH alone or with MLA/DBE. Results are expressed as means ± S.E.M. from at least three separate experiments run in triplicates. *, p < 0.05 and ***, p < 0.001 versus CTRL; #, p < 0.05 and ###, p < 0.001 versus METH group. Tukey's post hoc test.](image)
duces entrance of calcium and triggers a mechanism that modulates [3H]DA uptake.

After these initial results concerning MLA prevention of acute METH effects, we tested this drug for neuroprotective effects, in vivo and in vitro. The suitability of the in vivo neurotoxic model was demonstrated by the apparent loss of striatal dopaminergic terminals, which was reflected by a significant decrease in both [3H]WIN 35428 binding and tyrosine hydroxylase levels. Such terminal loss was attenuated by pretreatment with MLA, pointing to a neuroprotective effect. Microglial activation (evidenced by an increase in [3H]PK1 11195 binding) was also present. Increase in PBR (peripheral-type benzodiazepine receptors) has been postulated as an indirect marker of neuronal injury and subsequent reactive microgliosis (Stephenson et al., 1995; Vow- inckel et al., 1997; Escubedo et al., 1998). This microglial activation, evidenced 24 h post-treatment, was fully prevented by pretreatment with MLA, supporting the hypothesis of its neuroprotective effect.

Evidence suggests that the degree of METH-induced neurodegeneration is correlated with the degree of hyperthermia (Bowyer et al., 1992). When METH was administered to mice in this special dosage schedule, it originated a hyperthermic effect that was not prevented by MLA. Thus, a neuroprotective effect based on a hypothermic or anti-hyperthermic mechanism can be ruled out.

We also performed in vitro experiments, using a synaptosomal preparation from mouse striatum to study the effect of MLA on METH-induced ROS generation, which is thought to be the main factor responsible for neurotoxicity. METH increases DCF fluorescence when added to our preparation, which indicates that it induces ROS production. It must be pointed out that this increase is observed inside the synaptosomes. Because we wanted to reproduce the mechanisms implicated in the acute neurotoxic effect of METH, the METH concentration used was relatively high to obtain, after a short time of exposition (2 h), an effect that can be pharmacologically modulated (for further explanation, see Pubill et al., 2005).

As in rat striatal synaptosomes, the hypothesis was that METH, after displacing DA from its vesicular complexes, induces DA release to the cytosol by reversing the VMAT function. The inhibition obtained with reserpine, a VMAT blocker, points to DA coming from vesicles as the main source of METH-induced ROS that are detected by our procedure.

In view of the relevance of the VMAT blockade to METH-induced ROS production and due to lipophilicity of MLA, a possible effect of MLA on VMAT had to be evaluated. MLA was unable to inhibit [3H]DA uptake. The results with METH are in agreement with previous reports (Florin et al., 1995; Piif et al., 1995), arguing that METH, at low concentrations, mainly reverses the DAT function and, at higher concentrations, also induces DA release from synaptic vesicles to the cytoplasm. On the other hand, the fact that cocaine did not inhibit MET-induced ROS points to passive diffusion as the main way of entrance of METH inside the synaptosomes.

In our experiments, 7-NI completely abolished METH-induced ROS, demonstrating the role nNOS plays in METH oxidative effects. nNOS produces NO, which reacts with the peroxide radicals that originate from DA auto-oxidation (for review, see Davidson et al., 2001), producing the more toxic radical peroxynitrite (ONOO) (Demiryurek et al., 1998). In addition, peroxynitrite has been found to inhibit DAT (Park et al., 2002). Such an inhibition would favor cytosolic DA accumulation, which would increase oxidative species inside the synaptosomes.

On the other hand, in our model, the specific PKC inhibitor NPC 15437 completely prevented METH-induced ROS, corroborating the key role of PKC in this process. PKC and nitric-oxide synthase require Ca2+ to be activated and, in fact, when extracellular calcium was sequestered by EGTA, METH-induced ROS production was inhibited.

MLA inhibited METH-induced ROS production. This would implicate α7 receptors in the METH effect. However, at the concentration used (50 μM), MLA could also block the nicotinic receptors containing αβ2 and αβ2 subunits. Accordingly, we tested DBE. This compound failed to block METH-induced ROS production, whereas total prevention was obtained with α-bungarotoxin, a prototypic α7 antagonist, thus ruling out the possibility that the preventive effect of MLA is mediated by nAChRs other than α7. Calcium entry through activated α7 nicotinic receptors could activate calcium-dependent mechanisms such as PKC and nNOS that would be implicated in the changes in DAT function (Drew and Werling, 2001).

DA release in the striatum is modulated by several mechanisms. Kaiser and Wonnacott (2000), using perfused non-purified rat striatal synaptosomes and slices, provided evidence for a component of [3H]dopamine release in slices, but not in synaptosomes, that is sensitive to glutamate receptor antagonists and α7-selective nAChR antagonists. They proposed that α7 receptors localized on striatal glutamatergic terminals modulate dopamine release in the striatum by inducing glutamate release. However, in a previous work of ours using rat striatal synaptosomes, the METH oxidative effect was not blocked by PCP or NBQX but was totally prevented by MLA (Pubill et al., 2005), pointing that the activation of α7 nAChR directly induces the oxidative effect of METH. In contrast, the results obtained in the present study about the partial inhibition of METH-induced ROS production in the presence of NBQX would point to a modulating role for glutamate in the oxidative effect of METH, which is prevented by MLA and α-bungarotoxin.

Finally, the present results demonstrate that activation of α7 nAChR is a key step in both acute and long-term METH-induced neurotoxicity, pointing to an interaction of METH with this receptor type. Blockade of this nicotinic receptor subtype prevents microgliosis and attenuates the dopaminergic terminal loss induced by METH in mice. The importance of this study lies not only in the possible neuroprotective effect elicited by the blockade of α7 nicotinic receptors but also in that it proposes a new mechanism for studying METH-induced acute and long-term effects, such as the cognitive sequelae of its abuse (Nordahl et al., 2003).

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Methyllycaconitine Attenuates METH-Induced Effects

Methamphetamine use is associated with numerous neurotoxic effects, including the degeneration of dopaminergic neurons in the zona compacta of the substantia nigra. This process is thought to involve autophagy and apoptosis, and mechanisms that underlie these processes are currently being explored. In this study, the authors investigated the effects of methyllycaconitine, a neurotoxic agent, on METH-induced neuronal damage in culture systems.

**References**


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