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Title page

Brain disposition of *cis-para*-methyl-4-methylaminorex (*cis*-4,4'-DMAR) and its potential metabolites after acute and chronic treatment in rats: correlation with central behavioral effects

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Running title page

a) Running title: 4,4'-DMAR brain disposition: metabolism and central effects.

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Abtract: 235 words

Introduction: 666 words

Discussion: 932 words

d) Non-standard abbreviations: 2-Prop, alcol iso-propylic; ACN, acetronitrile; ANOVA, analysis of variance; AUC, area under curve; BBB, blood-brain-barrier; C_{max}, peak concentration; CPP, conditioning place preference; DAT, dopamine transporter; EIC, extracted ion chromatogram; ESI, electrospray ionization; FTMS, Fourier-transform mass spectrometry; HCOOH, formic acid; HPLC, high performance liquid chromatography; HQC, high quality control; IS, internal standard; LQC, low quality control; m/z, mass-to-charge ratio; MeOH, methanol; MF, matrix factor; MRM, multiple reaction mode; MS, mass spectrometry; MQC, mid quality control; NET, norepinephrine transporter; NPS, new psychoactive substance; PK, pharmacokinetics; SERT, serotonin transporter; $T_{1/2}$, half-life; T_{max}, time to reach the peak concentration.

e) Recommended section: Neuropharmacology

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Abstract

Para-methyl-4-methylaminorex (4,4′-DMAR) is a phenethylamine derivative with psychostimulant activity, whose abuse has been associated with several deaths and a wide range of adverse effects. We recently validated an HPLC-MS/MS method to measure the compound's concentrations in plasma, and applied it to describe the pharmacokinetic properties after single dose in rats. In this study we investigated the brain disposition and metabolism of *cis*-4,4'-DMAR after intraperitoneal injection, and its central behavioral effects. After a single injection of 10 mg/kg, locomotor activity increased, peaking at two hours and disappearing at five hours; in these conditions, brain absorption was very rapid, (t_{max} 30-60 minutes) and large (brain-to-plasma ratio 24); $t_{1/2}$ was ~50 min. After 14 daily doses, the compound's effect on locomotor activity was greater (~20% in comparison with the effect after the first dose), but not for pharmacokinetic reasons. By high resolution mass spectrometry we also identified four metabolites of *cis*-4,4'-DMAR in plasma and brain of treated rats. Semiquantitive analysis indicated low brain permeability and very low brain concentrations, suggesting that these metabolites do not contribute to central behavioral effects; however, the metabolite originating from oxidation of the *para*-methyl group (M2) persisted in plasma for longer time and at higher concentrations than the parent molecule, and could be used to evaluate drug intake in human consumers. Finally, we describe the rewarding effect of *cis*-4,4'-DMAR, in the conditioning place preference test, suggesting a high risk of addiction in humans.

Introduction

Para-methyl-4-methylaminorex (4,4'-DMAR) is a derivative of the scheduled psychostimulants 4-methylaminorex (4-MAR) (Bunker et al., 1990; Kankaanpaa et al., 2001; Kankaanpaa et al., 2002; Meririnne et al., 2004) and aminorex (Poos et al., 1963; Costa et al., 1971; Kay et al., 1971; Davis and Brewster, 1988; Hofmaier et al., 2014). 4,4'-DMAR was known as a "new psychoactive substance" (NPS) until it was added to schedule II of the 1971 United Nations Convention on Psychotropic Substances (March 2016) (UNODC, 2015; WHO, 2015; CND, 2016). Its appearance in Europe was notified for the first time in 2012 by the Netherlands national focal point. To begin with, it was mainly sold through illicit internet retailers as a "research chemical" under different names (e.g. Serotoni, 4-methyl-U4Euh or 4-methyl-euphoria), mainly in powder form. More recently, 4,4'-DMAR has been sold in the "street market", camouflaged like common illicit drugs, e.g. as tablets with logos similar to "ecstasy" tablets (EMCDDA, 2015).

From the first notification, and over a short period, the presence of 4,4'-DMAR has been analytically confirmed in 31 cases of death reported to the EU Early Warning System, but the presence of other psychoactive drugs in the blood of these patients, as well as in the materials seized, suggested that 4,4'-DMAR might not be the (only) cause of death (Cosbey et al., 2014; EMCDDA, 2015). Furthermore, a number of adverse effects, including agitation, hyperthermia, convulsions, breathing problems and cardiac arrest were reported in parallel (EMCDDA, 2015; Glanville et al., 2015).

Nausea, dysphoria, sweating, increased heart rate, dilated pupils, jaw clenching, restless legs, amnesia, feeling lethargic or "wiped out", foaming at the mouth, dry mouth, facial spasms, psychosis and hallucinations are also reported by users in Internet forums (EMCDDA, 2015; Glanville et al., 2015); these unwanted effects were similar to those of other sympathomimetic drugs such as 3,4-methylenedioxy-methamphetamine and mephedrone (EMCDDA, 2015; Glanville et al., 2015). The doses associated with adverse effects (5 - 200 mg, oral) overlap those required for the desired effects.

The molecular structure of 4,4′-DMAR involves two chiral centers within the oxazoline ring, resulting in four distinct (±)-*cis* and (±)-*trans* enantiomers (Brandt et al., 2014; EMCDDA, 2015). When the reference standard was available, the *cis* form was confirmed in 18 deaths from the UK and in seized materials, but the presence of the *trans* form in the drug market cannot be excluded (Brandt et al., 2014; EMCDDA, 2015). The activity of these two isomers was recently investigated *in vitro* on rat brain synaptosomes; *cis*-4,4'-

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DMAR is a potent, non-selective releaser of dopamine, norepinephrine and serotonin through interaction with the corresponding monoamine transporters (DAT, NET and SERT respectively), acting as a substrate at DAT and NET (EC_{50} = 8.6 and 26.9 nM) with potency comparable to aminorex and *d*-amphetamine. However, the potency to induce serotonin release via SERT (EC_{50} = 18.5 nM) was higher than these two psychostimulants (Brandt et al., 2014). The *trans*-isomer also induces dopamine and noradrenaline release (EC_{50} respectively 24.4 and 31.6 nM) but it acts as an uptake blocker at SERT (McLaughlin et al., 2015). The potency of *cis*-4,4'-DMAR for inducing the release of all three monoamines is reflected in its potential for serious side effects.

Information is scarce about the pharmacokinetics of 4,4'-DMAR and its metabolism *in vivo*. We recently validated a HPLC-MS/MS method for quantification of *cis*-4,4'-DMAR in rat and human plasma, and applied it for preliminary evaluation of the pharmacokinetic (PK) profile after a single intravenous injection in rats (Lucchetti et al., 2016). There was a rapid distribution phase, lasting about 1-2 h followed by a slow elimination phase with a terminal half-life of about 5 h. The bioavailability after intraperitoneal injection was about 20% (Lucchetti et al., 2016), lower than the analog 4-methylaminorex (Meririnne et al., 2004).

Here we focused on the PK profile in brain tissue, and in parallel its psychostimulant activity and its ability to induce motivational properties in conditioned place preference (CPP) test. The *in vivo* metabolites of the molecule were investigated for the first time in plasma and brain.

Material and Methods

Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), 2-propanol (2-Prop) and formic acid (HCOOH) were from Sigma-Aldrich Co. (Milan, Italy); all solvents were of HPLC-MS grade. HPLC-MS grade water was obtained in-house from a Milli-Q system (Millipore, Bedford, MA, USA). Carbamazepine hydrochloride (as internal standard, IS) was from Sigma-Aldrich (Milan, Italy). *Cis*-4,4'-DMAR was purchased from an internet vendor (http://www.chems-direct.org/serotoni-4-4-DMAR, March 2014). The powder contained 4,4'-DMAR in the *cis*-form with purity >99%, as previously confirmed (Lucchetti et al., 2016). On delivery, *cis*-4,4'-DMAR powder was stored at 4°C in dry conditions. Cocaine hydrochloride for behavioral studies was purchased from MacFarlan-Smith (Edinburgh, UK).

Cis-4,4'-DMAR stock solutions and working solutions for standard points (0.025, 0.05, 0.1, 0.5, 1, 5 and 10 μg/mL) and quality controls (0.025, 0.075, 3.0 and 7.5 μg/mL) were prepared in MeOH as previously described (Lucchetti et al., 2016). A stock solution of carbamazepine was also prepared in MeOH at a concentration of 1 mg/mL and diluted to 5 μg/mL in the same organic solvent as needed. Stock and working solutions were stored at -20°C until use.

Animals

Procedures involving animals were conducted at the IRCCS - Istituto di Ricerche Farmacologiche "Mario Negri" which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals was recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

Naïve male Wistar rats (Envigo RMS Srl, San Pietro al Natisone, Udine, Italy) were used. After arrival, rats weighing 175–200 g were housed two per cage at constant room temperature (21 ± 1°C) and 60% relative humidity with a 12h light cycle (light on 7:00 am – 7:00 pm) with food and water *ad libitum* (Global Diet 2018S, Envigo). Animals were allowed to adapt to laboratory conditions for at least two weeks and were handled daily during this period.

Pharmacokinetic studies in rats

Cis-4,4'-DMAR was dissolved in sterile 0.9% saline at the concentration of 5 mg/mL, and injected intraperitoneally (i.p.) in a volume of 2 mL/kg.

Single dose. A group of rats were treated i.p. with 10 mg/kg *cis*-4,4'-DMAR and were killed under deep anesthesia (5% isoflurane) at different times after injection (*N*=3-4 at 15min, 30min, 1h, 2h 4h, 6h, 8h and 24h). Blood samples were transferred to heparinized tubes and kept in ice; plasma was obtained by centrifugation at 2000g for 15 min, and stored at -20°C until analysis. Brain tissues were immediately removed after decapitation and kept on dry ice, then they were homogenized in 10 volumes of MilliQ and stored at -20°C until analysis.

Multiple dose. 8 rats were injected i.p. with 10 mg/kg *cis*-4,4'-DMAR, once a day; four were killed 24 h after the 13th dose, and the others 30 min after the 14th dose. Blood and brain tissues were removed and processed as described before.

Plasma and brain PK profiles were analyzed using a non-compartmental model to obtain PK parameters. The peak concentration (C_{max}) and the time taken to reach it (t_{max}) were taken directly from the data; half-life ($t_{1/2}$), area under the curve from 0 to the last time point (AUC_{0-t}) and from 0 to infinity (AUC_{0-inf}) were obtained with PKSolver, a freely available menu-driven add-in program for Microsoft Excel (Zhang et al., 2010).

Identification of cis-4,4'-DMAR metabolites in plasma and brain

Freely available software OECD QSAR Toolbox Ver. 3.2 (http://www.qsartoolbox.org/it) was used first to predict *cis*-4,4'-DMAR metabolites. The predicted ones were then checked in plasma and brain samples of treated rats (pool of samples at each time-point, as described above) using a high-resolution mass spectrometer (LTQ-Orbitrap XL) coupled with a liquid chromatography system (Agilent 1200 Series). *Cis*-

4,4'-DMAR metabolites were extracted from a pool of plasma and brain samples of 3-4 rats at each time-point by protein precipitation, as described below. HPLC separation was done with a Kinetex® C18 column (150 x 2.1 mm, 5 µm particle size) with a SecurityGuard™ ULTRA cartridge C18 precolumn (Phenomenex Inc), using an elution mixture composed of mobile phase A (MP-A, 0.1% formic acid in water) and mobile phase B (MP-B, acetonitrile). The elution gradient was from 2 to 50% of MP-B in 20 min; to 99% of MP-B in 1 minute, hold at 99% for 2 min and re-equilibration for 7 min at 2% of MP-B. Flow rate was set at 0.2 mL/min with an injection volume of 5 µL.

The LTQ-Orbitrap XL was equipped with an Ion Max Source used in electrospray positive mode (Thermo Scientific, Indianapolis, IN, USA); a complete scan at 60,000 resolving power, looking for molecules within the *m/z* range 50-1000, was done on extracted plasma and brain samples from *cis*-4,4'-DMAR-treated and untreated rats, used as control. Fragmentation spectra were acquired simultaneously (CID 35eV, resolving power 15,000) on the same samples, using a *targeted* method which selected the calculated *m/z* (±10 ppm) corresponding to metabolites previously predicted with the OECD QSAR ToolBox. No other peaks, corresponding to possible unpredicted metabolites, were found in the samples of treated rats.

HPLC-MS/MS quantification in plasma and brain

Plasma and brain levels of *cis*-4,4'-DMAR, and of metabolites found in significant levels, were measured by HPLC-MS/MS, using a triple quadrupole instrument to establish the time-course profiles in both matrices.

A recently validated method was used (Lucchetti et al., 2016). Briefly, plasma and brain samples were thawed at room temperature and processed in parallel with two replicates of QC samples at three concentrations (7.5, 300 and 750 ng/mL for plasma or 75, 3000 and 7500 ng/g for brain) and with freshly prepared calibration curves linear in the range 2.5 – 1000 ng/mL for plasma or 25 – 10000 ng/g for brain. After the addition of 5 µL of IS working solution (final concentration 500 ng/mL or 5000 ng/g) the solutions were mixed with 500 µL of cold ACN (with 1% HCOOH) to allow protein precipitation and then were centrifuged at 4°C for 10 min at 13000g. The supernatants were dried under nitrogen and the residues were re-suspended in 500 µL of water/CAN (98/2, v/v) containing 0.1% HCOOH. Ten µL were injected into an Alliance 2795 model HPLC system (Waters Corporation, Milford, MA, USA) coupled to a Quattro Micro API triple quadrupole mass spectometer (Waters Corp.). Auto-sampler temperature was 6°C. Separation was

on a Kinetex C18 column (150 × 2.1 mm, 5 μm particle size; Phenomenex Inc., Torrance, CA, USA) at 30°C with a SecurityGuard™ ULTRA cartridges C18 precolumn (Phenomenex Inc). The elution solvents were 0.1% HCOOH in water (mobile phase A, MP-A) and 0.1% HCOOH in acetonitrile (mobile phase B, MP-B). Elution started with 98% of MP-A and 2% MP-B for 2 min, followed by a 13-min linear gradient to 100% of MP-B, held for 2 min and a 2-min linear gradient to 98% of MP-A, which was maintained for 8 min to equilibrate the column; flow rate was 0.200 mL/min. The total run time was 25 min: retention times for *cis*-4,4'-DMAR and IS were 10.36 and 11.92 min, respectively.

The mass spectrometer, operating in ESI+ MRM mode, acquired the mass transitions *m*/*z* 191.4 → *m*/*z* 148.3 (quantification ion transition, collision energy 10 eV) and *m*/*z* 191.4 → *m*/*z* 131.3 (qualitative ion transition, collision energy 22 eV) for cis-4,4'-DMAR; for IS ion transitions *m*/*z* 237.3 → *m*/*z* 194.2 (quantification ion transition, collision energy 17 eV) and *m*/*z* 237.3 → *m*/*z* 165.2 (qualitative ion transition, collision energy 40 eV) were used. Capillary and cone voltage were set at 3.5 kV and 15 V, respectively. The HPLC-MS/MS system was controlled by a Masslynx® version 4.1 software (Waters Corp.).

To accept the analytical run, QCs and back-calculated calibrators had to satisfy the criteria indicated in EMA guidelines (EMA, 2011). Raw HPLC-MS/MS analysis data were processed with the GraphPad Prism® program (GraphPad Software, Inc. La Jolla, CA, USA) for plotting the calibration curve and quantifying *cis*-4,4'-DMAR plasma and brain levels. Since the method for *cis*-4,4'-DMAR quantification had been previously validated in plasma, the performance of the method was further examined in the brain homogenate, following EMA guidelines (EMA, 2011) (Validation results are reported in the Supplemental Tables 1-5 and Supplemental Figure S1). All calibration curves analyzed during method validation showed slopes highly reproducible with determination coefficients (r^2) always over 0.99; the accuracy of the back-calculated concentrations was always within the acceptance limits. The lower limit of quantification (LLOQ) was set at 25 ng/g and the selectivity of the methods was confirmed in six different matrices. Mean accuracy calculated on six replicates of QCs at four concentrations (25, 75, 3000 and 7500 ng/g) were in the range 93.9-108.0% (intra-day, in the same analytical run) and 100.7-105.1% (inter-day, over three different days), and precision, expressed as CV%, was 3.1-11.2% (intra-day) and 1.9-11.4% (inter-day). Recoveries of *cis*-4,4'-DMAR (75, 3000 and 7500 ng/g) and IS (5000 ng/g) were within the ranges 95.3-100.7% and 87.8-92.4%, respectively; ion suppression/enhancement (expressed as matrix factor, MF) of the assay was

calculated for 4,4'-DMAR (75, 3000 and 7500 ng/g) and IS (5000 ng/g) in six different lots of brain homogenate. The IS-normalized MF was consistent across the concentrations and the overall CV% ranged from 4.3% to 6.3%. We also confirmed the stability of *cis*-4,4'-DMAR (25 and 7500 ng/g, six replicates) in brain homogenate under four conditions; i) bench-top stability (2h at room temperature), ii) long-term storage stability (2 weeks at -20°C), iii) stability after two freeze–thaw cycles, and iv) auto-sampler stability (48 h at 6°C). All validation data are consistent with the requirements indicated in the EMA guidelines and comparable to those obtained in plasma (Lucchetti et al., 2016).

Behavioral studies

Cis-4,4'-DMAR was dissolved in sterile 0.9% saline at 0.5-5 mg/mL, and injected in a volume of 2 mL/kg to have the doses 1.0, 3.0 and 10 mg/kg. Cocaine hydrochloride was dissolved in sterile 0.9% saline at 5 mg/mL and injected in a volume of 2 mL/kg to have the dose of 10 mg/kg.

Locomotor activity

We tested the ability of *cis*-4,4'-DMAR to influence spontaneous motor activity during the light phase of the day (between 10 a.m. and 4 p.m.), according to published data (Dolezal and Krsiak, 2002), with some modifications. Rats were kept in individual transparent cages (42 x 28 x 21 cm, length x width x height) with sawdust bedding. Each cage was placed between metal frames (54 x 50 x 37 cm) holding two sets of parallel photo beams, crossing the cage 3 cm above the floor (Multiple Activity Cage, Cat. No 47420, Ugo Basile, Comerio, VA, Italy). The device counts the number of movements, by recording the number of infrared beam interruptions. On the testing day – after 1h of acclimation - rats were randomly allocated to the transparent cage and baseline motor activity counts were first automatically recorded in 10-min time bins for 1h using dedicated software (Basile, Italy). Afterwards, the rats were injected i.p. with vehicle or 3 and 10 mg/kg of 4,4'-DMAR, and their locomotor activity was recorded as the number of beam breaks in a 10-min time bin over a period of 5h.

Spontaneous locomotor activity was measured after the 1st and the 14th dose of a 14-day chronic treatment, and after a subsequent 48h wash-out period.

Conditioned Place Preference

Apparatus. Rats were conditioned and tested in standard place preference boxes (TSE Systems GmbH, Bad Homburg, Germany) consisting of three compartments. The two outer conditioning compartments (30 x 25 x 30 cm) were visually (black/white stripes versus gray) and structurally (grid floor versus smooth floor) different and separated by a smaller white middle compartment (10 x 25 x 30 cm, length x width x height) used for introducing the animal into the apparatus during the pre-test and test sessions. During the pretest and test sessions, arched gateways gave access to the two adjacent conditioning compartments, which allowed the animals to move freely around the entire apparatus. During conditioning sessions, the rats were placed in one of the two conditioning compartments and access to the other compartments were blocked by inserting dividers without a gateway between the compartments. All compartments were equipped with photo-sensors to detect the location of the rat; TSE Systems software was used to calculate the time spent in each compartment and the motor activity in the whole apparatus.

Procedure. Each experiment consisted of three phases during which each animal was always exposed to the same box:

- preconditioning phase (day 1): animals were allowed to freely explore the whole box for 15 min
- conditioning phase (days 2 to 9): animals were restricted to one of the outer compartments for the odd conditioning sessions, and to the other outer compartment for the even sessions.
- testing phase (day 10): animals were again allowed to freely explore the whole box for 15 min.

As previously described (Cervo et al., 2002; Cervo et al., 2005), on the first day, before any drug treatment, each rat was allowed to explore the apparatus for 15 min, and the time spent in the three compartments was recorded. Rats were then assigned to treatment groups and conditioning compartments, ensuring that all treatments were matched as closely as possible between compartments. The schedule during the conditioning phase consisted of i.p. injection of *cis*-4,4'-DMAR and cocaine or vehicle on alternate days, with a 24h-interval between conditioning days; this did not influence CPP in our experimental conditions (Cervo et al., 2002; Cervo et al., 2005).

Thus, on odd conditioning days rats were given i.p. *cis*-4,4'-DMAR or cocaine immediately before being confined to the randomly designated drug side. On the even conditioning days, they were given vehicle and confined to the opposite side. The conditioning session times were respectively 40 min and 1 hour for *cis*-

4,4'-DMAR and cocaine. These schedules were selected on the basis of data in figure 4 for 4,4'-DMAR, and published data for cocaine (Cervo et al., 2002; Cervo et al., 2005), and allowed the rats to be exposed at the drugs' peak effect.

This daily order of exposure to the drug under study and vehicle was counter-balanced for the rats in each group. Control animals received vehicle in both compartments.

On the test day neither drug nor vehicle was injected. Each rat was placed in the middle, neutral compartment with free access to the whole apparatus. The time spent in each compartment and the motor activity in the whole apparatus were recorded over a 15-min period. The difference in time spent in the drug- and vehicle-associated compartments, i.e. preference for the drug-associated side, was taken as a measure of place conditioning.

Results

Cis-4,4'-DMAR metabolites in plasma and brain

QSAR ToolBox predicted several potential metabolites *in silico* entering *cis*-4,4'-DMAR as a Simplified Molecular Input Line Entry System (SMILES) string, CC(N=C(N)O1)C1C2=CC=C(C)C=C2.

The *m/z* for the predicted molecular structures were searched for in full MS scan acquired from plasma and brain samples; to filter out interfering signals, plasma and brain samples of untreated rats were acquired the same way and common background peaks were not considered.

Four of the predicted metabolites were detected in both matrices and separated well by the 18-min gradients, with elution times between 6.0 and 12.0 min, as shown by the extracted ion chromatograms (EIC) (Figure 1); the retention time of *cis*-4,4'-DMAR was 11.72 min.

We confirmed the structures of cis-4,4,'-DMAR (Supplemental Figure 2) and of the metabolites identified, in plasma and brain, interpreting the MS and MS/MS spectra extracted from each chromatographic peak. We identified metabolite M1 (Supplemental Figure 3) as due to hydroxylation of the *para*-methyl group on the aromatic ring or direct hydroxylation of the aromatic ring. We detected other peaks corresponding to the EIC of M1 *m/z* of 207.1128 ± 10 ppm (*a*, *b* and *c*, Figure 1), probably due to other hydroxylated isomers of *cis*-4,4'-DMAR; however, no MS/MS spectra were obtained for these peaks because of their low intensities (data not shown). We also identified other metabolites whose MS spectra were consistent with i) oxidation of the *para*-methyl group leading to the corresponding carboxylic acid (M2, Supplemental Figure 4); ii) hydrolysis of the 2-amino-oxazoline ring (M3, Supplemental Figure 5); iii) oxidative deamination of the 2-amino-oxazoline ring (M4, figure Supplemental Figure 6)

Figure 2 shows the proposed *in vivo* metabolic pathway of *cis*-4,4'-DMAR, involving phase 1 metabolism reactions.

4,4'-DMAR absolute levels and semi-quantitative analysis of metabolites

The plasma PK profile of *cis*-4,4'-DMAR after a single i.p. injection (Figure 3A) gave the highest concentration (241.7 ± 94.2 ng/mL) at the first sampling time (15 min), with a rapid decline thereafter (no longer detectable 8h after the dose) with a $t_{1/2}$ of 56.8 min (calculated on the last three points); plasma AUC_{0-}

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$_{4h}$ was 0.319 µg/mL*h. In brain tissues (figure 3A) cis-4,4'-DMAR reached C_{max} (3569 ± 1195 ng/g) 30-60 min after the injection and then declined with a $t_{1/2}$ of 46.5 min until 8h, when levels were quantifiable only in one rat; AUC_{0-8h} in brain was 8.532 µg/g*h. The ratio brain AUC_{0-4h} / plasma AUC_{0-4h} was 24, indicating high penetration in the brain.

Metabolites M1, M3 and M4 had MS peak area < 10% of the parent compound, in plasma and brain at each time-point (data not shown); M2 had even higher values in plasma than those of *cis*-4,4'-DMAR (Figure 3C). Since no reference standard metabolite M2 was available, we could not determine its absolute plasma and brain levels, so the data are expressed as the ratio of analyte peak areas to IS peak area (response) normalized to mL plasma or g tissue.

The PK profile of M2 in plasma (Figure 3C) had a t_{max} of 30 min, while in the brain the maximum response was reached 1h after the injection (Figure 3D); in both matrices M2 was detectable up to 8h. Plasma and brain levels declined in parallel with $t_{1/2}$ 96 min and 104 min; M2 had a very low brain-to-plasma ratio ($AUC_{0-8h\ brain}$ / $AUC_{0-8h\ plasma}$ = 0.05) indicating weak ability to cross the BBB.

As shown in Table 1, the concentrations 30 min after the 14th dose were comparable to, or slightly lower than 30 min after the 1st dose (t-test, $p > 0.05$) indicating no accumulation after multiple doses. As expected, 24h after the 13th daily dose *cis*-4,4'-DMAR was not detectable in either plasma or brain. With *cis*-4,4'-DMAR (10 mg/kg/day i.p. for 14 days) there was no accumulation of the metabolites (data not shown).

Behavioral studies

Spontaneous locomotor activity

Figure 4A shows the spontaneous locomotor activity in the 5h after a single i.p. injection of 3 or 10 mg/kg *cis*-4,4'-DMAR, or vehicle. The number of beam breaks was dose-dependently increased by the compound, in comparison with the vehicle-treated rats. Motor activity already increased in the first hour post-treatment and remained significant for 2h with the dose of 3 mg/kg and 4h with 10 mg/kg.

After the 14th dose, the increase of locomotor activity occurred earlier and it was more sustained (Figure 4B), although this did not result in statistically different $AUC_{basal-5h}$ values (Figure 4D). Forty-eight hours after the 14th dose, rats were injected again with 3 and 10 mg/kg of *cis*-4,4'-DMAR and vehicle, to check for any re-bound effect. There was a further increase of motor activity after this challenge dose, with the

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corresponding $AUC_{basal\text{-}5h}$ after 10 mg/kg significantly higher than after the 1st and the 14th dose (one-way ANOVA analysis of matched measures, followed by multi comparisons with Newman–Keuls post-hoc test) (Figure 4C/D).

Conditioned Place Preference

Figure 5A shows the time spent by rats of the five experimental groups in the three compartments during the pre-conditioning phase; as required, there were no significant differences.

Figure 5B shows the effects of eight conditioning sessions with vehicle, *cis*-4,4'-DMAR (1, 3 and 10 mg/kg, i.p.) and cocaine (10 mg/kg, i.p.) on the time spent in the conditioned drug-associated side. One-way ANOVA followed by post-hoc comparisons showed that 10 mg/kg, but not 1 and 3 mg/kg of *cis*-4,4'-DMAR, and 10 mg/kg of cocaine induced a clear CPP ($P < 0.05$ vs. vehicle group, Newman–Keuls test).

Discussion

The main aim of this study was to investigate the behavioral effects of the psychoactive substance *cis*-4,4'-DMAR and to correlate them with its brain disposition in rats after single and repeated doses (10 mg/kg, i.p.); *in vivo* metabolism was investigated for the first time.

The pharmacokinetic profile of *cis*-4,4'-DMAR in plasma after i.p. injection confirmed previous data (Lucchetti et al., 2016), with very rapid absorption, C_{max} within 15 min, and rapid decline of the levels, undetectable afer 4h; An elimination half-time of about 5 hours was previously determined after intravenous injection (Lucchetti et al., 2016). *Cis*-4,4'-DMAR rapidly and extensively distributed to the brain after i.p. injection, with a t_{max} of 30-60 min and a brain-to-plasma ratio of 24, consistent with the lipophilicity (logP 1.5) of this small molecule. This ratio is three times that reported for the precursor *cis*-4-MAR (brain-to-plasma ratio ~7 after i.p. injection (Meririnne et al., 2004). An half-time of 46.5 min was estimated using the last three points until 8h, similar to that for the *cis*-4-MAR analog (Bunker et al., 1990; Kankaanpaa et al., 2001; Kankaanpaa et al., 2002; Meririnne et al., 2004).

Regarding the *in vivo* metabolism of *cis*-4,4'-DMAR, we identified metabolites due to hydroxylation (M1), hydrolysis (M3) and oxidative deamination (M4), i.e. metabolic reactions already described for the precursor 4-MAR (Henderson et al., 1995). The metabolite originating from hydrolysis is the *para*-methyl derivative of norephedrine, a psychoactive drug which is used as a stimulant, decongestant, and anorectic agent (Flavahan, 2005), suggesting that the M3 metabolite might have similar activities. Furthermore, both oxidations and aromatic hydroxylation are metabolic pathways typical of amphetamine-like compounds (Alleva, 1963; Dring et al., 1966; Ellison et al., 1966; Theobald and Maurer, 2007; Rohanova and Balikova, 2009; Maurer, 2010; Welter et al., 2014). However, the main metabolite found both in plasma and brain (M2) was due to oxidation of the *para*-methyl group of *cis*-4,4'-DMAR, a metabolic reaction not observed for 4-MAR. Although absolute quantification of this metabolite is not possible because of the lack of a reference standard, comparison of the MS signal intensities showed that M2 already had higher plasma levels than the parent compound at 15 min and at all the time-points thereafter; this metabolite reached C_{max} after 30 min and could be detected until 8h, i.e. longer than the parent compound. The higher plasma levels of M2 than the parent compound might be useful for more sensitive assessment of *cis*-4,4'-DMAR intake in humans. However, the BBB passage of M2 was very low (brain to plasma ratio 0.05), resulting in brain

concentrations 500 times lower than the parent compound, suggesting that M2 does not contribute to the pharmacological central effects of *cis*-4,4'-DMAR.

A single i.p. injection of 3-10 mg/kg *cis*-4,4'-DMAR increased spontaneous locomotor activity in a dose-dependent manner. This effect resembled those previously observed with amphetamine and its analog, such as phenethylamines/MDMA-like drugs (Wellman et al., 2009; Palenicek et al., 2011; Lopez-Arnau et al., 2012; Aarde et al., 2013; Miliano et al., 2016), and is very likely due to the fact that *cis*-4,4'-DMAR induces the release of biological monoamines, particularing dopamine (Charntikov et al., 2011; Brandt et al., 2014; McLaughlin et al., 2015). The rapid appearance and rapid decline of this behavioral effect is quite consistent with the pharmacokinetic profile of the compound in the brain, although a delay of about 1 h could be noted (Supplemental Figure 7). Several reasons could explain this delay (Louizos et al., 2014), e.g. time needed for receptor-activated signal transduction, a rapid sensitization of receptors, or formation of metabolites with agonist activity. The latter possibility is unlikely, considering the low BBB passage and the brain PK profile of the main metabolite (M2). As regards the second possibility, it has been reported that an immediate and long-lasting dopaminergic sensitization is already elicited after a single injection of amphetamine and other psychostimulants (Vanderschuren et al., 1999; Chinen et al., 2006; Kameda et al., 2011; Marinho et al., 2015). Consistently, after repeated treatment (once a day for 14 days), the compound's ability to stimulate locomotor activity occurred earlier and it was more sustained compared with that measured after the 1st dose, with a significant increase with a new *drug-challenge* after 48h wash-out. Since no drug accumulation or increase in drug bioavailability was observed after repeated injections, pharmacodynamics adaptation mechanisms probably underlie these observations. Behavioral sensitization and hyper-responsiveness of dopaminergic neurons has already been described after repeated methamphetamine, as well as with other phenethylamine derivatives (Nishikawa et al., 1983; Fukushima et al., 2007; Miliano et al., 2016).

We also describe for the first time the rewarding effect of *cis*-4,4'-DMAR, evaluated in the CPP test, an established method for studying the motivational properties of a substances of abuse (Mucha et al., 1982; Tzschentke and Schmidt, 1998; Tzschentke, 2007). In the CPP paradigm, repeatedly pairing the primary motivational properties of the drug (unconditioned stimulus, using Pavlov's terminology) - during the conditioning phase - with neutral environmental stimuli, the latter acquired secondary motivational effects likely to become conditioned stimuli (Mucha et al., 1982; Tzschentke and Schmidt, 1998; Tzschentke, 2007).

Specifically, after 10 mg/kg *cis*-4,4'-DMAR, rats showed a preference for the side associated with the drug and this effect resembled that seen with 10 mg/kg cocaine. These data clearly indicate that *cis*-4,4'-DMAR has positive motivational properties, suggesting a high risk of addiction in humans.

These data highlight the importance of studying psychoactive substances with an approach combining pharmacokinetic and behavioral investigations in animal models. In particular, the analysis of brain disposition and the drug's metabolites is vital to understand the time-course of the behavioral effects better, and to predict the consequences of repeated doses.

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Authorship Contributions

Participated in research design: Lucchetti, Marzo, Di Clemente, Gobbi and Cervo.

Conducted experiments: Lucchetti, Marzo, Moro and Di Clemente.

Performed data analysis: Lucchetti, Marzo, Passoni, Bagnati, Gobbi and Cervo.

Wrote or contribute to the writing of the manuscript: Lucchetti, Gobbi and Cervo.

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Footnotes

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Legends for figures

Figure 1. Explanatory high resolution ion chromatograms extracted for the *m/z* of *in silico*-predicted metabolites, found in plasma (panel **A**) and brain (panel **B**) analyzing a pool of plasma samples and brain homogenates from four rats killed 1h after i.p. injection of 10 mg/kg *cis*-4,4'-DMAR. Table reports *i)* the metabolic mechanism underlying each predicted metabolites; *ii)* the *m/z* of the predicted metabolites; *iii)* the corresponding *m/z* measured in biological tissues after *in vivo* treatment; *iv)* the molecular formula; *v)* the proposed metabolic reaction.

Figure 2. Proposed *in vivo* metabolic pathway of *cis*-4,4'-DMAR with metabolite identification; ambiguous assignments of functional groups are shown as Markush structures.

Figure 3. A) Time-course of *cis*-4,4'-DMAR concentrations in plasma (white circles) and brain (black circles) after i.p. injection of 10 mg/kg. **B)** Time-course (0-4h) of the brain-to-plasma ratio. Each point is the mean ± SD of 3-4 rats. Time-course of metabolite M2 in **(C)** plasma and **(D)** brain of rats injected with a single i.p. dose of 4,4'-DMAR 10 mg/kg and killed after 15 min, 30 min, 1h, 2h, 4h, 8h and 24h. The monitored ion transitions for M2 were *m/z* 221.1 → *m/z* 178.1 (quantifier, collision energy 10 eV) and *m/z* 221.1 →*m/z* 117.1 (qualifier, collision energy 25 eV). Levels of the metabolite M2 (black squares) are expressed as the peak area ratio of the analyte to the IS, normalized to mL plasma or g tissue. Each point is the mean ± SD of 3-4 animals. For comparison, the ratio of *cis*-4,4'-DMAR is reported in parallel (white triangles, dotted lines).

Figure 4. Spontaneous locomotor activity recorded over a 5h-period in rats treated with vehicle and 3 or 10 mg/kg of *cis*-4,4'-DMAR (*N*=8, mean ± SD). Analysis was done in the same rats **(A)** after the 1st dose, **(B)** after the 14th dose and **(C)** after a 15th dose given after 48h wash-out. Data were analyzed by mixed-factorial ANOVA (with treatment as between-subjects factor and time as within-subject factor) followed by Newman–Keuls test. **P* < 0.05, ***P* < 0.01 vs. vehicle. **Panel D** shows the $AUC_{basal\text{-}5h}$ of the number of interruptions in rats given 10 mg/kg. (Box and whiskers plot, min-max). These data were analyzed by one-way ANOVA on repeated measures followed by Newman–Keuls post-hoc test. Significant comparisons are

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indicated: $^{**}P < 0.01$, $^{*}P < 0.05$.

Figure 5. *Cis-4,*4'-DMAR induced conditioned place preference (CPP). **A)** During the pre-conditioning phase rats had no treatment and the groups indicated just refer to the planned treatments. Mean time (±SD, *N*=8) spent by rats in the three compartments of the CPP cage. Data were analyzed by two-way ANOVA followed by Newman–Keuls test, $^{*}P < 0.05$ vs. gray and black/white compartment. **B)** Data obtained on the test day, after the conditioning phase. Mean time (±SD, *N*=8) in the *cis*-4,4'-DMAR or cocaine-paired side, corrected for the time spent in the vehicle-paired side. Data were analyzed by one-way ANOVA followed by Newman–Keuls post-hoc test, $^{*}P < 0.05$ vs. vehicle-treated group

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Table 1. Plasma and brain concentrations of *cis*-4,4'-DMAR after i.p. injection of 10 mg/kg once a day for 14 days.

	30 min post 1st dose [a]	**30 min post 14th dose**	**24h post 13th dose**
Plasma (ng/mL)	241.71 ± 94.24	161.59 ± 17.61	Not detectable
Brain (ng/g)	3569.09 ± 1195.01	2363.71 ± 307.24	Not detectable

[a] For comparison the concentrations measured 30 min after a single dose (from Figure 3) are shown.

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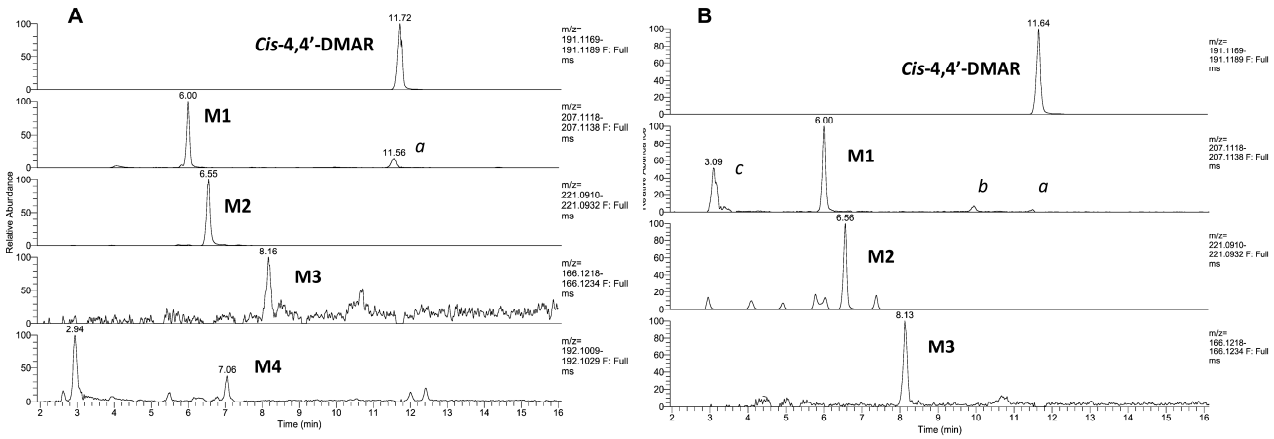

Peak ID	Simulator of metabolism	Calculated *m/z* (± 10 ppm)	Measured *m/z*	Molecular formula	Metabolic reaction
4,4'-DMAR	-	191.1179	191.1178	$C_{11}H_{14}N_2O$	-
M1	Rat liver S9	207.1128	207.1131	$C_{11}H_{14}N_2O_2$	Hyrdoxylation
M2	Rat liver S9	221.0921	221.0922	$C_{11}H_{12}N_2O_3$	Oxidation
M3	Hydrolysis (acidic or basic)	166.1226	166.1229	$C_{11}H_{15}N_2O$	Hydrolysis
M4	Microbial metabolism	192.1019	192.1017	$C_{11}H_{13}N_2O$	Oxidative deamination

Figure 1

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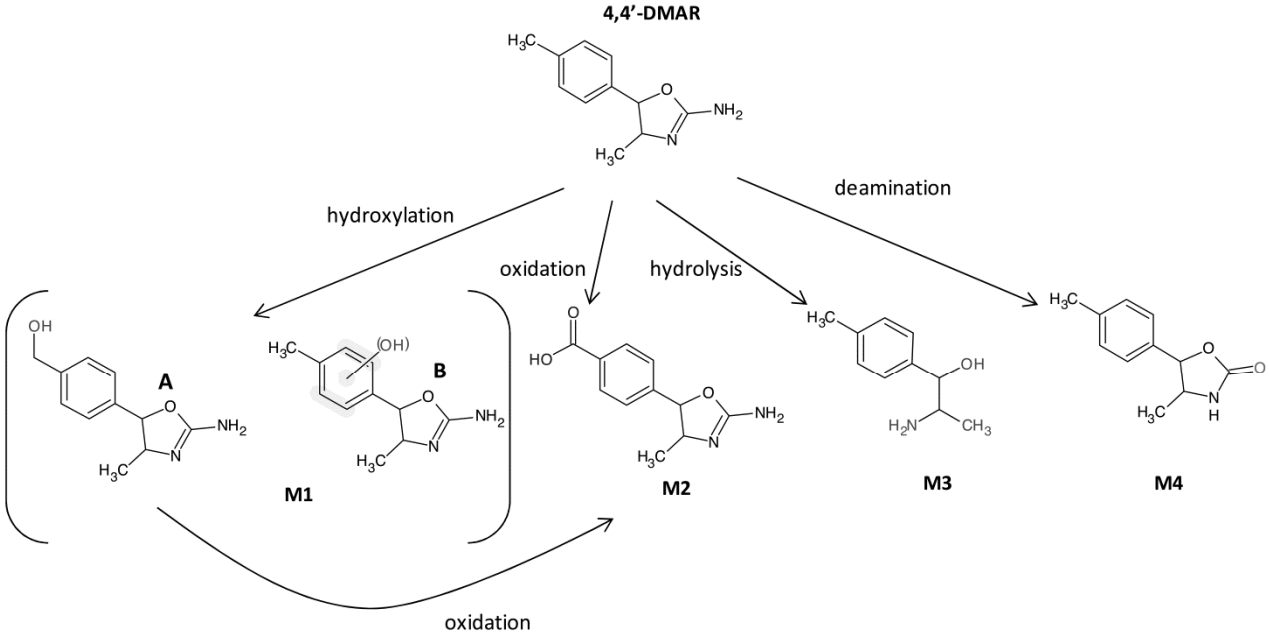

Figure 2

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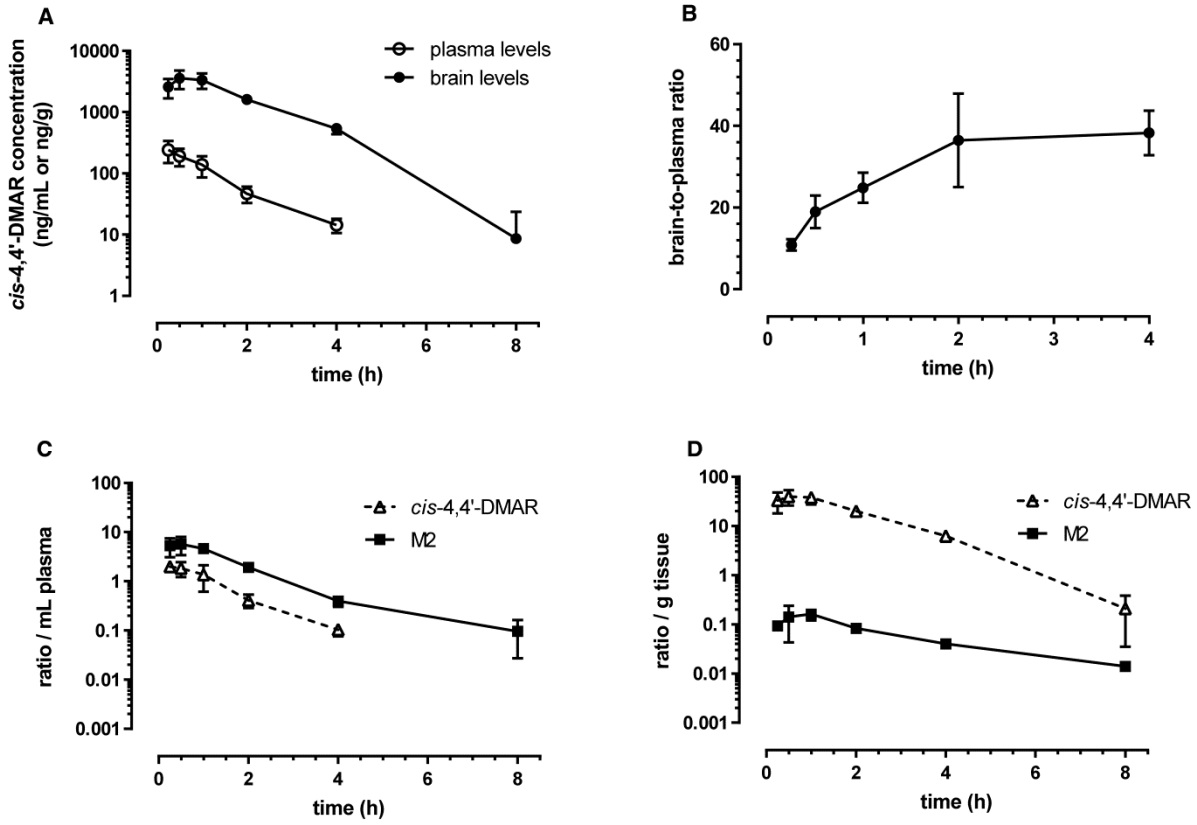

Figure 3

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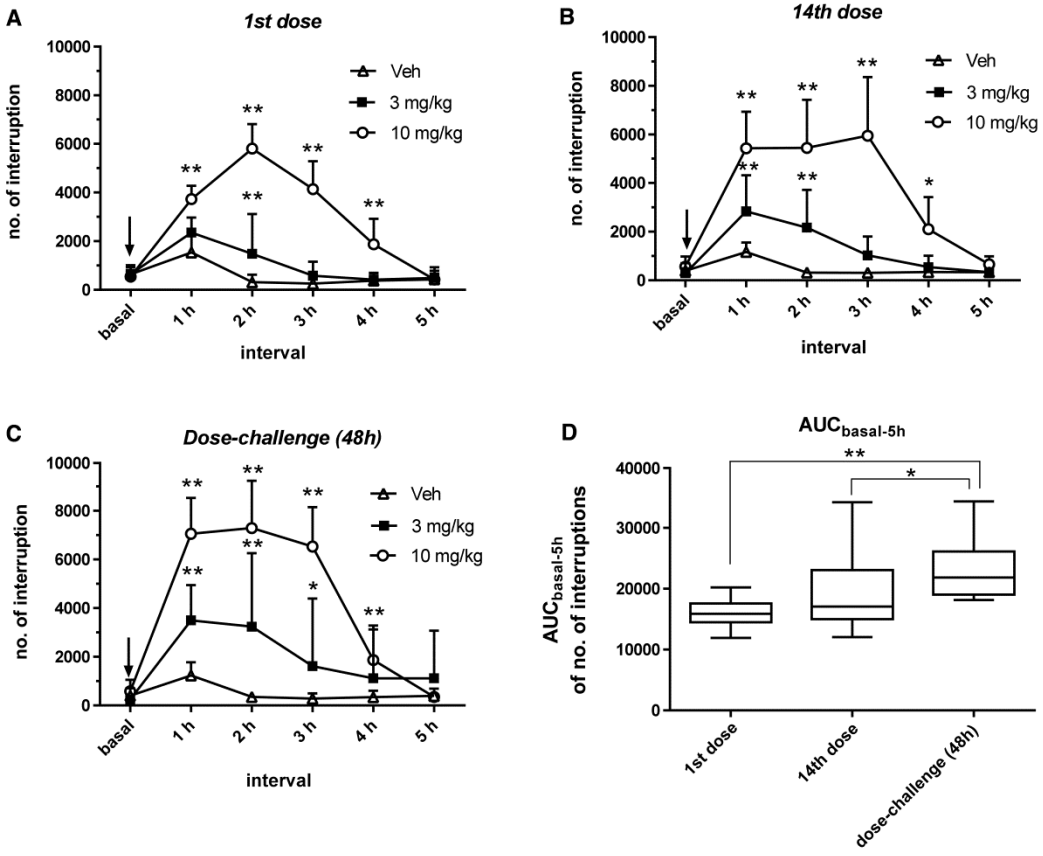

Figure 4

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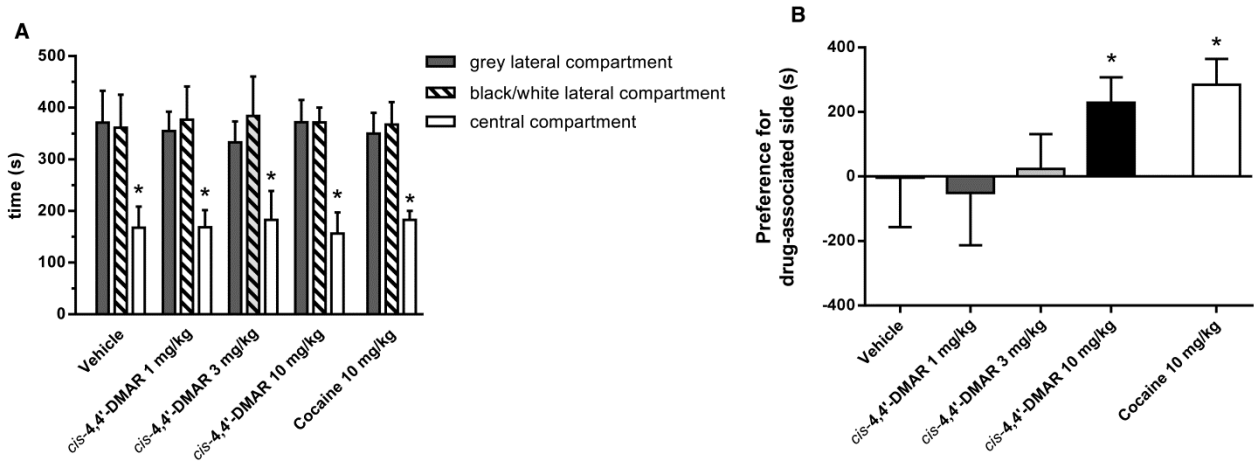

Figure 5