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4-Methylthioamphetamine Increases Dopamine in the Rat Striatum and has Rewarding Effects *In Vivo*

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Abstract: 4-Methylthioamphetamine (MTA) is a phenylisopropylamine derivative whose use has been associated with severe intoxications. MTA is usually regarded as a selective serotonin-releasing agent. Nevertheless, previous data have suggested that its mechanism of action probably involves a catecholaminergic component. As little is known about dopaminergic effects of this drug, in this work the actions of MTA upon the dopamine (DA) transporter (DAT) were studied *in vitro*, *in vivo* and *in silico*. Also, the possible abuse liability of MTA was behaviourally assessed. MTA exhibited an *in vitro* affinity for the rat DAT in the low micromolar range (6.01 μM) and induced a significant, dose-dependent increase in striatal DA. MTA significantly increased c-Fos-positive cells in striatum and nucleus accumbens, induced conditioned place preference and increased locomotor activity. Docking experiments were performed in a homology model of the DAT. In conclusion, our results show that MTA is able to increase extracellular striatal DA levels and that its administration has rewarding properties. These effects were observed at concentrations or doses that can be relevant to its use in human beings.

4-Methylthioamphetamine (MTA) is a phenylisopropylamine derivative originally synthesized and evaluated as an anorectic drug more than 40 years ago [1]. Subsequently, it was demonstrated that MTA is a potent, selective and non-neurotoxic serotonin (5-HT)-releasing agent *in vitro* [2,3] and *in vivo* [4], an effect that is mediated *via* the 5-HT transporter (SERT) [5,6]. MTA gained notoriety in the late 1990s as a street drug commonly known as 'flatliner', and its use has been associated with severe intoxications and several deaths [7–10]. Even though MTA is usually regarded as a selective serotonergic agent, it also potently inhibits monoamine oxidase-A (MAO-A) [4,11], and both hyperthermia [12] and aortic contraction [13] induced by MTA in rodent models can be blocked by α-adrenergic antagonists. In addition, it has been shown that MTA induces dopamine (DA) release from rat striatal synaptosomes pre-loaded with [^{3}H]-DA [5] at concentrations that might be relevant after recreational use in human beings [8,14]. This background indicates that the overall mechanism of action of MTA may be more complex than originally thought and probably involves a catecholaminergic component. Information about neurochemical effects of MTA is relatively scarce as compared with other abused amphetamine derivatives such as methylenedioxymethamphetamine (MDMA, 'ecstasy'). Also, despite its adverse effects, some potential clinical uses, for example related to its antidepressant and pro-apoptotic activities, have been suggested for this drug [4,15,16]. Consequently, we have now further characterized the dopaminergic effects of MTA. Thus, its actions upon the DA transporter (DAT) were studied *in vitro* and *in silico*. In addition, the consequences of its *in vivo* administration on striatal DA extracellular levels as well as its possible abuse liability were assessed.

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Methods

Animals and reagents. Animals. Male Sprague-Dawley rats weighing 250–285 g (average age, 75 days) were used in all experiments. Animals were kept in a controlled environment with a 12-hr light–dark cycle and 21°C room temperature. Food and water were provided *ad libitum*. In the case of experiments involving systemic drug administration, rats were divided randomly into 2–3 groups that received injections of MTA (2.5, 5.0 or 10.0 mg/kg, i.p.) or an equivalent volume of saline solution (1 mL/kg, i.p.).

Experimental procedures were approved by the Ethics Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and followed internationally accepted guidelines (NIH Guide for the Care and Use of Laboratory Animals). All efforts were made to minimize suffering and to limit the number of animals used.

Reagents. (±)-MTA hydrochloride was synthesized as previously reported [17]. Citalopram hydrobromide was purchased from Tocris Bioscience (Ellisville, MO, USA). Dopamine, EDTA and 1-octanesulfonic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). [^{3}H]WIN 35,428 (60–87 Ci/mmol), and [^{3}H]dopamine (59 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA, USA). Cocaine hydrochloride was kindly provided by the National Institute on Drug Abuse (NIDA), USA. All other reagents used were of analytical grade.

Binding of MTA at DAT. The ability of MTA to displace a radioligand from DAT was determined *via* a competitive binding assay, using [^{3}H]WIN 35,428 and a rat striatal membrane preparation, according to previously reported procedures [18,19]. Briefly, assays were carried out in a total volume of 0.5 mL, containing 10 mM phosphate buffer (pH 7.4) containing 0.32 M sucrose, 0.1 mg rat striatal tissue (original wet weight), 1 μM desipramine, 1 μM fluoxetine (to prevent drug interactions with noradrenalin transporter or SERT, respectively), 0.5 nM radioligand and MTA at different concentrations (10^{-10}–10^{-3} M). After 2 hr in an ice bath, incubations were terminated by filtration, with three 5-mL washes of ice-cold buffer, through Whatman GF/C filters that were previously soaked in 0.05% polyethyleneimine, performed with a cell harvester (Brandel Instruments, Gaithersburg, MD, USA). Radioactivity was counted in a Packard 1300 liquid scintillation counter with an efficiency of approximately 50%. Non-specific binding of [^{3}H]WIN 35,428 was defined in the presence of 30 μM cocaine.

[^{3}H]Dopamine uptake assay. The effect of MTA on [^{3}H]DA uptake was evaluated using a crude rat striatal synaptosome preparation, according to a previously published method [20] with minor modifications. Briefly, 100 μL of synaptosomes (1.5 mg original wet weight) was added to a solution (900 μL) of Krebs phosphate buffer (pH 7.4) containing 1 mg/mL ascorbic acid, 50 μM pargyline, [^{3}H] DA (5 nM), MTA at different concentrations (10^{-10}–10^{-3} M) and 1 μM desipramine and 1 μM fluoxetine (to prevent uptake into noradrenergic or 5-HT nerve terminals, respectively). After 15 min. at 25°C, incubations were terminated by adding wash buffer (10 mM Tris-HCl/150 mM NaCl, pH, 7.4) and filtering over Whatman GF/B filters previously soaked in 0.05% polyethyleneimine, performed with a cell harvester (Brandel Instruments). Radioactivity trapped in filters was counted using the liquid scintillation counter previously described. Non-specific uptake was determined in the presence of 0.5 mM cocaine.

In vivo microdialysis. The animals were anaesthetized deeply with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, MA, USA). Body temperature was maintained at 37°C with an electric blanket controlled by a thermostat. A quarter of the initial dose of chloral hydrate was given every hour to keep the animal anaesthetized during the course of the experiments. Concentric brain microdialysis probes (Microdialysis Probe, CMA 12, 100,000 Daltons cut-off, Sweden) were implanted in the striatum using the following coordinates according to the atlas of Paxinos and Watson [21]: 1.2 mm anterior to bregma, 3.0 mm lateral and 5.5 mm ventral to brain surface. Probes were perfused with Krebs–Ringer's phosphate buffer (pH = 7.4; KRP) at a rate of 2 μL/min using a Harvard infusion pump (Model 22; Dover, MA, USA). After a stabilization period of 90 min., samples were collected every 15 min. in 3 μL of perchloric acid (0.2 M). After the fourth basal fraction, MTA was administered systemically (2.5, 5.0 or 10.0 mg/kg i.p; dissolved in saline) or intra-striatally through the probe (10 μM for 15 min.; dissolved in KRP). In two additional experimental protocols, the effects of intra-striatal MTA were evaluated in the presence of either a 5-HT or a noradrenalin uptake blocker. In these cases, citalopram (10 μM) or desipramine (10 μM) was perfused through the cannula during the whole microdialysis procedure. In all cases, perfusion samples were maintained on ice during the experiment and stored at −80°C until analysis. At the end of each experiment, animals were killed and brains quickly removed and stored in formalin. Brain sections 50 μM thick were stained with cresyl violet to verify probe location, and the placement of the probe was examined microscopically.

DA analysis. HPLC-Electrochemical determination of DA was performed as described previously [22,23]. Briefly, 10 μL of dialysates was injected into an HPLC system with the following configuration: an isocratic pump (model PU-2080 Plus, Jasco Co. Ltd., Tokyo, Japan), a UniJet microbore column (MF-8949, BAS, West Lafayette, IN, USA) and an amperometric detector set at 650 mV and 0.5 nA (model LC-4C, BAS). The mobile phase, containing 0.1 M NaH_2PO_4, 1.2 mM 1-octanesulfonic acid, 1 mM EDTA and 3.8% CH_3CN (pH adjusted to 2.8), was pumped at a flow rate of 80 μL/min. The retention time for DA was 11.0 min., and the detection limit was 0.1 fmol/μL.

c-Fos immunohistochemistry in striatum and nucleus accumbens.

Brain tissue fixation. Two hours after injection of MTA (2.5 or 5 mg/kg i.p.) or saline, rats were deeply anaesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. Thereafter, brains were removed, cut into blocks and maintained in paraformaldehyde fixative solution for 2 hr. Tissue blocks were transferred and maintained in 20% sucrose in phosphate-buffered saline (PBS) 1× during 48 hr. Then, brain blocks were sectioned in 30-μM coronal slices by means of a cryostat (Leica CM 1510, Leica Microsystems Nussloch GmbH, Nussloch, Germany).

Immunological detection. Brain coronal sections were rinsed with PBS, and then endogenous peroxidase was inactivated with 0.5% H_2O_2. Later, the sections were incubated for 1 hr in a blocking solution containing 3% normal horse serum (Invitrogen), 0.2% TritonTM x-100 and 0.02% sodium azide in PBS and then incubated overnight in 1/1000 rabbit anti-cFos (sc-7202; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Biotinylated anti-rabbit secondary antibody (Vector Laboratory Inc., Burlingame, CA, USA) was used to detect the primary antibody. Detection was made using the Vectastain ABC kit (Vector Laboratory Inc.) and diaminobenzidine as substrate (Sigma-Aldrich). Finally, the sections were mounted on glass slides with gelatin (0.1%), dried overnight, cover-slipped and observed under light microscopy. Striatum and nucleus accumbens were photographed with a 10 × objective, bilaterally from eight sections per rat. Microphotographs were transformed to 8-bit format and threshold labelling, and c-Fos-positive cells were determined using Image J software, which is free and supported by the National Institutes of Health (http://rsbweb.nih.gov/ij/). The values of c-Fos-positive cells obtained from the eight sections of each rat were averaged and expressed as the number of c-Fos-positive cells/mm^2.

Locomotor activity. Basal locomotor activity was measured in control and MTA groups as previously described [24], with minor modifications. Briefly, animals were initially habituated to the test cages for 10 min. Immediately after the administration of MTA (2.5 or 5.0 mg/kg, i.p.) or saline solution, rats were transferred to test cages (15 × 47 × 26 cm) equipped with two pairs of infrared lights placed 2.5 cm above the floor. Crossovers in the test cage were monitored every 1 min. during 90 min., using a counting device programmed to count only when both infrared light beams were interrupted consecutively.

Conditioned place preference. Apparatus. A three-compartment place preference apparatus for rats was used (Model MED-CPP-RS, Med Associates Inc., St. Albans, VT, USA). It consisted of two equally sized outer compartments (21 × 21 × 28 cm) separated by a smaller central compartment with two guillotine doors. This central compartment served as a starting point during the place preference tests and the two outer compartments as experimental contexts for drug pairing. In one of the outer compartments, the inside of the walls was painted white with a grid rod style floor, whereas the inside of the walls of the other compartment was painted black with a mesh style floor. The inside of the central compartment was entirely grey with a smooth PVC floor. Infrared detectors allowed the automatic calculation of the time spent in each compartment by a computer.

Conditioned place preference procedure. Rats were divided into two groups (drug-treated and control groups) and housed four per cage. The conditioning protocol was divided into three periods: pre-test (1 day before starting the conditioning period), conditioning (7 days) and test (24 hr after the last conditioning session). During the pre-test phase, animals were placed in the neutral-grey centre compartment with both guillotine doors open. They were allowed to explore the entire apparatus for 15 min. for adaptation to this new environment. The time the animals spent in each compartment was recorded as pre-test preference baseline. Rats showed a spontaneous preference for the black compartment during the pre-test session. Therefore, we used a procedure in which the non-preferred compartment (the white one) was associated with MTA administration, while saline injection was paired with the preferred side. During the days of conditioning, rats from the drug-treated group received MTA (5.0 mg/kg i.p.) and rats from the control group received a saline injection. Thirty minutes after the injections (a delay period based on microdialysis results), the animals were confined for 40 min. to the respective compartment in the conditioned place preference (CPP) apparatus. For the test phase, carried out 24 hr after the last conditioning session, rats were placed in the central compartment of the apparatus with both guillotine doors open and allowed free access to the lateral compartments for 15 min. The time the animals spent in each compartment during this drug-free session was recorded and used as the preference score. In all cases, after each behavioural session, rats were returned to their home cages.

Molecular simulation. DAT modelling. The crystal structure of the leucine transporter from *Aquifex aeolicus* (LeuTAa) at 1.9 Å resolution (Protein Data Bank, PDB, code 2A65) [25] was used as a template to build a rat DAT (rDAT) model. The amino acid sequences of rDAT and a series of other solute carrier (SLC)6 gene family transporters were extracted from the National Center for Biotechnology Information (NCBI), and the crystal structure and amino acid sequence of LeuTAa were obtained from the PDB. The target protein and the template were aligned through a multiple alignment of different species sequences and by function. Then, manual modifications were introduced to align some amino acids that are conserved in the SLC6 family of transporters. MODELLER9v6 [26] was used to construct 3D models of rDAT. One hundred runs of MODELLER were carried out using standard parameters, and the outcomes were ranked on the basis of the internal scoring function of the program. The best model was chosen for further work. Two sodium atoms were placed in the rDAT model using the corresponding LeutAa crystal coordinates, and their positions were manually adjusted to maintain coordination bonds established with adjacent residues. The transporter modelled was submitted to the H++ server [27] to compute pKa values of ionizable groups and to add missing hydrogen atoms according to the specified pH of the environment. The transporter was then inserted into a POPC membrane, TIP3 solvated and ions were added creating an overall neutral system in approximately 0.02 M NaCl. The ions were equally distributed in a water box. The final system, which contained approximately 320,000 atoms, was subjected to a molecular dynamics (MD) simulation for 5 ns using NAMD 2.6 [28]. The NPT ensemble was used to perform MD calculations. Periodic boundary conditions were applied to the system in the three coordinate directions. A pressure of 1 atm was used, and temperature was kept at 310 K. The simulation time was sufficient to obtain an equilibrated system (RMSD < 2 Å).

Model evaluation involved analysis of geometry, stereochemistry and energy distribution. Firstly, the Visual Molecular Dynamics (VMD) program [29] was used to evaluate the 3D structure distribution and general physical chemistry characteristics. Then, stereochemical and energetic quality of the homology model were evaluated using PROSAII [30] and ANOLEA [31] servers and Procheck [32]. Finally, the potential rDAT binding sites were analysed using the ICMPocketFinder module from ICM [33].

Molecular docking. Dockings of (*S*)-MTA and DA, both on their protonated form, in the rDAT model, were performed with the AutoDock 4.0 suite [34]. The choice of the (*S*)-isomer for MTA docking experiments was made on the basis that, in most cases, (*S*)-amphetamine derivatives (which are always dextrorotatory) are the eutomers at the DAT [35]. In general, the grid maps were calculated using the autogrid4 option and were centred on the putative ligand-binding sites. The volumes chosen for the grid maps were made up of 50 × 50 × 50 points, with a grid-point spacing of 0.375 Å. The autotors option was used to define the rotating bond in the ligand and in flexible residues (see below). In the Lamarckian genetic algorithm dockings, an initial population of random individuals with a population size of 50 individuals, a maximum number of 1.5×10^6 energy evaluations, a maximum number of 27,000 generations, a mutation rate of 0.02 and a crossover rate of 0.80 were employed. The docked compound complexes were built using the lowest docked-energy binding positions. (*S*)-MTA and DA were built using Gaussian03, and the partial charges were corrected using ESP methodology. As both compounds showed similar binding modes, we further refined the simulation by performing a flexible docking protocol in which rotatable bonds, and amide torsions were allowed for all residues at 3 Å from the Asp79 and Tyr156. In these experiments, WIN 35,428, also known as β-CFT (2β-carbomethoxy-3β-(4-fluorophenyl)-tropane), which was built in the same conditions used for MTA and DA, was included for comparative purposes.

Statistical analysis. In all cases, results are presented as means ± S.E.M.

For binding and uptake experiments, inhibition curves were fitted performed with the sigmoidal dose–response curve (variable slope) equation built into GraphPad PRISM 5.01 (GraphPad Software Inc., San Diego, CA, USA). The IC_{50} presented corresponds to the results of 4–5 independent experiments, each in triplicate.

In the case of microdialysis studies, femtomoles of DA (uncorrected for recovery) were measured and values are presented as percentages of basal levels. Under the conditions used, the basal level of DA in the striatum was 2.35 ± 0.08 fmol/μL (n = 12). In experiments where citalopram or desipramine was included in the perfusion fluid, the DA basal level was 2.21 ± 0.10 fmol/μL (n = 6; $p > 0.05$ compared with basal levels in animals without citalopram) or 2.38 ± 0.28 (n = 4; $p > 0.05$ compared with basal levels in animals without desipramine), respectively.

In microdialysis, behavioural and immunohistochemical experiments, statistical analysis was performed using one-way ANOVA followed by the Newman–Keuls test.

Results

Binding of MTA to DAT and [^{3}H]DA uptake experiments. As shown in table 1, MTA binds to DAT and inhibits [^{3}H]DA uptake with an IC_{50} and potency, respectively, in the low

Table 1.
Comparative potencies of MTA at the rDAT or rSERT, assessed by displacement of selective transporter ligands and inhibition of the corresponding substrate uptake.

Transporter	IC_{50} on [^{3}H]ligand binding (μM)	IC_{50} on [^{3}H] monoamine uptake (μM)	Binding/uptake potency ratio
rDAT	6.01 ± 1.44	4.88 ± 1.12	1.2
rSERT	1.04[1]	0.10[1]	10

[1]Data from the study by Gobbi *et al.* [5] in which [^{3}H]citalopram was used as a SERT ligand and [^{3}H]5-HT was used as a SERT substrate.

micromolar range. These values are independent of MTA effects on 5-HT or noradrenalin transporters, and they agree very well with those reported for [^{3}H]DA uptake inhibition [2] and [^{3}H]DA release [5], as evaluated in similar experimental preparations. Previously reported values of MTA IC_{50} at rSERT as well as that for [^{3}H]5-HT uptake inhibition are included in table 1 for comparative purposes (see 'Discussion').

In vivo *microdialysis experiments*.
Fig. 1A shows that MTA produced a significant, dose-dependent increase in extracellular levels of DA in the striatum, after systemic administration. As seen in fig. 1B, intra-striatal perfusion of MTA (10 μM) also elicited a significant elevation in dialysate levels of DA. It should be noted that in view of the structural similarities between MTA and DA, one may assume that probe recovery for MTA would be similar to that for DA (5–10%). Therefore, an MTA concentration of 10 μM in the perfusion fluid would be expected to be approximately 1 μM in the proximity of the cannula.

As MTA is a potent 5-HT-releasing agent and could also affect noradrenalin transport and release, these actions might be contributing to the dopaminergic effects observed. Consequently, we assessed the effect of MTA upon DA levels in the presence of citalopram, a selective SERT blocker or desipramine, a selective noradrenalin transporter blocker. As shown in fig. 1B, the presence of either of these uptake blockers in the perfusion fluid did not modify the MTA-induced increase in striatal DA.

c-Fos immunohistochemistry.
As shown in fig. 2, the systemic administration of MTA (2.5 or 5 mg/kg) induced a significant, dose-dependent increase in the number of c-Fos-positive cells in the striatum. Interestingly, this effect was also observed in the nucleus accumbens, a limbic nucleus receiving dopaminergic input. Representative photomicrographs of c-Fos expression in both nuclei after MTA administration are provided in fig. S1.

Behavioural assessment.
Based on neurochemical and immunohistochemical results, variations on locomotor activity and possible rewarding effects of MTA were behaviourally assessed. Consistent with previous qualitative observations [12], the injection of MTA (2.5 and 5 mg/kg, i.p.) induced a significant increase in horizontal locomotor activity (fig. 3A).

In addition, a significant preference for the compartment associated with MTA administration (5 mg/kg, i.p.) was observed in the CPP paradigm (fig. 3B). This result indicates that MTA possesses rewarding properties.

Molecular docking.
In an effort to gain insight regarding the molecular interactions underlying the dopaminergic effects of MTA, a homology model of rDAT was built and MTA was docked into it. After analysis of the model with ICMPocketFinder, two potential binding sites for MTA were detected in rDAT (fig. 4A).

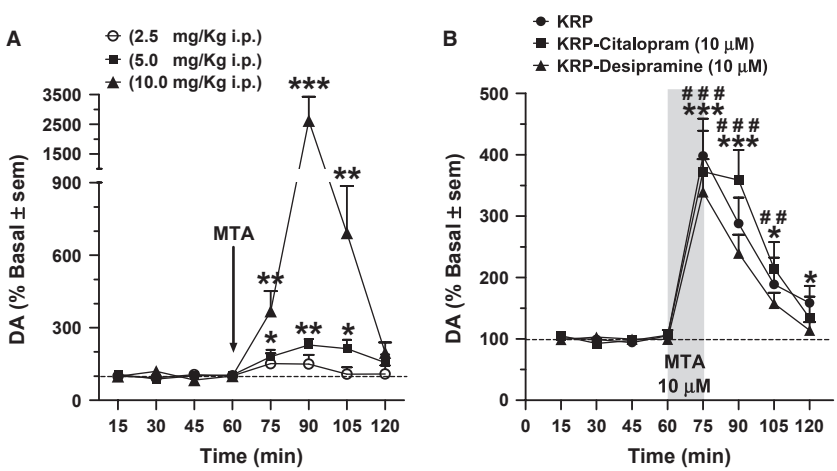

Fig. 1. Effects of MTA on extracellular DA levels in the striatum after systemic (A, n = 4 for each condition) or intra-striatal (B) administration. The grey bar (B) indicates the time during which MTA (10 μM) was perfused. In fig. 1A and B, asterisks (or the symbol #) indicate a significant difference (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) when comparing the effect of MTA with respective basal levels (one-way ANOVA followed by the Newman–Keuls multiple comparison test). Note that no significant differences were observed when comparing different treatments. KRP, Krebs–Ringer's phosphate buffer.

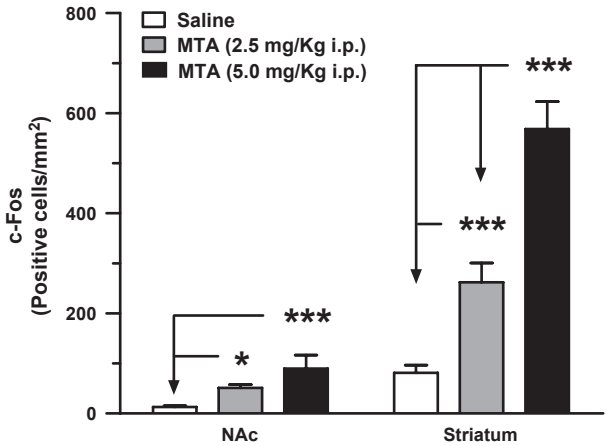

Fig. 2. Comparison of the effects of systemic MTA (2.5 or 5.0 mg/kg i.p.; n = 5 and 4, respectively) and saline (n = 8) upon c-Fos expression in the striatum and nucleus accumbens (NAc). * = $p < 0.05$ and *** = $p < 0.001$ (one-way ANOVA followed by the Newman–Keuls multiple comparison test).

The inner site (S1) was located in a position analogous to that occupied by leucine in the LeuTAa crystal (fig. S2). MTA and DA exhibited similar binding modes in this inner site. It should be noted that DA docked at this site with a similar orientation to that described in a previous report [36]. The other binding site (S2) was found in a location closer to the extracellular side of the transporter, which roughly coincides with the binding site of tricyclic antidepressants [37,38] and of the detergent *n*-octyl-β-D-glucopyranoside [39] in LeuTAa (fig. S2). Fig 4B and C show representative energy-minimized poses of rDAT model/MTA complex after docking of MTA at S1 and S2, respectively. At S1 (fig. 4B), MTA appeared in a position in which its amino group (protonated) generates an electrostatic interaction with Asp79 (located 1.9 Å from this residue). MTA binding is further stabilized by a hydrogen bond established with Phe319. Phe319 and Phe325 contribute to the hydrophobicity of this cavity, and their aromatic rings are oriented in such a way as to establish π interactions with the MTA aromatic group. Interestingly, the backbone carbonyl oxygen of Ala77 appears located 1.9 Å from the NH_3^+ group, which may further stabilize this binding mode. At S2 (fig. 4C), docking experiments revealed that MTA exhibits a binding mode where the amino group establishes an electrostatic interaction with Asp475. In addition, the centroids of Tyr469 and the ligand aromatic rings are separated by 4 Å, whereas Phe319 is positioned at 4 Å from the MTA sulphur atom, generating dispersive interactions that could contribute to the stabilization of MTA in this binding site.

Discussion

In the present study, we demonstrate that MTA is able to induce an increase in extracellular striatal DA levels and that the drug has rewarding effects. Furthermore, our results suggest that these effects are mediated, at least in part, by the interaction of MTA with the DAT.

Initial reports on the recreational use of MTA in human beings appeared in the late 1990s [7,40,41]. Its apparent slow onset of action and longer-lasting effects as compared for example with MDMA [42] might be a factor for the use of relatively high doses of the drug. This had been associated with several fatal and non-fatal intoxications, and in the cases where blood samples were measured, concentrations of MTA ranged from 0.7 to 28.9 μM [8,9,14,43]. Even though blood levels of MTA found in clinical determinations cannot be straightforwardly extrapolated to actual concentrations in the

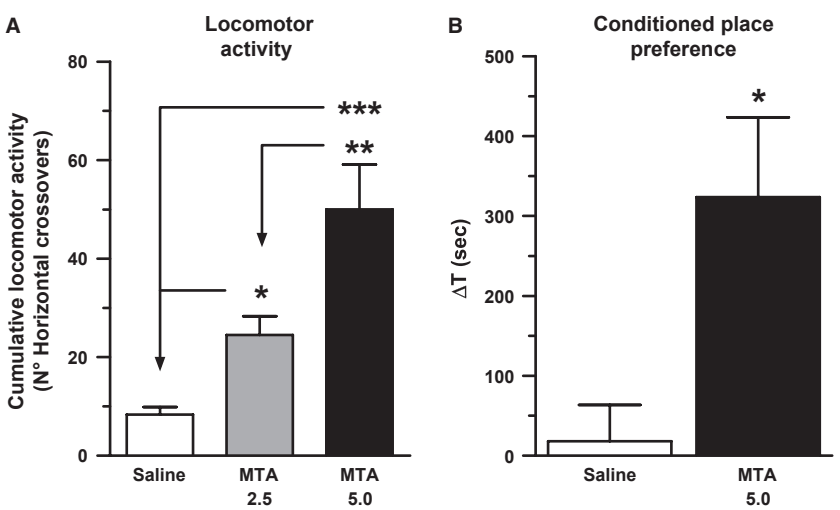

Fig. 3. Effect of acute administration of MTA (2.5 mg/kg i.p.; n = 6 or 5.0 mg/kg i.p.; n = 4) on locomotor activity (A) and of repeated MTA (5.0 mg/kg i.p.) administration (B) on conditioned place preference behaviour (n = 8 for both saline control and MTA treated groups). Time difference (ΔT) in fig. 3B was calculated as follows: time spent (in seconds) in MTA or saline-paired compartment post-conditioning (test) – time spent in the same compartment before conditioning (pre-test). Saline control rats in fig. 3A were n = 11. The statistical analysis for fig. 3A and B was performed by a one-way ANOVA followed by the Newman–Keuls (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) and unpaired *t*-test (*$p < 0.05$), respectively.

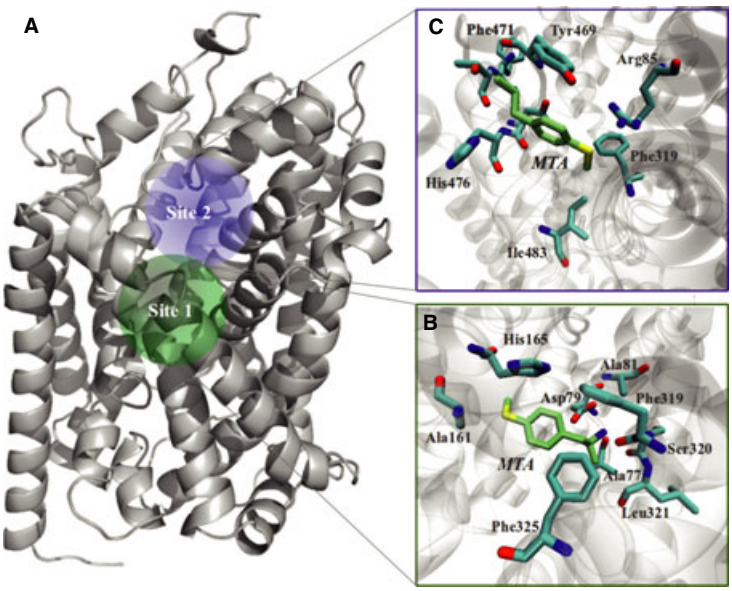

Fig. 4. Ribbon diagram of the rDAT model generated showing the putative binding sites of MTA (A). B and C show docking poses of MTA (green) at S1 and S2, respectively. Main active site aminoacid residues (cyan) are rendered as stick models.

brain, they give a reasonable range of possible MTA brain concentrations. In this context, it is of particular significance that dopaminergic effects of MTA were observed in rats at intra-striatal concentrations or systemic doses that could be relevant to its use in human beings.

In agreement with original reports on the MTA mechanism of action [2,3], MTA exhibited an *in vitro* affinity for rDAT (defined here as the ability to displace a selective ^{3}H-labelled transporter ligand) 5–6 times lower than that reported for rSERT [5] (table 1). Accordingly, the dopaminergic neurochemical effects we observed *in vivo* at doses of MTA of 5 mg/kg or lower were, although significant, much weaker than its effects on 5-HT. Thus, while a dose of 5 mg/kg induced an approximately 1.5 times striatal DA increase (this work), the same dose increases the extracellular concentration of hippocampal 5-HT more than 20 times, measured under similar conditions [4]. Interestingly, when a higher dose of MTA (10 mg/kg) was administered, a much stronger effect (>20 times increase) upon striatal DA levels was observed. Beyond possible mechanistic implications (see below), this abrupt rise of extracellular DA concentrations further supports the notion that a dopaminergic component should be considered when assessing the overall effects of MTA, particularly at relatively high doses.

As mentioned, the potent 5-HT-releasing action of MTA is related to its being a SERT substrate [44]. As some dopaminergic effects of compounds with similar properties (e.g. MDMA) have been shown to be mediated by the activation of 5-HT receptors [45,46], a possible serotonergic influence on the effects of MTA on striatal DA was evaluated by concomitantly perfusing the 5-HT uptake blocker citalopram. In addition, as some of the effects of MTA upon DA extracellular levels might be mediated by its actions upon noradrenergic transmission, drug effects were evaluated in presence of the noradrenalin uptake blocker desipramine. The lack of effect of either citalopram or desipramine on the MTA-induced increase in DA indicates that this MTA effect is largely independent of its actions on 5-HT and/or noradrenalin transport. As MAO-A inhibitory properties have been reported for MTA [11], further studies are necessary to evaluate the possibility that part of the effects of MTA are related to MAO-A inhibition.

Our immunohistochemical results demonstrated that MTA administration enhances neuronal activity in both the striatum and nucleus accumbens. This suggests that MTA also increases DA levels in the latter region and that therefore it might have rewarding properties. This hypothesis was behaviourally tested, and similarly to other abused amphetamine derivatives, MTA increased locomotor activity and induced CPP. Altogether, our results document reward-like effects of this recreational drug. This finding adds a further concern regarding MTA misuse in human beings and underlines the need for additional work on monoamine-releasing agents [10].

Even though, by definition, theoretical simulations yield speculative information, the two-pocket arrangement found for the rDAT is in accordance with the existence of two substrate binding sites experimentally found in LeuTAa [47]. In addition, both cavities were positioned in regions similar to those recently proposed as possible binding sites for DA (and other substrates and blockers) in the human DAT [48]. In general terms, the binding interactions proposed here for MTA in the rDAT are in good agreement with those suggested for structurally similar DAT substrates such as DA or amphetamine at S1 in the rDAT [36] and S2 in the human DAT [48,49]. Interactions between the ligand amino group and Asp79 and Asp475 (Asp476 in the human DAT) seem to be of particular importance for MTA binding at S1 and S2, respectively. This observation agrees with the role of both acidic residues in the

recognition of DAT substrates and blockers, as demonstrated by mutagenesis studies [50].

Regarding the possible mechanism of action of MTA, the IC_{50} values for radioligand displacement and for [^{3}H]DA uptake inhibition are very similar, yielding a binding/uptake ratio $\approx$ 1.2 (table 1). On the other hand, it has been shown that MTA is about 10 times more potent inhibiting [^{3}H]5-HT uptake than displacing [^{3}H]citalopram binding, as assessed using a rat hippocampal synaptosome preparation [5]. As discussed by Schmitt *et al.* [48] (and references therein), these differential potencies on binding/uptake inhibition are an indication of whether a ligand is a transporter substrate or a blocker. In this context, our results agree with the evidence showing that MTA is a substrate of SERT, while suggesting that it might be a DAT blocker. However, using superfused striatal synaptosomes (an experimental model which dissociates releasing effects from uptake inhibition), Gobbi *et al.* [5] have unequivocally shown that MTA behaves as a DA-releasing agent at concentrations of 10 μM or higher.

To theoretically evaluate this issue, we performed flexible docking experiments of MTA and DA at S1. The distance between Asp79 and Tyr156 after the docking of a given ligand has been proposed as indicative of the nature of its interaction with the DAT [48,51]. Our simulation study revealed that after docking of DA or MTA, the distances between these residues (as measured between the respective side-chain oxygen atoms) were 1.9 Å and 3.2 Å, respectively, which are indicative of a preserved hydrogen bond (fig. S3). On the other hand, docking of WIN 35,428, which was included to compare our docking results with those of previous studies [48,51], resulted in a distance of 4.2 Å, which is greater than the maximum distance (3.5 Å) proposed for a hydrogen bond (fig. S3). Therefore, these data also support the view that MTA could be a DAT substrate.

These experimental (binding/superfusion) and theoretical (flexible docking) observations agree with the profile we observed in microdialysis studies, where the effect of MTA on DA levels at 5 mg/kg resembles that of uptake blockers, while the effect at 10 mg/kg is more similar to that produced by DA-releasing agents [52]. In this context, it is tempting to speculate that the mechanism of action of MTA at the DAT might switch from an uptake blockade at low concentrations to a substrate-like behaviour at higher concentrations. Recent experimental and computational studies [47,49] have shown that transport by the DAT (and LeuTAa) requires not only the presence of the substrate in S1, but also the binding of a second substrate molecule at the more external S2. This latter event would trigger a series of conformational transitions of the protein, leading to the intracellular release of the substrate initially bound at S1 [49]. Based on these findings, we propose that MTA could block DAT by binding with higher affinity at S1 but, as the drug concentration increases, the binding of a second MTA molecule at S2 might trigger both the transport of the compound and the efflux of DA. Further experiments are necessary to test this hypothesis.

In conclusion, neurochemical effects on DA and their functional consequences have been demonstrated *in vivo* for MTA. Even though MTA can still be considered as a predominantly serotonergic drug, it also can modify dopaminergic neurotransmission, and this relative promiscuity should be considered while assessing the global effects of the drug, particularly when used by human beings.

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Conflict of interest

The authors of this paper declare that they have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative photomicrographs of c-Fos expression in the striatum (left column) and nucleus accumbens (right column) after administration of saline (A–D) or 2.5 (B–E) and 5.0 mg/kg i.p. (C–F) of MTA. Diagrams above show the areas where c-Fos-positive cells were counted in the striatum and nucleus accumbens (square and oval areas, respectively). Bar = 350 μM.

Fig. S2. Superimposition of the crystal structure of LeuTAa (grey) in complex with leucine (green) and imipramine (yellow) (PDB code 2q72) with the model of the rDAT (tan) with dopamine (blue) and MTA (red) docked at S1 and S2.

Fig. S3. Docking poses of DA (A), MTA (B) and WIN 35,428 (C) at S1. Residues Asp79 and Tyr156 as well as the distance between them are shown in all cases.

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