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Original Article

# Different glial response to methamphetamine- and methylenedioxymethamphetamine-induced neurotoxicity

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**Abstract** The consequences of the neurotoxic insult induced by 3,4-methylenedioxymethamphetamine (MDMA, an amphetamine derivative with specific action on the serotonergic system) were compared with those of methamphetamine (a derivative with specific action on dopaminergic system) in rats. Both drugs induced a very similar loss of body weight, especially evident 24 h after treatment. Their hyperthermic profile was also very similar and was dependent on ambient temperature, corroborating the thermo-dysregulatory effect of both substances. Methamphetamine (four injections of 10 mg $kg^{-1}$ s.c. at 2-h intervals) induced the loss of dopaminergic (35%) but not of serotonergic, terminals in the rat striatum and, simultaneously, a significant increase in striatal peripheral-type benzodiazepine receptor density, pointing to a glial reaction. Evidence for this drug-induced astrogliosis was the increased heat shock protein 27 (HSP27) expression in striatum, cortex and hippocampus. MDMA (20 mg $kg^{-1}$ s.c. b.i.d. for 4 days) induced a similar dopaminergic lesion in the striatum 3 days post-treatment, which reversed 4 days later. An important neurotoxic effect on serotonergic terminals was also observed in the cortex, striatum and hippocampus 3 days post-treatment, which partially reversed 4 days later in the striatum and hippocampus. No microglial activation was noticeable at either 3 or 7 days after MDMA treatment. This lack of effect on microglial cells was assessed by [$^3$H]PK 11195

binding and OX-6 immunostaining, which were unchanged in the striatum and cortex after MDMA treatment. A non-significant tendency to increase was noted in the hippocampus 3 days after MDMA treatment. Furthermore, in MDMA-treated rats, neither HSP27 expression nor an increase in HSP27 immunoreactivity were detected. This result, together with the lack of increase in glial fibrilliary acidic protein (GFAP) immunoreactivity, indicate no astroglial activation at either 3 or 7 days post-treatment. Without microglial activation, an inflammatory process would not accompany the lesion induced by MDMA. The differences in glial activation between methamphetamine and MDMA observed in the present study could have implications for the prognosis of the injury induced by these drugs.

**Keywords** Methamphetamine - MDMA - Ecstasy - Neurotoxicity - Glial activation - Peripheral-type benzodiazepine receptor - HSP27

# Introduction

Amphetamine-like drugs exert their powerful psychostimulant effects by promoting the release of monoamine neurotransmitters (carrier-mediated efflux) and by inhibiting their uptake. Although amphetamines have been used widely in clinical practice as anorectic agents to reduce body weight, the dopaminergic neurotoxicity induced by chronic administration of amphetamine to rats has been widely demonstrated (Eisch et al. *1992*). Methamphetamine (METH), a methyl derivative of amphetamine, is well known to decrease multiple indices of dopamine (DA) terminal integrity, especially in the striatum. The striatal changes produced by extended exposure of rats to METH include long-lasting decreases in DA content (Kogan et al. *1976*), DA metabolites (Ricaurte et al. *1982*), tyrosine hydroxylase activity (Ellison et al. *1978*) and DA uptake (Streit and Graeber *1993*).

The METH-induced reductions in specific biochemical parameters are accompanied by structural damage, as shown by alterations of two markers of neuronal injury: reactive gliosis [enhanced expression of glial fibrillary acidic protein (GFAP)] and the silver degeneration reaction, while loss of neuronal perikarya is not evident (O'Callaghan and Miller, *1994*).

Although the mechanisms underlying METH-induced damage are not fully understood, there is compelling evidence that amphetamines reverse the operational direction of the high-affinity transport sites present in monoaminergic terminals (Fleckenstein et al. *1997*), thus increasing the DA concentration in extracellular space (Bowyer et al. *1991*). The magnitude of METH-induced striatal overflow of DA correlates with the extent of subsequent injury (O'Dell et al. *1991*). Oxidative products of DA also play a key role in METH-induced toxicity (Sonsalla et al. *1989*).

The psychotropic amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") is also regarded as a serotonergic neurotoxin in rats and primates (Battaglia et al. *1988*; Ricaurte et al. *1988*). MDMA is a ring-substituted

phenylisopropylamine related to both amphetamines and mescaline. The acute effects of MDMA, like those of (+)-amphetamine and other drugs of abuse, include behavioural, neurochemical and electrophysiological changes that can be correlated with an increase in the extracellular levels of serotonin (5-HT) and DA. However, its action can be more prominent on 5-HT than on DA release (White et al. *1996*), depending on the animal species studied (Logan et al. *1988*). Recently, Ricaurte et al. (*2002*) have demonstrated that non-human primates exposed to several sequential doses of MDMA develop severe brain dopaminergic neurotoxicity, in addition to less-pronounced serotonergic neurotoxicity. It has also been suggested that MDMA-induced neurotoxicity results from MDMA or dopamine metabolites producing radicals that combine with NO to form peroxynitrites (Colado et al. *2001*).

Several markers can be used to assess neuronal damage. One is the peripheral-type benzodiazepine receptor (PBR). In the central nervous system (CNS), PBR are located primarily in glial cells. Numerous studies have shown that both astrocytes and microglia express PBR (Park et al. *1996*) and that the density of PBR can increase under conditions that result in glial activation, including inflammation, metabolic stress, trauma, ischaemia and chemically-induced brain injury (Casellas et al. *2002*). As some CNS injuries result in microgliosis (Stephenson et al. *1995*; Streit and Graeber, *1993*) early after injury, several studies on neurodegeneration have postulated that an increase in PBR density at this time reflects microglial activation and can be used as an indirect marker of neuronal damage (Benavides et al. *1987*; Escubedo et al *1998*). Vowinckel et al. (*1997*) have used [$^{3}$H]PK11195 binding to the PBR as a marker of microglia activation in multiple sclerosis and experimental autoimmune encephalomyelitis, whilst Stephenson et al. (*1995*) have colocalized PBR with activated microglia following transient global forebrain ischaemia in the rat. Since, however, studies using neuronal injury models have reported PBR localization also in astroglia (Kuhlmann and Guilarte *2000*), its use as an exclusively microglial marker could be misleading if it is not supported by other markers.

The 27-kD heat-shock protein (HSP27) is expressed in astroglia after several types of brain injury (Kato et al. *1994*; Plumier et al. *1996*) and can be used as an indicator of astroglial stress. It has been proposed that HSP27 is transferred from glia to neurones to exert neuroprotective effects (Renkawek et al. *1994*).

The aim of the present study was to investigate the consequences of the neurotoxic effect of MDMA in rats, comparing these with the extensively described effects of METH. These two amphetamine derivatives have specific effects on distinct neurochemical systems: methamphetamine on the dopaminergic, and MDMA on the serotonergic system. To this end, we evaluated the effect of both substances on body weight and body temperature, their neurotoxicity and the glial response, following the established dosage schedule for every derivative. Most authors described the neurotoxic effects of METH 3 days after treatment (Pu and Vorhees *1993*) and those of MDMA 7 days after treatment (Battaglia et al. *1988*). Because microglial activation can decline after a week, we also examined the neurotoxic injury induced by MDMA 3 days after finishing the treatment. Although it is well documented that MDMA induces serotonergic toxicity, we assessed

this toxicity to establish that the drug regimen that we use was indeed neurotoxic, regardless of the degree of glial activation.

# Materials and methods

*Chemicals* Aprotinin, bovine serum albumin, bupropion, clomipramine, methamphetamine, phenylmethylsulphonylfluoride (PMSF) and sodium orthovanadate were from Sigma (St. Louis, Mo., USA). MDMA was obtained from the National Health Laboratory (Barcelona, Spain); Ro5-4864 was from Fluka (Germany) and [$^3$H]GBR 12935, [$^3$H]paroxetine and [$^3$H]PK 11195 were purchased from New England Nuclear (Wilmington, Del., USA). All other chemicals were of analytical grade.

*Animal procedures and drug administration* The dosage regimens employed were chosen according to the literature, and are thought to be equivalent to a chronic administration (O'Callaghan and Miller *1994*; Battaglia et al. *1988*). Male Sprague-Dawley rats weighing 210–255 g (Harlan Ibérica, Barcelona, Spain), were used. The animals were housed one per cage. Before treatment, they were allowed to acclimatise to an environmental temperature of 26±2 °C. METH (10 mg/kg s.c.) or saline were given every 2 h, beginning at 10.00 a.m., for a total of four doses. One hour after the last dose, rats were returned to their housing. Animals were killed by decapitation 3 days after METH treatment. In the MDMA treatment schedule, animals were housed and treated under the same ambient conditions as in the METH protocol. They received two doses (of 20 mg/kg s.c.) per day (interval 7 h) for 4 days, and were sacrificed 3 days or 1 week later.

Body temperature was measured using a lubricated and flexible rectal probe inserted 2.5 cm into the rectum and attached to a digital thermometer (0331 Panlab, Barcelona, Spain), 1 h after the last dose and 1 h after the first injection of METH or the first daily injection of MDMA. If rectal temperature rose above 41 °C, animals were kept on ice for 5 min. Body weight was registered 24 h after the last dose of METH or daily in the MDMA treatment.

*Tissue homogenate preparation* Immediately after sacrifice, the brains were removed rapidly from the skull and the striatum, the hippocampus and the parietal cortex quickly excised, dissected out, frozen on dry ice and stored at –80 °C until use.

The same tissue homogenate preparation was used for all assays: tissue samples were thawed and homogenised in 10 vol buffer: 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 homogeniser. The homogenates were centrifuged at 15,000 *g* for 30 min at 4 °C. The resultant pellets were washed twice and the final pellets were resuspended in TRIS-HCl 50 mM buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl and protease inhibitors and stored at –80 °C until use. Protein content was determined by the method of Bradford (*1976*).

*Binding of [3H]GBR 12935* The density of dopaminergic terminals was assessed by [$^3$H]GBR 12935 equilibrium binding assays in the striatum. As affinity ($K_d$) values do not

differ significantly in control and treated rats (Escubedo et al. *1998*), a single concentration of 2 nM [$^3$H]GBR 12935 (specific activity: 53.5 Ci/mmol) was used.

Briefly, after thawing, membranes from several animals (100 µg protein/tube) were incubated separately with the radioligand in a final volume of 1 ml in buffer (50 mM TRIS-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4), containing 0.01% bovine serum albumin for 45 min at 25 °C. Specific binding was defined as the difference between the radioactivity measured in the absence (total binding) and in the presence (non-specific binding) of bupropion (30 µM).

*Binding of [3H]paroxetine* Serotonin uptake sites were quantified by measuring the specific binding of 0.05 nM [$^3$H]paroxetine (specific activity: 21.5 Ci/mmol) after incubation with 150 µg protein at 25 °C for 1.5 h in a TRIS-HCl buffer (50 mM, pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 µM) was used to determine non-specific binding.

*Binding of [3H]PK 11195* This binding was used as an indirect marker of microglial activation, although the possibility that astrogliosis contributes to the increase in PBR density cannot be ruled out. The ligand binds to PBR, which are transporters for cholesterol located in the mitochondrial membrane. The density of PBR increases when glial cells undergo activation, hypertrophy and proliferation (Biegon et al. *2002*). Equilibrium binding assays were performed at 0–4 °C for 120 min, using [$^3$H]PK 11195 (specific activity: 85 Ci/mmol), in a final volume of 0.25 ml (TRIS-HCl buffer 50 mM, pH 7.4) including 2 nM [$^3$H]PK 11195 and 100 µg protein per tube. Ro 5-4864 (10 µM) was used to assess non-specific binding.

In all binding experiments, after incubation, samples were filtered under vacuum through GF-51 glass fibre filters (Schleicher and Schuell, Dassel, Germany) presoaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly 3 times with 4 ml ice-cold TRIS-HCl buffer and the radioactivity trapped in the filters measured by liquid scintillation spectroscopy. Results are expressed either as femtomol specifically bound radioligand per milligram of protein or as a percentage of specific binding, considering 100% to be that obtained in samples from animals treated with saline.

*Western blot analysis of HSP27* The induction of HSP27 was used as an index of astroglial activation. Samples were thawed, diluted 1:1 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) and boiled for 5 min at 95–100 °C in a dry bath (Techne Dri-Block). Samples (30 µg) were then separated by electrophoresis on 10% acrylamide gels. One lane per gel was loaded with HSP27 standard (StressGen Biotechnologies, Victoria, B.C., Canada) as an internal control. Thereafter, proteins were transferred to polyvinylidene fluoride (PVDF) sheets (ImmobilonTM-P, Millipore Ibérica, Madrid, Spain). PVDF membranes were incubated with a primary rabbit polyclonal antibody anti-HSP27 (1:5,000, StressGen) and with a peroxidase-conjugated anti-rabbit IgG antibody (Amersham). Immunoreactive protein was visualised using a enhanced

chemoluminescence-based detection kit following the manufacturer's protocol (ECL Kit; Amersham).

*Immunohistochemistry* Saline- and MDMA-treated animals were anaesthetised with pentobarbitone sodium (60 mg/kg) and perfused through the heart with heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (1 ml $g^{-1}$ of body weight). Brains were removed and postfixed for 2 h in the same solution, cryoprotected by immersion in 30% sucrose/PBS and then frozen in dry ice-cooled isopentane. Serial coronal sections (40 $\mu$m thick) through the whole brain were cut in a cryostat and collected in PBS. Free-floating sections were preincubated for 1 h at room temperature in $H_2O_2$ (0.3% in PBS, 10% methanol). After blocking with 10% normal serum and 0.2% bovine serum albumin, sections were rinsed and incubated using the following antibodies: OX-6 (1:1,000, Serotec, Kidlington, Oxon., UK), a mouse monoclonal antibody that recognises MHC class-II antigens and is used as a marker of reactive microglia/macrophages (Gehrmann et al. *1992*); a monoclonal antibody against GFAP (1:100), (Boehringer Ingelheim, Ingelheim, Germany) and a primary rabbit polyclonal antibody against the 27-kDa heat shock protein (1:400, StressGen) (Kato et al. *1994*; Plumier et al. *1996*). After incubation with the first antibody overnight and washing, sections were incubated with a biotinylated secondary antibody (1:150, ABC Kit, Vector Laboratories; Burlingame, Calif., USA) for 1 h at room temperature. Thereafter they were incubated with avidin-biotin complex and developed with 0.05% diaminobenzidine and 0.02% $H_2O_2$.

*Data analysis* Results are expressed as mean±SEM between five and eight animals. Multiple mean comparisons were made by one-way ANOVA, followed by Tukey's test. Differences between values were considered significant when $P<0.05$.

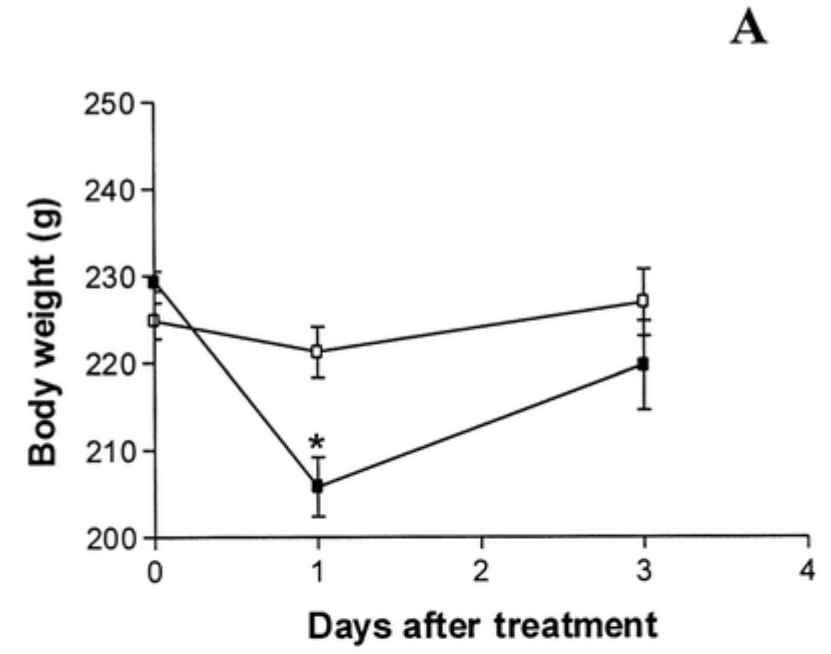
# Results

## Effect of METH and MDMA on animal body weight and temperature

Body weight at the beginning of the treatment is a key parameter influencing the lethality of amphetamine derivatives. At an ambient temperature of 26±2 °C, a body weight greater than 260 g (279.9±10.3 g) was associated with a lethality of about 30% in the METH-treated group and 55.5% in the MDMA-treated group. Lethality increased when ambient temperature rose above 28 °C. Body weight and the degree of hyperthermia and its maintenance were closely related. At an ambient temperature of 26±2 °C, a body weight <250 g was associated with a survival index of near 100% for METH- and 91% for MDMA-treated groups.

By 24 h after treatment with METH, body weight had decreased significantly (by 10.3%, from 229.4±1.2 to 205.8±6.7 g, $P<0.01$). By 72 h, when animals were killed, it had recovered partly (to 219.7±5.1 g) (Fig. 1). After the 1st day of treatment, MDMA-treated animals showed reduced body weight, but this did not differ significantly from body weight in saline-treated animals. The loss of body weight was significant 24 h after the 3rd day of treatment. This loss reached a maximum (9.4%) 24 h after the eighth dose

(from 256.1±5.1 to 232±6 g, $P<0.05$). The decrease in body weight of MDMA-treated rats was still significant 72 h post-treatment (240±6.3 g, $P<0.001$) but had disappeared by the time of sacrifice (321±6.1 g). In the METH studies, body weight in the saline-treated controls tended (not significant) to fall (1.6%) 24 h after the last dose, which can be attributed to the stress developed after receiving four subcutaneous injections. In contrast, for the MDMA studies, the saline-treated controls showed no decrease in body weight, probably because they were manipulated only twice a day (Fig. 1).



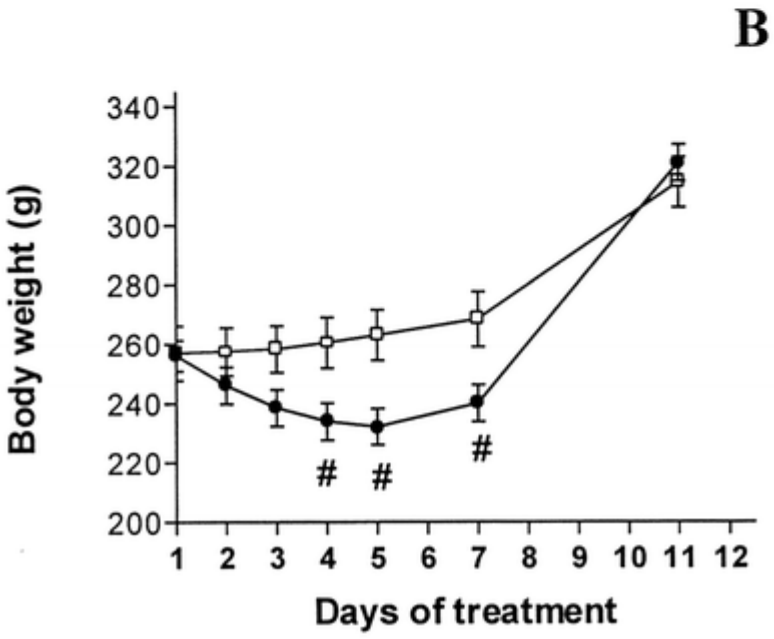



**Fig. 1.** Effect of methamphetamine (METH, *solid squares*, **A**) or 3,4-methylenedioxymethamphetamine (MDMA, *solid circles*, **B**) on rat body weight compared with saline (*open squares*). Means±SEM, $n$=6–8. *$P$<0.01, $^{\#}P$<0.05 vs. saline

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When ambient temperature was raised to 26 °C, MDMA and METH exerted a potent hyperthermic effect (Fig. 2), which was stronger for the first dose of MDMA than for the first dose of METH (40.4±0.2 °C vs. 38.6±0.2 °C, respectively, $P$<0.01). Subsequent doses of the two amphetamine derivatives had similar hyperthermic effects. The largest increases in body temperature were obtained 1 h after the first dose of MDMA and 1 h after the second dose of METH. When ambient temperature was maintained at 22 °C, no significant hyperthermic response (measured 1 h after the last dose) was obtained (37.1±0.2 °C for METH and 38.2±0.2 °C for MDMA, vs. 37.4±0.3 °C for the saline group)

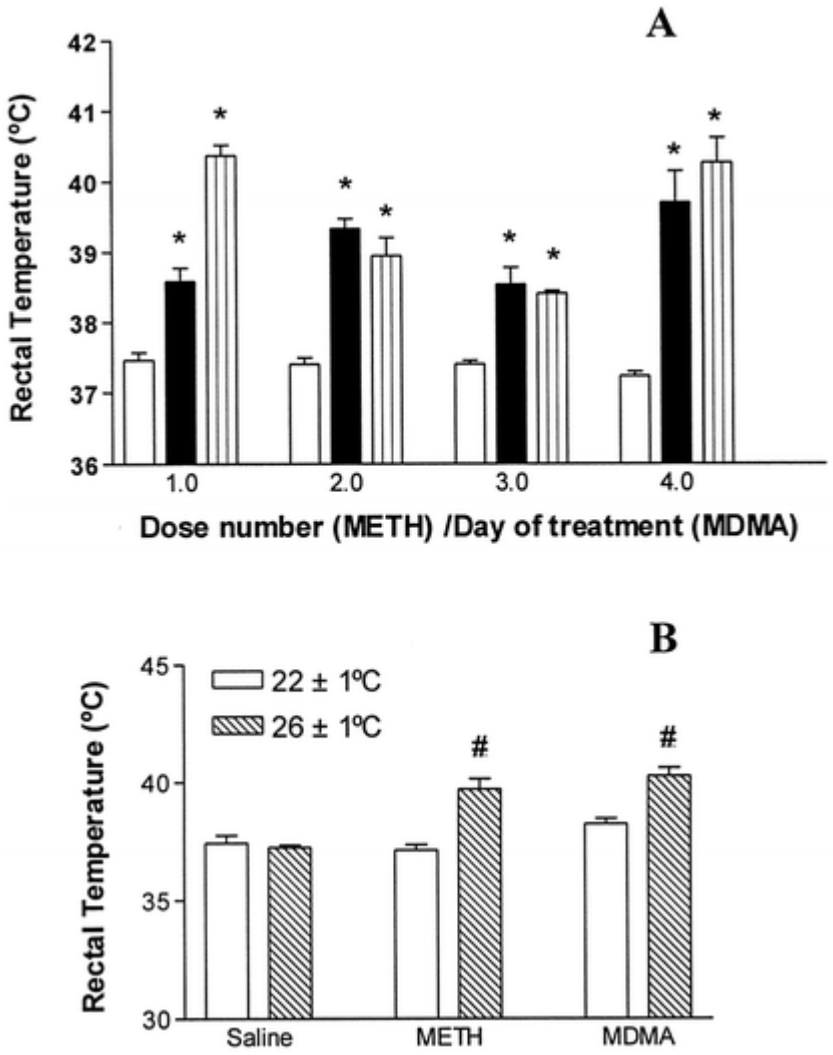


**Fig. 2A, B.** Response of body temperature in rats treated with METH or MDMA. **A** The hyperthermic effect, at an ambient temperature of 26±1 °C, induced by METH (*solid bars*, measured 1 h after each dose) or MDMA (*vertically hatched bars*, measured 1 h after the first daily dose). *$P$<0.001 vs. saline (*open bars*). **B** Dependency of the

hyperthermic effect induced by METH or MDMA (measured 1 h after the last dose of treatment), on ambient temperature. Means±SEM, $n$=6–8. $^{\#}P$<0.05 vs. 22±1 °C

## Assessment of dopaminergic and serotonergic uptake sites

METH strongly reduced (35%) the number of dopamine uptake sites (3,013±294 fmol $mg^{-1}$, $n$=5 vs. control 4,365±795 fmol $mg^{-1}$, $n$=5, $P$<0.05). We have shown previously that the decrease in dopamine transporter density peaks 72 h post-treatment and is maintained after 7 days post-treatment (Escubedo *1998*). Likewise, MDMA decreased the abundance of [$^3$H]GBR 12935 binding sites by about 40% (2,639±221 fmol $mg^{-1}$, $n$=8, $P$<0.01) 3 days after treatment (Fig. 3). Thus, the extent of dopamine terminal injury, 3 days after treatment, was similar for the two psychostimulants. However, while METH-induced dopaminergic toxicity is still present 7 days after treatment (Escubedo et al. *1998*) that of MDMA is reversed.

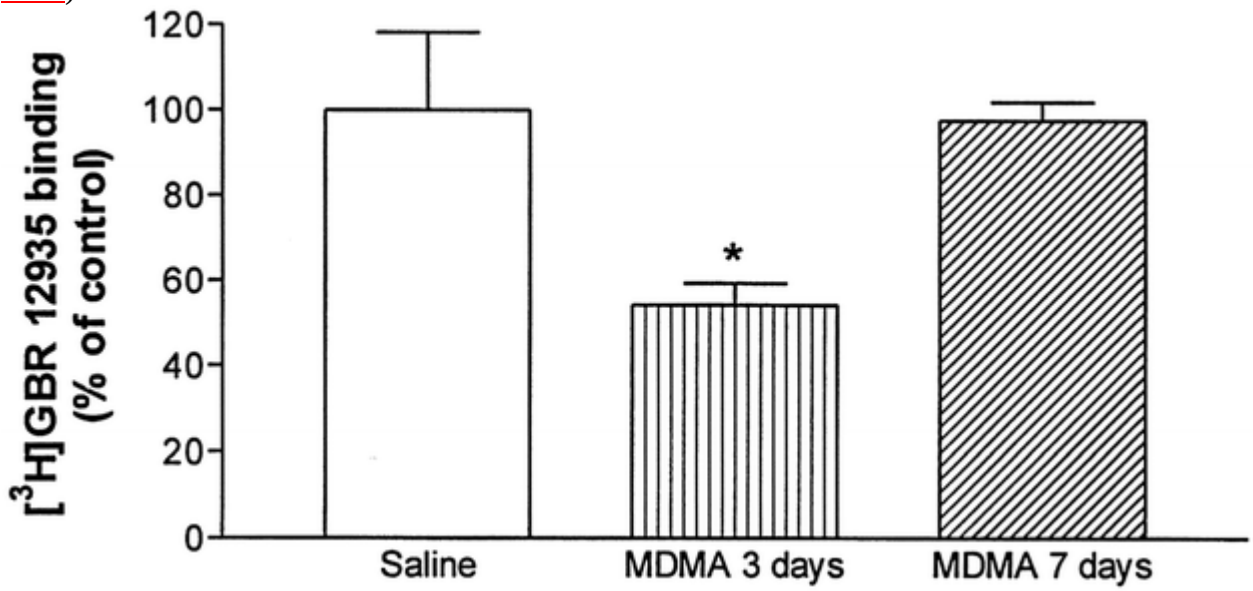


**Fig. 3.** Changes in [$^3$H]GBR 12935 binding site density in striatum induced by MDMA treatment 3 and 7 days after the last dose. Means±SEM, $n$=5–8. *$P$<0.05 vs. saline

[$^3$H]Paroxetine binding was measured in the cortex, hippocampus and striatum in METH-treated rats. [$^3$H]Paroxetine binding did not decrease significantly in the cortex (287±35 vs. 236±54 fmol $mg^{-1}$) or striatum (398±24 vs. 384±80 fmol $mg^{-1}$), whereas it was reduced by 33% in the hippocampus (488±46 vs. 328±66 fmol $mg^{-1}$, $P$<0.05) (control vs. METH, respectively). In contrast, MDMA-treated rats showed a significant ($P$<0.001) decrease in [$^3$H]paroxetine binding 3 days post-treatment in all the cerebral areas studied: from 261±9 to 107±12 fmol $mg^{-1}$ in the cortex; from 437±8 to 97±7 fmol $mg^{-1}$ in the striatum and from 449±32 to 120±19 fmol $mg^{-1}$ in the hippocampus; (control, $n$=6 vs. MDMA, $n$=8 group, respectively). The decrease in [$^3$H]paroxetine binding was still present 7 days post-treatment in the cortex (from 375±43 fmol $mg^{-1}$ to 132±19 fmol $mg^{-1}$). In the striatum and the hippocampus, although the decrease in [$^3$H]paroxetine binding was still significant 7 days post-treatment (from 352±61 fmol $mg^{-1}$ to 218±82 fmol $mg^{-1}$ in striatum, and from 455±37 to 277±67 fmol $mg^{-1}$ in hippocampus), the values were

significantly higher than those obtained after 3 days post-treatment ($P$<0.001 vs. binding reduction at 3 days, in both areas, Fig. 4).

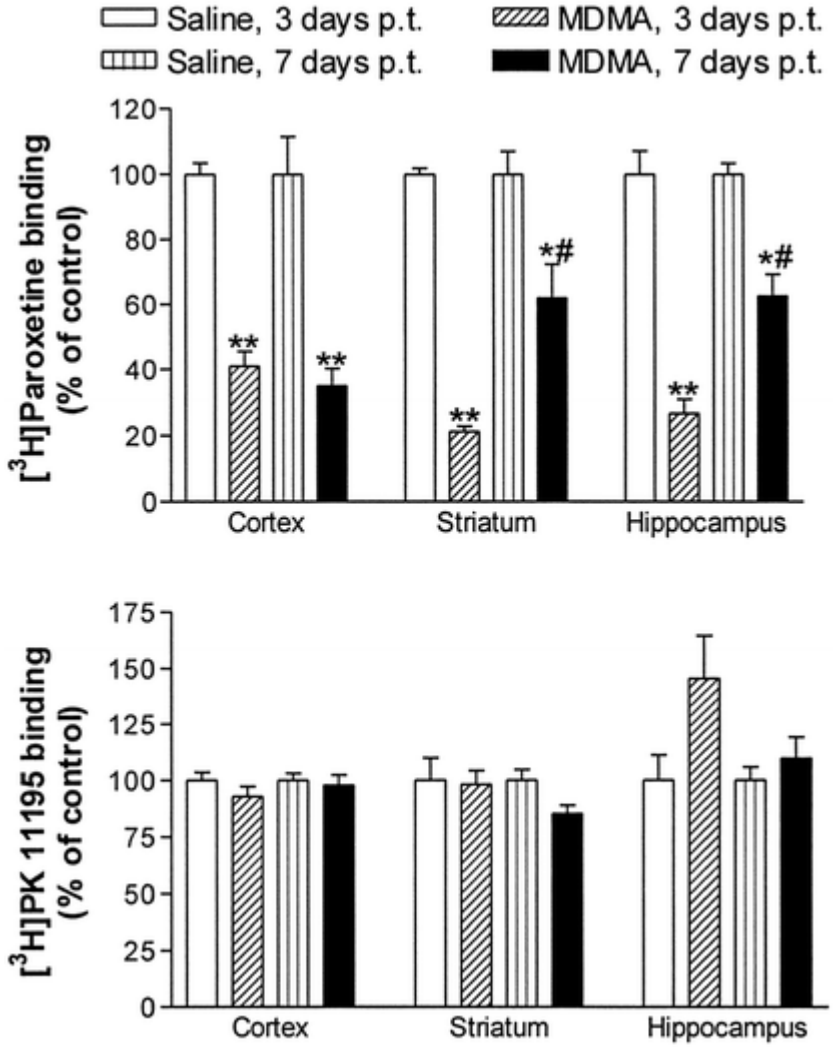


**Fig. 4.** *Upper panel*: decrease in [$^3$H]paroxetine binding sites in the cortex, striatum and hippocampus induced by MDMA 3 and 7 days post-treatment (*p.t.*). *Lower panel*: levels of [$^3$H]PK 11195 binding (indirect marker of microgliosis) in the same animals. Means±SEM, $n$=6–8 animals. *$P$<0.05, **$P$<0.001 vs. corresponding saline; $^{\#}P$<0.001 vs. 3 days post-MDMA

## Microglial activation

PBR density markedly increased in the striatum (39%, from 185±16 fmol mg$^{-1}$, $n$=5, to 258±12 fmol mg$^{-1}$, $n$=8) and cortex (32%, from 150±1 fmol mg$^{-1}$, $n$=4 to 199±8 fmol mg$^{-1}$, $n$=8) in METH-treated animals compared with saline-treated animals ($P$<0.01), indicating microglial activation. This increase in PBR density is no longer present 7 days post-treatment (Escubedo et al. *1998*). In the hippocampus, although there was a reduction in paroxetine binding, no significant differences in PBR levels were detected between the saline- and METH-treated groups.

In the striatum of MDMA-treated animals, there were no significant differences in PBR levels either 3 or 7 days post-treatment. Also, no significant differences in PBR density between saline- and MDMA-treated groups were detected 3 and 7 days post-treatment in the cortex. PBR density tended to increase in the hippocampus 3 days post-treatment (control 1055±123 fmol $mg^{-1}$, *n*=5, vs. MDMA 1534±199 fmol $mg^{-1}$, *n*=7, n.s.) and returned to normal values 7 days post-treatment (Fig. 4).

To assess the absence of microglial activation after treatment with MDMA, the induction of the specific antigen OX-6, which is not normally expressed by quiescent microglia, was determined by immunohistochemistry. OX-6 was not detected in either cortex or striatum 3 or 7 days post-treatment. In the hippocampus, some immunoreactivity was present 3 days post-treatment in the dentate gyrus but not in the CA1 region (Fig. 5). This result is in agreement with the tendency for PBR density to increase in this cerebral region 3 days post-treatment. No OX-6 expression was detected in hippocampus 7 days post-treatment.

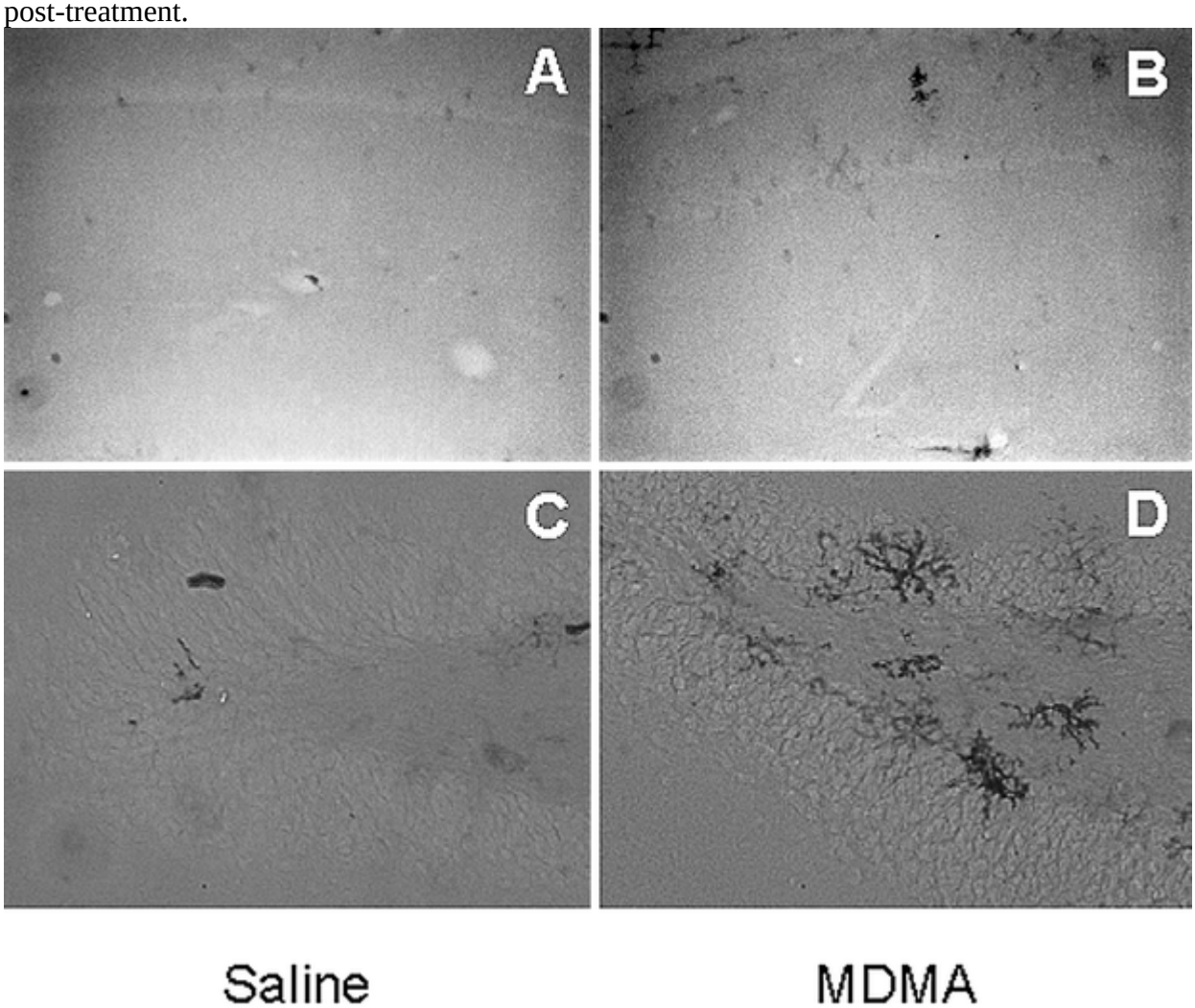


**Fig. 5A–D.** Immunohistochemical identification of OX-6 expression in the hippocampus. **A**, **B** CA1 region (×10); **C**, **D** dentate gyrus (×20) from animals treated with saline (**A**, **C**) or MDMA (**B**, **D**) and sacrificed 3 days after the last dose

## Astroglial activation

METH treatment induced the expression of HSP27 in striatum, cortex and hippocampus, whereas tissues from saline-treated animals did not express this protein. In the animals treated with MDMA, HSP27 expression was absent in all areas studied 3 and 7 days post-treatment. This lack of HSP 27 expression was corroborated by the absence of HSP27 immunoreactivity in brain slices (Fig. 6).

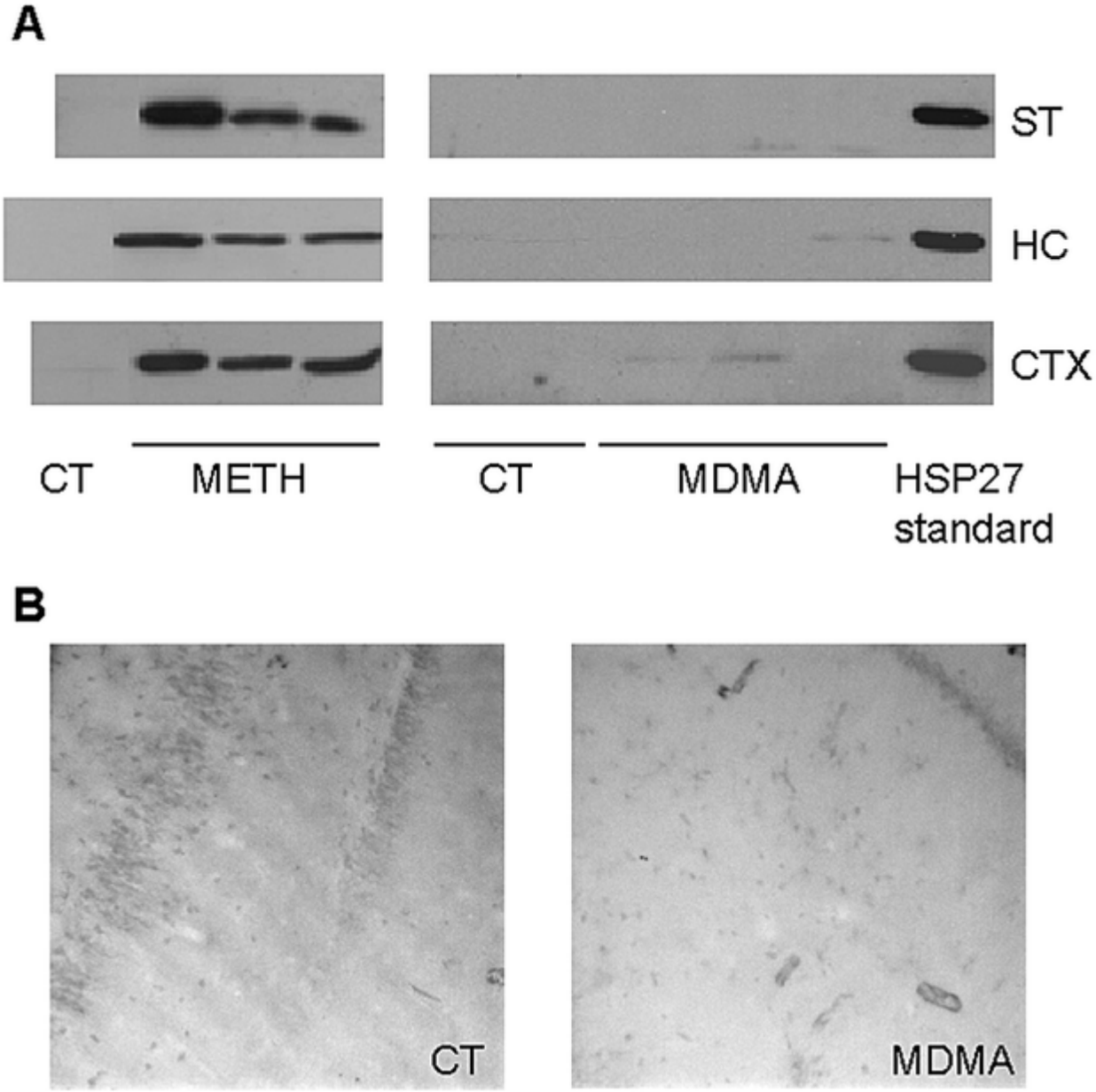


**Fig. 6A, B.** Expression of the 27-kDa heat shock protein (*HSP27*) in the brain. **A** Representative Western blots showing HSP27 in striatum (*ST*), hippocampus (*HC*) and cortex (*CTX*) of rats after METH or MDMA treatment. *CT*: samples from control animals. HSP27 standard was also loaded as an internal control. **B** Immunohistochemical localization (×10) of HSP27 in CA1 regions of the hippocampus from a control and a MDMA-treated rat

To confirm the lack of glial activation after MDMA treatment, immunohistochemistry studies were carried out with the glial-specific marker, GFAP. There were no signs of striatal or cortical astroglial activation in saline- or MDMA-treated animals, 3 or 7 days post-treatment. In the hippocampus, some astrocytes with the typical stellate morphology were observed in control animals, but no enhanced expression of GFAP was noticeable 3 days after MDMA treatment. In the MDMA-treated animals sacrificed 7 days after the last dose, a slight increase in GFAP immunoreactivity could be seen in the CA1 region but not in the dentate gyrus (Fig. 7).

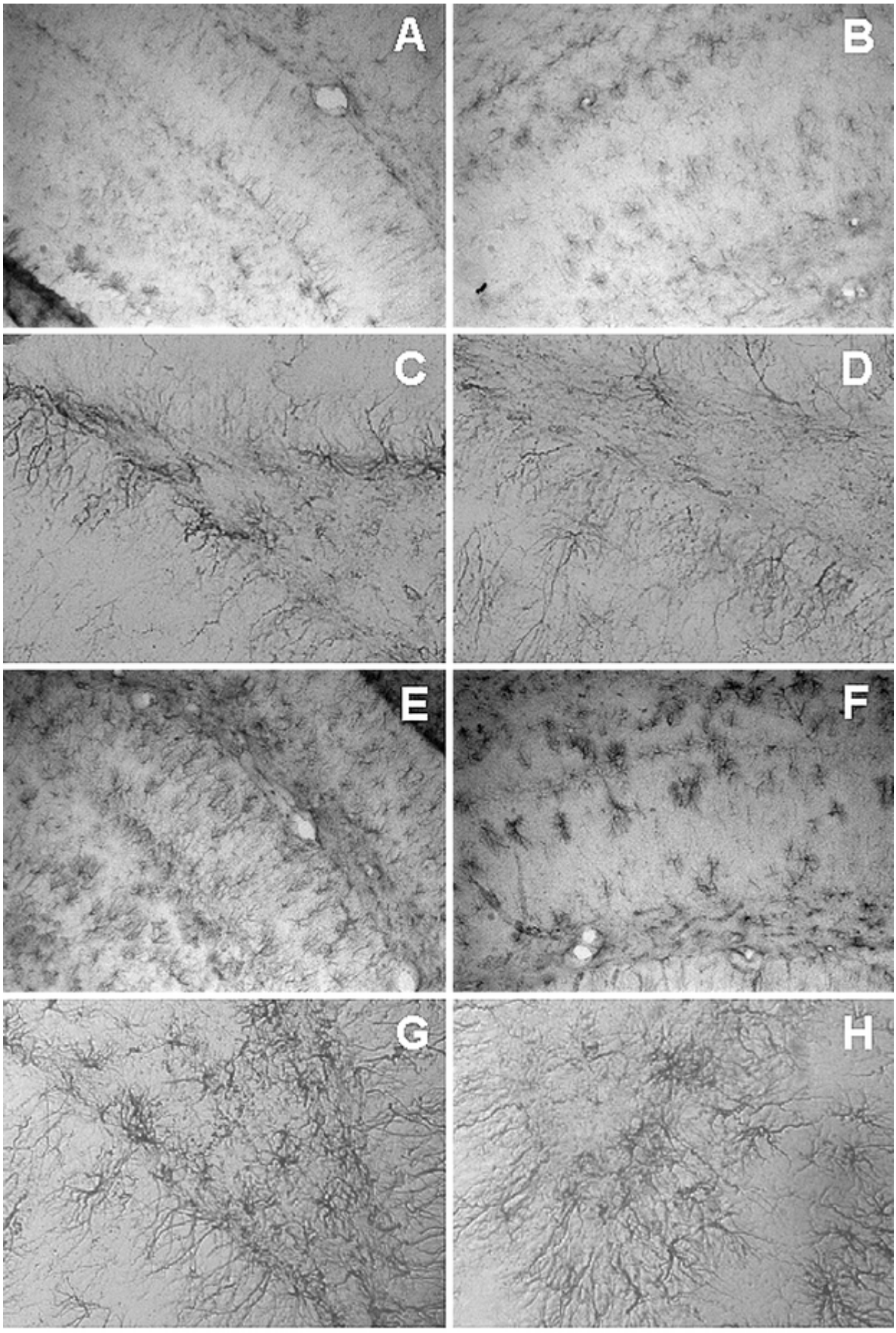
A
B
C
D
E
F
G
H
Saline
MDMA

**Fig. 7A–H.** Hippocampal expression of glial fibrilliary acidic protein (GFAP). Sections of the CA1 region (×10; **A**, **B**, **E, F**) and dentate gyrus (×20; **C**, **D**, **G, H**) from rats treated with saline (*left*) or MDMA (*right*). The animals were sacrificed 3 (**A–D**) or 7 days (**E**–**H**) after the last dose. **F** shows increased GFAP immunoreactivity of astrocytes from the CA1 layer in rats treated with MDMA

# Discussion

Centrally acting appetite suppressant drugs stimulate brain catecholamine and serotonin pathways in the hypothalamus. Since METH and MDMA exert distinct effects on dopaminergic and serotonergic systems, they may induce specific anorectic effects. The weight loss induced by amphetamine derivatives is largely due to reduced food intake and, to a lesser extent, to an increase in metabolism (Samanin and Garattini *1993*). Amphetamine exerts its anorectic action through a central adrenergic mechanism involved in the control of food intake, especially in the lateral hypothalamus (Blundell and Leshem *1973*). However, fenfluramine, an amphetamine derivative that has been used as anorectic in clinical trials, mediates its effect through serotonergic mechanisms in the lateral hypothalamus. As MDMA specifically affects serotonergic transmission in the CNS, its anorectic effect may be the same as that of fenfluramine. The maximal anorectic effect of both treatments was similar (10.3% vs. 9.4% loss of body weight) and especially evident 24 h after finishing the treatment. The progressive effect of MDMA on body weight is probably due to the cumulative effect of the longer treatment (4 days vs. 1 day for METH).

High doses of METH induce a thermo-dysregulatory effect in rats exposed to low or high ambient temperatures. It is well known that body temperature is involved in METH neurotoxicity. In particular, METH toxicity increases with ambient temperature (Ali et al. *1994*). Similarly, a neurotoxic dose of MDMA impairs thermoregulation when animals are exposed to high ambient temperatures (Mechan et al. *2001*), owing to impaired heat dissipation (Mechan et al. *2002*). These authors suggested a link between the MDMA-induced increase in dopamine release, $D_1$ receptor activity and hyperthermia in the hypothalamus. We observed a similar hyperthermic effect of METH and MDMA and a similar dependence on ambient temperature, which reflects the thermo-dysregulatory effect of both substances. In rats with higher body weights (>250 g), the hyperthermic effect was more evident, probably because heat dissipation is hindered (Mechan et al. *2002*).

The present study compared the neurotoxic effects of METH and MDMA. The extent of METH injury was determined 72 h after concluding a dosage regimen that mimics chronic administration. Using this schedule, subcutaneous administration of METH caused the degeneration of dopaminergic terminals in the striatum, reflected by the substantial decrease in the density of dopamine transporters. With regard to serotonergic toxicity, we only detected a decrease in the density of serotonin transporters in hippocampus after METH treatment. Simultaneously, METH significantly increased striatal and cortical PBR density, pointing to microgliosis 3 days post-treatment. This increase declines 4 days later (Escubedo et al. *1998*). No increases in PBR density were

detected in the hippocampus, the only brain area showing loss of serotonergic terminals. These results show that the increase in PBR density induced by METH is not a direct consequence of the neurotoxic effect of METH on serotonergic nerve terminals.

MDMA-induced neurotoxicity in rats is usually determined 7 days post-treatment (Battaglia et al.*1988*; Colado et al *2001*; Logan et al. *1988*; Ricaurte et al. *2000*). When neuronal injury is accompanied by reactive microgliosis, this glial response is maximal 2–3 days post-treatment and gradually decreases. Recovery can be achieved about 7 days post-treatment. To take this glial kinetic profile into account, the effects of MDMA in neuronal and glial cells were also determined 3 days post-treatment. Subcutaneous administration of MDMA, in a schedule imitating chronic administration, produced a dopaminergic lesion in the striatum 3 days after the last dose, quantitatively similar to that induced by METH but, in contrast to METH, it reversed 7 days post-treatment. Recently, Ricaurte et al. (*2002*) have described dopaminergic toxicity in non-human primates after treatment with MDMA. MDMA also had a potent and persistent neurotoxic effect on serotonergic terminals in the cortex. This neurotoxic effect is also present in the striatum and hippocampus, although it tends to recover 7 days post-treatment. Scanzello et al. (*1993*) have shown that serotonergic neurons in most (but not all) rats recover from MDMA injury 32 weeks after treatment.

Having assessed the neurotoxic effect induced by our MDMA dosage schedule, we then examined the effects related to a possible glial response. MDMA-treated rats did not show any glial reaction 3 or 7 days post-treatment. [$^{3}$H]PK 11195 binding remained unchanged in the striatum and cortex after MDMA treatment and only tended (non significant) to increase in the hippocampus at 3 days. Tissue samples from MDMA-treated animals showed no OX-6 expression (a specific indicator of microglial activation) in the striatum and cortex, and only a slight increase was noticeable in dentate gyrus at 3 days, which was not present 4 days later. The slight and non-homogeneous expression of OX-6 in the hippocampus explains the lack of significance of the increase obtained in PBR density.

Similarly, MDMA treatment did not induce astroglial activation in any of the studied areas, apart from increased GFAP immunoreactivity 7 days after treatment in the CA1 region of the hippocampus only. The CA1 region is considered to be the main termination field of the serotonergic innervation to the rat hippocampus (Steinbusch *1981*). The lack of HSP27 expression observed by Western blot was confirmed by immunohistochemistry in that HSP27 stained astrocytes were not detected. Thus, it can be concluded that there are differences between the brain responses to the neurotoxicity induced by METH and MDMA as far as glial activation is concerned.

If glial activation does not occur in MDMA-treated animals, the decrease in [$^{3}$H]paroxetine binding sites could be due to the internalisation of serotonin transporters or to their down-regulation, rather than to the destruction of serotonergic terminals. However, Ricaurte et al. (*2000*) reported that the abundance of type-2 vesicular monoamine transporters is reduced in MDMA-treated animals, further supporting the MDMA serotonergic neurotoxic effect. It can thus be concluded that in MDMA-treated

rats, although there is an important reduction of serotonin terminals, no marked changes in glial activity occur.

Astrocytes and microglia are thought to be the immune effector cells of the CNS, and to protect its integrity. Astrocytes stabilise and maintain homeostatic repair of tissues, control neurotoxins, regulate intercellular calcium signalling, and contribute to early wound repair (Eddlestone and Mucke *1993*). Under certain conditions, astrocytes are thought to participate in the removal of myelin and neuronal debris from injured areas. However, they may have pathological effects by interfering with the function of residual neuronal circuits, by preventing remyelination, or by inhibiting axonal regeneration. As the intrinsic neuronal support cells of the CNS, astrocytes may play a pivotal role in maintaining neuronal survival under pathological situations through the delivery of specific neurotrophic factors (Rudge *1993*). The most characteristic feature of microglia is their rapid activation in response to even minor pathological changes within the CNS (Kreutzberg *1996*). Although microglial cells have been implicated in lesion progression following various CNS injuries, their activation is a key factor in the defence of the brain parenchyma against infectious diseases, inflammation, trauma, ischaemia, tumours and neurodegeneration. Microglia are mainly scavenger cells but have also various functions related to tissue repair and neuronal regeneration (Xiao and Link *1998*).

In the CNS, the PBR are located primarily in glial cells and their density can increase after brain injury. Microglial proliferation or migration to the site of injury may contribute to an increased number of PBR-expressing cells. Usually, the microglial response precedes reactive astrocytosis and resolves much sooner after injury. In fact, in most cases reactive astrocytes cannot be detected until 2 or 3 days after neuronal insult (Kuhlmann and Guilarte *2000*) while activated microglia appears in the first hours. In the case of METH-induced neurotoxicity, we have reported that PBR levels peak 3 days post-treatment in the striatum (Escubedo et al. *1998*) and return to basal levels 4 days later. Thus, such a temporal evolution parallels that of the microglia. This indicates that, in this model of neurotoxicity, increased PBR would correlate with microgliosis. PBR primarily transport cholesterol from the outer to the inner mitochondrial membrane and may be increased in injured brain tissue to provide trophic support for either glial cell activation or neuronal recovery through the production of neurosteroids. They increase neuronal survival both in culture and following various forms of neuronal injury (Baulieu *1998*; Lacor et al. *1999*). Additionally, they may regulate astrocyte reactivity (Del Cerro et al. *1995*). In the peripheral nervous system, it has been suggested that PBR are involved in nerve regeneration (Lacor et al. *1999*) and that this mechanism may be linked to steroid biosynthesis (Papadopoulos et al. *1997*; Veenman and Gavish *2000*).

METH treatment induced the expression of HSP27 in striatum, cortex and hippocampus. In contrast, in tissues from MDMA treated animals, HSP27 expression was absent in all areas studied 3 and 7 days post-treatment. HSPs have been implicated in cellular resistance to injury. HSP27 has been shown to increase cell resistance to oxidative injury and thermal stress. Recently, Currie et al (*2000*) have demonstrated that brief and benign ischaemia (used for ischaemic preconditioning) is associated with very significant and prolonged astrogliosis as well as with a robust increase in the expression of HSP27 in

activated astrocytes 1–7 days after injury. This HSP27 expression is detected in occasional neurons among numerous astrocytes and probably participates in the neuroprotection/brain tolerance induced by focal preconditioning.

Glia appears to play a dual role, amplifying the effects of inflammation and maintaining neuronal survival. Without astroglial and microglial activation, the lesion by MDMA, especially in cortex and striatum, would probably not be accompanied by an inflammatory process but also would be deprived of the functions related to tissue repair and neuronal regeneration. The two possibilities can coexist and have different consequences depending on the affected brain area. In fact, the recovery of both areas from serotonergic lesion at 7 days post-treatment is different. Therefore, the differences in glial activation between METH and MDMA described in the present study may condition the lesion and prognosis of the injury induced by these psychostimulants.

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