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Complete List of Authors:	López-Arnau, Raúl; University of Barcelona, Pharmacology and Therapeutic Chemistry Martínez-Clemente, José; University of Barcelona, Pharmacology and Therapeutic Chemistry Abad, Sonia; University of Barcelona, Pharmacology and Therapeutic Chemistry Pubill, David; University of Barcelona, Pharmacology and Therapeutic Chemistry Camarasa, Jorge; University of Barcelona, Pharmacology and Therapeutic Chemistry Escubedo, Elena; University of Barcelona, Pharmacology and Therapeutic Chemistry
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Repeated doses of methylone, a new drug of abuse, induce changes in serotonin and dopamine systems in the mouse.

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

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

(*) Corresponding author:

Jorge Camarasa

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: jcamarasa@ub.edu

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Abstract

Rationale Methylone, a new drug of abuse sold as "bath salts" has similar effects to ecstasy or cocaine.

Objective We have investigated changes in dopaminergic and serotoninergic markers, indicative of neuronal damage, induced by methylone in the frontal cortex, hippocampus and striatum of mice and according two different treatment schedules.

Methods Methylone was given subcutaneously to male Swiss CD1 mice and at an ambient temperature of 26ºC. Treatment A: three doses of 25 mg/Kg at 3.5 h interval between doses for two consecutive days. Treatment B: four doses of 25 mg/Kg at 3 h interval in one day.

Results Repeated methylone administration induced hyperthermia and a significant loss in body weight. Following treatment A, methylone induced transient dopaminergic (frontal cortex) and serotoninergic (hippocampus) impairment. Following treatment B, transient dopaminergic (frontal cortex) and serotonergic (frontal cortex and hippocampus) changes 7 days after treatment were found. We found evidence of astrogliosis in the CA1 and the dentate gyrus of the hippocampus following treatment B. The animals also showed an increase in immobility time in the forced swim test, pointing to a depressive-like behavior. In cultured cortical neurons, methylone (for 24 and 48 h) did not induce a remarkable cytotoxic effect.

Conclusions The neural effects of methylone differ depending upon the treatment schedule. Neurochemical changes elicited by methylone are apparent when administered at an elevated ambient temperature, four times per day at 3 h intervals, which is in accordance with its short half-life.

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

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Introduction

The illegal status of the classic psychostimulants (particularly MDMA and cocaine) has encouraged users to seek newer drugs that have become increasingly available through the Internet, which allows effective marketing, sale and distribution and is the major reason for the increase in their availability (Brandt et al., 2010; McElrath and O' Neill, 2011; Karila and Reynaud, 2010).

Synthetic modifications of cathinone, structurally similar to amphetamine and extracted from the leaves of khat, have led to a number of so-called designer cathinone derivatives sold as “bath salts”. The most commonly available cathinones appear to be mephedrone (4-methyl-methcathinone) and methylone (3,4-methylene-dioxymethcathinone) (Brunt et al., 2011). Methylone was first synthesized as an antidepressant but, around 2004, emerged as a recreational drug under the trade name “explosion”, and was one of the first products of this nature to be marketed on-line (Bossong et al., 2005). It is taken by the oral or intranasal route.

Methylone shows a strong structural and pharmacological similarity to MDMA, but little is known about its particular pharmacology. Results from in vitro studies hypothesized that methylone acted similarly to d-amphetamine (Cozzi et al., 1999; Baumann et al., 2012), by binding to monoamine transporters (Nagai et al., 2007; Simmler et al., 2013). Recently, studies have been published on the pharmacological targets of cathinones by our group (Martínez-Clemente et al., 2012; López-Arnau et al., 2012) and others (Kehr et al., 2011; Hadlock et al., 2011; Motbey et al., 2011) demonstrating that methylone acts on monoaminergic systems. Moreover, in vitro studies reveal that cathinone derivatives are non-selective substrates for monoamine transporters, which lead to reuptake blockage and enhancement of the release of monoamines by reversing the flow of the transporter, which results in elevated synaptic neurotransmitter levels (Sogawa et al., 2011; Baumann et al., 2012; Simmler et al., 2013; Eshleman et al., 2013). This is a critical point since only transporter substrates (and not uptake inhibitors) are capable of causing long-term deficits in monoamine cells (Fleckenstein et al., 2007).

Human data on methylone abuse are obtained from consumer reports (Shimizu et al., 2007; Boulanger-Gobeil et al., 2012). On-line reports indicate that 100-250 mg in each intake is a common oral dose of methylone and users evidenced a desire to re-dose, leading them to ingest large quantities of the drug. Fatal intoxications due to methylone have been described (Pearson et al., 2012) with elevated body temperature and symptoms similar to sympathomimetic toxicity, including metabolic acidosis, rhabdomyolysis, acute renal failure and disseminated intravascular coagulation. The

authors concluded that peripheral blood methylone concentrations greater than 0.5 mg/L may be lethal.

Not only is the rise in abuse of methylone of concern, but so is the lack of experimental data on the neurotoxicity of methylone in rodents. MDMA produces species-dependent neurotoxicity (Logan et al., 1988), and there is reason to suspect that methylone would display a similar effect. In keeping with this, some species differences in the sensitivity to long-term neurochemical effects of methylone have been shown. Hollander et al. (2013) described no effects of methylone on serotonin (5-HT) levels in mice, but a widespread depletion of 5-HT and 5-HT transporter levels in rats was evidenced. Moreover, Baumann et al. (2012) found no effect of methylone on monoamine levels two weeks after treatment, also in rats, although they concluded that the effects of methylone and other cathinones should be evaluated in assays measuring 5-HT deficits, for example, with high-dose administrations.

The aim of this paper is to investigate methylone-induced neurochemical changes in mice that are indicative of neurotoxicity, addressing some of the limitations found in the literature on the subject to date. Any information that may lead to suspicion regarding the neurotoxicity or safety of methylone is critical. Experiments were carried out at a high ambient temperature simulating hot conditions found in dance clubs where amphetamine derivatives are usually consumed (Senn et al., 2007). We have evaluated the in vivo effect of this cathinone using different dosage schedules and in different brain areas assessed by decreases in the density of DA or 5-HT uptake sites and the enzyme that catalyzes the first and rate-limiting step in the biosynthesis of both neurotransmitters. Other outcome measures included drug-induced changes in body weight, core body temperature, depressive behavior and glial activation. Although mice or rats do not provide an exact model of methylone-induced neurotoxicity for humans, the present study intends to broaden our understanding of the adverse effects of this cathinone derivative.

Materials and methods

Drugs and reagents

Racemic and pure methylone HCl was synthetized and characterized by us in our department's organic chemistry laboratory, under authorization from the University of Barcelona as described previously (López-Arnau et al., 2012). The rest of 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.

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Animals

The 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, following the guidelines of the European Community Council (86/609/EEC). Male Swiss CD-1 mice (Charles River, Spain) weighing 25-30 g and aged 4-5 weeks were used. Animals were housed at 22 ± 1 ºC under a 12 h light/dark cycle with free access to food and drinking water. All the endpoints were taken in different animals.

In vivo neurotoxicity assays

The doses used in the present study were chosen according to available Internet information (www.erowid.org) and literature (Hollander et al., 2013; Simmler et al., 2013) and were calculated following to the FDA (Food and Drug Administration, Center for Drug Evaluation and Research, 2005) guidelines. No information is available on subcutaneous doses in humans. A dose of 150-200 mg in a 60-70 kg human yields a 2-3 mg/kg dose equivalent to 25-35 mg/kg in mice.

Mice (8-12 animals per group) were treated with methylone applying a regimen of three subcutaneous doses of 25 mg/kg, with a 3.5 h interval between each injection for 2 consecutive days (treatment A; total daily dose: 75 mg/kg); or four subcutaneous injections of 25 mg/kg in one day with a 3 h interval between each injection (treatment B; total daily dose: 100 mg/kg). Another group also received saline (5 ml/kg). Rectal temperatures were measured by inserting a lubricated, flexible rectal probe (1.5 cm) attached to a digital thermometer (0331 Panlab, Barcelona, Spain) into the rectum. In preliminary studies we found that maximum hyperthermia was achieved 45 min after administration. However, in order to reduce animal stress we chose to record body temperature after the second dose of each day's treatment. Mice were lightly restrained by hand during the procedure, with a steady read-out of temperature obtained approximately 40 s after the probe insertion. During the treatments, the animals were maintained in an ambient temperature of 26 ± 2ºC and kept under these conditions until 1 h after the last daily dose.

Tissue sample preparation

Crude membrane preparations were prepared as described elsewhere (Escubedo et al., 2005) with minor modifications. Mice were killed by cervical dislocation at 3 or 7 days post-treatment, and the brains were rapidly removed from the skull. Hippocampus, striatum, frontal or parietal cortex were quickly dissected out, frozen on dry ice, and stored at -80℃ until use. When required, tissue samples were thawed and

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homogenized at 4℃ in 20 volumes of buffer consisting of 5 mM Tris-HCl, 320 mM sucrose, and protease inhibitors (aprotinin 4.5 µg/µl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4. The homogenates were centrifuged at 1,000 x *g* for 15 min at 4℃. 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℃. The pellets were resuspended in buffer and incubated at 37ºC for 5 min to remove endogenous neurotransmitters. The protein samples were then recentrifuged and washed two more times. The final pellets (crude membrane preparation) were resuspended in the appropriate buffer and stored at -80℃ until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent.

DA and 5-HT transporter densities

The density of DA transporters in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding assays. 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℃ and non-specific binding was determined in the presence of 30 µM bupropion. All incubations were finished by rapid filtration under vacuum through GF-B glass fiber filters (Whatman, Maidstone, UK) pre-soaked in 0.5% polyethyleneimine. 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.

The density of 5-HT transporters in the hippocampal and frontal cortex membranes was quantified by measuring the specific binding of 0.05 nM [^{3}H]paroxetine after incubation with 150 µg of protein at 25ºC 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.

5-HT$_{2A}$ receptor density

The density of 5-HT$_{2A}$ receptors in mice parietal or frontal cortex membranes was measured by [^{3}H]ketanserin binding assays. Membranes were resuspended in 50 mM Tris–HCl buffer, pH 7.4 at 4 ℃ to a concentration of 1 µg/µl. Assays were performed in glass tubes containing 1 nM [^{3}H]ketanserin and 100 µg of membranes. Incubation was carried out at 37 ℃ for 30 min in a 50 mM Tris–HCl buffer to a final volume of 0.5 ml. Methysergide (10 µM) was used to determine non-specific binding.

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Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2) levels. For each sample, 20 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 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-buffered saline buffer plus 0.05% Tween-20 and incubated for 2 h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Laboratories, Lexington, KY, USA) diluted 1:5000 or with a primary rabbit polyclonal antibody against TPH2 (Millipore, USA) diluted 1:1000. After washing, membranes were incubated with a peroxidase-conjugated antimouse IgG antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2500 or with a peroxidase-conjugated antirabbit IgG antibody (GE Healthcare) diluted 1:5000. 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 Labs.,Hercules, CA, USA). Scanned blots were analyzed using BioRad Image Lab software and dot densities were expressed as a percentage of those taken from the control. Immunodetection of β-actin (mouse monoclonal antibody, dil.1:2500) served as a control of load uniformity for each lane and was used to normalize differences in TH or TPH2 expression due to protein content.

Immunohistochemistry

Seven days after treatment B animals were anaesthetized with pentobarbital sodium (60 mg/kg) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (1 ml/g of body weight). 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 cut in a cryostat and collected in phosphate buffer solution. Free-floating coronal sections 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℃ using a monoclonal antibody against fibrillary acidic protein (GFAP, 1:1000)

(Dako, Denmark). Following this, sections were washed and incubated with a biotinylated secondary antibody (1:200 Sigma-Aldrich) for 2 h at room temperature. Afterwards sections were incubated with avidin-biotin-peroxidase complex (ABC; 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).

Neuronal cell cultures

Primary neuronal cultures of cerebral cortex were obtained from mouse embryos (E-16-18). The cerebral cortex was dissected, meninges were removed, and tissue was incubated for 20 min in trypsin (0.05%) at 37℃. Trypsin was inactivated with fetal bovine serum and tissue was triturated with a fire-polished Pasteur pipette. Dissociate cells were washed with phosphate buffer containing 0.6% glucose and centrifuged at 500 x *g* for 5 min to remove debris. The cells were redissociated in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) with 0.5 mM L-glutamine, sodium bicarbonate (0.04%) and 1 μg/ml penicillin and streptomycin, containing B27 supplement and 10% horse serum. Neurons were plated at 0.4 million cells/ml in 96-well plates precoated with 1 mg/mL poly-L-lysine. Cultures were maintained at 37℃ in a humidified incubator with 5% CO_2 and 95% air. Twenty-four hours later, cells were treated with arabinosylcytosine (10μM) to prevent the growth of glial cells. The culture medium was changed every 3 days to Neurobasal medium with B27 without antioxidants and the concentration of horse serum was reduced gradually down to 1%. The cultures were used for experiments after 8-9 days in vitro with different concentrations of methylone (80-1000 μM) and different times of drug exposure (24 and 48 h).

Cell Viability Assay

Cell viability was assessed by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide) assay. MTT was added to the cells to a final concentration of 250 μM and incubated for 2 h (Hansen et al., 1989). The media were removed and cells were dissolved in dimethylsulphoxide. Formation of formazan was tested by measuring the amount of reaction product by absorbance change (595 nm) using a microplate reader (BioRad Laboratories, CA, U.S.A.). Viability results were expressed as a percentage of the absorbance measured in untreated cells.

Forced swimming test (FST)

The immobility time in the FST was measured by an observer blind to the treatment using the procedure described by Porsolt et al. (1978). Briefly, mice were placed individually in a glass cylinder (height 21 cm, diameter 12 cm) containing water at 25 ± 1℃ up to a height of 15 cm. Mice do not try to dive or explore the water surface, which explains the ease in use of mice that do not need a previous session (Petit-Demouliere et al., 2005). Animals were randomly divided into two groups (12-16 animals per group) and treated with saline or methylone and tested 3 or 7 days after treatment. Each animal was recorded for 6 min and the total period of immobility, in seconds, was measured. A mouse was judged to be immobile when it remained floating in water, making only the necessary movements to keep its head above water. Each mouse was only tested once (Calapai et al., 2001). Increase in the duration of immobility was considered to reflect a depressant-like effect of the drug.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using one-way ANOVA or Student-t test for independent samples where appropriate. Significant ($P<0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons where appropriate. All statistic calculations were performed using Graph Pad Instat (GraphPad Software, San Diego, USA).

Results

Lethality

Initial experiments were carried out with 4-6 animals per cage (48x25x13 cm). Under these conditions, lethality was at an approximately 75% level. Given that, all the treatments carried out in this study were performed with a single animal per cage (35x14x13 cm) housed 4 days before dosing.

The number of fatalities of methylone-treated mice was similar in treatment A (of about 25%) and treatment B (of about 20%) and occurred 1-2 h after the third or the fourth dose. To obtain an accurate cause of death a veterinarian necropsy was performed. Immediately after the animal's death, the veterinarian did an overall examination of some of the animals, as well as looking at individual organs within the body. Final diagnostics evidenced hepatomegaly, and acute hemorrhagic pericarditis as the cause of death.

Effect of methylone on body temperature and body weight

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Mehylone induced a significant increase in the body temperature in treatment A and treatment B (Fig. 1A). Since the hyperthermia induced by methylone at the second day of treatment A was significantly lower than that induced at the first day, tolerance to hyperthermic effects of methylone can be concluded.

In both treatments, methylone produced significant loss in body weight (Fig. 1B). At the end of treatment A methylone-treated animals showed an overall decrease in body weight of -0.60 ± 0.16 g.

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

Treatment A: methylone-treated mice showed a transient decrease in [^{3}H]WIN35428 specific binding in the frontal cortex that resumed a normal level four days later (Fig. 2A). This decrease was not accompanied by a change in TH expression (3 days: saline: 100.00 ± 22.60%; methylone: 110.06 ± 12.70%; 7 days: saline: 100.00 ± 11.72%; methylone: 116.00 ± 9.49%).

In contrast, in the striatum, methylone neither affected [^{3}H]WIN35428 binding (saline: 100.00 ± 10.27%; methylone: 94.38 ± 8.20%) nor TH expression (saline: 100.00 ± 11.10%; methylone: 82.40 ± 4.20%), even after 3 days following the end of exposure. Thus no further determinations were performed at 7 days.

With regards to 5-HT transporters, methylone did not modify [^{3}H]paroxetine binding in the frontal cortex (Fig 2B). However, in the hippocampus, methylone induced a slight reduction in [^{3}H]paroxetine binding, measured 3 days after treatment, which was reverted 4 days later (Fig 2C). The levels of THP2 remained unchanged after the treatment (saline: 100.00 ± 3.80%; methylone: 83.20 ± 5.70%, $P>0.05$).

Treatment B: As above, methylone treatment induced a transient decrease in the specific binding of [^{3}H]WIN35428 of about 27% in the frontal cortex that returned to control values four days later (Fig. 3A). Similarly, no changes in TH expression were evidenced (3 days: saline: 100.00 ± 24.47%; methylone: 94.75 ± 15.47%; 7 days: saline: 100.00 ± 10.52%; methylone: 97.43 ± 29.88%). As in treatment A, methylone did not affect any of these dopaminergic markers in the striatum ([^{3}H]WIN35428 binding: saline, 100.00 ± 7.57%; methylone, 93.91 ± 4.26%. TH expression: saline: 100.00 ± 3.53%; methylone: 97.06 ± 4.70%; 3 days after drug exposure).

As regards serotonergic markers, at three and seven days post-treatment, methylone induced a diminution of 5-HT reuptake sites of about 30-20% in frontal cortex and 20-12% in hippocampus respectively (Fig. 3B and 3C). Additionally, 7 days after treatment, TPH2-immunoreactivity levels were decreased in both brain areas in the methylone-treated mice that correlates with the diminution of 5-HT reuptake sites (Fig. 4A and 4B).

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6 Effect of methylone on 5-HT_{2A} receptor density.
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8 According to the treatment A schedule, methylone-treated, animals showed a decrease
9 in the number of 5-HT_{2A} receptors in the frontal (saline: 100.00 ± 4.80%; methylone:
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11 81.69 ± 2.65%, P<0.01) and parietal cortex (saline: 100.00 ± 4.75%; methylone: 80.60
12 ± 5.35%, P<0.05), measured as [^{3}H]ketanserin binding, 3 days after treatment, which
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14 returned to basal values four days later (105.26 ± 10.23% and 119.13 ± 8.49%,
15 respectively), pointing to a homeostatic process. In contrast, following schedule B,
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17 methylone did not modify the density of 5-HT_{2A} receptors in the two cortical areas either
18 at 3 (frontal cortex: 85.79 ± 5.40%; parietal cortex: 96.40 ± 3.58%) or 7 days after drug
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20 exposure (frontal cortex: 111.23 ± 12.96%; parietal cortex: 113.16 ± 3.45%).
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23 Effect of methylone on astroglial activation.
24 Because methylone induced neuronal damage 7 days after treatment B schedule, the
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26 next experiment was carried out to assess the presence of astroglial activation.
27 Accordingly, immunohistochemistry studies were performed with the glial-specific
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29 marker GFAP in brains from animals killed 7 days after the treatment. There were no
30 signs of striatal or cortical astroglial activation in methylone-treated animals. However,
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32 in the hippocampus, an increase in GFAP immunoreactivity was observed in the CA1
33 and dentate gyrus of the methylone group, compared with that from saline-tested mice.
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35 This suggests the presence of a slight reactive astrocytosis (Fig. 5).
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38 Effect of methylone on cultured cortical neuron viability
39 The exposure of cultured cortical mouse cells to various concentrations of methylone
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41 (from 80 µM to 1 mM) for 24 h or 48 h caused a weak concentration- or time-dependent
42 decrease in metabolically active cells, as assessed by the MTT assay (Fig. 6). Cell
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44 viability was only affected by methylone concentrations above 300 µM and the
45 corresponding calculated LD_{50} value for methylone after 24 h or 48 h exposure was
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47 over 1 mM, ruling out cell toxicity in cortical neurons.
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50 Depressant-like effect of methylone
51 Methylone administration (treatments A and B) increased the immobility time in the
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53 FST 3-day post-treatment as compared with the saline group. This time period
54 coincided with the observed impairment of both dopaminergic and serotonergic
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56 markers. These effects vanished in both schedules one week following drug exposure,
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58 when only the serotonergic markers remained decreased (Fig. 7).
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Discussion

There is little information regarding methylone and its potential toxicity. The initial status of cathinones as legal highs may have contributed to their increasing popularity as drugs of abuse. Because of the relatively short history of the use of cathinones as recreational drugs, their long-term effects have not yet been determined.

Very few studies exist, even in rodents, on the dosing schedules or doses required to induce damage (Baumann et al., 2012; Hollander et al., 2013). Therefore, the primary goal of this study was to evaluate the risk of neuronal changes linked to methylone abuse in mice. Methylone is a close structural analogue of MDMA, differing only by the addition of a β-ketone group. Consequently, it is also known as beta-keto-MDMA. As with MDMA, it might affect the DA or 5-HT system differently, depending on the animal species used for the experiment. Most authors described the maximum neurotoxic effects of methamphetamine three days after treatment (Pu and Vorhees, 1993) and those of MDMA seven days after treatment (Battaglia et al., 1988). Thus, we examined the neurotoxic injury induced by methylone at 3 and 7 days after following the end of the treatment. In addition, a close relationship was already established between the hyperthermic response and the severity of the brain lesion induced by amphetamines (Sánchez et al., 2004), supporting the hypothesis that MDMA is neurotoxic when a binge dosing schedule is employed and the animals are in a hot environment. Accordingly, present experiments were carried out at a high ambient temperature simulating hot conditions found in dance clubs. We administered the drug at 3-3.5h interval, in accordance with our previous paper characterizing the pharmacokinetics of methylone, distinguished by its short half-life (López-Arnau et al., 2013). To model recreational methylone use, we simulated the widespread practices of "stacking" (taking multiple doses at once in order to increase the desired effect and/or offset tolerance from prior use) and "boosting" (taking supplemental doses over time in order to maintain the drug's effect). Thus, we chose to administer multiple doses/day of methylone during each treatment.

Overall, our data demonstrate a slight serotonergic toxicity of methylone, one week after treatment, only when four doses are administered in a day. This toxicity is substantiated by decreases in density of 5-HT terminal marker and reduction in TPH2 expression, both more apparent in frontal cortex than in hippocampus. Post-mortem necropsies showed evident signs of hepatomegaly and hemorrhagic pericarditis that could be the cause of death. Further pathology studies are needed to affirm that these

signs resulted directly from methylone toxicity but methylone has been described as a cause of cardiac arrest leading to human death (Cawrse et al., 2012).

Our initial experiments indicated that methylone toxicity is exacerbated in group-housed animals like other amphetamines (Fantigrossi et al., 2003). Baumann et al., (2013) already described that, in humans, adverse effects of bath salts could be intensified in hot crowded spaces, such as rave party venues where these drugs are often used. Present experiments were performed in singly-housed mice. We have studied the evolution of body weight during methylone treatments. Like other amphetamine derivatives such MDMA, the animals treated with methylone, but not with saline, showed a weight loss, probably due to an anorectic effect of the drug.

Several factors, and particularly hyperthermia, contribute to MDMA-induced neurotoxicity. In this regard, the influence of ambient temperature on MDMA-induced thermal responses has been shown in earlier studies that noted a hyperthermic response when experiments were carried out at high ambient temperatures (26-28℃). Hyperthermia is, in fact, a commonly reported acute adverse effect of beta-ketoamphetamine ingestion in humans (Borek and Holstege, 2012; Prosser and Nelson, 2012). In the present study, experiments were performed at ambient temperature of 26 ± 2ºC. Under these conditions, methylone induced hyperthermia. This effect was more apparent on the first day of treatment and diminished when the drug was administered on a second day, indicating the possibility of tolerance that could be due to a depletion of neurotransmitter stores.

Following the two treatments performed in this study, methylone induced a transient loss of the DA transporter in the frontal cortex. The initial decline and later recovery of DA transporter points to a biochemical down-regulation in the absence of tissue damage but we can also assume that a methylone-induced dopamine transporter structural modification could occur, explaining the reduction in binding experiment. This hypothesis is in agreement with our previous results, which demonstrate that methylone inhibits [^{3}H]DA uptake after drug withdrawal, pointing to alterations in the transporter that are more complex than a simple blocking of the carrier (López-Arnau et al., 2013). Unlike MDMA (Chipana et al., 2006), methylone did not alter DA transporter radioligand binding or TH levels in striatum in any of the performed treatments. The main difference between treatments was found in 5-HT terminal markers. When exposure to methylone was performed over two consecutive days with three doses per day, we registered a transient reduction of these markers, but when treatment consisted of four doses in a single day, a more persistent effect appeared, affecting frontal cortex and hippocampus. The final reduction in 5-HT

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transporter marker correlates with the decrease in TPH2 expression. Astrocytes stabilize and maintain homeostatic tissue repair and contribute to early wound repair (Eddleston and Mucke, 1993). In the present study methylone-treated animals with schedule B showed an increase in GFAP immunoreactivity in hippocampal CA1 and dentate gyrus that corresponds to real terminal injury in these areas.

Only one study has been published concerning the neurotoxic effect of methylone in DA and 5-HT systems in mice (Hollander et al., 2013). Authors demonstrated that methylone exposure (30 mg/kg, twice daily for 4 days) had no effect on neurotransmitter levels in C57BL/J6 mice two weeks after treatment. Our results demonstrate that methylone neurotoxicity in mice depends on the number of doses and intervals between each dose, as occurs with other cathinones. Furthermore, we suspect that the high room temperature, used in this study, could play an important role in methylone-induced neurotoxicity if we compare our findings with those of Hollander et al. (2013). Nonetheless, further research is necessary in order to assess whether the role of hyperthermia and room temperature is complementary or essential in the advent of methylone-induced neurotoxicity. Some differences among neurotoxicity cathinone literature can be partially due to differences in the employed dosing-regimen and the time of sacrifice. However, due to the mechanism of action and structural similarity between methylone and MDMA, we used similar doses and time of sacrifice assessed in MDMA neurotoxicity studies (Chipana et al., 2006; Granado et al., 2008; Sánchez et al., 2003; Mueller et al., 2013).

Mice differ from other animal species because they display deficits in DA neurotransmission greater than 5-HT after binge MDMA exposure. Present results demonstrated that methylone acts on contrary. The methylone neurochemical profile could be explained by the fact that this drug acts preferentially as an inhibitor for the 5-HT transporter than for the DA transporter (Baumann et al., 2012; López-Arnau et al., 2012; Sogawa et al., 2011), implying a better access of this drug to the 5-HT nerve terminals leading to the corresponding injury. Moreover, a well-recognized hypothesis of MDMA neurotoxicity involves some metabolite participation that has not been demonstrated for methylone.

Methylone increased the immobility time in the FST following both treatments, which indicates an increase in stress-related depressive behavior. This effect was evidenced 3 days after treatment and correlates with the reduction of DA and 5-HT markers assayed. This is in accordance with results from McGregor et al. (2003), who demonstrated that MDMA-treated animals show a higher immobility and fewer active escape attempts in the forced swimming model. To our knowledge, the present studies provide the first preclinical data to shed light on this issue, suggesting that mice

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3 exposed to a stacking and boosting regime of methylone could be more prone to suffering from depressive-like symptoms. This effect disappeared 7 days after treatment, when only serotonergic neurotransmission remained impaired. It must be noted that depression pathophysiology may also involve changes in 5-HT_2 receptor in brain regions selectively implicated in mood regulation. In this regard, in treatment A we found a significant transient decrease in the number of cortical 5-HT_{2A} receptors three days after administration, possibly resulting from a neuroadaptative response to the massive 5-HT release induced by methylone. These results hark back to those published by Scheffel et al. (1992) regarding MDMA. In treatment B, we detected a similar, but non-significant, reduction in frontal cortex.

The impairment induced by methylone on 5-HT and DA terminals is limited to frontal cortex and hippocampus when exposure is clustered in four doses in a day. This mild cathinone neurotoxicity correlates with results of our in vitro studies in cortical cultured cells, where we describe that methylone did not show concentration- and time-dependent deleterious effect on neuronal viability. The data reveal that doses up to 1000 µM for 24 to 48 h do not appreciably affect cell viability. This is a remarkable finding, which confirms previous studies that found methylone alone is not cytotoxic even at high doses (Nakagawa et al., 2009; Sogawa et al., 2011). In this regard, other studies assessing the effects of MDMA on cortical or hippocampal cultured cell viability reported no or low cell death following exposure to high MDMA concentrations (Capela et al., 2006).

In conclusion, our results demonstrate that methylone-induced brain consequences differ according to treatment schedule (dose, number of doses and dose interval). Neurochemical changes elicited by methylone are apparent when administered at an elevated ambient temperature, four times per day at 3h intervals. This schedule is related with patterns used by humans and agree with methylone's half-life in rodents (López-Arnau et al., 2013). Following this, we found decrease in frontal cortex and hippocampal serotoninergic nerve ending markers around 20-25% together with hippocampal astrogliosis suggesting nerve ending injuries. No effect in striatum was evidenced. Methylone did not show a cytotoxic effect in cortical cultured neurons. The limited neurotoxicity found in this study, however, should not preclude advice concerning the high risk of acute fatal effects affecting the cardiovascular system and thermoregulation.

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Conflicts of interest. The authors declare that they have no financial or commercial conflicts of interest.

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Legends of Figures

Figure 1.- Effect of methylone treatments A and B in body temperature measured after the second dose of each day's treatment (panel A) and in body weight (panel B; empty bars represent saline and striped bars represent methylone). Results are expressed as mean ± S.E.M. from 8-10 animals. **P<0.01 and ***P<0.001 vs. saline. ## P<0.01 vs. methylone treatment A day 2.

Figure 2.- Effect of a methylone treatment (3 doses of 25 mg/kg, sc at 3.5 h interval for 2 days) in dopamine transporter density, measured as [^{3}H]WIN35428 binding in mouse frontal cortex (panel A) and serotonin transporter density, measured as [^{3}H]paroxetine binding in frontal cortex (panel B) and hippocampus (panel C). Results are expressed as mean ± S.E.M. from 8-10 animals. *P<0.05 and **P<0.01 vs. saline.

Figure 3.- Effect of a methylone treatment (4 doses of 25 mg/kg, sc at 3 h interval) in dopamine transporter density, measured as [^{3}H]WIN35428 binding in mouse frontal cortex (panel A) and serotonin transporter density, measured as [^{3}H]paroxetine binding in frontal cortex (panel B) and hippocampus (panel C). Results are expressed as mean ± S.E.M. from 8-10 animals. *P<0.05; **P<0.01 and ***P<0.001 vs. saline.

Figure 4.- Effect of a methylone treatment (4 doses of 25 mg/kg, sc at 3 h interval) on tryptophan hydroxylase 2 expression in mouse frontal cortex (panel A) and hippocampus (panel B) 7 days after treatment. Below each bar graph, representative Western blots of TPH-2 expression in frontal cortex and hippocampus, respectively. **P<0.01 and *P<0.05 vs. saline.

Figure 5.- Representative hippocampal expression of glial fibrilliary acidic protein (GFAP). Sections of the dentate gyrus from mice treated with saline (panels A and C) or methylone (4 doses of 25 mg/kg, sc 3 h interval) (panels B and D). The animals were sacrificed 7 days after treatment.

Figure 6.- Effect in cell viability of methylone on mouse cortical cultured neurons. Cells were exposed to different concentrations of methylone for 24 or 48 h and cell viability was assessed by the MTT assay. Data are expressed as mean ± S.E.M. from 3 different cultures.

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Figure 7.- Effect of methylone on immobility time in mouse forced swim test. Animals (12-16 animals/group) were randomly divided and treated subcutaneously with saline (5 ml/kg) or methylone (3 doses of 25 mg/kg, sc at 3.5 h interval for 2 days (panel A) or 4 doses of 25 mg/kg, sc 3 h interval (panel B)) and tested 3 or 7 days after treatment. Each animal was recorded for 6 min and the total period of immobility was registered. Each mouse was used only once for each experimental session. Each bar represents mean ± S.E.M. immobility time in seconds. **P<0.01 and *P<0.05 as compared with respective saline-treated group (one-way ANOVA and post hoc Tukey's test).

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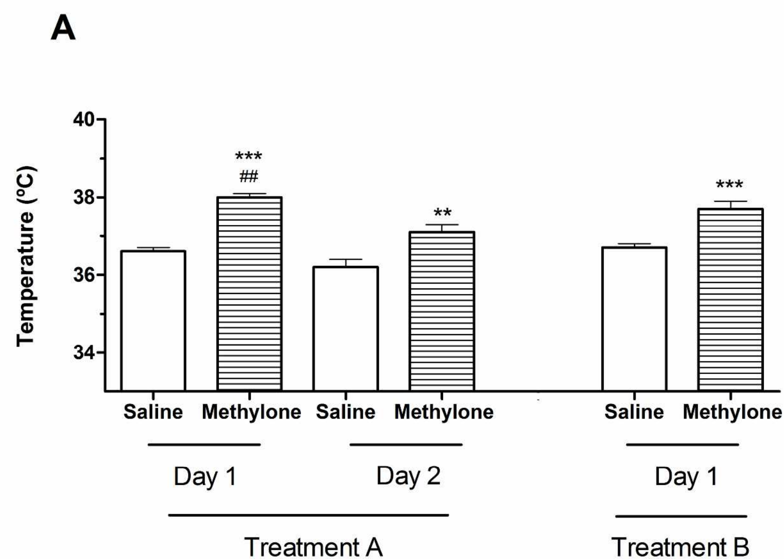

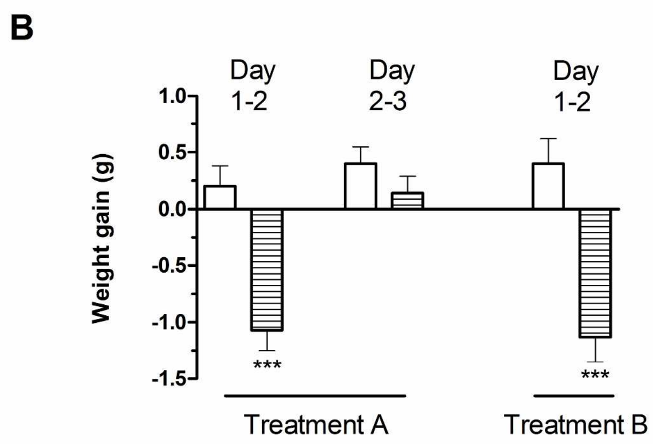

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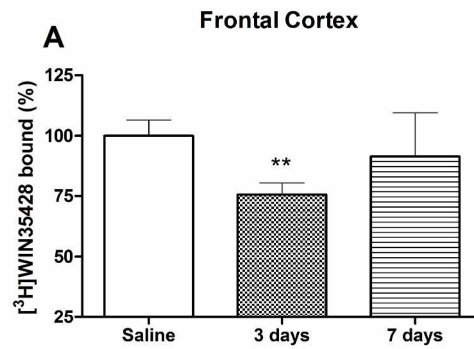

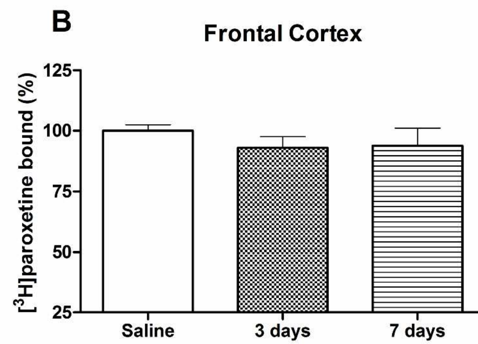

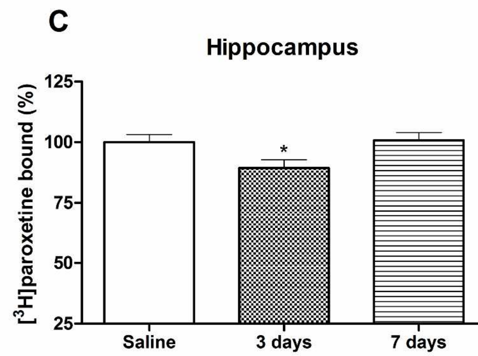

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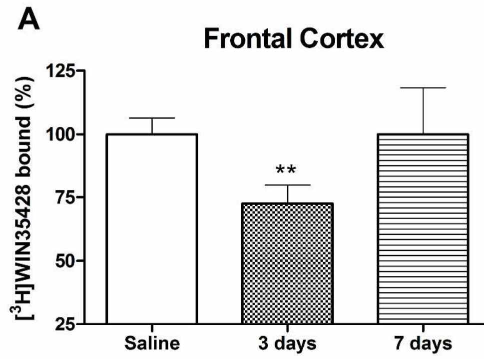

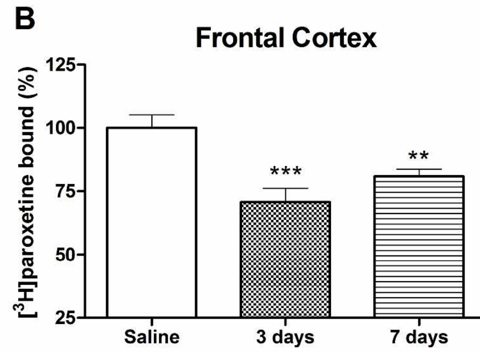

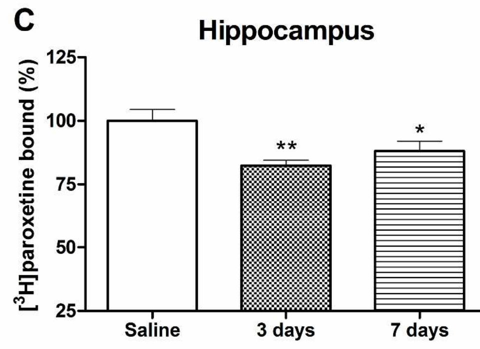

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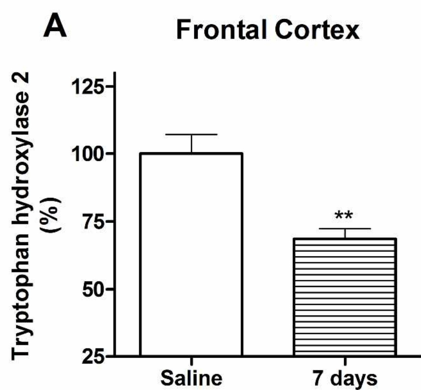

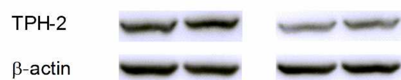

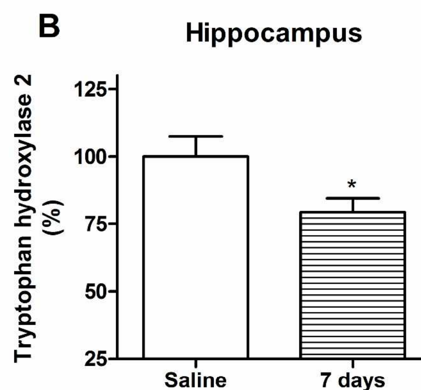

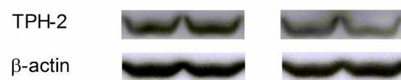

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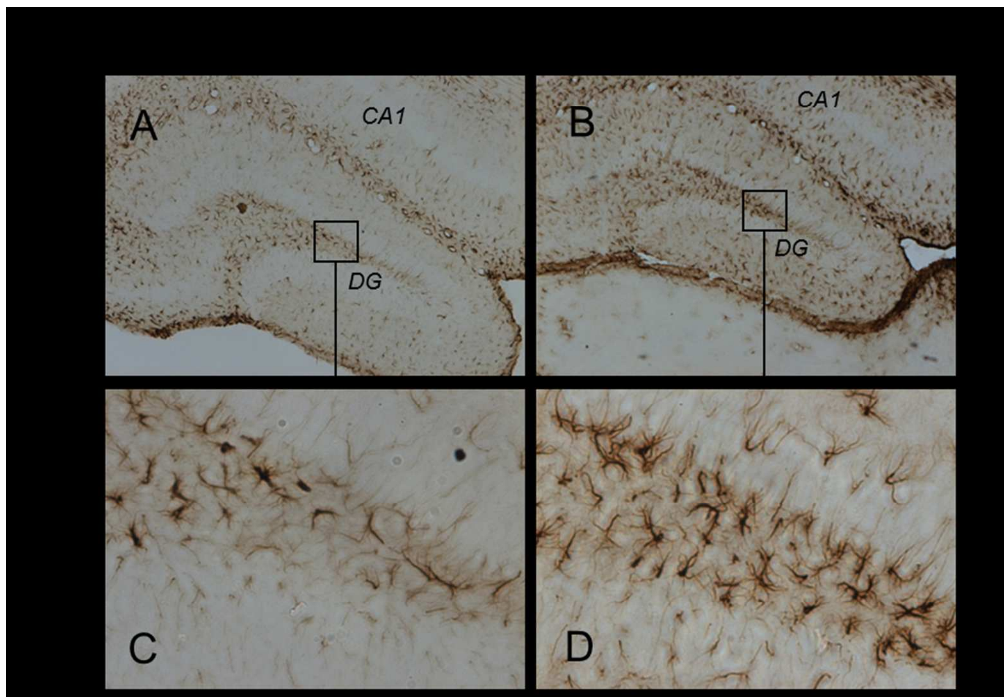

153x105mm (150 x 150 DPI)

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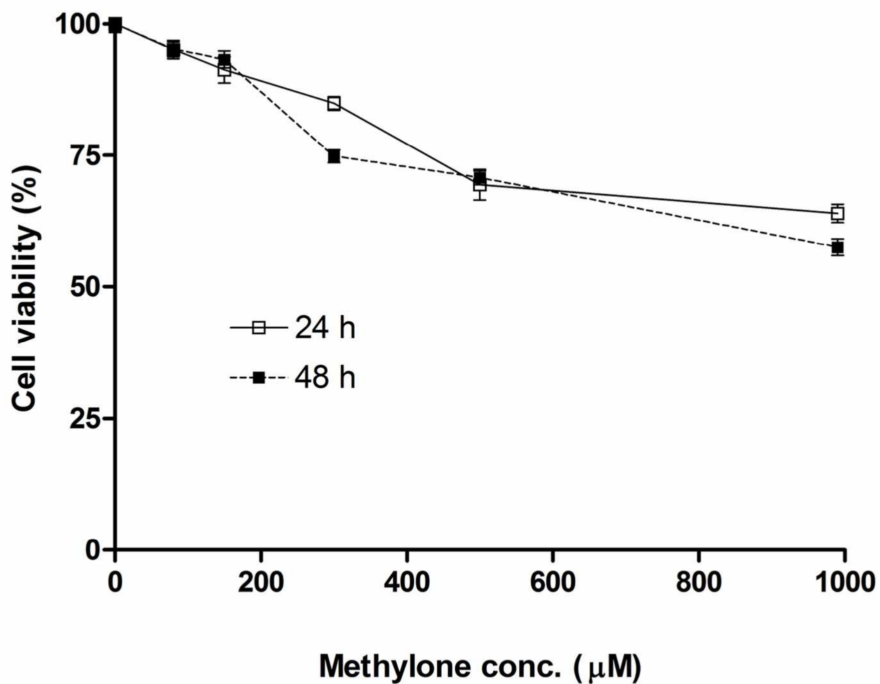

132x103mm (300 x 300 DPI)

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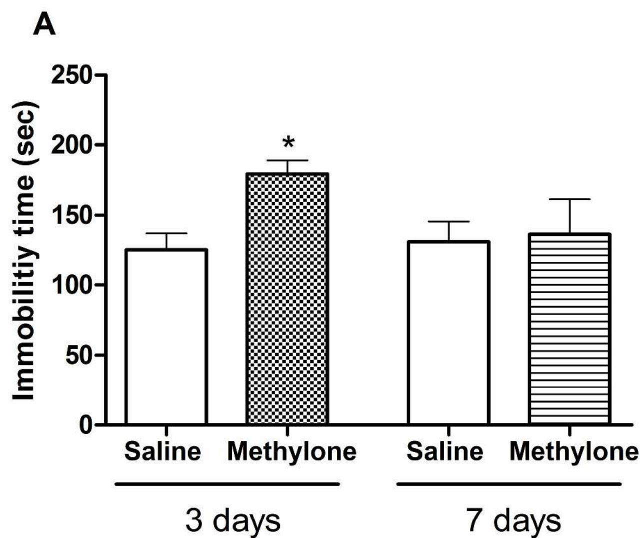

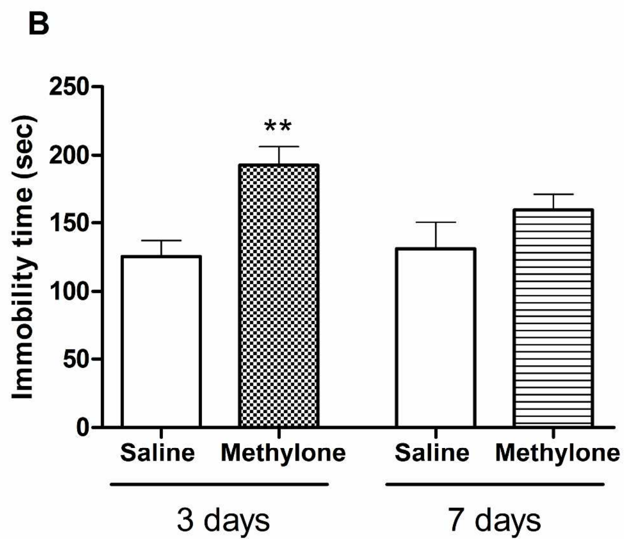

263x412mm (300 x 300 DPI)