European Neuropsychopharmacology (2011) xx, xxx–xxx

www.elsevier.com/locate/euroneuro

Interaction of mephedrone with dopamine and serotonin targets in rats

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Received 20 May 2011; received in revised form 3 July 2011; accepted 6 July 2011

KEYWORDS
Mephedrone;
Dopamine;
Serotonin

Abstract

Introduction: We described a first approach to the pharmacological targets of mephedrone (4-methyl-methcathinone) in rats to establish the basis of the mechanism of action of this drug of abuse. *Experimental procedures:* We performed *in vitro* experiments in isolated synaptosomes or tissue membrane preparations from rat cortex or striatum, studying the effect of mephedrone on monoamine uptake and the displacement of several specific radioligands by this drug. *Results:* In isolated synaptosomes from rat cortex or striatum, mephedrone inhibited the uptake of serotonin (5-HT) with an IC_{50} value lower than that of dopamine (DA) uptake (IC_{50} = 0.31 ± 0.08 and 0.97 ± 0.05 μM, respectively). Moreover, mephedrone displaced competitively both [^{3}H] paroxetine and [^{3}H]WIN35428 binding in a concentration-dependent manner (Ki values of 17.55 ± 0.78 μM and 1.53 ± 0.47 μM, respectively), indicating a greater affinity for DA than for 5-HT membrane transporters. The affinity profile of mephedrone for the 5-HT_2 and D_2 receptors was assessed by studying [^{3}H]ketanserin and [^{3}H] raclopride binding in rat membranes. Mephedrone showed a greater affinity for the 5-HT_2 than for the D_2 receptors. *Discussion:* These results provide evidence that mephedrone, interacting with 5-HT and DA transporters and receptors must display a similar pattern of other psychoactive drugs such as amphetamine-like compounds.

1. Introduction

In the last years, a decrease in the availability of chemical compounds used in the synthesis of amphetamine derivatives, mainly 3,4-methylenedioxymethamphetamine or ecstasy, associated with also a decrease (by more than 50%) in the purity of ecstasy pills (Measham et al., 2010; Winstock et al., 2011), lead to the appearance in the illicit market of a new

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doi:10.1016/j.euroneuro.2011.07.009

Please cite this article as: Martínez-Clemente, J., et al., Interaction of mephedrone with dopamine and serotonin targets in rats, Eur. Neuropsychopharmacol. (2011), doi:10.1016/j.euroneuro.2011.07.009

generation of designer drugs, known as "legal highs" or "beta-keto (bk)" since until 2010 these compounds could buy on-line, are legal to use or possess and supply and are characterized by the presence of a ketone in the side chain. Mephedrone (4-methyl-methcathinone) (with street names such as "miaow miaow") and other cathinone derivatives were banned in Scandinavian countries in 2009 and later (April 2010) in UK and classified as Class B, Schedule I under the UK's Misuse of Drugs Act 1971. Following this, other European countries also banned these derivatives.

Mephedrone has comparable abuse potential to cocaine or ecstasy (McElrath and O'Neill, 2011). Based in its chemical structure it is likely to postulate that this drug acts as a psychoactive compound that elicits stimulant effects similar to amphetamine derivatives (Schifano et al., 2011). However, very little is known about the pharmacology of mephedrone. Cozzi et al. (1999) performed in vitro studies with methcathinone and methylone (cathinone derivatives) and confirm that the main mechanism of action could be similar to that of amphetamine. Moreover, methylone is able to bind to noradrenalin, dopamine and serotonin transporters (Nagai et al., 2007).

Mephedrone is expected to act as a central nervous system stimulant. The limited information available comes from user self-reports, although these are unsubstantiated with no toxicological analysis to confirm mephedrone use.

To date, only a very recent paper of Kehr et al. (2011) studied the psychostimulant effect of mephedrone using a microdyalisis technique. The aim of this paper is to characterize *in vitro* some pharmacological targets of mephedrone in rats in order to establish the basis of the mechanism of action of this cathinone derivative and hypothesize about its effects in humans.

2. Experimental procedures

2.1. Animals

The Experimental protocols were approved by the Animal Ethics Committee of the University of Barcelona, following the guidelines of the European Community Council (86/609/EEC). Male Sprague–Dawley rats (Janvier, France) weighing 225–250 g were used. Animals were housed at 22 ± 1 °C under a 12-h light/dark cycle with free access to food and drinking water.

2.2. Drugs

Mephedrone was synthesized by us in the Laboratory of Organic Chemistry of our Department, under permission of our University, according to those described by Camilleri et al. (2010). D-amphetamine, aprotinin, ascorbic acid, bupropion, clomipramine, fluoxetine, ketanserin, methysergide, pargyline, phenylmethylsulfonyl fluoride, pindolol, sodium orthovanadate and sulpiride were from Sigma-Aldrich. Cocaine was provided by the National Health Laboratory (Barcelona, Spain). [^{3}H]DA, [^{3}H]5-HT, [^{3}H]ketanserin, [^{3}H]paroxetine, [^{3}H]raclopride and [^{3}H]WIN35428 were from Perkin Elmer Life Sci. All buffer reagents were of analytical grade.

2.3. Tissue membrane and synaptosome preparation

Animals were killed by decapitation under isoflurane anaesthesia and the striatum and cortex were quickly dissected out, frozen on dry ice and stored at −80 °C until later use. When required, samples were homogenized in buffer with a Polytron homogenizer. The homogenates were centrifuged at 15,000×*g* for 30 min at 4 °C. The resulting pellets were washed twice and resuspended in the appropriate buffer and stored at −80 °C for use in radioligand binding experiments Moreover, pure synaptosomes suspensions were obtained as described previously (Pubill et al., 2005; Chipana et al., 2006).

2.4. Serotonin and dopamine uptake

To obtain evidence of the direct blockade of [^{3}H]5-HT uptake in the presence of mephedrone, synaptosomes from the cortex were prepared as described above and the protein content was equivalent to 10 mg of tissue (wet weight) per ml. Reaction tubes consisted of 0.85 ml of mephedrone at different concentrations in buffer and 0.1 ml of synaptosome suspension. Tubes were warmed 10 min at 37 °C before the addition of 0.05 ml of [^{3}H]5-HT (15 nM), after which incubation was carried out for a further 5 min. The reaction was stopped by rapid filtration under vacuum through Whatman GF/B glass fibre filters. Filters were washed rapidly 3 times with 4 ml ice-cold 50 mM Tris–HCl. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Non-specific uptake was determined at 4 °C in parallel samples containing fluoxetine (10 μM), ketanserin (10 nM) and pindolol (0.5 nM).

Similarly, to obtain evidence of the direct blockade of [^{3}H]DA uptake, synaptosomes from the striatum were prepared. The experiments were carried out as described above, using a concentration of [^{3}H]DA of 5 nM. Non-specific uptake was determined at 4 °C in parallel samples containing cocaine 100 μM. Competitive blockade of [^{3}H]DA uptake was assessed in the presence of mephedrone at different concentrations.

Previous studies (Hrometz et al., 2004) demonstrated that DA can enter 5-HT neuron terminal through the 5-HT transporter. To measure this uptake, experiments were carried out as above, but using synaptosomes from the rat cortex and a final concentration of [^{3}H]DA of 5 nM. In these experiments both [^{3}H]DA and mephedrone at different concentrations were present in the medium. D-amphetamine (1 μM) was also present in the medium to assess that [^{3}H]DA uptake was carried out only by the 5-HT transporter.

2.5. Interaction with 5-HT and DA transporters

Competition [^{3}H]paroxetine binding experiments were carried out using the membrane preparations from rat cortex. These experiments were performed in tubes containing 0.05 nM [^{3}H]paroxetine, mephedrone at increasing concentrations, and 150 μg of brain membranes. Incubation was carried out at 25 °C for 2 h in a Tris–HCl buffer to a final volume of 1.6 ml. Clomipramine (100 μM) was used to determine non-specific binding.

Competition [^{3}H]WIN35428 binding experiments were carried out using the membrane preparations from rat striatum. Binding assays were performed in tubes containing 200 μl of [^{3}H]WIN35428 (5 nM), mephedrone at increasing concentrations and 50 μl of membrane suspension (1 μg/ml). Incubation was performed for 2 h at 4 °C and non-specific binding was determined in the presence of 30 μM bupropion.

2.6. Interaction with 5-HT and DA receptors

Competition [^{3}H]ketanserin binding experiments were carried out using the membrane preparations from rat cortex. These experiments were performed in tubes containing 1 nM [^{3}H]ketanserin, mephedrone at increasing concentrations, and 100 μg of brain 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.

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Competition [^{3}H]raclopride binding experiments were carried out using the membrane preparations from rat striatum. These experiments were performed in tubes containing 2 nM [^{3}H]raclopride, mephedrone at increasing concentrations, and 50 μg of brain membranes. Incubation was carried out at 25 °C for 1 h in a Tris–HCl buffer to a final volume of 0.5 ml. Sulpiride (300 μM) was used to determine non-specific binding.

2.7. Statistics

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using two-tailed one-way analysis of variance (ANOVA). Significant ($p<0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons, where appropriate. Competition binding curves were plotted and calculated by nonlinear regression analysis using GraphPAD Prism software The Ki values were calculated using the equation by Cheng and Prusoff: $Ki = IC_{50}/(1+(L/K_D))$, where L is the total radioligand concentration and K_D is the dissociation constant of the radioligand.

3. Results

3.1. Effect of mephedrone on 5-HT uptake

Mephedrone (10^{-8} to 10^{-4} M), inhibited competitively 5-HT transporter function, in a concentration-dependent manner (Fig. 1A), with an IC_{50} value of 0.31 ± 0.08 μM. To evaluate the long-term effects of mephedrone on 5-HT uptake, rat synaptosomes were pre-incubated with different concentrations of mephedrone for 1 h. After this, mephedrone was removed from the synaptosomal preparation and [^{3}H]5-HT uptake was measured as described. In these experimental conditions, mephedrone (from 1 to 1000 μM) did not significantly inhibit [^{3}H]5-HT uptake.

3.2. Effect of mephedrone on DA uptake

Mephedrone (10^{-8} to 10^{-4} M) inhibited [^{3}H]DA uptake in a concentration-dependent manner (Fig. 1B), with an IC_{50} value of 0.97 ± 0.05 μM. As above, to evaluate the long-term effects of mephedrone on DA uptake, after 1 h of pre-incubation with different concentrations of mephedrone, this was removed and the [^{3}H]DA uptake was then measured. In these experimental conditions, mephedrone (from 1 to 1000 μM) did not significantly inhibit [^{3}H]DA uptake.

3.3. Effect of mephedrone on DA uptake by the 5-HT transporter

[^{3}H]DA at a concentration of 5 nM enters the dopaminergic terminal through the 5-HT transporter (full inhibition was found with fluoxetine 1 μM). This uptake was concentration-dependent inhibited by mephedrone (10^{-6} to 10^{-3} M). At the highest concentration tested, mephedrone inhibited about 40% of DA uptake (Fig. 2).

3.4. Interaction of mephedrone with the 5-HT and DA transporters

Mephedrone (10^{-7} to 10^{-4} M) displaced [^{3}H]paroxetine binding in a concentration-dependent manner (Fig. 3A). This displacement occurred with a Ki value of 17.55 ± 0.78 μM; Hill coefficient = 0.72 ± 0.04, $p<0.05$). Similarly, mephedrone (10^{-7} to 10^{-4} M) also displaced the [^{3}H]WIN35428 bound in a concentration-dependent manner (Fig. 3B). This displacement occurred with a Ki value in the low micromolar range, (Ki = 1.53 ± 0.47 μM; Hill coefficient = 0.93 ± 0.04, ns *vs.*1).

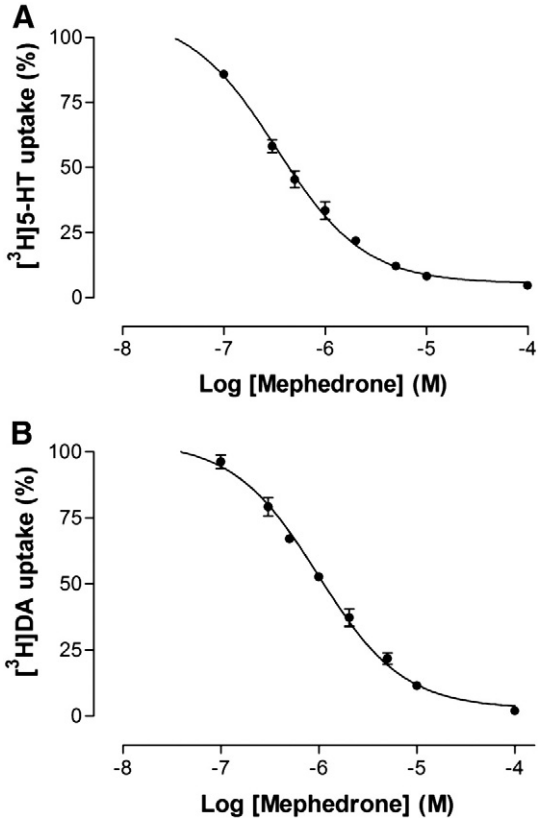

Figure 1 Panel A: Effect of different concentrations of mephedrone on [^{3}H]5-HT (15 nM) uptake in rat synaptosomes. Non-specific [^{3}H]5-HT uptake was determined at 4 °C in parallel samples containing 10 μM fluoxetine. Panel B: Effect of different concentrations of mephedrone on [^{3}H]DA uptake in rat synaptosomes. Non-specific [^{3}H]DA uptake was determined at 4 °C in parallel samples containing 100 μM cocaine. Data represent mean ± S.E.M. of duplicates and the experiments were performed in triplicate.

3.5. Interaction of mephedrone with the 5-HT_2 and D_2 receptors

The affinity of mephedrone for the 5-HT_2 receptors was assessed by studying [^{3}H]ketanserin binding in rat cortical membranes. Mephedrone (10^{-7} to 5×10^{-4} M) displaced [^{3}H]ketanserin binding in a concentration-dependent manner (Fig. 4A). This displacement occurred with a Ki value in the low micromolar range (Ki = 3.96 ± 0.22 μM; Hill coefficient = 0.77 ± 0.03, $p<0.05$).

The affinity profile of mephedrone for D_2 receptors was assessed by studying the displacement of [^{3}H]raclopride binding in rat striatal membranes. The results demonstrate that mephedrone (10^{-7} to 10^{-4} M) also displaced the [^{3}H]raclopride bound in a concentration-dependent manner (Fig. 4B). This

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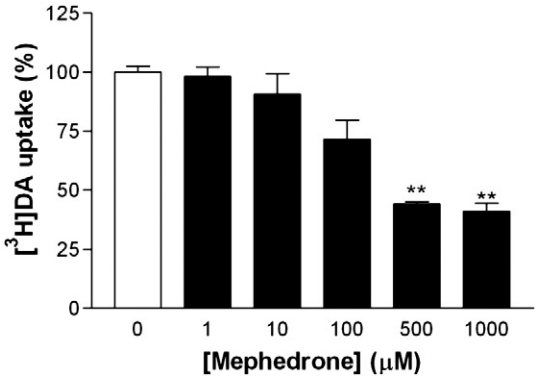

Figure 2 Effect of different concentrations of mephedrone on [^{3}H]DA (5 nM) uptake by the 5-HT transporter in isolated synaptosomes from rat cortex. Data are expressed as mean ± S.E.M. from 7 to 10 different experiments. ** $p<0.01$ *vs*. saline.

displacement occurred with a Ki value in the micromolar range (Ki = 50.86 ± 3.45 μM; Hill coefficient = 0.83 ± 0.21, $p=0.05$).

4. Discussion

Due to the chemical similarity of mephedrone to amphetamines and its use as an alternative to these drugs, a stimulant effect of the so-called beta-keto designer drugs could be postulated. In the present study we have synthesized pure mephedrone (yield of about 98%) as racemate, because is the form reported by users.

We attempt to characterize the pharmacological targets of mephedrone that could be involved in its psychostimulant effect. Methamphetamine produces an increase in the dopaminergic transmission in the nucleus accumbens that is directly related to the blockade of DA uptake and non-exocytotic transporter-mediated DA release (Escubedo et al., 2005). MDMA shares this hyperdopaminergic property but has been found that it shows a higher affinity for the 5-HT than for the DA transporter, blocking 5-HT uptake and inducing 5-HT

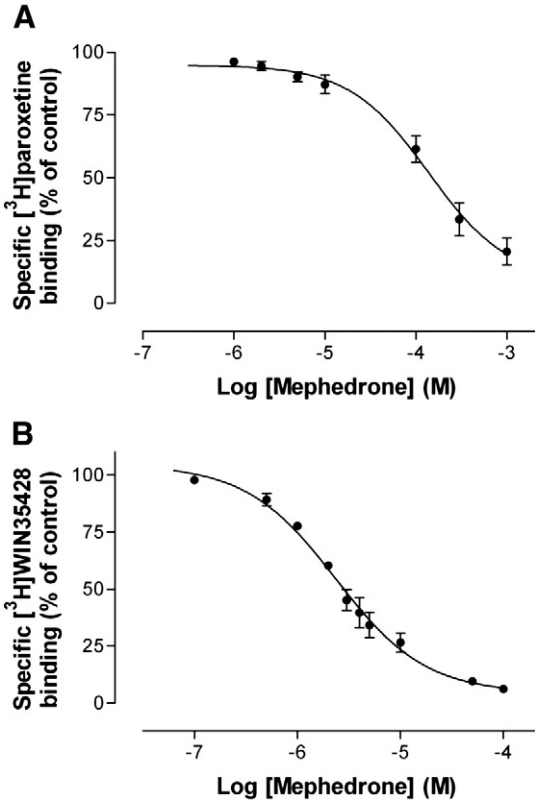

Figure 3 Panel A shows the representative competition curves of the inhibition of [^{3}H]paroxetine binding by mephedrone in cortical membranes from Sprague–Dawley rats. Membranes were incubated at 25 °C for 2 h with 0.05 nM [^{3}H]paroxetine in the presence of increasing concentrations of mephedrone. Panel B shows the inhibition of [^{3}H]WIN35428 binding by of mephedrone in striatal rat membranes incubated for 2 h at 4 °C with 5 nM [^{3}H] WIN35428 also in the presence of increasing concentrations of mephedrone. Inhibition curves were calculated using the nonlinear least squares method and adjusted to a one-site model. Data represent mean ± S.E.M. of duplicates and the experiments were performed in triplicate.

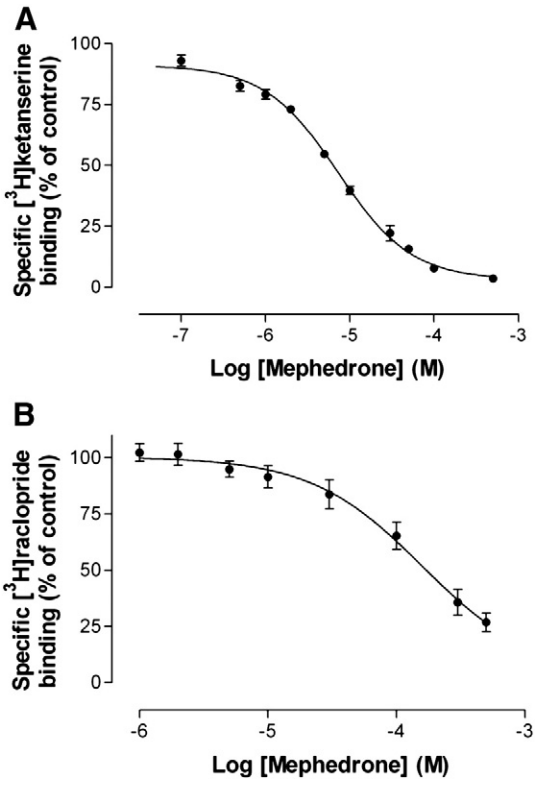

Figure 4 Panel A shows the representative competition curves of the inhibition of [^{3}H]ketanserine binding by mephedrone in cortical membranes from Sprague–Dawley rats. Membranes were incubated at 25 °C for 2 h with 1 nM [^{3}H]ketanserine in the presence of increasing concentrations of mephedrone. Panel B shows the inhibition of [^{3}H]raclopride binding by mephedrone in striatal rat membranes incubated for 2 h at 4 °C with 2 nM [3H]raclopride also in the presence of increasing concentrations of mephedrone. Inhibition curves were calculated using the nonlinear least squares method and adjusted to a one-site model. Data represent mean ± S.E.M. of duplicates and the experiments were performed in triplicate.

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release (Capela et al., 2009). Consequently, we have studied the interaction of mephedrone with 5-HT and DA targets.

In the present study, [^{3}H]5-HT uptake in synaptosomes from rat cortex and [^{3}H]DA uptake in rat striatal synaptosomes were measured as indicative of an indirect serotonergic and dopaminergic effect (Chipana et al., 2006). Preincubation of synaptosomes with low concentrations of mephedrone induced a significant concentration-dependent reduction in both [^{3}H]5-HT and [^{3}H]DA uptake that is related with a direct interaction with transporter molecule. Moreover, Kehr et al. (2011) described that mephedrone preferentially increases serotonin levels over the dopamine levels, in nucleus accumbens. In agreement with these results we found that mephedrone inhibits serotonin uptake with an IC_{50} value lower than that for dopamine uptake.

Saunders et al. (2000) found that amphetamines reduce transporter function not only by direct competition for uptake but also reducing transporter capacity by inducing a molecular change that remains after removal of amphetamines from the medium, i.e. PKC-mediated phosphorylation and internalization of the transporter. Our [^{3}H]5-HT and [^{3}H]DA uptake experiments demonstrate that this mechanism cannot be extended to mephedrone as the decrease in the uptake disappears when the drug is removed by washout.

We demonstrate the affinity of mephedrone for the DA transporter by the displacement of [^{3}H]WIN35428 bound with a Ki value in the low micromolar range. A good correlation between the displacement of [^{3}H]WIN35428 binding and [^{3}H]DA uptake was found. This result indicates that the inhibition of DA uptake by mephedrone is due to a competitive (a Hill coefficient non-significant different from the unity was obtained) interaction with DA transporter. The potency of mephedrone inhibiting DA uptake is similar to that described for methamphetamine (Cozzi et al., 1999) and MDMA (Escubedo et al., 2011).

Cozzi et al. (1999) demonstrated that methamphetamine and MDMA are less potent inhibiting 5-HT uptake than DA uptake. Our results demonstrate that mephedrone inhibits 5-HT uptake with an IC_{50} value lower than that for DA uptake. However, the affinity of mephedrone for the 5-HT transporter is lower than the IC_{50} value of mephedrone inhibiting 5-HT uptake, indicating that an additional mechanism to the reversible interaction with the 5-HT transporter is probably involved in the inhibition of 5-HT uptake by mephedrone. In this case, a competition of mephedrone with 5-HT could be hypothesized.

MDMA induces a selective serotonergic neurotoxicity in rats (Green et al., 2003) but the exact mechanism remains unknown. Although some authors (Colado et al., 1999) ruled out the role of DA in the serotonergic neurotoxicity induced by MDMA, Sprague et al. (1998) suggested that following MDMA treatment, extracellular DA present in high amounts, may be taken up into the depleted 5-HT terminals, where monoamine oxidase-B metabolizes it producing hydrogen peroxide which is proposed to be responsible of MDMA selective neurotoxicity. The present results demonstrate that mephedrone can inhibit this entry of DA through the 5-HT transporter only at very high concentrations that probably cannot be reached *in vivo*. Consequently, the above mentioned hypothesis about the mechanism of neurotoxicity of MDMA could be extended to mephedrone.

Moreover, we studied the affinity of mephedrone for the 5-HT_2 and D_2 receptors. From the obtained values of Ki, a 15 times greater affinity of mephedrone for 5-HT_2 than for D_2 receptors can be deduced. This is in agreement with results obtained by Battaglia et al. (1988) with MDMA and suggests that mephedrone, like MDMA, could display cardiotoxicity and hallucinogenic properties derived from this 5-HT receptor affinity.

To sum up, in this paper, we provide evidence that mephedrone, interacting with DA and 5-HT transporters can block the uptake of these neurotransmitters. The interaction of mephedrone with 5-HT_2 receptors could contribute to an increase in the dopaminergic activity that could be probably related with its psychostimulant effect.

Role of the funding source

Funding for this study was provided by grants from Generalitat de Catalunya (SGR977), Plan Nacional sobre Drogas (2008/003; 2010/005) and Ministerio de Ciencia e Innovación (SAF2010-01456). Any of these institutions had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

Authors Martinez-Clemente and Escubedo performed uptake and transporter experiments. Author Pubill performed receptor radioligand binding experiments. Author Camarasa designed the study, undertook statistical and non-linear regression analysis and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflicts of interest

J. Martínez-Clemente, E. Escubedo, D. Pubill and J. Camarasa: None declared.

Acknowledgements

The authors are grateful to Drs. Bosch and Amat (Laboratory of Organic Chemistry) for their helpful assistance in the mephedrone synthesis. Authors acknowledge the Language Advisory Service of the University of Barcelona for revising the language of the manuscript. J. Martínez-Clemente is a fellowship of Plan Nacional sobre Drogas.

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Please cite this article as: Martínez-Clemente, J., et al., Interaction of mephedrone with dopamine and serotonin targets in rats, Eur. Neuropsychopharmacol. (2011), doi:10.1016/j.euroneuro.2011.07.009