Serotonergic impairment and memory deficits in adolescent rats after binge exposure of methylone

Raúl López-Arnau, José Martínez-Clemente, David Pubill, Elena Escubedo*, Jorge Camarasa

Department of Pharmacology and Therapeutic Chemistry (Pharmacology Section) and Institute of Biomedicine (IBUB). Faculty of Pharmacy. University of Barcelona. Spain.

(*) **Corresponding author:**

Elena Escubedo

Department of Pharmacology and Therapeutic Chemistry.

Faculty of Pharmacy. University of Barcelona.

Av. Joan XXIII s/n

08028 Barcelona. Spain

Tel: +34 934024530

Fax: +34 934035982

E-mail: eescubedo@ub.edu

Abstract

Methylone is a cathinone derivative that has recently emerged as a designer drug of abuse in Europe and the USA. Studies on the acute and long-term neurotoxicity of cathinones are starting to be conducted. We investigated the neurochemical/enzymatic changes indicative of neurotoxicity after methylone administration (4 x 20 mg/kg, s.c. a day with 3 h intervals) to adolescent rats, to model human recreational use. In addition, we studied the effect of methylone on spatial learning and memory using the Morris water maze (MWM) paradigm. Our experiments were carried out at a high ambient temperature to simulate the hot conditions found in dance clubs where the drug is consumed. We observed a hyperthermic response to methylone that reached a peak 30 min after each dose. We determined a serotonergic impairment in methylone-treated rats, especially in the frontal cortex, where it was accompanied by astrogliosis. Some serotonergic alterations were also present in the hippocampus and striatum. No significant neurotoxic effect on the dopaminergic system was identified. Methylone-treated animals only displayed impairments in the probe trial of the MWM, which concerns reference memory, while the spatial learning process seemed to be preserved.

Keywords: Methylone. Neurotoxicity. Striatum. Frontal cortex. Hippocampus. Rat

Introduction

Methylenedioxymethcathinone (methylone), together with other substances such as methylenedioxypyrovalerone and 4-methylmethcathinone (mephedrone), is a derivative of cathinone, a naturally occurring stimulant found in the leaves of the khat plant. These compounds have recently emerged as designer drugs of abuse in Europe and the USA (McElrath and O'Neill, 2011; Spiller et al., 2011; Vardakou et al., 2011), and are marketed under deceptively benign names, including the term "bath salts", and sold over the internet and in head shops worldwide (Davies et al., 2010). Synthetic cathinones are also phenylalkylamine derivatives often termed ''bk-amphetamines'' for the beta-ketone moiety. Methylone was first synthesized as an antidepressant, but it, emerged as a recreational drug in about 2004 under the trade name "Explosion" and was one of the first products of this type to be marketed on-line (Bossong et al., 2005). In April 2013, the Drug Enforcement Administration included it in Schedule I of the Controlled Substances Act (Drug Enforcement Administration, 2013).

Methylone has a strong structural and pharmacological similarity to 3,4,-methylenedioxymethamphetamine (MDMA). Studies on the pharmacological targets of cathinones have recently been published by our group (Martínez-Clemente et al., 2012) and by others (Hadlock et al., 2011; Kehr et al., 2011; Motbey et al., 2012), and have demonstrated that methylone acts on monoaminergic systems. This substance, like MDMA, stimulates the non-exocytic release of serotonin (5-HT) and, to a lesser degree, dopamine (DA) (Hadlock et al., 2011; Sogawa et al., 2011). Furthermore, it is a potent nonselective monoamine uptake inhibitor that blocks 5-HT and DA uptake competing with the substrate, and its effects partially persist after drug withdrawal (López-Arnau et al., 2012).

Methylone has been shown to increase spontaneous locomotor activity in mice and rats in a dose-dependent manner (Baumann et al., 2013; Kehr et al., 2011; Marusich et al., 2012; Motbey et al., 2012). This effect is prevented by ketanserin or haloperidol pre-treatment (López-Arnau et al., 2012).

The repeated administration of amphetamine derivatives such as methamphetamine and MDMA to rodents has also been found to cause

neurotoxicity (Halpin et al., 2014). Because cathinones are relatively new, research on their acute and long-term neurotoxicity of cathinones is starting to be conducted. A recent report (Blum et al., 2013) stated that cathinones ‘‘have been known to cause intensive cravings for the substances and users have been reported to go on multiday binges that often cause medical problems necessitating medical intervention’’.

Due to its similarity to MDMA, there is reason to suspect that methylone could also display a species-dependent neurotoxicity. MDMA is toxic to the 5-HT system in both rats and humans, whereas it affects the DA system in mice (Logan et al., 1988; Reneman et al., 2001). For this reason, the rat is generally considered to be a better rodent model for amphetamines than the mouse, although it is currently unclear whether rats are also a better model for methylone neurotoxicity, as the long-term behavioural and neurochemical effects of this drug have not yet been characterized in humans. Den Hollander et al. (2013) found that methylone had no effects on 5-HT levels in mice, but demonstrated a widespread depletion of 5-HT and 5-HT transporter (SERT) levels in rats two weeks after the last administration. By contrast, Baumann et al. (2012) found that methylone had no effects on monoamine levels in rats two weeks after treatment. These inconsistencies may be partially due to differences in the dosage-regimen and recovery period used. In a recent paper (López-Arnau et al., 2014), we described neuronal disturbances in methylone-treated mice depending on the drug schedule, but it is necessary to study whether these changes also occur when rats are treated using a similar regimen.

There is also ample evidence linking the use of amphetamines with decreased memory function and increased neuropsychiatric symptoms in animal models and humans (McCardle et al., 2004; McGregor et al., 2003; Moon et al., 2007; Parrott et al., 2000; Piper and Meyer, 2004; Volkow et al., 2001); this could be a consequence of neurotoxicity or robust neurochemical changes.

The aim of this study was to investigate the neurochemical changes indicative of neurotoxicity after methylone administration in rats and, to address some of the limitations found in the current literature. We administered

methylone to rats using a schedule intended to model human recreational use, and assessed the possible changes in the density of DA or 5-HT uptake sites and tyrosine hydroxylase (TH) and tryptophan hydroxylase-2 (TPH-2) expression. In addition, we studied whether a glial response took place as a result of cathinone-induced neuroinflammation (Blum et al., 2013). Since a close relationship between the hyperthermic response, which increases in a high ambient temperature, and the severity of the brain lesion induced by MDMA has been established (Sanchez et al., 2004), our experiments were carried out in a raised ambient temperature to simulate the hot conditions found in dance clubs, where the drug is consumed. Finally, we also assessed the effect of methylone on spatial learning and memory using the Morris water maze (MWM).

Materials and methods

Animals and treatment

Male Sprague-Dawley rats (125 – 175 g, aged 4-6 weeks) (Janvier, Lé Genest, France) were used. The animals were housed in a regulated temperature environment (21 ± 1 ºC; 12 h light/dark cycle, lights on at 08:00 h) with free access to food and water. With the exception of experiments registering body temperature, other experiments took place between 09:00 and 18:00 h. Experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia, and following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used. Animals were administered subcutaneously with four doses of saline (1 ml/kg) or methylone (20 mg/kg) a day with 3 h intervals. There is good evidence that, at least from the perspective of neurotoxicity of MDMA, the subcutaneous injection route yields to results similar to those observed after oral administration (Slikker et al., 1988; Finnegan et al., 1988). Therefore, for the sake of convenience, easy of drug delivery and safety we chose to dose the animals by s.c. injection. During the treatments, the animals remained one per cage in a room with a

controlled ambient temperature of 26 ± 1ºC until 1h after the last dose. A different set of animals was used for behavioural, neurochemical or immunohistochemistry experiments. No information about the subcutaneous doses in humans is available. In our case, the dose of 20 mg/kg in rats corresponds to a 3 mg/kg in a human. This rat equivalent dose was calculated following the body surface area normalization method (Reagan-Shaw et al., 2008). Moreover, the interval of 3 h between doses was chosen according to our previous published paper on methylone pharmacokinetics in rats (López-Arnau et al., 2013).

Drugs and reagents

Pure racemic methylone hydrochloride was synthetized and characterized by us as described previously (López-Arnau et al., 2012). Methylone solutions for injection were prepared in sterile 0.9% NaCl (saline) immediately before administration. Isoflurane was from Laboratorios Dr. Esteve (Barcelona, Spain). The other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]ketanserin, [^{3}H]paroxetine, and [^{3}H]WIN35428 were from Perkin Elmer (Boston, MA, USA). All buffer reagents were of analytical grade.

Surgical procedures and telemetric acquisition of body temperature

The animals (n=6-8/group) were allowed 1 week after arrival to acclimatize before surgery. Subsequently, they had implanted an electronic device (Thermo Tracker, I.D.C., Barcelona, Spain), enabling continuous measurement of core body temperature. The implant was placed in the abdominal cavity as follows: the rats were anesthetized with isoflurane, the abdomen was opened by making a 2-cm midline incision and the device was placed in the abdominal cavity, along the sagittal plane. The abdominal and the skin wound were then closed with absorbable suture material. After surgery, animals were individually housed, received analgesic therapy and were allowed to recover for 7 days before saline or methylone administration avoiding a possible influence of this type of manipulation.. The device registered the core temperature every 5 min and values were downloaded to a computer after removal of the device, once the animals had been sacrificed, using the interface and software provided by

the manufacturer. Data were acquired from 24 h prior to until 24 h after drug administration.

Morris water maze

Spatial learning and memory were assessed in a MWM one week after treatment. Male Sprague-Dawley rats (n=9-11/group) were trained in the water maze which consisted of a circular pool (160 cm in diameter and 45 cm high) that was filled with water (22 ± 1ºC) to a depth of 25 cm and rendered opaque by the addition of a non-toxic latex solution. The pool was in an isolated room and black curtains were closed around it to minimize static room cues. Four positions around the edge of the tank were designated as north (N), south (S), east (E), and west (W) and also defined the division of the tank into four quadrants: NE, SE, SW, and NW, providing alternative start positions A Plexiglas escape platform (11 cm diameter) was submerged to a depth of 1 cm from the water surface and was not visible at water level. The escape latency or total time needed by the rats to find the platform (in seconds) was measured using an appropriate computer software (Smart, Panlab SL, Barcelona, Spain) connected to a video camera placed over the pool that allowed to trace the tracking of each animal.

In the spatial learning (acquisition trials) task, four objects or landmarks were suspended from a false ceiling at 30 cm above the water surface in N, S, E and W positions. The platform was always in the NW quadrant. Rats received a training session, consisting of five trials per day, by using a semi-random set of start locations that were not equidistant from the goal, thus creating short and long paths to the platform. Moreover, they were designed so that the animal was not able to learn a specific order of right or left turns to locate the platform. Animals were tested on five consecutive days (a total of 25 trials per animal were performed to reach asymptotic performance).

A trial was started by placing a rat in the desired start position in the maze, facing the tank wall. Rats were allowed to swim to the hidden platform, and the escape latency was measured. If an animal did not escape within 120 s, it was gently placed on the platform or guided to it. Rats were allowed to rest for 30 s (inter-trial interval) on the platform (even those that failed to locate it). This

procedure was repeated with each animal over the trials (Vorhees and Williams, 2006).

To assess reference memory at the end of learning, a probe trial (free swimming without platform for 60 s), was carried out 24 h after the last training session. We used a novel start position during the probe trial to ensure that its spatial preference is a reflection of the memory of the goal location rather than for a specific swim path. We used as start position the opposite quadrant (SE) to the original platform position. Different parameters of the rat's performance were analysed: The total time spent swimming and the number of entries in the target (where the platform should be located) and the opposite quadrants.

Tissue sample preparation

Crude membrane preparation was prepared as described (Escubedo et al., 2005) with minor modifications. Animals (n=5-8/group) were killed by decapitation under isoflurane anaesthesia at 7 days after treatment, and the brains were rapidly removed from the skull. Hippocampus, striatum and frontal cortex were quickly dissected out, frozen on dry ice, and stored at -80°C until use. When required, tissue samples were thawed and homogenized at 4°C in 20 volumes of buffer (5 mM Tris-HCl, 320 mM sucrose) with protease inhibitors (aprotinin 4.5 µg/µl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4 using a Polytron homogenizer. The homogenates were centrifuged at 1,000 x *g* for 15 min at 4°C. Aliquots of the resulting supernatants were stored at -80ºC until use for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000 x *g* for 30 min at 4°C. The pellets were resuspended in buffer and incubated at 37ºC for 5 min to remove endogenous neurotransmitters. The protein samples were recentrifuged. The final pellets (crude membrane preparations) 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, according to the manufacturer's instructions.

DA and 5-HT transporter density

The density of the DA transporter (DAT) in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding assays, as a marker of dopaminergic nerve terminals. Assays were performed in glass tubes containing 250 or 500 µl of [^{3}H]WIN35428 diluted in phosphate-buffered 0.32 M sucrose (final radioligand concentration, 5 nM) and 50 or 100 µg of membranes, respectively. Incubation was done for 2 h at 4°C and non-specific binding was determined in the presence of 30 µM bupropion.

The density of SERT, a marker of serotonergic nerve terminal, was assessed in membranes from hippocampus, striatum and frontal cortex by measuring the specific binding of 0.1 nM [^{3}H]paroxetine after incubation with 150 µg of protein at 20-22ºC for 2 h in a Tris-HCl buffer (50 mM, pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration under vacuum through Whatman GF/B glass fibre filters, previously pre-treated with 0.05% polyethylenimine. Tubes and filters were washed rapidly three times with 4 ml of ice-cold buffer, and the radioactivity in the filters was measured by liquid scintillation spectrometry.

[^{3}H]-paroxetine saturation binding assay

This assay was performed to assess whether the decrease in [^{3}H]paroxetine binding is due to a change in affinity or a decrease in SERT density. Saturation binding assay was measured as described by Hewitt and Green (1994) with some modifications. Frontal cortex membrane preparation was prepared as described above. The density of SERT in frontal cortex membranes was quantified by measuring the specific binding of [^{3}H]paroxetine (0.01- 3 nM) after incubation with 150 µg of protein at 20-22ºC for 2 h in a Tris-HCl buffer (50 mM, pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 2 ml. 5-HT (100 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration as described for [^{3}H]paroxetine and [^{3}H]WIN35428 binding assays.

Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine the expression of TH and TPH-2 in animals exposed to the treatment. For each sample, 15 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), boiled for 5 min, and loaded onto a 10% acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF membranes were blocked overnight with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated for 2 h at room temperature with a primary mouse monoclonal antibody against TH (dil. 1:5000) (Transduction Labs, Lexington, KY, USA) or a primary rabbit polyclonal antibody TPH-2 (dil. 1:1000) (Millipore, Billerica, MA, USA).

After washing, membranes were incubated with a peroxidase-conjugated antimouse IgG antibody (dil. 1:2500) or a peroxidase-conjugated antirabbit IgG antibody (dil. 1:5000) (GE Healthcare, Buckinghamshire, UK). Immunoreactive protein was visualized using a chemoluminescence-based detection kit following the manufacturer's protocol (Immobilon Western, Millipore, USA) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Hercules, CA, USA). Scanned blots were analysed using BioRad Image Lab software and dot densities were expressed as a percentage of those taken from the control. Immunodetection of beta-actin (dil.1:2500) (mouse monoclonal antibody, Sigma-Aldrich) served as a control of load uniformity for each lane and was used to normalize differences in the corresponding enzyme expression due to protein content.

5-HT_{2A} receptor density

The density of 5-HT_{2A} receptors was measured in frontal cortex membranes in rat one week after their exposure to the treatment, using [^{3}H]ketanserin binding. Assays were performed in glass tubes containing 1 nM [^{3}H]ketanserin and 100 µg of membranes. Incubation was carried out at 37°C for 30 min in a Tris–HCl buffer to a final volume of 0.5 ml. Methysergide (10 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration under vacuum, washed rapidly and the radioactivity was measured by liquid scintillation spectrometry as described previously.

Iba-1 and GFAP immunohistochemistry

Animals (n=3/group) used in ionized calcium binding adaptor molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) immunohistochemistry were anaesthetized with pentobarbital sodium (60 mg/kg) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (1ml/g of body weight) 48 hours and seven days after treatment, respectively. Brains were removed and postfixed for 2 h in the same solution, cryoprotected by immersion in 30% sucrose/phosphate buffer solution for 24 h and frozen in dry ice-cooled isopentane. Serial coronal sections (30 µm thick) through the whole brain were incubated for 15 min at room temperature in H_2O_2 (0.3% in phosphate buffer with 10% methanol). Thereafter, sections were incubated in a blocking solution (1% of fetal bovine serum, and 0.2 M glycine plus 0.5% Triton X-100 in phosphate buffer). After blocking with 10% normal serum and 0.2% bovine serum albumin, sections were rinsed and incubated overnight at 4°C using a polyclonal antibody against Iba1 (dil. 1:1000) (Wako, USA) or monoclonal antibody against GFAP (dil. 1:1000) (Dako, Denmark). Following this, the sections were washed and incubated with a biotinylated secondary antibody (dil. 1:200) for 2 h at room temperature. Afterwards they were incubated with avidin-biotin-peroxidase complex (dil. 1:200; Vector, Burlingame, CA). Peroxidase reaction was developed with 0.05% diaminobenzidine in 0.1 M phosphate buffer and 0.02% H_2O_2, and immunoreacted sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61) and quantified using Image J software.

Data analysis

Results are given as the mean ± S.E.M. (standard error of the mean). One-way or two-way (repeated measures) ANOVA, followed by Tukey's post-hoc tests were used. Saturation-binding curves were fitted by nonlinear regression using GraphPAD Prism (GraphPAD software, San Diego, CA, USA); *p* values less than 0.05 were considered significant.

Results

Lethality

Adolescent rats were used in all experiments. Under these conditions, the number of fatalities in methylone-treated rats was 16%. Deaths were mostly produced after the fourth dose but never after the first or second dose. It is important to note that all methylone treatments induced the appearance of head-dipping stereotypy and salivation.

Effect of methylone on body weight and core temperature

Like amphetamines and MDMA, methylone produced a significant loss in body weight. In the saline group, weight gain was found to be 3.2 ± 0.9%, while the weight of methylone-treated rats decreased by 4.0 ± 1.5% (p<0.001) 24 hours after treatment.

We used an electronic implant that recorded core temperature every 5 min. Through this system we observed a robust hyperthermic response that reached a peak between 25 and 35 min after each drug administration. Analysis of the average core temperature demonstrated a significant effect of treatment (p<0.001). When the curve profile analysis was carried out, it showed four significant peaks corresponding to the four maximal increases after drug administration (Figure 1).

Analysis of the core body temperature demonstrated an overall effect of treatment ($F_{1,36}$ = 20.14; p<0.001) and treatment x dose ($F_{3,36}$ = 6.84; p<0.001). Saline-treated rats exhibited a non-significant (p=0.074) increase in temperature after each injection, probably due to stress, and this decreased slightly in the two last administrations. Conversely, the effect of methylone increased significantly with the dose, so that the last dose induced a higher increase in body temperature than the first one (p<0.05) (Table 1).

Effect of methylone on different in vivo markers of DA and 5-HT terminals

Methylone treatment did not affect the specific binding of [^{3}H]WIN35428 either in the striatum (saline: 100.00 ± 15.83%; methylone: 105.67 ± 6.35%, n.s.) or in the frontal cortex (saline: 100.00 ± 15.72%; methylone: 81.79 ± 11.20%, n.s.) of animals killed seven days after drug administration. Also, although TH expression decreased, it showed no significant differences between the

treatment groups in any of the areas studied (striatum: saline: 100.00 ± 3.35%; methylone: 90.62 ± 4.83%, n.s.; frontal cortex: saline: 100.00 ± 19.69%; methylone: 69.09 ± 16.85%, n.s.).

In terms of 5-HT markers, methylone induced a decrease in 5-HT reuptake sites assessed by the specific binding of [^{3}H]paroxetine: 13 % in the striatum, 24 % in the hippocampus and 48 % in the frontal cortex (Figures 2A, 2C and 2E). In the same animals, TPH-2-immunoreactivity levels, also a marker of serotonergic terminal integrity, decreased significantly in the three areas of the methylone-treated rats, compared with saline-injected animals (Figures 2B, 2D and 2F). The decrease in these parameters ran in parallel, except in the frontal cortex, where the SERT reduction was higher than that of TPH-2.

Effect of methylone treatment on the saturation binding isotherm of [^{3}H]paroxetine in the frontal cortex

To assess the nature of the reduction in [^{3}H]paroxetine binding sites, saturation binding experiments were performed. Figure 3 shows a representative saturation binding isotherm of [^{3}H]paroxetine in the frontal cortex of saline- and methylone-treated rats. In both groups, a non-linear regression analysis of specific binding revealed a single saturable site (r^2 = 0.99). Methylone administration produced no changes in the dissociation constant (K_D). However, a reduction in the maximal number of binding sites (B_{max}) was evidenced seven days after treatment.

Effect of methylone treatment on 5-HT$_{2A}$ receptor density

Based on the reduction in serotonergic parameters, we determined the density of 5-HT$_{2A}$ receptors in the hippocampus and frontal cortex by measuring the specific binding of [^{3}H]ketanserin. None of the areas studied showed a significant change in this parameter (hippocampus: saline 100.00 ± 16.05%; methylone: 83.33 ± 6.30%, n.s.; frontal cortex: saline: 100.00 ± 5.11%; methylone: 112.00 ± 3.63%, n.s.).

Effect of methylone on microglial and astroglial activation

Because methylone exposure induces disturbances in 5-HT terminal markers, the next experiment was carried out to assess the presence of microglial and astroglial activation. In previous studies we have characterized the glial response to amphetamine derivatives (Pubill et al., 2003) and no microglial activation was noticeable at either 3 or 7 days after treatment. On the contrary, astrogliosis was evidenced at 7 days after treatment. To this end, immunohistochemistry studies were performed with the microglial- and glial-specific markers Iba-1 and GFAP on the brains from animals killed two or seven days after treatment, respectively. There were no signs of striatal, hippocampal or cortical microgliosis in methylone-treated animals (data not shown).

However, we found a significant increase (65 %) in this marker in the frontal cortex of methylone-treated rats when assaying GFAP (Figure 4). By contrast, no significant differences were found in the striatum or any subregion of the hippocampus (dentate gyrus, CA1 or CA3) in the GFAP-immunohistochemistry study (data not shown).

Effect of methylone on performance in the Morris water maze

We investigated the effect of treatment on spatial learning and reference memory seven days after the end of the binge exposure to saline or methylone using the MWM paradigm. An analysis of the mean swimming speed in the overall maze presented no differences between groups (saline: 22.43 ± 1.04 cm/s; methylone: 22.67 ± 1.24 cm/s), so the latency time to reach the hidden platform was used as the variable to measure performance.

Overall, both saline- and methylone-treated rats demonstrated a good ability to learn the task. In the acquisition phase, a repeated measures ANOVA showed a significant effect of the variable training days ($F_{4,72}$ = 12.29, p<0.001). There was no effect of the treatment ($F_{1,18}$ = 0.54, n.s.) or treatment x training days ($F_{4,72}$ = 0.68, n.s.). These results indicate that the escape latency diminished as the trials progressed (from day 1 to day 5) and that the animals learned the platform location (Figure 5), but the treatment did not affect the rats' ability to learn such a location.

A probe test in which the escape platform was removed from the pool was performed on day 6. Two-way ANOVA (repeated measures) analysis of time

spent in the target or the opposite quadrant showed a significant effect of the quadrant ($F_{1,18}$ = 4.42, p<0.05) and the interaction quadrant x treatment ($F_{(1,18)}$ = 8.29, P<0.01). This allowed us to conclude that the treatment only has an effect in the target quadrant. Post-hoc comparisons showed that the saline group displayed better reference memory, as the animals in this group swam in the target quadrant for 37.88 ± 3.68% of total time while methylone-treated rats did so for 22.96 ± 2.82% of the time (Figure 6A) (p<0.01 vs. saline).

There was a similar behavioural profile of memory performance when we analysed the number of entries in the target and the opposite quadrant ($F_{1,18}$ = 4.50, p<0.05). Pot hoc comparisons revealed significantly fewer entries in the target quadrant in the methylone group (p<0.05) (Figure 6B).

Discussion

Excessive consumption of cathinones may lead to toxicity with severe neurologic and peripheral symptoms, including death. There is not any study devoted to the mortality rate induced by methylone in humans. However, some reports identified the relationship between methylone and sudden death associated with an elevated body temperature and seizure activity (Carbone et al., 2013; Cawrse et al., 2012; Pearson et al., 2012). It is very difficult to know the correlation between the amount of methylone detected post-mortem in blood and the ingested doses. So, there is insufficient forensic evidence to determine how, how many and when methylone has been consumed and the corresponding amount found post-mortem. It is therefore important to study these effects in depth in order to provide society with evidence-based information on the risks involved.

Some studies reveal that methylone is non-selectively taken up by monoamine transporters leading to competitive monoamine uptake inhibition and stimulates the non-exocytic release of 5-HT and, to a lesser extent, DA, resulting in elevated synaptic neurotransmitter levels (Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013; Sogawa et al., 2011). This is a critical point, since only uptake inhibitors that are transporter substrates (as amphetamines), but not direct transport blockers (as cocaine) cause long-term deficits in monoamine cells (Fleckenstein et al., 2007). In this sense, the

potential for this cathinone to produce neurotoxic effects in various brain regions requires intensive investigation.

In the present study, we found that the neuronal disturbances induced by methylone in rats are closely related to those found in mice (López-Arnau et al., 2014), using a similar drug schedule.

To model human recreational methylone use, we simulated the widespread practices of “stacking” and “boosting”. Thus, we chose to administer four doses/day of methylone during each treatment period. It is important to note that, in contrast to previous published papers (den Hollander et al., 2013), we performed our experiments in adolescent rats, to establish a relationship with human abuse.

Rats receiving methylone showed an incidence of death around 16%. This mortality rate was similar or lower to that reported in MDMA neurotoxicity studies (Ho et al., 2004; Itzhak et al., 2003; Mueller and Korey, 1998). Furthermore, there is only a single description of methylone-induced lethality in rats that reached 100% at 60mg/kg (den Hollander et al., 2013).

It is known that hyperthermia plays a key role in MDMA-induced neurotoxicity (Sanchez et al., 2004). For this reason, we performed the experiments at an ambient temperature of 26 ± 1ºC to simulate the hot conditions found in dance clubs and under these conditions, methylone induced hyperthermia.

In our study we used electronic implants, which allowed us to measure body temperature more accurately over time. This revealed a hyperthermic response to methylone that peaked 30 min after administration.

Using our drug schedule, methylone caused significant changes in the specific marker of 5-HT terminals (SERT density) in the three brain areas studied, to a higher extent in the frontal cortex. In the same areas, methylone significantly decreased TPH-2 expression. With regard to DA terminal markers, we only detected a slight decrease in DA transporter density and TH expression in the frontal cortex, but no change was found in the striatum. We have reported that the increase in locomotor activity induced by methylone is directly correlated with blockade of dopamine uptake and with a non exocytotic, transporter-mediated, dopamine (López-Arnau et al., 2012). In the present work

we do not see a neurotoxic injury in dopamine nerve terminals leading to an integrity of these neuronal pathway.

Previous to the present study, den Hollander et al. (2013) studied the neurotoxicity of methylone in rats using a schedule of 30 mg/kg twice daily for four days, and reported a decrease in SERT markers in the frontal cortex and hippocampus, as well as a profound decrease in 5-HT levels in these brain areas plus the striatum. However, Baumann et al. (2012) examined monoamine levels two weeks treatment and reported that there were no changes. This contrasts with the results of our experiment, but this might be explained by the fact that the doses used in that study were lower than in ours, and these authors did not observe a pronounced hyperthermic response, as occurs with MDMA. We can conclude from these comparisons that hyperthermia, together with dosing (both variable probably related), plays a crucial role in methylone's impairment of 5-HT.

There is substantial evidence that MDMA doses that produce substantial, long-term reductions in rat brain 5-HT and other serotonergic markers do not reliably provoke astroglial (Biezonski and Meyer, 2011) or microglial responses (Herndon et al., 2014; Pubill et al., 2003), and thus it is not clear whether such reductions truly reflect structural damage to the serotonergic system. We did not detect any significant microglial responses in any of the brain regions processed. Nevertheless, methylone induced significant astroglial activation in the frontal cortex, the area where changes in the biochemical parameters of the serotonergic system are more apparent.

Whether these biochemical changes reflect an actual serotonergic injury requires further discussion. SERT depletion may occur in the absence of axotomy. In fact, SERT can be regulated by endocytic translocation (Carneiro and Blakely, 2006). In early studies we corroborated that the membrane preparation used also contains the transporter internalized by endocytosis, despite the fact that the loss of a small amount of this fraction cannot be completely ruled out. Consequently, the decrease in binding density is not the result of transporter redistribution. Moreover, the parallel decrease in TPH-2, a cytosolic serotoninergic terminal marker, leads us to suggest that a real terminal injury exists.

Furthermore, the actual number of transporters could be higher than the apparent score because of a decrease in the affinity for [^{3}H]paroxetine induced by the interaction of methylone with SERT. In fact we previously found that the inhibition of 5-HT uptake by methylone can persist after drug withdrawal (López-Arnau et al., 2012). To clarify this point we carried out the saturation binding experiments with [^{3}H]paroxetine in samples from methylone- and saline-treated animals. This study revealed that the affinity for the radioligand remains unchanged, whereas the B_{max} value reduced significantly in the methylone group. This allowed us to conclude that the decrease in [^{3}H]paroxetine binding is not due to a decrease in affinity, but to a decrease in SERT density.

Moreover, other authors (Cuyas et al., 2014; Kirilly et al., 2008) have suggested that brain SERT gene expression may be negatively regulated seven days after MDMA exposure, which could lead to a reduction in paroxetine binding in the absence of physical damage. Conversely, they found an increase in the expression levels of the TPH-2 gene (Cuyas et al., 2014). Both regulations are considered a compensatory mechanism in order to overcome the decrease in 5-HT induced by MDMA treatment. In this study we have shown that both the density of SERT and the expression of TPH-2 are reduced in all of the brain areas studied. Thus, the fact that TPH-2 decreases precludes a regulatory response to 5-HT reduction and reflects damage to serotonergic terminals.

As a result of an initial massive release of 5-HT followed by a damage-induced serotonergic depletion (Simmler et al. 2013), the compensatory regulation of post-synaptic receptor could be expected. For this reason, we assessed the 5-HT_{2A} receptor density. Although a slight increase was observed in the frontal cortex, non-significant changes were ascertained, which ruled out a compensatory mechanism seven days after drug exposure.

In the MWM, methylone-treated rats do not show spatial learning deficits one week after binge exposure. However, when a single probe trial was conducted 24 h after the acquisition phase to assess reference memory, the methylone group displayed a poorer performance, which would suggest that this drug affects this memory type.

Den Hollander et al. (2013observed an improved performance in methylone-treated mice during a reversal probe trial eight weeks after treatment. The authors explained this result by suggesting that the drug-treated mice forgot the former location of the platform more quickly, so they had no preference for either quadrant. This assertion is consistent with our results in the probe trial with methylone-treated rats.

MWM performance appears to depend on the coordinated action of different brain regions and neurotransmitter systems (D'Hooge and De Deyn, 2001). These systems do not support learning and memory in a task-dependent manner, but some predominate over others in a specific task. Glutamate, GABA and DA seem to be the most critical in MWM performance (Myhrer, 2003), while reducing 5-HT synthesis with p-chlorophenylalanine has no effect on such performance unless combined with a treatment that reduce cholinergic transmission (Richter-Levin and Segal, 1989).

We found serotonergic impairment in methylone-treated rats, especially in the frontal cortex, where it was accompanied by astrogliosis. Some serotonergic alterations were also present in the hippocampus and striatum. However, these animals only displayed impairments in the probe trial of the MWM. Further investigation is needed to determine the effects of binge administration of methylone in rats on different memory tasks, but the present results indicate that learning processes are unaffected, although there are some memory deficits.

In conclusion, the present results demonstrate that, in contrast to the species-dependent neurotoxicity of MDMA, methylone-induced brain alterations in rats do not qualitatively differ from those observed in mice using a similar drug schedule. Methylone elicited neurochemical changes when it was administered in an elevated ambient temperature, four times a day at 3 h intervals. These include loss of 5-HT neuronal markers in the frontal cortex, hippocampus and striatum, as well as cortical astrogliosis, which would indicate the presence of injuries in nerve endings in this area. However, other additional mechanisms, such as the regulation of SERT gene expression or transporter recycling cannot be completely ruled out. No significant effect on the dopaminergic system was observed. While these changes occur, the animals

perform well during a spatial learning task, but show memory impairment in the probe trial.

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Conflicts of interest

None declared

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Legends for figures

Figure 1. Effect of methylone on core body temperature. Rats (n=6-8 per group) were treated with saline (—) (1ml/kg, s.c.) or methylone (---) (4 x 20 mg/kg, s.c., 3 h intervals) and core body temperature was recorded using an electronic implant at 5 min-intervals for 12 h. Results are presented as group means. SEM are omitted for the sake of clarity. Injections times are indicated by arrows.

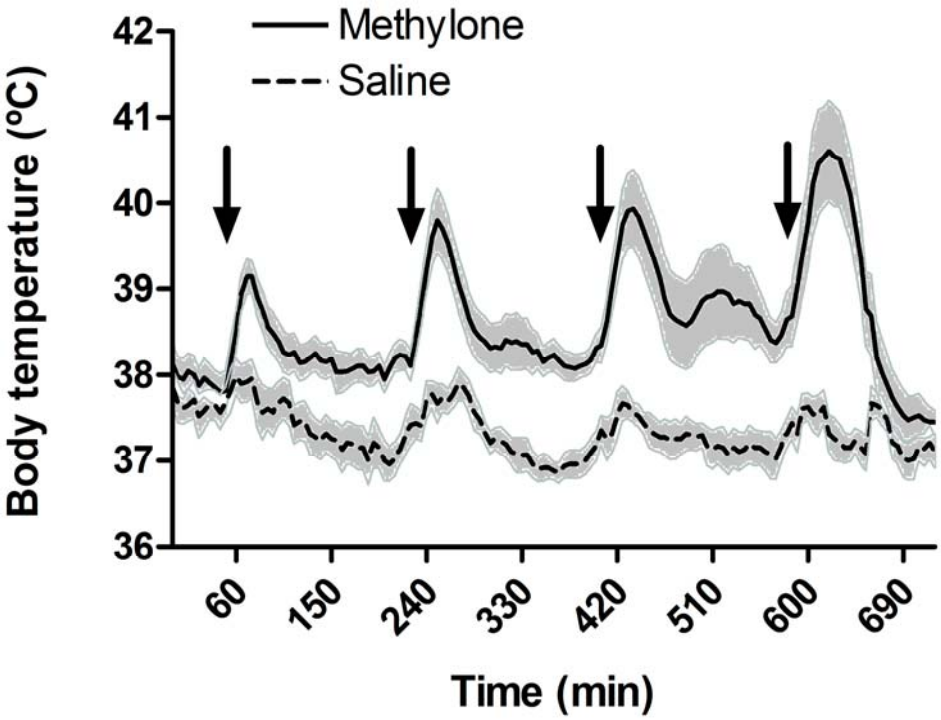
Figure 2. Effect of methylone treatment (4 x 20 mg/kg, s.c., 3 h intervals) in serotonin transporter density, measured as [^{3}H]-paroxetine binding in the striatum (panel A), frontal cortex (panel C) and hippocampus (panel E); effect in tryptophan hydroxylase 2 (TPH-2) expression in the striatum (panel B), frontal cortex (panel D) and hippocampus (panel F). The values correspond to animals killed seven days after treatment. Below each bar graph, the corresponding representative Western blots of TPH-2 expression in the striatum, frontal cortex and hippocampus are shown. Results are expressed as mean ± S.E.M. from 5-8 animals per group. *p<0.05 and **p<0.01 vs. saline.

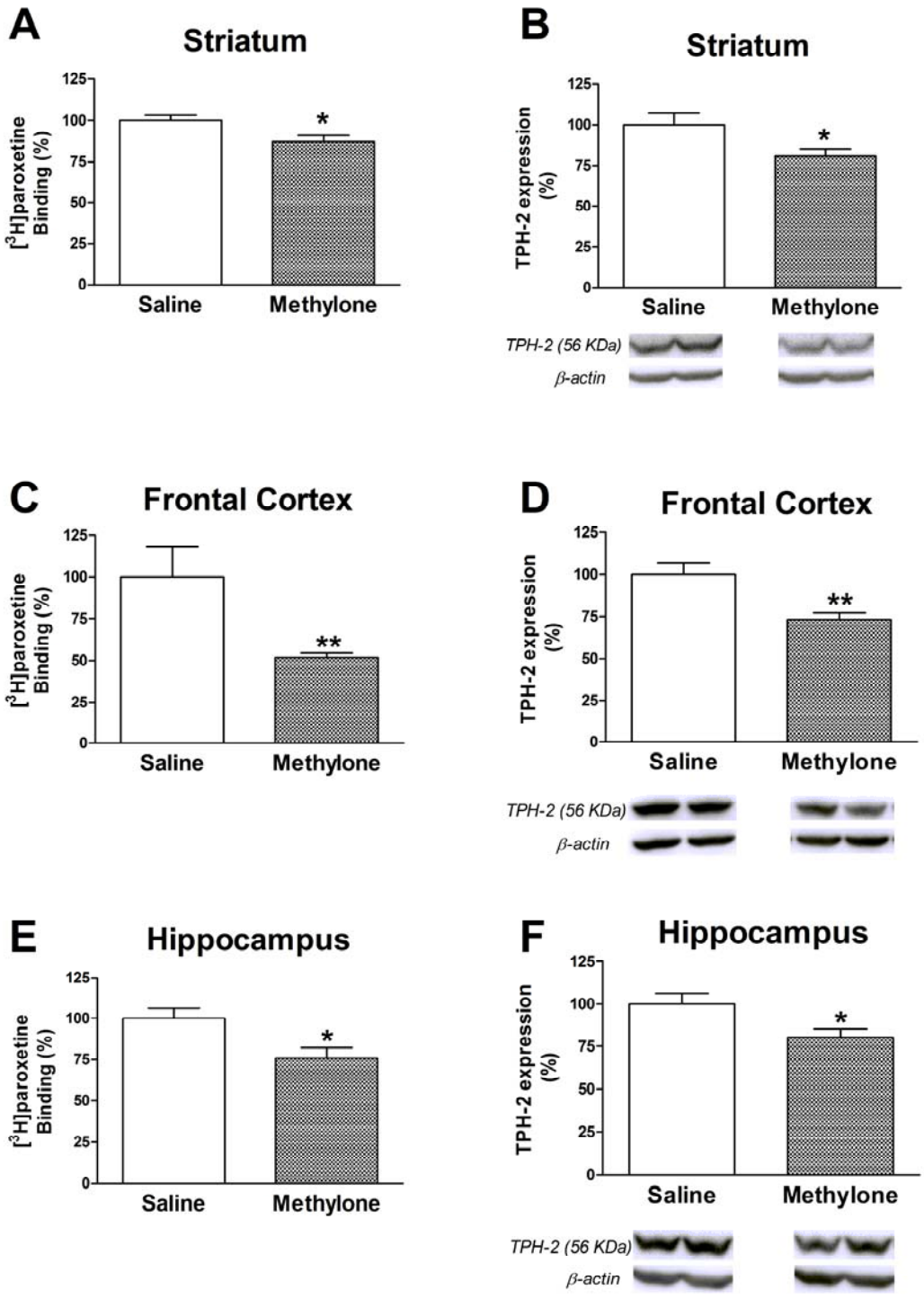
Fig 3. Representative saturation binding assay of [^{3}H]-paroxetine in the frontal cortex of saline- and methylone-treated rats. B_{max} and K_D values are expressed as mean ± S.E.M. of 4-5 experiments. **p<0.01 vs. saline (see box).

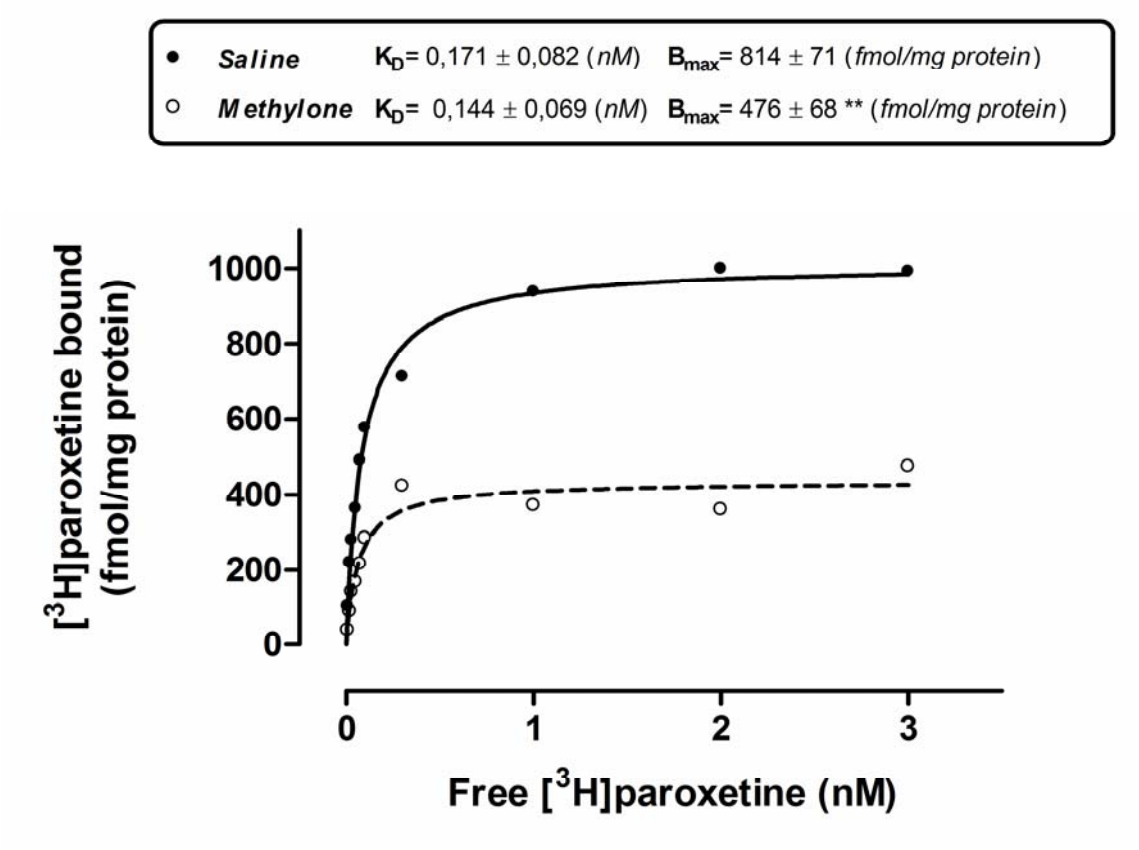
Figure 4. Representative immunohistochemistry for GFAP expression in horizontal sections of the frontal cortex for animals treated with saline or methylone (x2 and x20) (Panel A). Effect of methylone treatment on GFAP expression in rat frontal cortex (Panel B) seven days after treatment. Quantification was carried out using Image J software. Results are expressed as mean ± S.E.M. from 3 animals per group. *p<0.05 vs. saline.

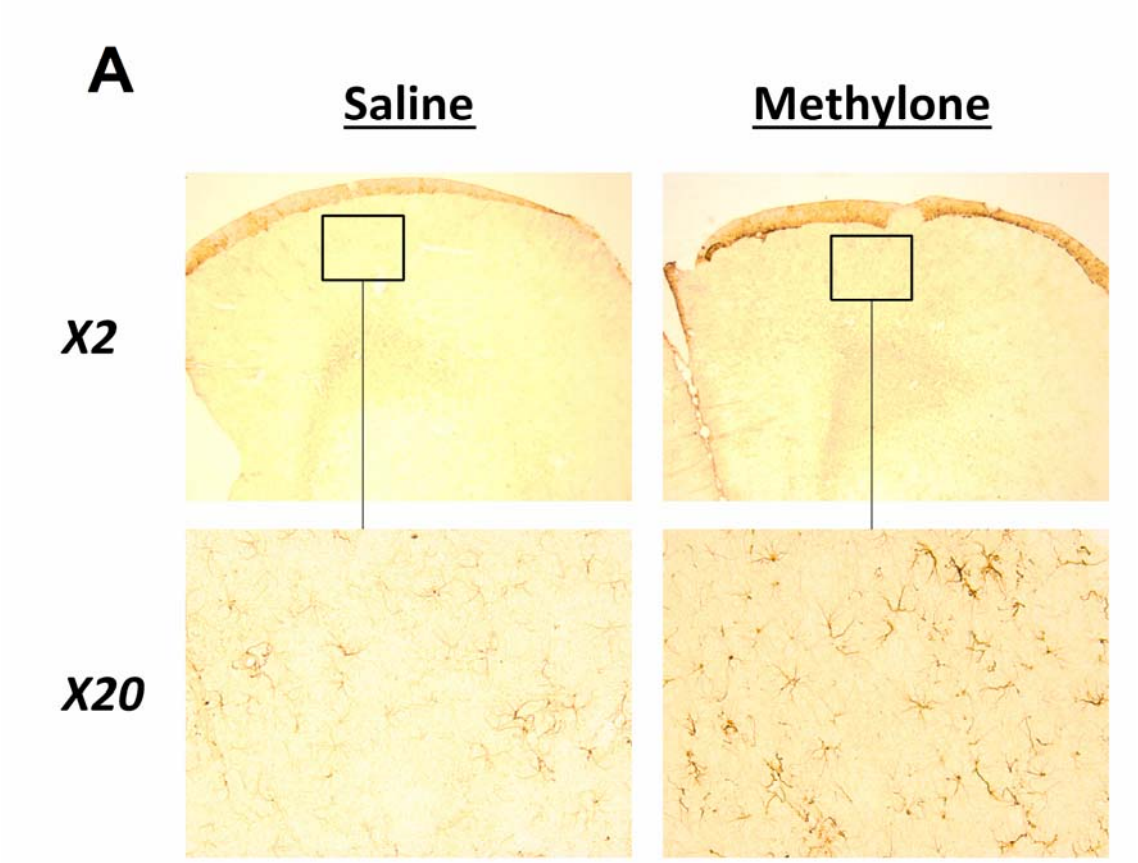
Fig 5. Effect of treatment with saline or methylone in the acquisition phase of spatial learning in the Morris water maze. The escape latency (the time required for rats to locate the platform) for the five different days is expressed as mean ± S.E.M. of 9-11 animals per group.

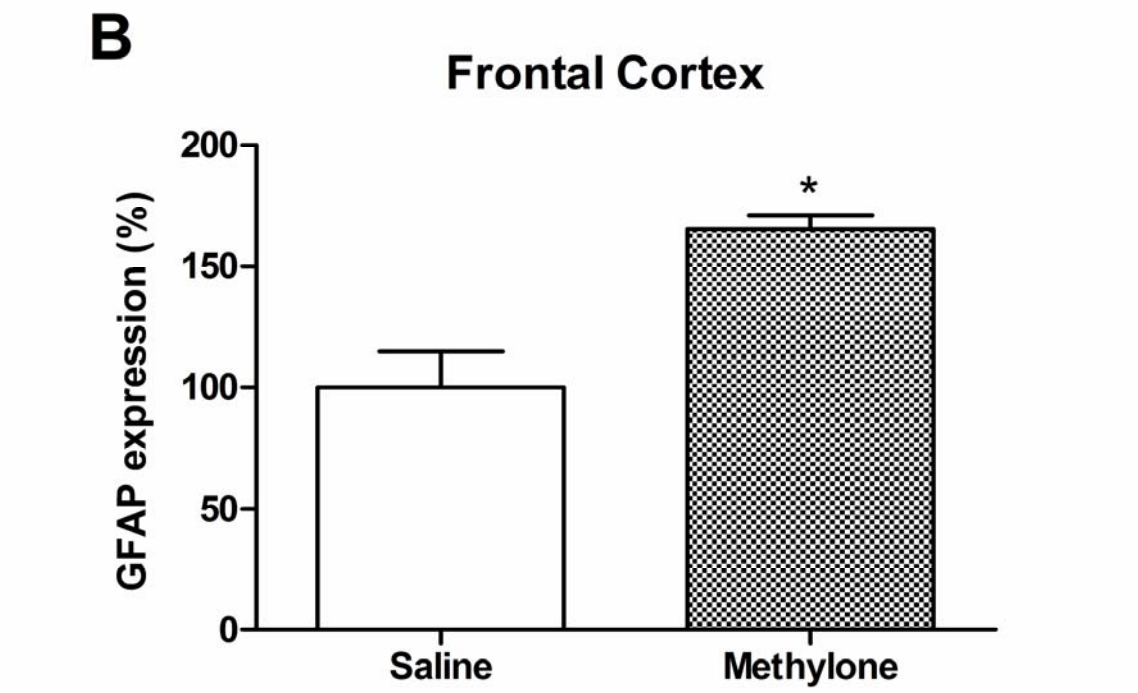
Fig 6. Effect of methylone treatment on the probe trial in the Morris water maze. Panel A shows % of time spent by the animals in the target (T) and the opposite (O) quadrant. Panel B shows the number of entries in the target and opposite quadrant. Results are expressed as mean ± S.E.M. from 9-11 animals per group. ***p<0.001 vs. saline (two-way ANOVA of repeated measures followed by Tukey's post-hoc test).

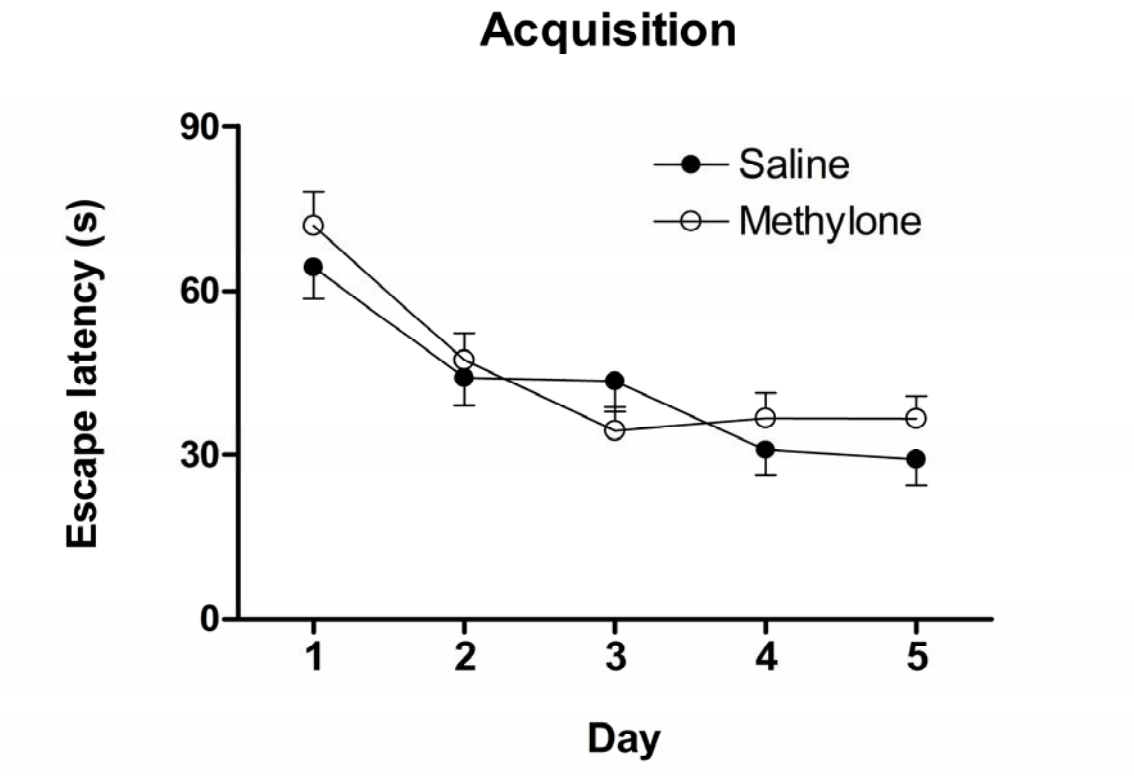

Methylone
Saline
Body temperature (°C)
42
41
40
39
38
37
36
60
150
240
330
420
510
600
690
Time (min)

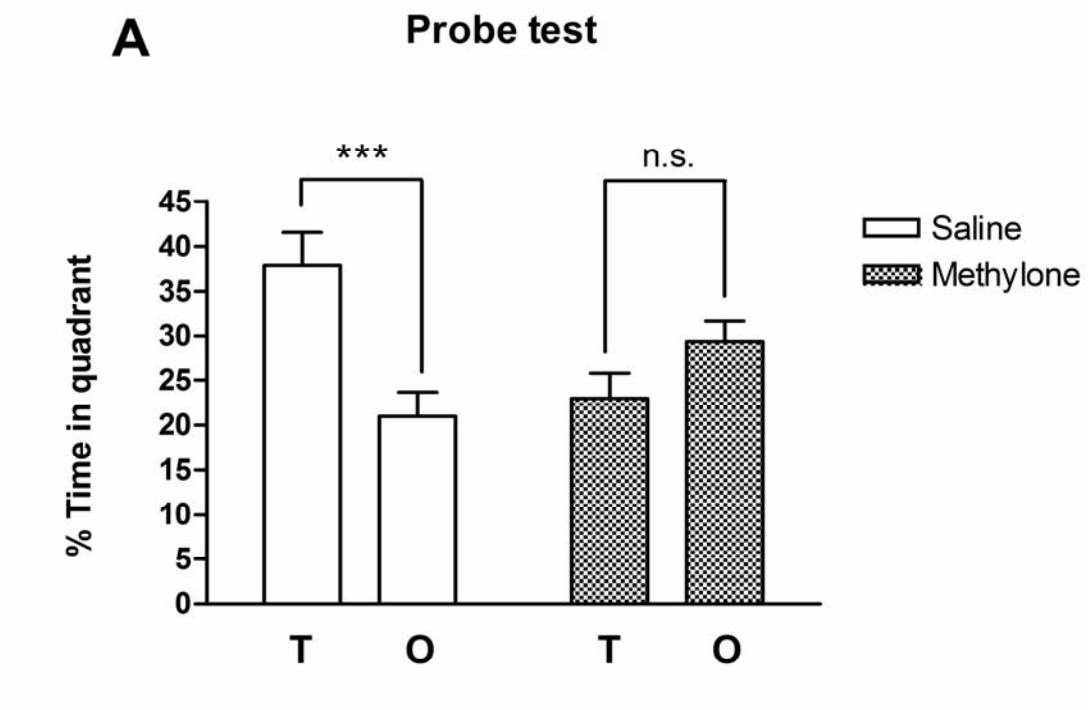

A
Striatum
[3H]paroxetine Binding (%)
125
100
75
50
25
0
*
Saline
Methylone
B
Striatum
TPH-2 expression (%)
125
100
75
50
25
0
*
Saline
Methylone
TPH-2 (56 KDa)
β-actin
C
Frontal Cortex
[3H]paroxetine Binding (%)
125
100
75
50
25
0
**
Saline
Methylone
D
Frontal Cortex
TPH-2 expression (%)
125
100
75
50
25
0
**
Saline
Methylone
TPH-2 (56 KDa)
β-actin
E
Hippocampus
[3H]paroxetine Binding (%)
125
100
75
50
25
0
*
Saline
Methylone
F
Hippocampus
TPH-2 expression (%)
125
100
75
50
25
0
*
Saline
Methylone
TPH-2 (56 KDa)
β-actin


Saline K_D= 0,171 ± 0,082 (nM) B_{max}= 814 ± 71 (fmol/mg protein)
Methylone K_D= 0,144 ± 0,069 (nM) B_{max}= 476 ± 68 ** (fmol/mg protein)
[3H]paroxetine bound (fmol/mg protein)
1000
800
600
400
200
0
0
1
2
3
Free [3H]paroxetine (nM)


A
Saline
Methylone
X2
X20


B
Frontal Cortex
GFAP expression (%)
200
150
100
50
0
*
Saline
Methylone


Acquisition
Escape latency (s)
90
60
30
0
1
2
3
4
5
Day
Saline
Methylone


A
Probe test
***
n.s.
% Time in quadrant
45
40
35
30
25
20
15
10
5
0
T
O
T
O
Saline
Methylone

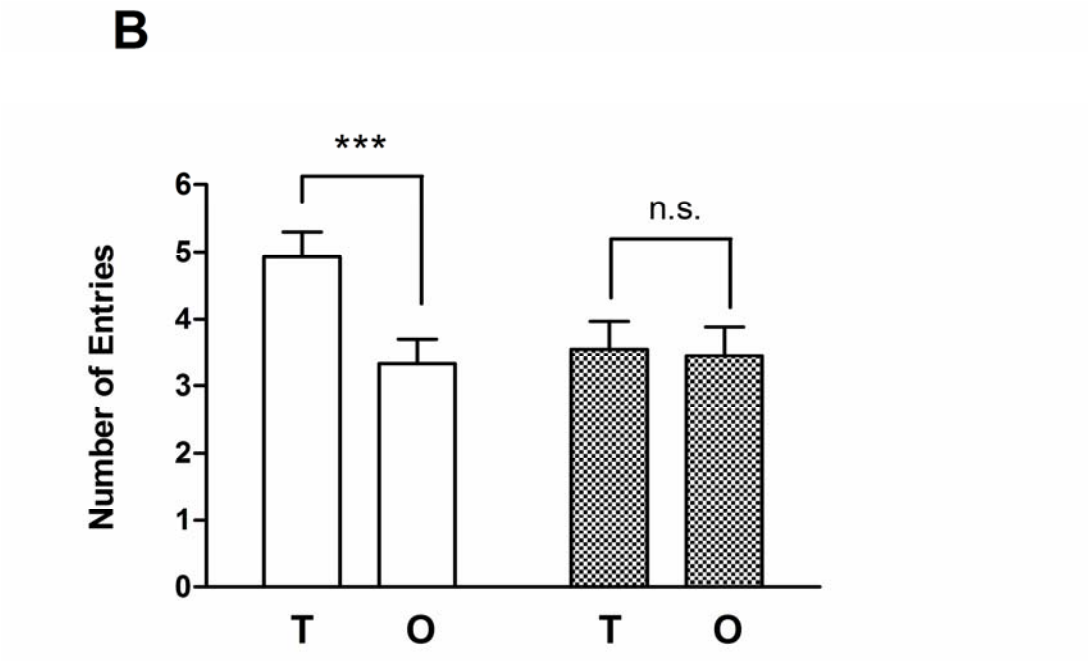

B
***
n.s.
Number of Entries
6
5
4
3
2
1
0
T
O
T
O

Table 1. Peak rat core body temperatures, in centigrade, after each saline (1ml/kg, s.c.) or methylone (4x20mg/kg, s.c.) administration at 3h interval. Results are expressed as mean ± SEM from n=6-8 animals.

Treatment	*Basal*	*1st dose*	*2nd dose*	*3rd dose*	*4th dose*
Saline	37.69±0.24	37.98 ± 0.21	37.90 ± 0.20	37.67 ± 0.20	37.63 ± 0.16
Methylone	37.98±0.23	39.14 ± 0.21**	39.80 ± 0.38**	39.94 ± 0.45**	40.61 ± 0.59***

**P<0.01 and ***P<0.001 vs. saline. Two-way ANOVA followed by post-hoc test.