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TITLE PAGE

Title:

METHYLLYCACONITINE PREVENTS METHAMPHETAMINE-INDUCED EFFECTS IN MOUSE STRIATUM: INVOLVEMENT OF ALPHA-7 NICOTINIC RECEPTORS.

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RUNNING TITLE PAGE

Running title: Methyllycaconitine attenuates METH-induced effects.

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Abbreviations: α7 nAChR, α7 neuronal nicotinic acetylcholine receptors; AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor; CNS, central nervous system; DA, dopamine; DAT, dopamine transporter; DBE, dihydro-β-erythroidine; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DCF, 2',7'-dichlorofluorescein; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; MLA, methyllycaconitine; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide, 7-NI, 7-nitroindazole; nNOS, neuronal nitric oxide synthase; NPC 15437, (S)-2,6-Diamino-N-[(1-(1-oxotridecyl)-2-piperidinyl)methyl]hexanamide dihydrochloride; PBR, peripheral-type benzodiazepine receptors; PCP, phencyclidine, PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter.

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Abstract

In a previous study we demonstrated that in rat striatal synaptosomes METH-induced 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 1μM METH also included a decrease in striatal synaptosome 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 [^{3}H]PK11195 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- nNOS- and PKC-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 nicotinic receptors by MLA, but also in that it proposes a new mechanism with which to study METH-induced acute and long term effects.

INTRODUCTION

Amphetamine derivatives such as methamphetamine (METH) and methylenedioxymethamphetamine (MDMA) are drugs that are widely abused in the USA 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 (see Davidson et al., 2001 for a review). 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 psycho-stimulant effects by promoting the release of monoamine neurotransmitters (carrier-mediated efflux) and by inhibiting their uptake, thus increasing the extracellular 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).

Neurotoxic effects after high doses of METH in rodents and other species include long-lasting depletion in the striatal content of dopamine (DA) and its metabolites (Ricaurte et al., 1982), decrease in tyrosine hydroxylase activity (Ellison et al., 1978) and loss of DA transporters (Escubedo 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 paper (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 can interact also with α4β2 and α6β2 nAChR (Mogg et al., 2002). In rat synaptosomes, 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 (nAChRs), homomeric α7 nAChRs have the highest fractional Ca^{2+} current. In fact, Liu et al. (2003) found that D-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 [^{3}H]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 [^{3}H]DA uptake was evaluated *in vitro*. Assessment of neurotoxicity markers after an *in vivo* treatment with a neurotoxic schedule of METH and *in vitro* METH-induced ROS production was used to determine the neuroprotective effect of MLA.

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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 ºC under a 12-h light/dark cycle with free access to food and drinking water.

1. Climbing behavior

Climbing behavior was measured using Gerhardt's method (Gerhardt et al., 1985) as modified by us. Briefly, mice of 20–26 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 minute period of exploratory activity, stereotypy measurement was performed for a period of 30 minutes. 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 Spain) and expressed as the percentage of the total time (30 min).

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2. 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 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, Barcelona, Spain) 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 (SedaCom32, Letica, Barcelona, Spain). 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 a.m. and 3:00 p.m. 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 120 min.

3. *In vivo* treatments for neurotoxicity assessment

Mice weighing 28–32 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

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its pharmacokinetics and affinity for the α-7 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 ºC 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 [^{3}H]WIN 35428 binding and tyrosine hydroxylase studies, and 24 h after the last dose for [^{3}H]PK 11195 binding studies.

3.1. Tissue sample preparation

Immediately after sacrifice, mice were decapitated and the brains 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 ºC in 10 volumes of buffer consisting of 5 mM Tris-HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 µg $µl^{-1}$, 0.1 mM PMSF and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at 1,000 x g for 15 min at 4^{o}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 15,000 x g for 30 min at 4^{o}C. The pellets were resuspended in buffer and

incubated at 37 ºC for 10 min in order to remove endogenous neurotransmitters. Then the protein samples were re-centrifuged 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, USA) according to the manufacturer's specifications.

3.1.1. 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% (W/V) SDS, 5% (V/V) 2-β-mercaptoethanol, 0.05% bromophenol blue, final concentrations), boiled for 10 min and loaded onto a 10 % acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P, Millipore). PVDF membranes were blocked overnight with 5 % defatted milk in Tris-buffered saline buffer plus 0.05 % Tween-20 (TBS-T) and incubated for 2 h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Laboratories, Lexington, KY, USA) diluted 1:5,000. After washing, membranes were incubated with a peroxidase-conjugated anti-mouse IgG antibody (Amersham). Immunoreactive protein was visualized using a chemoluminiscence-based detection kit following the manufacturer's protocol (ECL kit; Amersham) and exposing x-ray film. After exposure, developed films were scanned and semi-quantitative analysis was performed using video-densitometric software (IMAT program, Scientific and Technical Services, University of Barcelona). Immunodetection of β-actin (mouse monoclonal antibody from Sigma, 1:2,500 dilution) served as a control of load uniformity for each lane and was used to normalize

differences in TH expression due to protein content. TH levels are expressed as percent of those of saline-treated animals.

3.1.2. Radioligand binding experiments

Microglial activation was assessed by [^{3}H]PK 11195 binding. Crude membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.4. Equilibrium binding assays were performed at 4°C for 2 h in borosilicate glass tubes containing 2 nM [^{3}H]PK 11195 (specific activity: 85 Ci/mmol), and 50 µg of protein in a final volume of 0.25 ml. 10 µM of unlabelled PK 11195 was used to determine nonspecific binding.

The density of DAT in striatal membranes was measured by [^{3}H]WIN 35428 equilibrium binding assays. Membranes were resuspended in phosphate-buffered 0.32 M sucrose, pH 7.9 at 4 ºC (Coffey and Reith, 1994) to a concentration of 1 µg µl^{-1}. Binding assays were performed in borosilicate glass tubes containing 200 µl of [^{3}H]WIN 35428 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 ºC. Nonspecific binding was determined in the presence of 30 µM bupropion.

All incubations were finished by rapid filtration under vacuum through GF-51 glass fiber filters (Schleicher and Schüell, Dassel, Germany). Tubes and filters were washed rapidly 3 times with 4 ml 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 non-labeled ligand.

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4. 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 1,000 X *g* at 4 ºC 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 13,000 X *g* for 30 min at 4 ºC. 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, composition in mM: 140 NaCl, 5.37 KCl, 1.26 $CaCl_2$, 0.44 KH_2PO_4, 0.49 $MgCl_2 \cdot 6H_2O$, 0.41 $MgSO_4 \cdot 7H_2O$, 4.17 $NaHCO_3$, 0.34 $Na_2HPO_4 \cdot 7H_2O$, 5.5 glucose and 20 HEPES-Na), 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 to perform 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.

4.1. Measurement of methamphetamine-induced ROS

The formation of intrasynaptosomal ROS was measured using fluorochrome 2',7'-dichlorofluorescin 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'-dichlorofluorescine, 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.

50 μM 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 ºC in the dark and methamphetamine was then added at the desired concentration. Incubation was continued in the dark for 2 h and finally stopped by centrifugation at 13,000 X *g* for 30 min, 4 ºC. 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 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 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 500-900 synaptosomes s^{-1} and each sample was measured for one minute. Fluorescence data were analyzed using Elite software (Coulter, USA). 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 without METH). Results are mean ± SEM of at least three separate experiments run on triplicates.

In order 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 pre-loaded with the dye, then washed and incubated with METH while 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 non-washed preparations, the percentage of increase in fluorescence was the same in both cases. Thus all the experiments were carried out on non-washed preparations in order to avoid an additional centrifugation step.

5. Plasmalemmal and vesicular [^{3}H]dopamine uptake

For measuring [^{3}H]DA uptake via plasmalemmal transporters, synaptosomes were obtained as described above and preincubated in a shaking water bath at 37 ºC 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 13,000 x g for 20 min, resuspended in 5 mM Tris-HCl/320 mM sucrose buffer and re-centrifuged. Final pellets were resuspended in a volume of HBSS buffer containing 10 µM pargyline so that final protein content was approximately equivalent to 10 mg of tissue (wet weight) per ml. Reaction tubes consisted of 0.85 ml of HBSS buffer (plus 10 µM pargyline and 1 mM ascorbic acid), 0.1 ml of synaptosome suspension and 0.05 ml of [^{3}H]DA (final concentration 5 nM) added at the start of incubation. Tubes were warmed 10 min at 37ºC before the addition of [^{3}H]DA, after which incubation was carried out for a further 5 min. Uptake reaction was stopped by rapid filtration as described for binding experiments. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Nonspecific uptake was determined at 4 ºC in parallel samples containing 100 µM cocaine. Specific DA uptake was calculated subtracting nonspecific uptake values from those of total uptake (37ºC).

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The remaining synaptosomal preparation (i.e., not used for the uptake assay) was kept and protein was determined as described above. Specific DA uptake for each condition was normalized dividing by the protein concentration and expressed as percentage of the uptake in control tubes.

For measuring [^{3}H]DA uptake via Vesicular Monoamine Transporters (VMAT) the method described by Hansen et al. (2002) was used with minor modifications. Briefly, mouse striatal synaptosomes were resuspended and lysed in cold distilled deionized water. Osmolarity was restored by addition of HEPES and potassium tartrate to final concentrations of 245 mM and 100 mM, respectively, and samples were centrifuged for 20 min at 20,000 x g (4 ºC) to remove synaptosomal membranes. $MgSO_4$ (1 mM, final concentration) was added to the supernatant, which was then centrifuged for 45 min at 100,000 x g (4 ºC). The resulting vesicular pellet was resuspended in wash buffer (see below for composition) at a concentration of 50 mg ml^{-1} (wet tissue weight). Vesicular [^{3}H]DA uptake measurement was performed by incubating 100 µl of vesicles at 30 ºC for 3 min in assay buffer (final concentration: 25 mM HEPES, 100 mM potassium tartrate, 1.7 mM ascorbic acid, 0.05 mM EGTA, 0.1 mM EDTA, 2 mM ATP-Mg^{2+}, pH 7.5) in the presence of 30 nM [^{3}H]DA. The reaction was terminated by addition of 1 ml of cold wash buffer (assay buffer containing 2 mM $MgSO_4$ instead of ATP-Mg^{2+}, pH 7.5) and rapid filtration followed by three 1 ml-washes as described for binding assays. Radioactivity trapped in filters was quantified using a liquid scintillation counter. Reserpine (10 µM) was tested in each experiment as a positive control for vesicular uptake inhibition. Non-specific incorporation was determined by measuring uptake at 4 ºC in wash buffer. Calculations were the same that those described for plasmalemmal uptake.

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). 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. All calculations were performed using Graph Pad Instat (GraphPad software, San Diego, USA).

Drugs and reagents

Drugs and reagents were obtained from the following sources: α-bungarotoxin, (+)-methamphetamine hydrochloride, reserpine, methyllycaconitine, vitamin E ((±)-α-tocopherol nicotinate), 7-nitroindazole, EGTA, NBQX, NPC 15437 ((S)-2,6-Diamino-N-[(1-(1-oxotridecyl)-2-piperidinyl)methyl]hexanamide dihydrochloride), PCP and dihydro-β-erythroidine, were purchased from Sigma-Aldrich . Cocaine was provided by the National Health Laboratory (Barcelona, Spain). DCFH-DA was obtained from Molecular Probes (Leiden, The Netherlands) and [^{3}H]PK 11195 (1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide), [^{3}H]WIN 35428 ((-)-2-beta-carbomethoxy-3-beta-(4-fluorophenyl)tropane 1,5-napthalenedisulfonate), and [^{3}H]dopamine from Perkin-Elmer Life Sciences (Boston, MA, USA). All buffer reagents were of analytical grade.

Drugs were dissolved in bi-distilled water and added at a volume of 10 µl to each ml of synaptosomal preparation. DCFH-DA, vitamin E, NI and reserpine were dissolved in DMSO. The final concentration of DMSO was less than 0.5%. This concentration had no effect on METH-induced ROS generation.

RESULTS

Climbing behavior

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 behaviour when compared to 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 *vs* 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 stimulant response, and a dose of 0.4 mg kg^{-1} was chosen for further studies. Horizontal locomotor activity profile was registered for 360 minutes 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 minutes. 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.

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[^{3}H]DA uptake in mouse striatal synaptosomes

Preincubation of synaptosomes with METH (1 μM) for 30 min significantly reduced [^{3}H]DA uptake (80%). MLA (0.1 μM) did not affect basal uptake values and prevented the inhibition of [^{3}H]DA uptake induced by METH. In experiments carried out with the calcium chelator EGTA (5 mM), the basal [^{3}H]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 [^{3}H]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 [^{3}H]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. 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 ºC, n = 5, saline group *vs* 38.5 ± 0.3 ºC, 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 ºC, n = 5) or reduce the METH-induced hyperthermia (38.2 ± 0.4 ºC, n = 6, MLA+METH group, n.s. *vs*. METH group).

METH-treated animals showed a significant loss in body weight (-9.0 ± 1.8 % METH group, n = 6 *vs*. 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 pre-treatment 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. *vs*. METH group).

Effect on tyrosine hydroxylase levels

Treatment with METH induced a marked loss (by 90%) of the levels of striatal tyrosine hydroxylase measured by Western blot analysis(Fig. 4). This decrease was attenuated in mice pretreated with MLA, showing an average loss of about 45%. Treatment with MLA alone had no effect on tyrosine hydroxylase levels.

METH-induced ROS production in synaptosomes

METH (0.05–10 mM) increased DCF fluorescence in striatal mouse synaptosomes, indicating ROS production, in a concentration-dependent manner. The fluorescence histogram shifted to

the right (Fig. 5A) and individual synaptosomes showed increased fluorescence when compared with untreated synaptosomes (Fig. 5B and 5C). Incubation with a concentration of 2 mM METH was chosen for further experiments. Hydrogen peroxide (200 μM) was used as positive control (240.67 ± 2.93% increase in ROS production) in order rule out a nonspecific antioxidant effect of some compounds.

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 *vs.* 100 ± 5,2% CTRL, $p < 0.001$). Incubation of striatal mouse synaptosomes with METH 2 mM in the presence of vitamin E (100 μM) did not induce a significant increase in ROS production (56.9 ± 1.9% vitamin E *vs.* 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 (NI) (100 μM), a nNOS inhibitor, significantly prevented the effect of METH while 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).

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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 *vs.* 100 ± 5.2% CTRL), but the oxidative effect of METH 2 mM was significantly abolished (189.7 ± 8.7% METH *vs.* 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 *vs.* 105.3 ± 5.4% Cocaine) or METH-induced ROS production (144.4 ± 5.3% METH *vs.* 143.1 ± 10% Cocaine+METH, n.s.).

Effect of reserpine in vitro and in vivo

In order 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 reserpine 10 μM, 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 2mM or 5 mM, Fig. 7B).

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Effect of nicotinic antagonists

Methyllycaconitine (MLA) is an antagonist of homomeric α7 nicotinic receptors. MLA (10 and 50 μM) prevented the oxidative effect of METH 2mM (Fig. 8). At the concentrations used in the present paper (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 μM 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. *vs.* 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 nM 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 *vs.* 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

In order to study the implication of glutamate ionotropic receptors, the effect of NBQX (AMPA/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,

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reversing partially the METH effect (204.3 ± 5.6% METH *vs*. 146.8 ± 3.2% NBQX + METH, $p < 0.001$).

[^{3}H]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, in order to rule out an effect of this specific α7 nAChR antagonist on VMAT. Incubation with reserpine 10 μM, as expected, fully abolished [^{3}H]DA vesicular uptake (100 ± 7% CTRL *vs.* 0 ± 4.6% reserpine, $p < 0.001$). METH 500 μM, but not 1 μM, prevented [^{3}H]DA vesicular uptake (0.6 ± 6.0% METH 500 μM, $p < 0.001$ *vs.* CTRL). MLA had no effect on this transport (92 ± 12.4% MLA 50 μM, n.s. *vs*. CTRL).

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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 paper (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 in order to determine if 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 (see Hogg et al., 2003 for a review). Among the homomeric type, only α7 receptors are widely distributed in the mammalian CNS. 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 (K_D 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). Also, 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 non-exocytotic 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, pre-incubation 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 induces entrance of calcium and triggers a mechanism that modulates [^{3}H]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 [^{3}H]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 [^{3}H]PK 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; Vowinckel 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 antihyperthermic 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 in order to obtain, after a short time of exposition (2 h), an effect that can be pharmacologically modulated (see Pubill et al., 2005 for further explanation).

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 [^{3}H]DA uptake. The results with METH are in agreement with previous reports (Pifl et al., 1995, Florin 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.

As in *in vivo* studies (Imam et al., 1999; Yamamoto and Zhu, 1998), antioxidants protected *in vitro* against METH-induced ROS production in synaptosomes. Several *in vivo* studies demonstrate the involvement of nNOS in METH neurotoxicity (Deng and Cadet, 1999; Sanchez et al., 2003). 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 (see Davidson et al., 2001 for a review), 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

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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 NOS require Ca^{2+} 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 α4β2 and α6β2 subunits. Accordingly, we tested DBE. This compound failed to block METH-induced ROS production while 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 [^{3}H]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 paper 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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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Panel 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 ± SEM (n = 7-11 per group) of total time spent in climbing (as percentage) *** $p < 0.001$ *vs.* saline-treated group; ## $p < 0.01$ *vs.* METH group. One-way analysis of variance (ANOVA) was used for statistical analysis. Significant differences were then analyzed by Tukey's *post hoc* test for multiple means comparisons. Panel 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. *** $p < 0.001$ *vs* saline-treated group. One-way analysis of variance (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 [^{3}H]DA uptake in mouse striatal synaptosomes. After preincubation, synaptosomes were washed and [^{3}H]DA uptake performed (5 min). Data are presented as mean ± S.E.M. percentage of control [^{3}H]DA uptake from at least three separate experiments run in duplicates. Nonspecific [^{3}H]DA uptake was determined at 4 ºC in parallel samples containing 100 µM cocaine and was about 7% of total [^{3}H]DA uptake. * $p < 0.05$ and ** $p < 0.01$ *vs.* control [^{3}H]DA uptake; Tukey's *post-hoc* test.

Fig. 3. Panel 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

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specific binding of [^{3}H]WIN 35428. * $p < 0.05$ and ** $p < 0.01$ *vs.* saline-treated group, # $p < 0.05$ *vs.* METH group. Panel 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 [^{3}H]PK 11195. Increase in binding indicates microglial activation. . * $p < 0.05$ *vs.* saline-treated group; # $p < 0.05$ *vs.* METH group. In both panels values are expressed as means ± S.E.M. of those obtained from 5 - 6 animals in each group; Tukey's *post-hoc* test.

Fig. 4. Panel A: Representative Western blot showing individual tyrosine hydroxylase expression in mouse striatum 72 h after treatment with METH, MLA or MLA + METH. Panel B shows quantification of tyrosine hydroxylase levels obtained from all the Western blots performed. MLA pretreatment attenuated the decrease on tyrosine hydroxylase levels induced by METH. β-actin expression was used as a gel load control and to normalize the results as the ratio TH/β-actin expression. Data are presented as percent of saline-treated values ± S.E.M. Western blots were performed with samples originating from at least five animals of each treatment group. One-way analysis of variance (ANOVA) was used for statistical analysis. Significant differences were then analyzed by Tukey's *post hoc* test for multiple means comparisons. *** $p < 0.001$ and ** $p < 0.01$ *vs.* saline; ## $p < 0.01$ *vs.* METH.

Fig. 5. Representative flow cytometry histograms (panel A) and dot plots showing the change in dichlorofluorescein fluorescence of mouse striatal synaptosomes after 2 h incubation at 37 ºC alone (Control, CTRL, panel B) or with METH (2 mM, panel C).

Fig. 6. Effect of the neuronal nitric oxide synthase inhibitor 7-nitroindazole (NI) (100 μM) and the protein kinase C inhibitor NPC 15437 (NPC) (100 μM) on METH (2 mM)-induced

ROS production in mouse striatal synaptosomes. Results are expressed as means ± S.E.M. from at least three separate experiments run in triplicates. * p <0.05 and *** p <0.001 *vs.* CTRL (no drugs). ns: non significant. Tukey's *post-hoc* test.

Fig. 7. Panel 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 *vs*. CTRL; ns: non significant. Results are expressed as means ± S.E.M. of three separate experiments run in triplicates. Tukey's *post-hoc* test. Panel B: Effect of *in vivo* catecholamine depletion on METH-induced ROS production in mouse striatal synaptosomes. Animals were pretreated as described in 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 *vs*. CTRL group. Tukey's *post-hoc* test.

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 *vs*. CTRL; # p < 0.05 and ### p <0.001 *vs*. METH group. Tukey's *post-hoc* test.

Figure 1

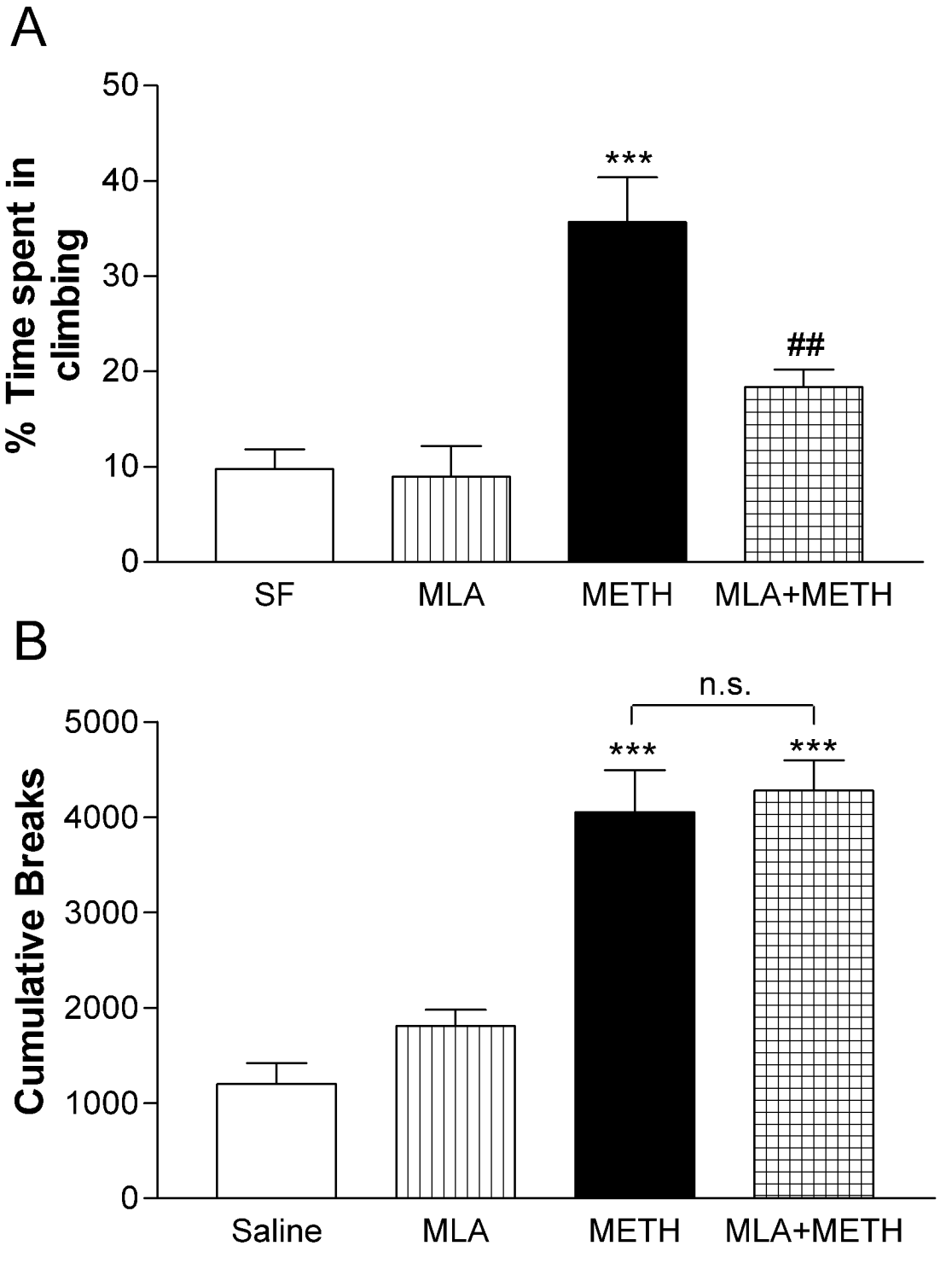

A
% Time spent in climbing
50
40
30
20
10
0
***
##
SF
MLA
METH
MLA+METH
B
Cumulative Breaks
5000
4000
3000
2000
1000
0
n.s.
***
***
Saline
MLA
METH
MLA+METH

Figure 2

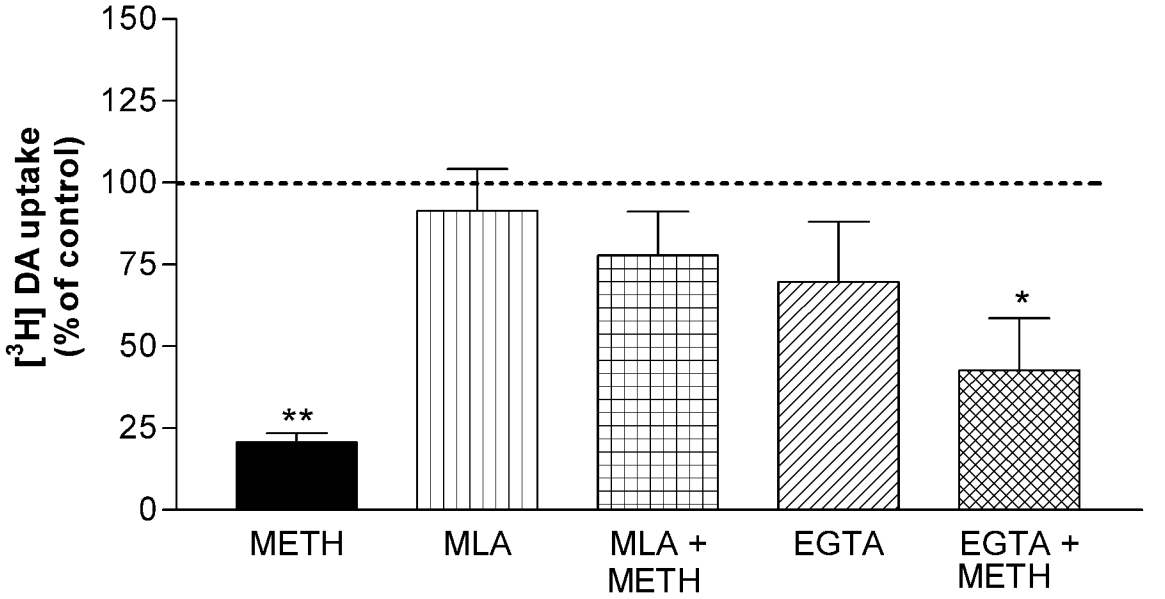

150
125
100
75
50
25
0
[3H] DA uptake
(% of control)
**
*
METH
MLA
MLA +
METH
EGTA
EGTA +
METH

Figure 3

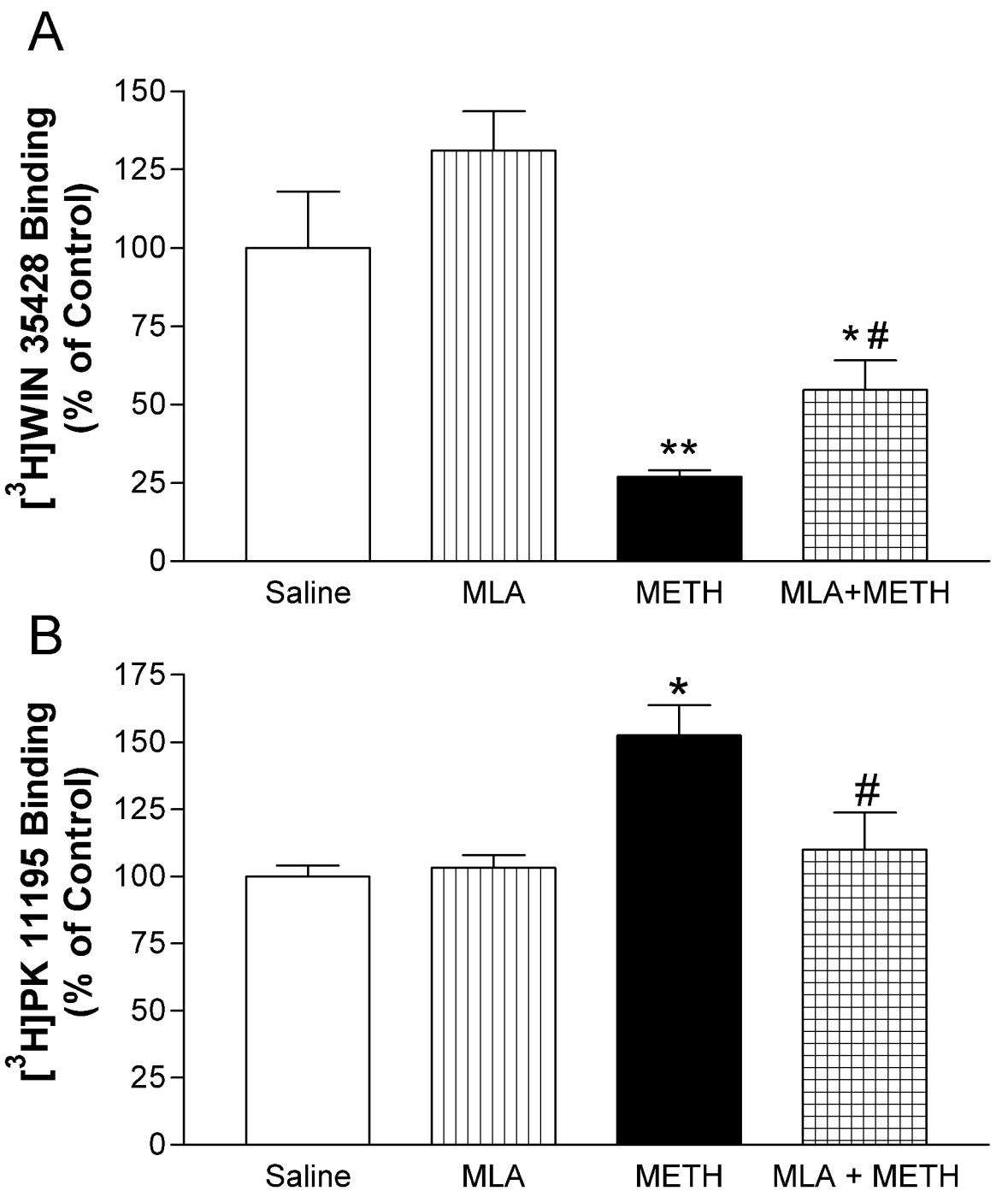

A
[3H]WIN 35428 Binding
(% of Control)
150
125
100
75
50
25
0
**
*#
Saline
MLA
METH
MLA+METH
B
[3H]PK 11195 Binding
(% of Control)
175
150
125
100
75
50
25
0
*
#
Saline
MLA
METH
MLA + METH

Figure 4

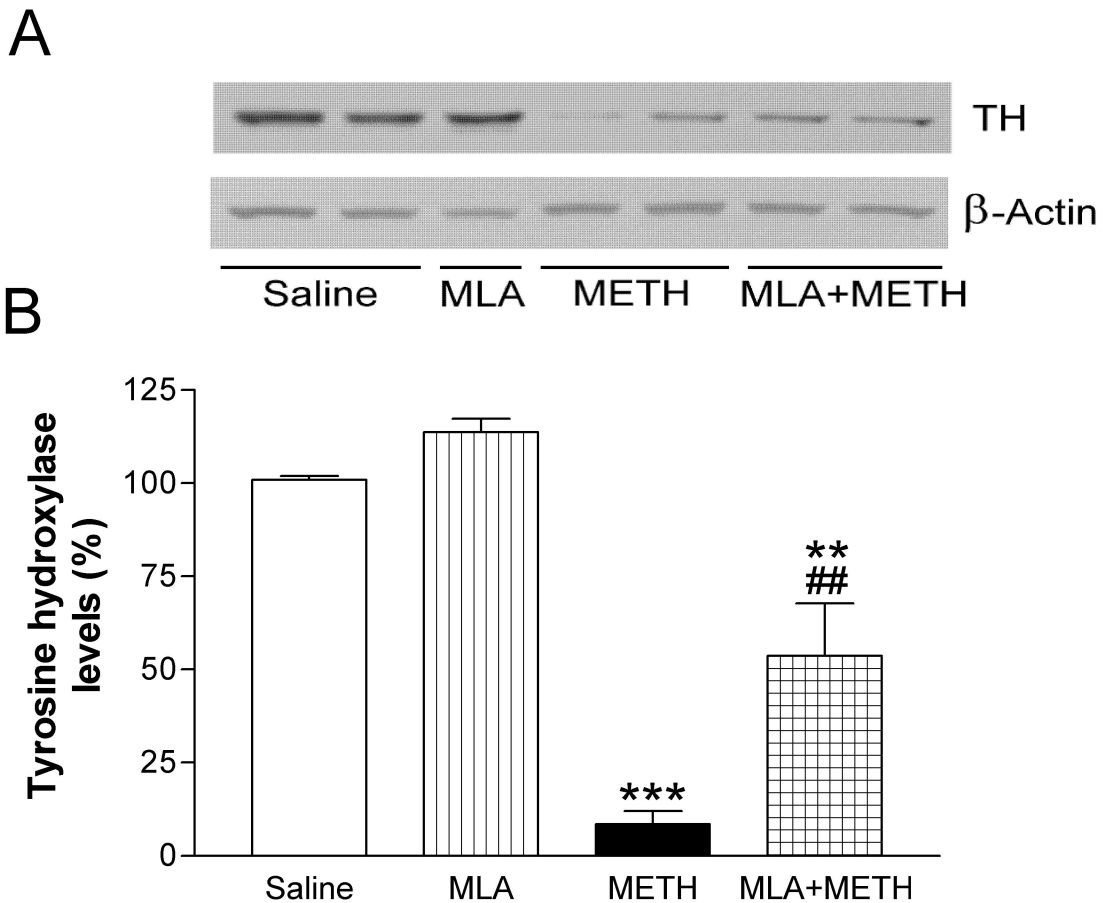

A
TH
β-Actin
Saline
MLA
METH
MLA+METH
B
Tyrosine hydroxylase levels (%)
125
100
75
50
25
0
**
##
***
Saline
MLA
METH
MLA+METH

Figure 5

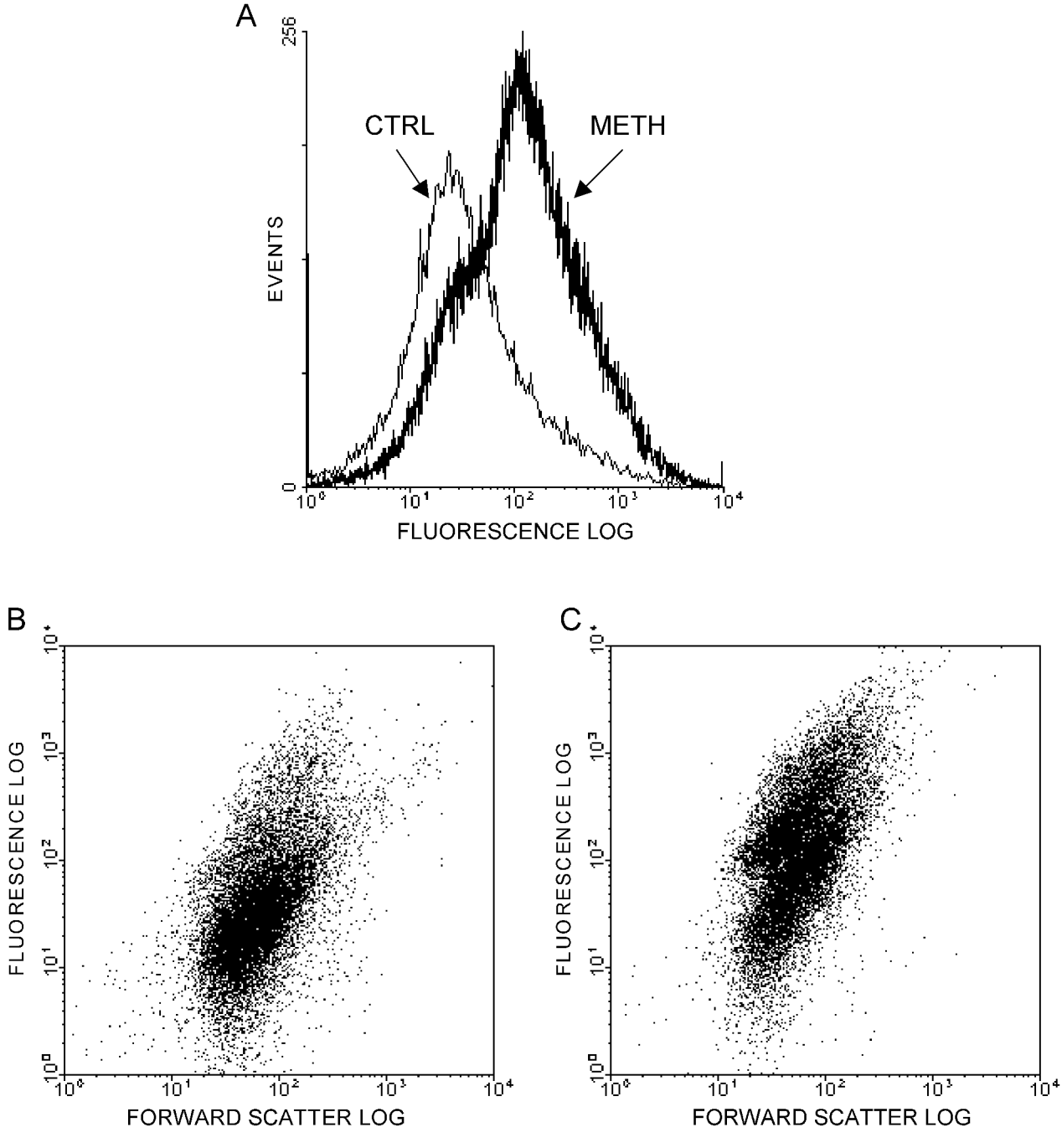

A
256
0
CTRL
METH
EVENTS
10^0
10^1
10^2
10^3
10^4
FLUORESCENCE LOG
B
FLUORESCENCE LOG
FORWARD SCATTER LOG
C
FLUORESCENCE LOG
FORWARD SCATTER LOG

Figure 6

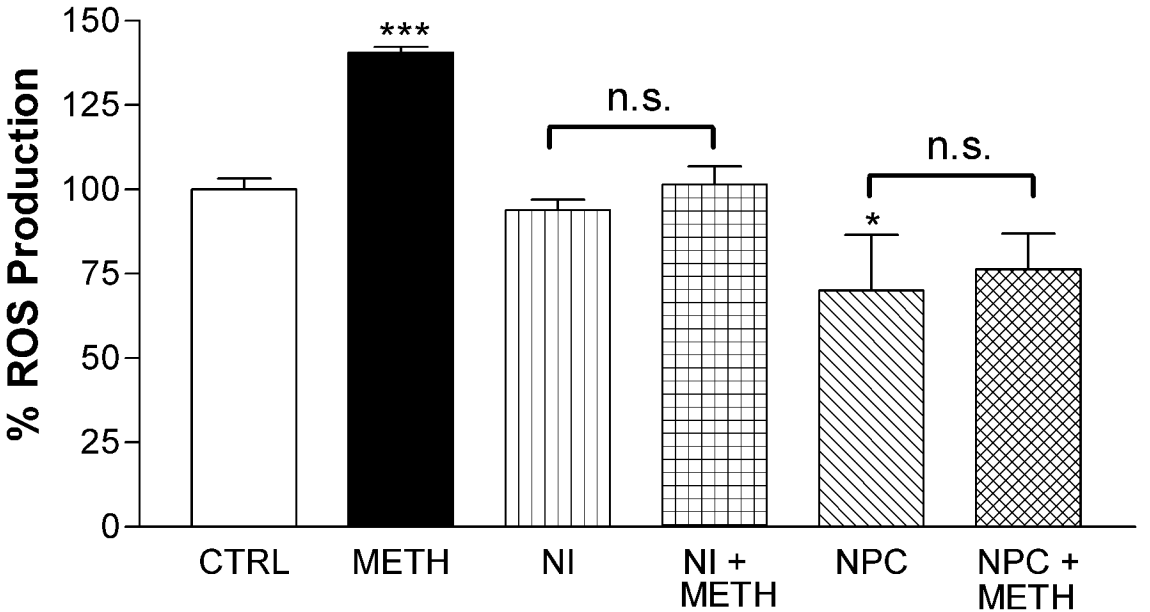

% ROS Production
150
125
100
75
50
25
0
***
n.s.
n.s.
*
CTRL
METH
NI
NI + METH
NPC
NPC + METH

Figure 7

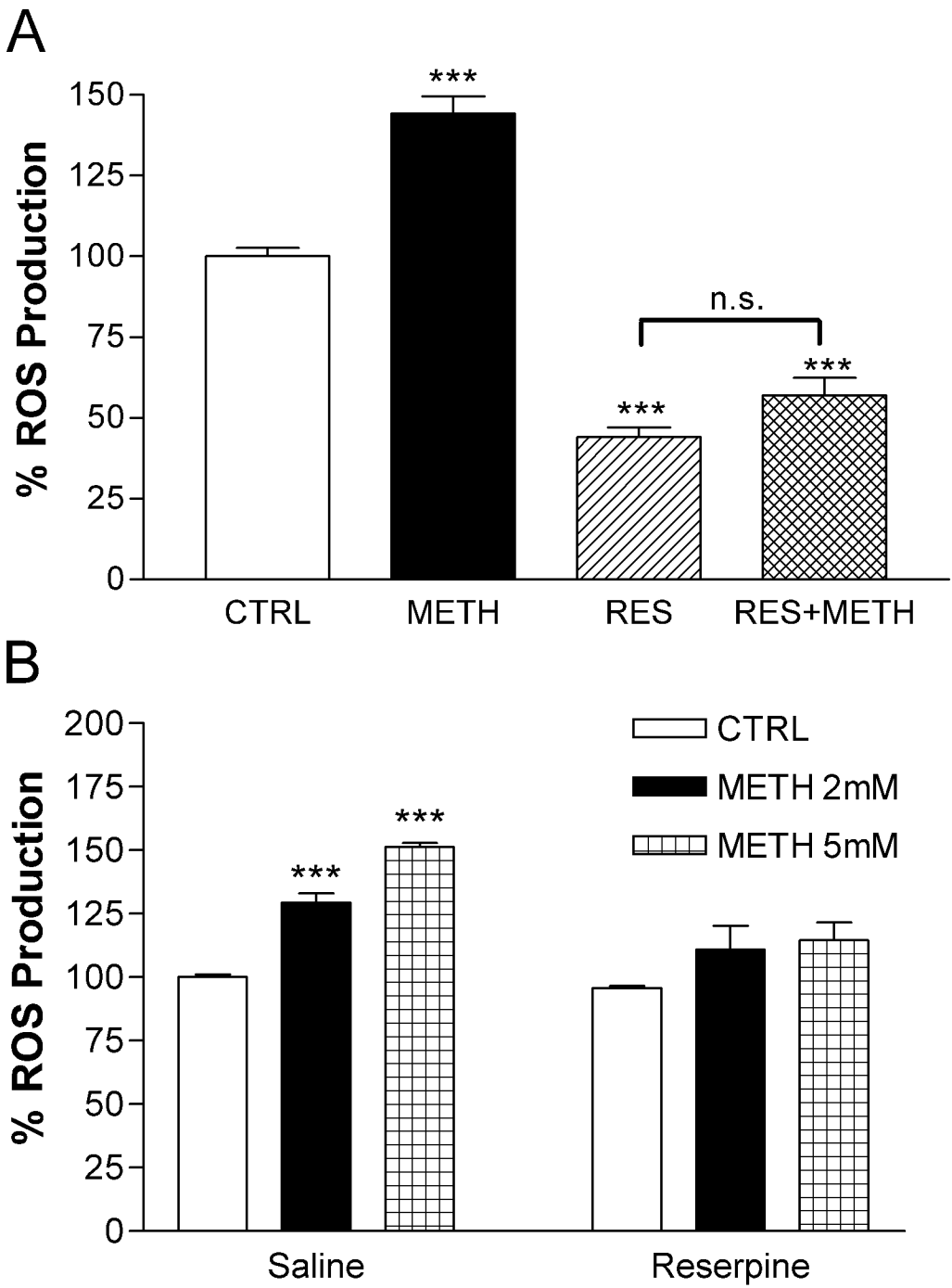
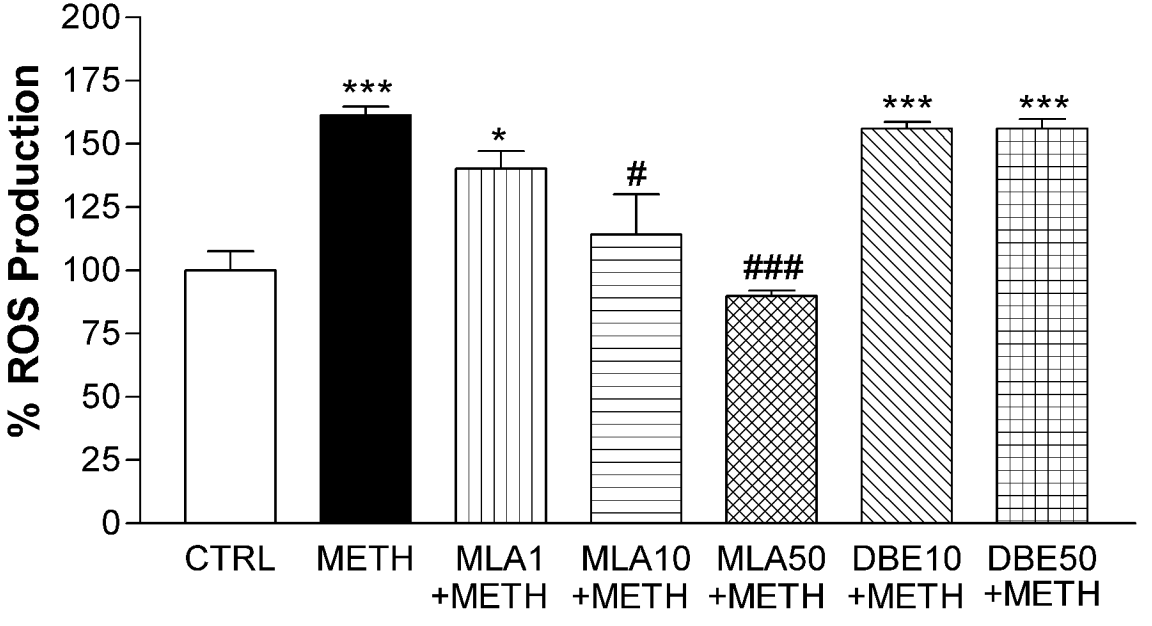

A
% ROS Production
150
125
100
75
50
25
0
***
***
***
n.s.
CTRL
METH
RES
RES+METH
B
% ROS Production
200
175
150
125
100
75
50
25
0
***
***
CTRL
METH 2mM
METH 5mM
Saline
Reserpine

Figure 8


% ROS Production
200
175
150
125
100
75
50
25
0
***
*
#
###
***
***
CTRL
METH
MLA1 +METH
MLA10 +METH
MLA50 +METH
DBE10 +METH
DBE50 +METH