JPET Fast Forward. Published on December 20, 2012 as DOI: 10.1124/jpet.112.200915

JPET #200915

Title Page

ADX71743, a potent and selective negative allosteric modulator of metabotropic glutamate receptor 7 ($mGlu_7$): *in vitro* and *in vivo* characterization.

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Running Title: Characterization of an $mGlu_7$ negative modulator ADX71743

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A recommended section: Drug Discovery and Translational Medicine

A list of abbreviations:

ACTH (adrenocorticotropic hormone)

ADX (Addex)

ADX71743 ((+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one)

AMN082 (N,N'- dibenzhydrylethane-1,2-diamine dihydrochloride)

AUC (area under the curve)

CAR (conditioned avoidance response)

CSF (cerebrospinal fluid)

DOI (2,5-dimethoxy-4-iodoamphetamine)

EPM (elevated plus maze test)

fEPSP (field excitatory postsynaptic potential)

FLIPR (fluorometric imaging plate reader)

FST (Forced Swim Test)

FU (function unbound)

GPCR (G-protein coupled receptor)

HEK (human embryonic kidney)

HPA (hypothalamic-pituitary-adrenal axes)

HPLC (high performance liquid chromatography)

L-AP4 (L(+)-2-amino-4-phosphono butyric acid)

MB (marble burying)

MED (minimum effective dose)

MMPIP (6- (4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one)

NAM (negative allosteric modulator)

OCD (obsessive compulsive disorder)

PTSD (post-traumatic stress disorder)

Abstract

Metabotropic glutamate receptor 7 ($mGlu_7$) has been suggested to be a promising novel target for treatment of a range of disorders including anxiety, PTSD, depression, drug abuse and schizophrenia. Here we characterized a potent and selective $mGlu_7$ negative allosteric modulator (NAM) ADX71743. *In vitro*, Schild plot analysis and reversibility tests at the target confirmed the NAM properties of the compound and attenuation of L-AP4-induced synaptic depression confirmed activity at the native receptor. The pharmacokinetic analysis of ADX71743 in mice and rats revealed that it is bioavailable following subcutaneous administration and brain-penetrant (CSF concentration/total plasma concentration ratio at C_{max} = 5.3%). *In vivo*, ADX71743 (50, 100, 150 mg/kg s.c.) caused no impairment of locomotor activity in rats and mice or activity on rotarod in mice. ADX71743 had an anxiolytic-like profile in the marble burying and elevated plus maze tests, dose-dependently reducing the number of buried marbles and increasing open-arm exploration, respectively. While ADX71743 caused a small reduction in amphetamine-induced hyperactivity in mice, it was inactive in the mouse DOI-induced head twitch and the rat conditioned avoidance response tests. Also, the compound was inactive in the mouse forced swim test. These data suggest that ADX71743 is a suitable compound to help unravel the physiological role of $mGlu_7$ and understand better its implication in CNS diseases. Our *in vivo* tests using ADX71743, reported here, suggest that pharmacological inhibition of $mGlu_7$ is a valid approach for developing novel pharmacotherapies to treat anxiety disorders, but may not be suitable for treatment of depression or psychosis.

Introduction

Metabotropic glutamate receptor 7 ($mGlu_7$) has been suggested to be one of the most important receptors for multiple CNS functions among eight mGluRs, based on it having the widest distribution in the brain and the highest degree of evolutionary conservation across species (Flor et al., 1997). In the brain, $mGlu_7$ is most abundant in anatomical regions involved in emotional reactivity and cognitive functioning, such as hippocampus, amygdala, and the locus coeruleus (Kinoshita et al., 1998; Swanson et al, 2005).

Development in understanding the function of $mGlu_7$ and its use as a potential target for drug discovery has been hampered by the lack of bio-available and brain-penetrant pharmacological tools. High conservation of the orthosteric binding site has been a serious challenge for developing molecules with a high selectivity at this receptor. Initial evidence on possible role of $mGlu_7$ in the CNS largely came from a series of studies involving $mGlu_7$ knock-out (KO) mice and those implementing gene silencing techniques (Cryan et al., 2003; Masugi et al., 1999; Sansig et al., 2001). Compared to wild type controls, $mGlu_7$ KO mice showed consistent reductions in anxiety- and depression-like responses in a variety of behavioral tests (Callaerts-Vegh et al., 2006: Cryan et al., 2003). Also, $mGlu_7$ KO mice had signs of reduced reactivity of the hypothalamic-pituitary-adrenal (HPA) axis to stress (Mitsukawa et al., 2006), impaired fear extinction response and a deficit in the conditioned taste aversion paradigm (Callaerts-Vegh et al., 2006; Masugi et al., 1999).

The discovery of N,N'- dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), presumably the first orally active and brain-penetrant $mGlu_7$ allosteric agonist promised to provide a much needed pharmacological tool for assessing effects of direct activation of the receptor (Mitsukawa et al., 2005; Conn and Niswender 2006). However, *in vivo* evaluation of

AMN082 provided much conflicting results. On the one hand AMN082 reduced open arm exploration in the elevated plus maze (EPM) test in rats, indicative of an anxiogenic-like response (Palazzo et al., 2008) and elevated the plasma levels of corticosterone and ACTH in mice, indicative of a stress response (Mitsukawa et al., 2005). On the other hand, AMN082 exhibited an anxiolytic-like profile in the rat stress-induced hyperthermia (SIH) and four plate tests (Stachowicz et al., 2008) and antidepressant-like profile in the mouse forced swim test (FST) and tail suspension test (TST; Palucha et al., 2007); an outcome that was not expected from the $mGlu_7$ KO studies. Suggested explanations for these discrepancies are the possibilities that the receptor may be internalized upon activation by AMN082 (Pelkey et al., 2007) resulting in a functional antagonism, as well as activity of its metabolite, Met-1, at serotonin (SERT), dopamine (DAT) and norepinephrine (NET) transporters (Sukoff Rizzo et al., 2011).

Recent discovery of the systemically-active negative allosteric modulator (NAM) of $mGlu_7$ 6- (4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP) with inverse agonist activity was another step towards uncovering the role of this receptor (Nakamura et al., 2009; Suzuki et al., 2007). However, when tested *in vivo*, MMPIP impaired non-spatial and spatial memory in the object recognition and in the radial arm maze tests, respectively, and reduced social interaction in rats, while having no effects in a battery of tests relevant for motor function, anxiety, depression, sensorimotor gating, nociception and seizure threshold (Hikichi et al., 2010). An extensive pharmacological study of MMPIP and close analogs has revealed that these compounds show a context-dependent activity when expressed in recombinant cell lines, but were found inactive in a physiological setup (Niswender et al., 2010). Further studies are needed to understand the *in vivo* effects, or lack of effects, of MMPIP.

Here we provide a comprehensive characterization of ADX71743 ((+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one), a potent, selective, and brain-penetrant $mGlu_7$ NAM (Figure 1A). It was developed through chemical lead optimization of a hit compound (Tang et al., submitted), which was identified from a high-throughput screening campaign of our corporate chemical library using a Ca^{2+} mobilization assay. *In vitro,* we performed Schild plot analysis and reversibility tests at the target confirming the NAM properties of the compound and demonstrated activity at the native receptor using electrophysiological measures in the mouse hippocampus. After confirming selectivity of ADX71743, we performed pharmacokinetic evaluation of the compound in mice following subcutaneous (s.c.) administration confirming its suitable profile for *in vivo* testing. *In vivo,* after confirming normal motor activity in mice and rats using locomotor activity and rotarod tests, ADX71743 was evaluated in tests relevant for anxiety (mouse marble burying (MB) and EPM tests), depression (mouse FST) and psychosis (amphetamine-induced hyperactivity, DOI-induced head twitch tests in mice and conditioned emotional response (CAR) test in rats).

Materials and Methods

Stable Cell Lines. The cDNAs encoding the human or the rat $mGlu_7$ ($hmGlu_7$ and $rmGlu_7$, respectively) were subcloned into an expression vector containing also the hygromycin resistance gene. For intracellular calcium flux measurement, the cDNA encoding a chimeric Gα protein allowing redirection of the activation signal to intracellular calcium flux was subcloned into a different expression vector containing also the puromycin resistance gene, and both these vectors were co-transfected into HEK293 cells with PolyFect reagent (Qiagen, Basel, Switzerland). Subsequently, hygromycin and puromycin treatment allowed selection of antibiotic resistant clones that had stably integrated one or more copies of both plasmids. Alternatively, the $hmGlu_7$ containing expression vector was transfected into HEK293 cells expressing Phoenyx™, a cAMP biosensor allowing a dynamic real-time cAMP measurement in live cells described previously (Lütjens et al, 2010). Positive functional cellular clones expressing $mGlu_7$ were identified based on using the reference group III mGluR agonist L-2-amino-4-phosphonobutyric acid (L-AP4) and the non-selective mGlu orthosteric antagonist LY341495. HEK293 cells expressing rat or human $mGlu_7$ were maintained in media containing Dulbecco's modified Eagle medium (DMEM), fetal calf serum (10%), penicillin (100 units/mL), streptomycin (100 μg/mL), geneticin (100 μg/mL), hygromycin B (40 μg/mL), and puromycin (1 μg/mL) at 37°C with 5% CO_2 in a humidified atmosphere.

Fluorescent Cell – Based Ca^{2+} Mobilization Assay. This assay was performed in a pH 7.4 buffered solution containing 20 mM HEPES, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.125 mM sulfinpyrazone, and 0.1% glucose. Twenty-four hours prior to the

pharmacological experiment, hmGlu$_7$ or rmGlu$_7$-transfected HEK293 cells were plated out at a density of 2.5x10^4 cells/well in black-well/clear-bottomed and poly-l-ornithine – coated 384-well plates in a glutamine/glutamate-free DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin, supplemented with 1 µg/mL of doxycycline. Cells were incubated overnight at 37°C with 5% CO_2 in a humidified atmosphere. On the day of the assay, the cells were loaded with a 3-µM dye solution of Fluo4-AM (Invitrogen, Lucerne, Switzerland) in assay buffer containing 0.03% pluronic acid. After 1 h at 37°C with 5% CO_2 in a humidified atmosphere, the extracellular dye was removed by washing the cell plate three times with 1X phosphate buffered saline (PBS; Invitrogen, Lucerne, Switzerland). Assay buffer was added to cells and calcium flux was measured using a FLIPR (Molecular Devices, Sunnyvale, USA). After 10 seconds of basal fluorescence recording, compounds to be tested were added to cells in a concentration-dependent manner, and left for incubation on cells for 170 seconds. During that time, changes in fluorescence levels were monitored in order to detect any agonist activity of the compounds. The cells were then stimulated by L-AP4 EC_{80} (concentration giving 80% of the maximal L-AP4 response) for an additional 170 seconds in order to measure inhibiting activities of the compounds. In the reversibility experiments, cells were either washed three times with PBS (1X) after compound addition, or not. Then cells were stimulated by L-AP4 EC_{80}.

Phoenyx cAMP Assay. This assay was performed in the pH 7.4 1X buffered Hanks' Balanced Salt Solution (HBSS; Invitrogen, Lucerne, Switzerland). Twenty-four hours prior to the experiment, human mGlu$_7$-Phoenyx- transfected HEK293 cells were plated out at a density of 2x10^4 cells/well in black-well/clear-bottomed and poly-l-ornithine – coated 384-well plates in

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media containing DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL geneticin, 40 μg/mL hygromycin B, and 1 μg/mL puromycin, supplemented with 1 μg/mL of doxycycline. Cells are incubated overnight at 37°C with 5% CO_2 in a humidified atmosphere. On the day of the assay, the cells were first starved for 1h30 in the assay buffer at 37°C with 5% CO_2 in a humidified atmosphere. Then the cells were loaded with a 4-μM solution of coelenterazine H (Dalton Pharma, Toronto, Canada) in assay buffer, and the intracellular cAMP level was measured using a FLIPR (Molecular Devices, Sunnyvale, USA). After 63 seconds of basal signal recording, compounds to be tested were added to the cells in a concentration-dependent manner, and left for incubation on the cells for 95 seconds. The cells were then co-stimulated by 10 μM forskolin (Sigma-Aldrich; Buchs, Switzerland), and glutamate or L-AP4 EC_{80} (concentration giving 80% of the maximal agonist response) for an additional 10 minutes in order to measure inhibiting activities of the tested compounds. Schild-Plot experiments were performed using the same protocol, by testing L-AP4 in a concentration-dependent manner in the absence or presence of increasing concentrations of tested compounds.

Selectivity. ADX71743 was functionally tested up to 30 μM as agonist, and positive or negative allosteric modulator of other rat or human members of mGlu family ($mGlu_1$, $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$, $mGlu_6$, $mGlu_8$) using the fluorescent cell-based Ca^{2+} mobilization assay described above. In addition, ADX71743 was tested in agonist and antagonist mode in the CEREP P27 cellular functional GPCR profile containing 29 targets (Cerep; Poitiers, France).

Serum protein binding. Serum protein binding was measured by equilibrium dialysis using 96-well plates specifically designed for this purpose (HT Dialysis, Gales Ferry, USA). This reusable

96-well plate is assembled so that each well is divided vertically in two parts by a dialysis membrane. MWCO regenerated dialysis cellulose-membranes (Dialysis Membrane Strips, HT Dialysis, Gales Ferry, USA) with a molecular-weight cut off 12 to 14,000 Da were conditioned according to the manufacturer and used for all experiments. 1 µL of a 1 mg/ml DMSO solution of ADX71743 was added to mouse serum to reach the final concentration of 1 µg/mL. Portions (150 µL) of the serum solution are added to one side of the membrane and the pH 7.4 phosphate buffer solutions is added to the other side of the well. The addition in the two compartments is made simultaneously. Experiments were carried out in duplicate for each time point. Individual wells were used for each time point. The plates were sealed and set in an incubator at 37 °C under gentle shaking. Samples were taken from the serum and buffer compartments at the start of the experiments and after 6 and 7 hours they were immediately stored at 4°C. The serum and buffer samples were subsequently analysed by a specific LC-MS method to measure ADX71743 concentrations. The measured-concentration of ADX71743 in serum and buffer is determined. The portion of bound ADX71743 in serum was calculated according to the following equation (Wright et al., 1996):

$$\%\ \text{new chemical entities (NCE)bound} = (C_{serum} - C_{buffer}) \times 100/C_{serum}$$

Where C_{serum} is the total (bound + free) concentration of ADX71743 in serum after equilibrium is reached and C_{buffer} is the concentration of ADX71743 measured in the buffer solution at the same time. This equation is valid only when the equilibrium between the serum and buffer solutions is completed. Previous experiments demonstrated that, for the reference substances, the equilibrium between the serum and buffer solution is reached after 5-6 hours.

Animals. Unless otherwise specified below, the studies used adult male C57Bl6/J mice (24-30g) and Sprague-Dawley rats (250-350 g; Charles River; L'Arbresle, France). Upon arrival to the animal facility mice were group-housed (5 per cage) in type II cages (16 x 22 x 24 cm), whereas rats were group-housed (2 per cage) in type III cages (22 x 37 x 18 cm). Animals were maintained on a 12-h light/dark cycle (lights on from 07:00 to 19:00 h) under constant temperature (22 ± 2 ºC) and humidity (>45 %) conditions. Standard chow and water were available *ad libitum*. Animals were acclimated for at least 10 days before experimentation. All experimental procedures and conditions were approved by the Ethical Committees of Addex Therapeutics and performed in full compliance with international European ethical standards (86/609-EEC), the French National Committee (décret 87/848) for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 and the Declaration of Helsinki.

Hippocampal electrophysiology. Adult (>8 weeks) female C57Bl6/J mice (Harlan, UK) were killed by decapitation and the brain was removed and placed into ice-cold oxygenated sucrose Krebs' medium containing (mM): sucrose 202, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 10, $CaCl_2$ 0.5, $NaHCO_3$ 26, glucose 10. The brain was hemisected along the midline and 300µm parasagittal slices were prepared with an oscillating microtome (Integraslice; Campden Instruments Ltd., Loughborough, UK). Slices were then transferred to a recovery chamber at room temperature containing oxygenated Krebs' solution (mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10. Following at least 1h of recovery, individual slices were transferred to an interface recording chamber where they were perfused with Krebs' solution (21.5-21.9 °C). Extracellular field potential recordings were made with an Axoprobe 1A amplifier (Axon Instruments Ltd., Inverurie, UK) via a Krebs'-filled glass micropipette (resistance 8-10 MΩ)

positioned in the stratum radiatum of the CA1, digitized (5kHz) via a CED1401 interface and stored on a computer with Spike2 software (Cambridge Electronic Design Ltd., Cambridge, UK). Field excitatory postsynaptic potential (fEPSP) responses were evoked (0.1ms pulses applied every 10s; 3.2-4.5V adjusted to approximately 80% of the maximal spike-free response) by a bipolar stimulating electrode positioned in the stratum radiatum near the CA3-CA1 border.

L-AP4 was prepared as a 100 mM stock solution in water and ADX71743 (10 mM) was prepared in 100% DMSO. Stock solutions were stored in aliquots at -20°C and individual aliquots were thawed on the day of experiment and diluted to the desired concentration in Krebs' solution. DMSO was present throughout the recordings at a concentration of 0.1%. For the electrophysiological studies the average peak amplitude of the fEPSPs was measured over 6 consecutive trials with CED Spike2 software. Data are presented as mean effect ± SEM. Statistical significance was determined by paired t-test using GraphPad Prism (GraphPad Inc., San Diego, USA).

***In vivo* pharmacokinetic studies.** Pharmacokinetic studies following s.c. administration of ADX71743 to mice and rats where performed with a suspension of the drug in a 50% water solution of CD. The volume of administration was 3 mL/kg. ADX71743 was administered at 12.5 and 100 mg/kg in order to cover the range of doses to be used in *in vivo* behavioral studies. Blood was collected at 0.25, 0.5, 1 and 2 hours after administration as terminal samples (n=3 per time point). Samples at 6, 8 and 24 hours were additionally collected in animals receiving the dose of 100 mg/kg. Additionally, brain and cerebrospinal fluid (CSF) samples were collected in animals receiving the dose of 12.5 mg/kg. Blood samples were collected in 1.5 mL polyethylene Eppendorf tubes containing 4 µL of 15% EDTA solution and immediately placed on ice.

Samples were centrifuged at 4°C for 12 minutes at 5900 x g for 3 min (equivalent to 8000 rpm if using an Eppendorf type 5415 centrifuge). Plasma was transferred to 1.5 mL Eppendorf tubes and stored at -20°C until analysis. Brain samples were collected into plastic 24-well plates. CSF was collected into 20-µL heparin-coated capillary tube. For sample preparation, CSF samples were checked carefully for blood contamination and transferred into Eppendorf tubes containing 20 µL of control rat plasma. Subsequently, samples were placed on ice and, if necessary, kept frozen at -20°C until analysis. CSF sample extraction was identical to the procedure described for plasma samples (see below).

Plasma analysis. The plasma sample analysis was performed using a tandem liquid chromatography – mass spectrometry (LC/MS/MS) method (UPLC, Waters coupled with API 3200 Qtrap; Applied Biosystems, Switzerland). Acquisition in mass spectrometry was done in MSMS electrospray-positive mode (MRM), monitoring the following MH transition: 270.09/138.15. The high performance liquid chromatography (HPLC) conditions used were a 1.5-min gradient with ammonium formate 10 mM pH 3.5/acetonitrile formic acid (15 mM) at 0.8mL/min starting with 5% of buffer to 100% acetonitrile mobile phase. To prepare plasma sample, 150 µL of acetonitrile (protein precipitation) was added to 50 µL of plasma spiked respectively with 10 µL DMSO for unknown samples or 10 µL of ADX71743 for calibration and QC samples. After vortexing and centrifugation (15 min, 4 °C, 13200 rpm), a portion (100 µL) was transferred into the 384-well analytical plate. Five microliters of the supernatant were injected into the system. Quantification process was performed using the Analyst software (AB SCIEX; Framingham, USA). The concentrations of the test item in the samples were calculated from the corresponding peak areas produced and using the calibration equation curve (10

calibration points in duplicate). Three levels of QC samples (in duplicate) were added to validate the run. The assay was qualified valid if at least 75% of the calibration points and at least 66% of the QC samples were determined with accuracy equal or better than 20% of the nominal concentrations. The limits of quantification (LOQs) for the compound in plasma were 1.57 and 0.6 ng/mL at 12.5 and 100 mg/kg doses, respectively.

Rotarod Test in Mice. A mouse rotarod apparatus (MED associates; St. Albans, USA) with constant speed (16 rotations per minute) was used in this experiment as described previously (Campo et al., 2011). On the day of the experiment mice (n=10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD) and were tested on the rotarod in two, 3-min sessions performed 30 and 90 min following treatment. Two additional groups of mice (n=10/group) were treated p.o. with either (*R*)-Baclofen (10 mg/kg) or its vehicle (saline) and were tested 60 and 120 min following treatment. The time spent on rotarod (sec) was analysed by Kruskal-Wallis test followed by Dunn's multiple comparisons.

Spontaneous Locomotor Activity Test in Mice. Spontaneous locomotor activity, assessed as horizontal distance travelled (cm), was monitored using Plexiglas arenas (35 X 35 X 50 cm) in conjunction with a video tracking and computerized analysis systems (Viewpoint; Lyon, France) as described previously (Campo et al., 2011). Mice (n=10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD). Two additional groups of mice (n=10/group) were treated p.o. with either (*R*)-baclofen (10 mg/kg) or its vehicle (saline). Thirty and sixty min after administration of ADX71743 and (*R*)-baclofen, respectively, mice were individually placed into arenas and their locomotor activity was monitored for 60 min. The total

distance travelled during the test (cm) was analysed by one-way ANOVA followed by planned comparisons.

Spontaneous Locomotor Activity Test in Rats. Spontaneous locomotor activity, assessed as horizontal distance travelled (cm), was monitored using Plexiglas arenas (50 X 50 X 50 cm) in conjunction with a video tracking and computerized analysis systems (Viewpoint; Lyon, France). Rats (n=9-10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD). Two additional groups of mice (n=8-9/group) were treated p.o. with either (*R*)-baclofen (10 mg/kg) or its vehicle (saline). Thirty and sixty min after administration of ADX71743 and (*R*)-baclofen, respectively, mice were individually placed into arenas and their locomotor activity was monitored for 60 min. The total distance travelled during the test (cm) was analysed by one-way ANOVA followed by planned comparisons.

Marble-Burying Test in Mice. A set of type II cages (with clear Plexiglas covers) used in the experiment contained extra amounts (5-cm high) of sawdust bedding and had ten marbles evenly spaced against the walls of the cage. Mice (n=10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg), or its vehicle (CD). Two additional groups of mice (n=10/group) were administered p.o. either chlordiazepoxide (30 mg/kg) or its vehicle (saline). Thirty and sixty min after administration of ADX71743 or chlordiazepoxide, respectively, animals were individually placed in experimental cages and were left undisturbed for 30 min. At the end of this period, animals were removed from the cage and numbers of buried marbles were counted. The marble was considered to be buried if it had at least 2/3 of its surface covered in sawdust. Terminal blood samples were collected from all ADX71743-treated animals at the end of the experiment

and plasma was analysed as described for the pharmacokinetic studies. The number of buried marbles was analysed by Kruskal-Wallis test followed by Dunn's multiple comparisons.

Elevated Plus-Maze Test in Mice. The elevated plus maze was made of opaque plastic and consisted of 4 arms of equal lengths and width (15 x 4 cm) arranged in the form of a plus sign. Two opposite arms, referred to as closed arms, were enclosed by 12 cm high walls, whereas two remaining arms, referred to as open arms, had no walls. The maze was raised 50 cm above the floor. A mouse was placed in the centre of the maze facing one of the closed arms and was left to explore the maze for 5 min. The arms were cleaned with 35% ethanol between each test session. The experiment was performed under dim light conditions (~70 Lux). The number of open and closed arms entries and time animals spent on open arms were recorded. Mice (n=10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD). Two additional groups of mice (n=10/group) were administered p.o. either chlordiazepoxide (30 mg/kg) or its vehicle (saline). Thirty and sixty min after administration of ADX71743 and chlordiazepoxide, respectively, animals were individually placed on the maze and left to explore it for 5 min. Percept entries into open arms as well as time (sec) spent on open and closed arms of the maze was analysed by one-way ANOVA followed by planned comparisons.

Forced-Swim Test in Mice. The procedure was performed as described previously by Campo et al., (2011). Briefly, one day before the experiment, mice were pre-exposed to swim session using individual glass cylinders (height: 25 cm, diameter: 10 cm) containing water 10 cm deep at 24 $\pm$ 1C° for 15 min. On the test day, 24h later, animals were treated with s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD; n=10/groups). Two additional groups (n=10/group)

were treated i.p. with imipramine (30 mg/kg) or its vehicle (saline). Thirty or sixty min following administration of ADX71743 or imipramine, respectively, animals were exposed to the test swim session for 6 min under identical conditions. All test sessions were recorded by a video camera positioned on the side of the cylinder. A trained observer blind to the treatment scored the tapes. The behavioural measures scored included the duration of immobility exhibited during the last 4 min of the 6-min test. An animal was considered to be immobile as it remained floating motionless in the water making only the movements necessary to keep its head above the water. Terminal blood samples were collected from all ADX71743-treated animals at the end of the experiment and plasma was analysed as described for the pharmacokinetic studies. The time spent immobile (sec) in the FST was analysed with a one-way ANOVA, followed by planned comparisons.

Amphetamine-induced Hyperactivity in Mice. The experiment was performed in the locomotor arenas used for the assessment of spontaneous locomotor activity in mice. Mice (n=10/group) were treated s.c. with ADX71743 (50, 100, 150 mg/kg), or its vehicle (50% cyclodextrine). Two additional groups (n=10/group) received p.o. risperidone (0.3 mg/kg) or its vehicle (saline). After treatment, animals were individually placed into activity arenas for 30 min of habituation. At the end of this period they were challenged i.p. with either amphetamine (3 mg/kg) or its vehicle (saline), returned to the arenas and monitored for activity for the additional 60 min. The total distance travelled during the test (cm) was analysed by one-way ANOVA followed by planned comparisons.

DOI-induced Head Twitches in Mice. Adult male C57Bl6/J mice were purchased from Charles River (Margate, UK) and group-housed under standard laboratory conditions at RenaSci Ltd. (Nottingham, UK). Mice (n=8/group) were treated with ADX71743 (50, 100, 150 mg/kg, s.c.), its vehicle (CD; s.c.) or olanzapine (0.1 mg/kg i.p.). Sixty (ADX71743 or CD) or thirty (olanzapine) minutes later animals were challenged (i.p.) with either vehicle (saline) or DOI (3 mg/kg). Each animal was then placed into a novel cage and the number of head twitches was counted for a 6 minutes immediately after administration of DOI or saline by an observer blind to drug treatment. Terminal blood samples were collected from all ADX71743-treated animals at the end of the experiment and plasma was analysed as described for the pharmacokinetic studies. The number of DOI-induced head twitches was analysed by one-way ANOVA followed by planned comparisons.

Conditioned Emotional Response (CAR) Test in Rats. Adult male Wistar rats were purchased from Charles River (Margate, UK) and group-housed under standard laboratory conditions at RenaSci Ltd. (Nottingham, UK). Conditioned avoidance behavior was assessed using automated shuttle boxes (42 x 16 x 20 cm; Med Associates; St. Albans USA), partitioned into two compartments and equipped with infrared-sensitive photocells. Each box was placed into a sound-attenuated chamber. Animals were trained to move to the adjacent compartment within 10s upon administration of the conditioned stimulus (tone and light), in order to avoid exposure to the unconditioned stimulus (footshock, 0.5mA for a maximum duration of 10s) via the grid floor. In the first phase of training, each animal underwent 30 trials in a 30 min test session with a variable inter-trial interval of 20-30s. If an animal crossed to the other compartment to avoid the shock, this was recorded by the apparatus as an avoidance response. If the animal crossed to

the other compartment during presentation of the shock, this was recorded by the apparatus as an escape response. The training continued until 80% avoidance responses (i.e. 24 correct trials) were obtained for the group. In the second phase of training each animal underwent 10 trials in a 10 min session with a variable inter-trial interval of 20-30s. This was continued until 80% (i.e. 8 correct trials) avoidance responses were obtained for the group. The day prior to the experiment a baseline (pre-test) session was run. In this session all animals were dosed with vehicle 60 minutes prior to the test. Animals then underwent the test protocol (10 trials in a 10 minute session with a variable inter-trial interval of 20-30s). Animals that exhibited stable performance (>80% avoidance responses for last 3 drug-free CAR session), underwent drug (or vehicle) testing the next day. Any animals that did not meet the success criteria underwent further training on the day of the experiment. As a part of the pharmacological validation of the model animals trained in the CAR were tested with antipsychotic drugs (haloperidol, aripiprazole, risperidone and olanzapine) administered in weekly intervals. All drugs dose-dependent inhibited avoidance response and increased escape responses (data not shown). One week after the last validation experiment with an antipsychotic drug, animals were tested with a vehicle (50% CD) and the day after received ADX71743 (10, 30, 100 mg/kg, s.c.), its vehicle (50% CD, s.c.) or olanzapine (0.1 mg/kg p.o.) 60 min before being evaluated in the test protocol as described above. At the end of the experiment blood from all ADX71743-treated animals was collected via tail vein and plasma was analysed as described for the pharmacokinetic studies.

Drugs. L-AP4 (L(+)-2-amino-4-phosphono butyric acid; suspended in 0.1N NaOH) was purchased from Abcam Biochemicals (Cambridge, UK). Glutamate, or L-glutamic acid hydrochloride, ((*S*)-2-Aminoglutaric acid, (*S*)-2-Aminopentanedioic acid ammonium salt), (*R*)-

baclofen ((R)-4-amino-3-(4-chloro-phenyl) butanoic acid), D-amphetamine, chlordiazepoxide, imipramine, olanzapine and risperidone were purchased from Sigma-Aldrich (Buchs, Switzerland). LY341495 ((2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid; $mGlu_{2/3}$ orthosteric antagonist; Kingston et al., 1998) was purchased from Tocris Bioscience (Bristol, UK). DOI (2,5-dimethoxy-4-iodoamphetamine) was purchased from Steroplast Ltd. (Manchester, UK).

ADX71743 ((+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one) was synthesized at Addex Therapeutics. The compound was suspended in water containing a 50% hydroxyl-propyl-β-cyclodextrine (CD). The suspensions were homogenised with stainless steel balls for 30 min at 30 Hz in a 2mL Eppendorf tube, then vortexed and sonicated for 10 min. D-amphetamine was dissolved in saline and administered intraperitoneally (i.p.) at 3 mL/kg volume. (*R*)-Baclofen was dissolved in saline and administered orally (p.o.). Chlordiazepoxide and risperidone were suspended in saline and administered p.o. Olanzapine was suspended in saline and administered i.p. Imipramine was suspended in distilled water and administered i.p. All drugs dosed s.c. or i.p. were administered at 3 mL/kg volume. All drugs dosed p.o. were administered either at 10 mL/kg or at 5 mL/kg volumes when given to mice and rats, respectively. All solutions and suspensions were prepared fresh daily. All doses of pharmacological agents are expressed as free base.

Results

Identification and *in vitro* pharmacological characterization of ADX71743 on rat and human recombinant mGluRs and native mouse receptor. Following a high throughput screening campaign of Addex' corporate library using a HEK293 cell line stably coexpressing $mGlu_7$ with a chimeric $G_{i\alpha}$ protein, several compounds inhibiting the calcium flux induced by L-AP4 were identified. Following a hit confirmation and lead optimization processes, ADX71743 was identified (Tang et al, submitted; Figure 1A). This compound tested in cell lines expressing $hmGlu_7$ or $rmGlu_7$together with a chimeric Gα protein, allowing redirection of receptor activation onto calcium signalling was found to fully inhibit an EC_{80} of L-AP4 (2 mM), with full efficacy and an IC_{50} of 63 ± 2 nM and 88 ± 9 nM respectively (Table 1, Figure 1B). In comparison, the IC_{50} of reference compound LY341495, a non-selective orthosteric $mGlu_{2/3}$ antagonist was 345 ± 9.5 nM and 449 ± 42 nM for the $hmGlu_7$ and $rmGlu_7$ clones, respectively (Table 1, Figure 1B), in agreement with values previously reported (Kingston et al., 1998).

$mGlu_7$ is naturally coupled to the adenylate cyclase through Gi/o. Therefore to demonstrate activity of ADX71743 on the physiological signalling of $mGlu_7$, the compound was tested on cells co-expressing $hmGlu_7$ and a cAMP biosensor Phoenyx^{TM} (Lütjens et al., 2010). In this assay, a full concentration response curve with glutamate, the natural ligand of $mGlu_7$ receptor, saturates, allowing calculation of an EC_{50} of 264 ± 37 μM (Table 1). ADX71743 could therefore be tested against an EC_{80} of glutamate (IC_{50} of 22 ± 4 nM; Table 1) as well as against an EC_{80} of L-AP4 (IC_{50} of 125 ± 17 nM; Table 1, Figure 1C). The IC_{50} of LY341495 when tested against L-AP4 was 2262 ± 268 nM (Table 1, Figure 1C).

To further characterize the pharmacological profile of ADX71743, its mode of action was analyzed by Schild-plot experiments in the Phoenyx™ cAMP assay and compared with the effect of the orthosteric antagonist LY341495. ADX71743 induced a concentration-dependent rightward shift of the L-AP4 concentration-response curve (a 3-fold-shift of the EC_{50} of L-AP4 with 0.1 μM of compound on average, and a maximum 13.7-fold-shift observed with 10 μM of compound) together with a decrease of L-AP4 efficacy (Figure 2B). In a similar protocol, LY341495 induced a concentration-dependent rightward shift of the L-AP4 concentration-response curve without any impact on maximal efficacy of the agonist as expected for a competitive antagonist- competitive agonist pair (Figure 2C; calculated pA_2=5.92, slope=0.83). In order to test the reversibility of ADX71743 activity, experiments were performed in the calcium assay using the $hmGlu_7$ clone. Cells were either washed 3 times or were kept without washing following addition of ADX71743, followed by measurement of calcium levels. ADX71743 was found active on an EC_{80} of L-AP4 as expected in non-washed cells, whereas the inhibitory effect was not observed in washed cells, demonstrating the reversible effect of the compound on $mGlu_7$ (Figure 2A).

We also investigated the selectivity of ADX71743 versus other mGlu-expressing cells in series of FLIPR experiments and observed that it had no detectable activity (agonist or allosteric effects) in cell lines expressing $hmGlu_3$, $hmGlu_4$, $rmGlu_5$, $hmGlu_6$, and $hmGlu_8$. A negligible inhibition of $rmGlu_1$ (32% at 30 μM) and a weak positive allosteric modulator (PAM) effect on $hmGlu_2$ (EC_{50} of 11 μM) was measured. When further tested in a functional GPCR screen against 27 targets (Cerep profile P27, excluding muscarinic M_2 and M_4 receptors, unavailable at the time of the test; Cerep, Poitiers, France) in agonist and antagonist mode, no stimulation or inhibition above 27% was observed (data not shown).

Hippocampal electrophysiology. In the presence of L-AP4 (300 µM) the hippocampal CA1 fEPSP amplitude was reversibly depressed to 62±1 (n=6) % of control values. Co-application of ADX71743 and L-AP4 resulted in a concentration-dependent reversal of the L-AP4–induced depression, with 0.1 µM ADX71743 reversing the effects of L-AP4 by 11±1% (n=3; $P<0.01$) and 10 µM resulting in a 20±3% reversal (n=6; $P<0.001$; Figure 3 A-B).

Serum protein binding; plasma and brain pharmacokinetic profile of ADX71743. In mice and rats s.c. administration of 100 mg/kg ADX71743 resulted in a similar pk profile (Table 2). After s.c. administration of 12.5 ADX71743 in mice, plasma reached high C_{max} between 0.25 and 0.5 hours, then declining rapidly, following a half-life of approximately 0.5 hours (Figure 4A; Table 2). After s.c. administration of 12.5 mg/kg, CSF and brain concentrations of ADX71743 also reached C_{max} rapidly. Thereafter, brain concentrations decline rapidly, while the CSF concentrations were sustained for a slightly longer period (Figure 4 A; Table 2). The ratio between the CSF and plasma concentration at C_{max} was 5.3, which is consistent with the free plasma concentration available for diffusion in the brain, as determined in the serum protein binding studies (fu = 0.044). The AUC and C_{max} values of plasma exposure show linear and similar increases in relation to the dose following s.c. administration of 12.5 and 100 mg/kg ADX71743 in mice (Figure 4B).

Spontaneous Locomotor Activity Test in Mice and Rats. ADX71743 (50, 100, 150 mg/kg) had no effect on spontaneous locomotor activity in mice, while baclofen markedly supressed (80%; $P<0.001$) the total distance travelled by animals when compared to vehicle treatment

(Figure 5A). ADX71743 (50, 100, 150 mg/kg) also had no effect on spontaneous locomotor activity in rats, while baclofen resulted in a virtually full ($p<0.001$) suppression of activity (Figure 5B).

Rotarod Test in Mice. ADX71743 (50, 100, 150 mg/kg) had no effect on latencies to fall from rotarod (sec) when animals were tested 30 and 90 min following administration (Table 3). In contrast, baclofen resulted in robust reduction of latencies to fall (75%; $P<0.001$) when animals were tested 60 and 120 min following administration (Table 3).

Marble Burying Test in Mice. ADX71743 resulted in ~60% reduction in the number of buried marbles at 50 and 100 mg/kg ($P<0.01$ and $P<0.05$, respectively), with further reduction (~75% $P<0.001$) at 150 mg/kg when compared to its vehicle (Table 4). The corresponding concentrations of ADX71743 in plasma in animals treated at 50, 100 and 150 mg/kg were 3451, 6990 and 10430 ng/mL, respectively. These plasma concentrations resulted in CSF/IC_{50} values of 4, 9, and 14, respectively (Table 4) based on fu determined in independent experiments. Chlordiazepoxide-treated mice buried ~60% ($P<0.01$) fewer marbles compared to corresponding vehicle-treated controls (Table 4).

Elevated Plus Maze Test in Mice. ADX71743 resulted in dose-dependent increases in the percentage of entries into open arms [$F(5, 54)=6.02$, $P<0.001$]. Specifically, at 100 and 150 mg/kg ADX71743 resulted in 2.3- ($P<0.05$) and 2.5-fold ($P<0.01$) increases in open arm entries compared to corresponding vehicle-treated controls (Figure 6A). ADX71743 also increased the time spent on open arms [$F(5, 54)=6.65$, $P<0.001$]. Specifically, at 100 and 150 mg/kg

ADX71743 resulted in 2.3- and 2.5-fold (both P <0.05) increases in time spent on open arms compared to corresponding vehicle-treated controls (Figure 6A). Diazepam-treated animals exhibited similar, 2.5-fold (P<0.001) increases in the percentage of entries into open arms and in time spent on those arms compared to vehicle-treated control (Figure 6A, B). There was no effect of treatment on the number of closed arm entries (data not presented).

Forced Swim Test in Mice. ADX71743 (50, 100, 150 mg/kg) had no effect on the time animals spent in immobility, while imipramine-treated animals exhibited over 50% reduction (P<0.001) in the immobility time (Table 5). The corresponding plasma concentrations of ADX71743 in animals treated at 50, 100 and 150 mg/kg were 7265, 12607 and 13107 ng/mL, respectively. These plasma concentrations resulted in CSF/IC_{50} values of 9, 16, and 17, respectively (Table 5).

Amphetamine-induced Hyperactivity Test in Mice. ADX71743 (50, 100, 150 mg/kg) dose-dependently reduced amphetamine-induced hyperactivity [F(5, 95)=46.2, P <0.001; Figure 7]. Specifically, at 100 and 150 mg/kg there was approximately 20% (P<0.01) and 30% (P<0.001) reductions in hyperactivity compared to corresponding vehicle-pretreated controls (Figure 7). Risperidone-treated animals exhibited robust reduction in amphetamine-induced hyperactivity (~ 80%, P<0.001; Figure 7).

DOI-induced Head Twitches in Mice. ADX71743 (50, 100, 150 mg/kg) had no effect on the number of DOI-induced head twitches, while olanzapine resulted in nearly 80% reduction (P<0.001) in this number (Table 6). The corresponding plasma concentrations of ADX71743 in

animals treated at 50, 100 and 150 mg/kg were 3233, 4273 and 6311 ng/mL, respectively. These plasma concentrations resulted in CSF/IC_{50} values of 4, 6, and 8, respectively (Table 6).

Conditioned Emotional Response Test in Rats. ADX71743 (10, 30, 100 mg/kg) had no effect on numbers of avoidances or escapes (Table 7). In contrast, olanzapine resulted in ~ 60 % (P<0.001) reduction in the number of avoidances and more than 3-fold (P<0.01) increases in the number of escapes (Table 7). None of the animals, except one, showed escape failures during the test (data not included). The corresponding plasma concentrations of ADX71743 in rats treated at 10, 30 and 100 mg/kg were 1188, 3351 and 9800 ng/mL, respectively. These plasma concentrations resulted in CSF/IC_{50} ratio of 1, 3, and 9, respectively (Table 7).

Plasma concentration of ADX71743 in *in vivo* studies. Concentrations of ADX71743 in plasma following s.c. administration at 50, 100, and 150 mg/kg in the DOI-induced head-twitch, MB and the FST in mice are shown in Figure 8. Plasma concentrations of ADX71743, measured in the FST study, were higher than those measured in the DOI and MB study, probably due to different post-dosing sampling time (30 min vs 60 min) and the rapid clearance of the compound (Figure 8).

Discussion

Over the last decade data supporting the hypothesis that $mGlu_7$ receptor plays a pivotal role in the CNS have been accumulating. While initial studies using inactivation of $mGlu_7$ receptor provided important first evidence of its role, those involving direct and selective engagement of the receptor have not been possible due to the absence of subtype selective and brain-penetrant molecules, like ADX71743.

Here we describe the *in vitro* and *in vivo* pharmacological properties of ADX71743, a potent and selective $mGlu_7$ NAM. We identified ADX71743 through chemical lead optimization after a high-throughput screening campaign of the corporate chemical library using a FLIPR assay. ADX71743 was found to completely block the $mGlu_7$ agonist-induced signal, tested in recombinant systems measuring either changes in intracellular calcium or changes in intracellular levels of cAMP. However, in curve shift analysis using the cAMP assay, the degree of inhibition of the L-AP4 response appeared to saturate at around 40% for L-AP4 concentrations above 300 μM, suggesting a weak degree of cooperativity between ADX71743 and L-AP4. It also suggests that at high concentrations of glutamate, some $mGlu_7$-mediated signalling may persist even in presence of high concentrations of the NAM. The Schild plot experiments clearly indicate the non-competitive nature of ADX71743 inhibition, whereas simple wash experiments seem to indicate that its binding to the receptor is readily reversible. Activity of ADX71743 at the native $mGlu_7$ was demonstrated in the *in vitro* preparation of mouse hippocampus, where ADX71743 attenuated the depression of synaptic transmission induced by the Group III mGlu agonist L-AP4. ADX71743 also exhibited good exposure following s.c. administration in mice and rats making it suitable for *in vivo* testing. The

pharmacokinetic profile of ADX71743 was found to be similar in mice and rats when the compound was given s.c. at 100 mg/kg. Considering an IC_{50} of 125 nM, and an average CSF/total plasma concentration ratio of 0.05 or more, plasma concentration above 1 μg/mL would be necessary to cover 50% of the receptor activity. A pharmacokinetic analysis of the compound showed that a C_{max} just above this value was reached for a very short period of time when ADX71743 was administered at 12.5 mg/kg s.c. Therefore we chose higher doses (50, 100 and 150 mg/kg, s.c.) for *in vivo* studies in mice and rats and confirmed that pharmacological activity can be observed when plasma concentrations are above 1 μg/mL.

In vivo efficacy of ADX71743 was evaluated in rodent models of anxiety, depression and psychosis based on earlier evidence, but also reflecting regional localization of $mGlu_7$ within the mammalian CNS. While being widely distributed throughout the brain, $mGlu_7$ receptor shows particularly high abundance in the neocortex, piriform and entorhinal cortices, hippocampus, amygdala, globus pallidus, ventral pallidum, and the locus coeruleus (Kinoshita et al., 1998). Abnormalities in these regions have been linked to anxiety disorders (Walker and Davis 2002), depression (Sanacora et al., 2012) and psychosis (Moghaddam and Adams 1998; Schoepp and Marek 2002) among other CNS disorders.

ADX71743 showed anxiolytic-like profile in the MB and the EPM tests in mice. Both tests are known to be sensitive to typical anxiolytic drugs (Lister, 1990; Nicolas et al 2006; Pellow et al., 1985), the latter also being relevant to obsessive compulsive disorder (OCD; Thomas et al., 2009). ADX71743 resulted in robust reductions in numbers of buried marbles to near maximal levels at lower doses (50 and 100 mg/kg) and similar to those produced by an anxiolytic drug, chlordiazepoxide. The plasma analysis confirmed that approximately 60% reduction in the number of buried marbles corresponded to CSF/IC_{50} values of 4 to 9 (at 50 and

100 mg/kg), followed by further reductions (73%) at CSF/IC_{50} = 14 (at 150 mg/kg). In accordance with our findings, $mGlu_7$ KO mice exhibited approximately 60% reduction in the number of buried marbles compared to their wild type controls (Callaerts-Vegh et al., 2006). In the EPM test ADX71743 dose-dependently increased open-arm entries and the time spent on these arms, without producing non-specific changes in activity on closed arms. In accord with our findings, $mGlu_7$ KO mice showed increases in open arm entries and time spent on these arms compared to their wild type controls (Callaerts-Vegh et al., 2006).

At this point we can only speculate on how the reduction in activity of the $mGlu_7$ receptor can lead to reduced anxiety-like reactivity. The $mGlu_7$ receptor is located presynaptically and, depending on the type of neuron it is located on, can regulate the release of glutamate, GABA or other (e.g. norepinephrine) neurotransmitters (O'Connor et al., 2010). Glutamate has low affinity to $mGlu_7$ receptor (Okamoto et al., 1994), which remains inactive under normal conditions, only becoming active under the conditions of excessive glutamate release (Ferraguti and Shigemoto 2006). While an $mGlu_7$ NAM is expected to reduce receptor-mediated inhibitory control, the net outcome of this disinhibition will depend on a specific brain region. Future studies involving site-specific injections of ADX71743 can aid in further understanding the question of modulation of anxiety by the $mGlu_7$ receptor. In addition there is a possibility that reduced activity of $mGlu_7$ will impact anxiety-like reactivity via modulating several downstream targets, especially those involved in reactivity to stress. According to Mitsukawa et al (2006) $mGlu_7$ KO mice exhibit signs of HPA axis dysregulation, including upregulation of glucocorticoid (GR) and 5-HT_{1A} receptors in the hippocampus, increased sensitivity to GR-mediated negative feedback and increases in BDNF protein in the hippocampus (Mitsukawa et al., 2006). These changes correlate well with reduced anxiety- and depression-like reactivity of

$mGlu_7$ KO mice (Cryan et al., 2003). Whether inhibition of $mGlu_7$ with a NAM can lead to alterations in HPA axis seen in $mGlu_7$ KO animals remains to be investigated.

ADX71743 failed to show antidepressant-like profile in the mouse forced swim test despite reaching CSF/IC_{50} values of 9, 16 and 17 at 50, 100 and 150 mg/kg doses, respectively. Also, ADX71743 failed to show a clear antipsychotic-like profile in models relevant to psychosis. The amphetamine-induced hyperactivity, DOI-induced head twitch and CAR tests in rodents have been shown to be sensitive to typical and atypical antipsychotic drugs (Ellenbroek 1993; Wadenberg 2010, Wettstein et al., 1999). The rationale for testing ADX71743 in models of psychosis, in part, came from several independent studies showing a link between polymorphisms in $mGlu_7$ and schizophrenia (Ganda et al., 2009; Ohtsuki et al., 2008; Shibata et al., 2009). ADX71743 resulted in dose-dependent, albeit modest reduction of amphetamine-induced hyperactivity in mice. However, the follow up mouse DOI-induced hyperactivity and the rat CAR tests revealed no activity of the compound despite it reaching adequate concentrations in plasma and CSF for *in vivo* activity (see Tables 6 and 7).

We can only speculate on reasons why $mGlu_7$ NAM ADX71743 was inactive in tests relevant to depression and psychosis. The outcome of *in vivo* studies is unlikely to be impacted by regional distribution of $mGlu_7$ in mice and rats, which is virtually identical in these species (Kinoshita et al., 1998). In fact, only in the medial habenula, $mGlu_7$ is present in high abundance in rats, while being absent in mice, while the opposite trend is seen in the cerebellar nuclei (Kinoshita et al., 1998). One explanation of *in vivo* results is involvement of $mGlu_7$ in anxiety-like reactivity, but not in depression or psychosis. Future studies involving more disease-relevant animals models, such as genetic line of “helpless” mice (El Yacoubi et al., 2003) and Flinders Sensitive line rats (Overstreet et al., 2005) for depression as well as rats exposed in

utero to mitotoxin methylaxosymethanol acetate (MAM) for schizophrenia (Lodge and Grace 2009), can shed light on this question. There is also a possibility that the effect of ADX71743 was linked to $mGlu_7$ concentration in a specific neural circuit recruited in the *in vivo* test. Such possibility is unlikely, since both amphetamine-induced hyperactivity (where a weak effect was seen) and CAR test (where there was no effect) rely on the nucleus accumbens and the ventral tegmental area (Geyer and Ellenbroek 2003, Wadenberg 2010), anatomical regions that have similar low concentration of $mGlu_7$ (Kinoshita et al., 1998).

In conclusion, here we present *in vitro* and *in vivo* characterization of ADX71743 as a centrally-active compound suitable for investigation of the role of $mGlu_7$ receptor. This compound shows potent NAM activity at the $mGlu_7$ receptor with a clean selectivity profile at other subtypes of the mGlu family and other GPCRs, and a pharmacokinetic profile making it suitable for *in vivo* profiling. *In vivo*, ADX71743 shows anxiolytic-like efficacy in the mouse MB and EPM tests. Interestingly, the compound did not demonstrate antidepressant-like activity in the FST – suggesting that reduction of $mGlu_7$ via NAM mechanism is more relevant for anxiety than depression. The compound also was largely inactive in models predictive antipsychotic-like activity. Further optimization of this and other series of $mGlu_7$ NAMs is underway to provide even more potent and better exposed compounds for future studies. In short, these data suggest that $mGlu_7$ inhibition merits further study as a novel approach for the treatment of OCD and other anxiety disorders.

Acknowledgments

The authors would like to thank Sonia Manganiello for determining chirality of ADX71743, as well as Jean-Philippe Rocher and Christelle Bolea for reviewing the manuscript. Also, the authors thank Steven Vickers and Sharon Cheetham at RenaSci Ltd. (Nottingham, UK) for their support in DOI-induced head twitch and conditioned emotional response tests.

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Figure Legends

Figure 1 The chemical structure of ADX71743 ((+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one), A), concentration-response curves of ADX71743 and LY341495 on L-AP4-induced intracellular Ca^{2+} mobilization (B) and concentration-response curves of ADX71743 and LY341495 on intracellular cAMP decrease induced by L-AP4 (C). Data are presented as a percentage of the response to 3 mM of L-AP4.

Figure 2 Reversibility of the antagonistic activity of ADX71743 toward $hmGlu_7$ in intracellular Ca^{2+} mobilization assay (A). Effect of ADX71743 and LY341495 on concentration-response curves of L-AP4 in the Phoenyx cAMP assay (B, C). Agonist concentration-response curves for L-AP4 induced intracellular cAMP decrease were generated in the absence or presence of various concentrations of ADX71743 (B) or LY341495 (C).

Figure 3 Reversal of L-AP4-induced depression of synaptic transmission in the presence of ADX71743. Representative traces (averaged fEPSPs in response to Schaffer collateral stimulation) from a single hippocampal slice experiment taken at different time points (i-iv) as indicated in the time course plot below (A). For clarity the stimulus artifacts have been truncated. Mean percentage reversal of the depression of fEPSPs amplitude induced by L-AP4 (300 µM) by ADX71743 (B; n=3-6; error bars represent SEM; $^{**}P<0.01$; $^{***}P<0.001$).

Figure 4 Plasma (ng/mL), CSF (ng/mL) and brain (ng/g) concentrations of ADX71743 in mice following s.c. administration at 12.5 mg/kg and sample collection 0.25, 0.5, 1 and 2 hours post-

administration (A). The C_{max} (ng/mL) and area under the curve with extrapolation to infinity (AUC_{inf}; ng.h/mL) values following s.c. administration of ADX71743 (0, 12.5 and 100 mg/kg) in mice (B).

Figure 5 Locomotor activity (distance travelled; cm) of male C57Bl6/J mice (n=10/group; A) and Sprague-Dawley rats (n=8-10/group; B) during 60 min expressed as total distance. Animals were pretreated with ADX71743 (50, 100, 150 mg/kg s.c.), 50% hydroxyl-propyl-β-cyclodextrine (CD, s.c.), (*R*)-Baclofen (10 mg/kg, p.o.) or saline (Sal; p.o.). ADX71743 and its vehicle were administered 30 min, while (*R*)-Baclofen and its vehicle, 60 min before test. Each point represents the observed mean (± SEM). ***P<0.001 compared to Sal.

Figure 6 Percent entries into open arms (A) and time spent (sec) on open arms (B) by male C57Bl6/J mice (n=10/group) during 5 min of the elevated plus maze (EPM) test. Animals were pretreated with ADX71743 (50, 100, 150 mg/kg s.c.), 50% hydroxyl-propyl-β-cyclodextrine (CD, s.c.), diazepam (1.5 mg/kg, p.o.) or saline (Sal; p.o.). ADX71743 and its vehicle were administered 30 min, while diazepam and its vehicle, 60 min before test. Each point represents the observed mean (+SEM). * P <0.01, ** P <0.001 compared to CD. ###P<0.001 compared to Sal

Figure 7 Total distance (cm) traveled by male C57Bl6/J mice (n=20/group) during 60 min of the amphetamine-induced hyperactivity test. Animals were pretreated with ADX71743 (50, 100, 150 mg/kg s.c.), 50% hydroxyl-propyl-β-cyclodextrine (CD, s.c.), or risperidone (0.3 mg/kg, p.o.) and habituated to the locomotor arenas for 30 (ADX71743/CD) or 60 (risperidone) min. At

the end of the habituation period animals were challenged i.p. with either amphetamine (3 mg/kg) or saline and tested for further 60 min. Each point represents the observed mean (+SEM). $^{**}P<0.01$, $^{***}P<0.001$ compared to Ref.

Figure 8 Plasma concentration (ng/mL) of ADX71743 following s.c. administration at 50, 100 and 150 mg/kg in mice across 3 experiments including DOI-induced head twitches (DOI), marble burying (MB) and forced swim test (FST). Samples were taken approximately 30 (FST) or 60 (DOI and MB) min following administration of the compound.

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Tables

Table 1. Summary of functional potency of reference agonists L-AP4 and glutamate (EC_{50} values), and of ADX71743 and LY341495 (IC_{50} values) on recombinant $mGlu_7$.

Compound	FLIPR Ca^{2+} assay		Phoenyx cAMP assay
	Human $mGlu_7$ recombinant	Rat $mGlu_7$ recombinant	Human $mGlu_7$ recombinant
L-AP4 EC_{50}, μM[a]	630 ± 15	646 ± 61	133 ± 8
Glutamate EC_{50}, μM[a]	not saturating	not saturating	264 ± 37
ADX71743 IC_{50}, vs EC_{80} of L-AP4, nM[a]	63 ± 2	88 ± 9	125 ± 17
ADX71743 IC_{50}, vs EC_{80} of glutamate, nM[a]			22 ± 4
LY341495 IC_{50}, nM[a]	345 ± 9.5	449 ± 42	2262 ± 268

[a]EC_{50} values (μM) and IC_{50} values (nM) are expressed as mean ± SEM from at least five independent experiments performed in duplicate.

Table 2. Summary of the pharmacokinetic properties of ADX71743 after s.c. administration to mice and rats

	mice		rats
Route	**s.c.**	**s.c.**	**s.c.**
Dose (mg/kg)	12.5	100	100
$T_{1/2}$ (h)	0.68	0.40	1.5
C_{max} (ng/mL)	1308	12766	16800
T_{max} (h)	0.25	0.5	0.7
CSF $_{conc}$/Plasma $_{conc}$ (%)*	5.3%		5.3
Brain/Plasma*	1.0		

*at C_{max}

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Table 3. Activity of ADX71743 in the mouse rotarod test.

				1st session		2nd session	
Treatment	**Route**	**Dose (mg/kg)**	**Animal n**	**Mean ± SEM**	**% of Change**	**Mean ± SEM**	**% of Change**
CD	s.c.		10	178 ± 2.3	0	176 ± 3.8	0
ADX71743	s.c.	50	10	173 ± 6.7	-2.5	170 ± 9.9	-3.5
ADX71743	s.c.	100	10	174 ± 4.9	-1.9	180 ± 0.0	2.1
ADX71743	s.c.	150	10	159 ± 11.4	-10.6	180 ± 0.0	2.1
Saline	p.o.		10	180 ± 0.0			
Baclofen	p.o.	10	10	47 ± 6.7***	-74.1	43 ± 3.4***	-75.9

***P<0.001 compared to corresponding vehicle.

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Table 4. Activity of ADX71743 in the mouse marble burying (MB) test and measured plasma, CSF concentrations and CSF conc/$IC_{50\ (in\ vitro)}$ of ADX71743 in mice at the end of the marble burying experiment.

Treatment	Route	Dose (mg/kg)	Animal n	Marbles buried (Mean ± SEM)	% of Change	Plasma exposure (ng/mL)	Plasma exposure (nM)	CSF exposure[a] (ng/mL)	CSF exposure[a] (nM)	CSF/ $EC_{50\ (in\ vitro)}$
CD	s.c.		10	6.3 ± 0.5						
ADX71743	s.c.	50	10	2.4 ± 0.6**	-62	3451	12814	152	564	4
ADX71743	s.c.	100	10	2.7 ± 0.8*	-57	6990	25958	308	1142	9
ADX71743	s.c.	150	10	1.7 ± 0.7***	-73	10430	38732	459	1704	14
Saline	p.o.		10	6.3 ± 0.9						
Chlordiazepoxide	p.o.	10	10	2.3 ± 0.6**	-63					

*P <0.05, **P <0.01, ***P <0.001 compared to corresponding vehicle. [a]CSF exposure was calculated from the plasma exposure using mice plasma protein binding (PPB; fu=4.4%).

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Table 5. Activity of ADX71743 in the forced swim test (FST) and measured plasma, CSF concentrations and CSF conc/$IC_{50\ (in\ vitro)}$ of ADX71743 in mice at the end of the experiment.

Treatment	Route	Dose (mg/kg)	Animal n	Immobility (Mean ± SEM)	Plasma exposure (ng/mL)	Plasma exposure (nM)	CSF exposure[a] (ng/mL)	CSF exposure[a] (nM)	CSF/ $EC_{50\ (in\ vitro)}$
CD	s.c.		10	164 ± 10					
ADX71743	s.c.	50	10	155 ± 11	7265	26977	320	1187	9
ADX71743	s.c.	100	10	145 ± 10	12607	46813	555	2060	16
ADX71743	s.c.	150	10	159 ± 11	13107	48671	577	2142	17
Saline	s.c..		10	137 ± 15					
Imipramine	i.p.	30	10	63 ± 14**					

***P* <0.01 compared to corresponding vehicle. [a]CSF exposure was calculated from the plasma exposure using mice plasma protein binding (PPB; fu=4.4%).

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Table 6. Activity of ADX71743 and olanzapine in the DOI-induced head twitches test in mice together with the measured plasma, CSF concentrations and CSF conc/$IC_{50\ (in\ vitro)}$ of ADX71743 in mice at the end of the experiment.

Pretreatment	Dose (mg/kg)	Route	Animal n	DOI-induced twitches (Mean ± SEM)	Plasma exposure (ng/mL)	Plasma exposure (nM)	CSF exposure[a] (ng/mL)	CSF exposure[a] (nM)	CSF/ EC_{50} (in vitro)
CD / Saline	0 / 0		8	0.7 ± 0.3					
CD / DOI	0 / 3	s.c. / i.p.	8	23.1 ± 1.8					
ADX71743 / DOI	50 / 3	s.c. / i.p.	8	18.1 ± 1.6	3233	12007	142	528	4
ADX71743 / DOI	100 / 3	s.c. / i.p.	8	21.1 ± 1.5	4273	15865	188	698	6
ADX71743 / DOI	150 / 3	s.c. / i.p.	8	21.8 ± 2.0	6311	23436	278	1031	8
Olanzapine / DOI	0.1 / 3	p.o. / i.p.	8	5.0 ± 0.4***					

***P<0.001 compared to corresponding vehicle. [a]CSF exposure was calculated from the plasma exposure using mice plasma protein binding (PPB; fu=4.4%).

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Table 7. Activity of ADX71743 in the conditioned emotional response (CAR) test together with the measured plasma, CSF concentrations and CSF conc/$IC_{50\ (in\ vitro)}$ of ADX71743 in rats at the end of the experiment.

Treatment	Route	Dose (mg/kg)	Animal n	Avoidances (Mean ± SEM)	Escapes (Mean ± SEM)	Plasma exposure (ng/mL)	Plasma exposure (nM)	CSF exposure[a] (ng/mL)	CSF exposure[a] (nM)	CSF/ $EC_{50\ (in\ vitro)}$
CD	s.c.		8	8.1 ± 1.0	1.9 ± 1.0					
ADX71743	s.c.	10	8	9.0 ± 0.4	1.0 ± 0.4	1188	4411	37	137	1
ADX71743	s.c.	30	8	9.1 ± 0.4	0.9 ± 0.4	3351	12444	104	386	3
ADX71743	s.c.	100	8	9.3 ± 0.3	0.8 ± 0.3	9800	36391	304	1128	9
Olanzapine	p.o.	3	8	3.0 ± 1.0**	6.5 ± 1.0**					

**P<0.01 compared to corresponding vehicle. [a]CSF exposure was calculated from the plasma exposure using mice plasma protein binding (PPB; fu=4.4%).

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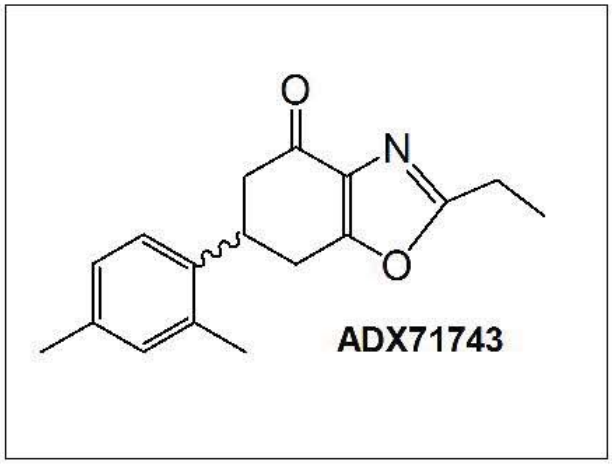

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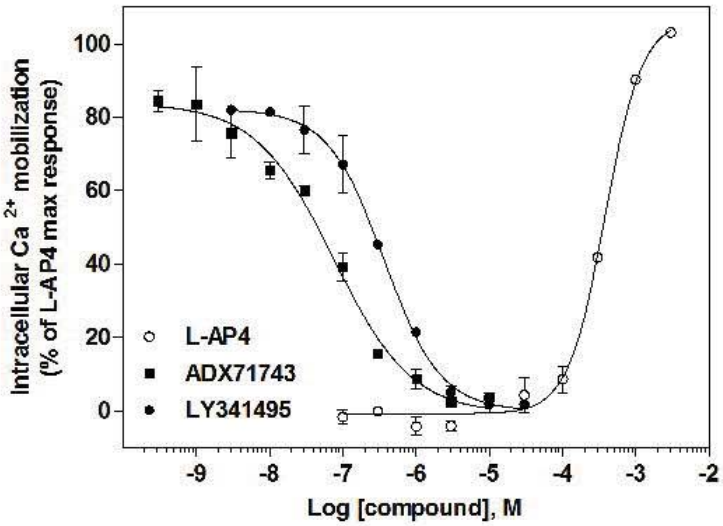

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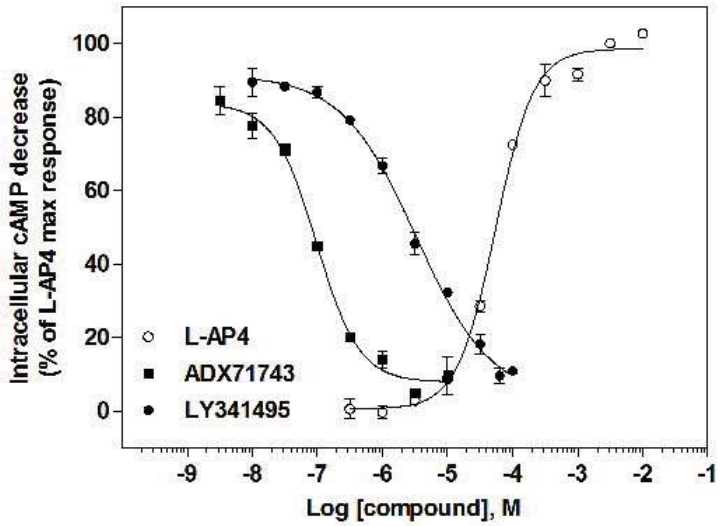

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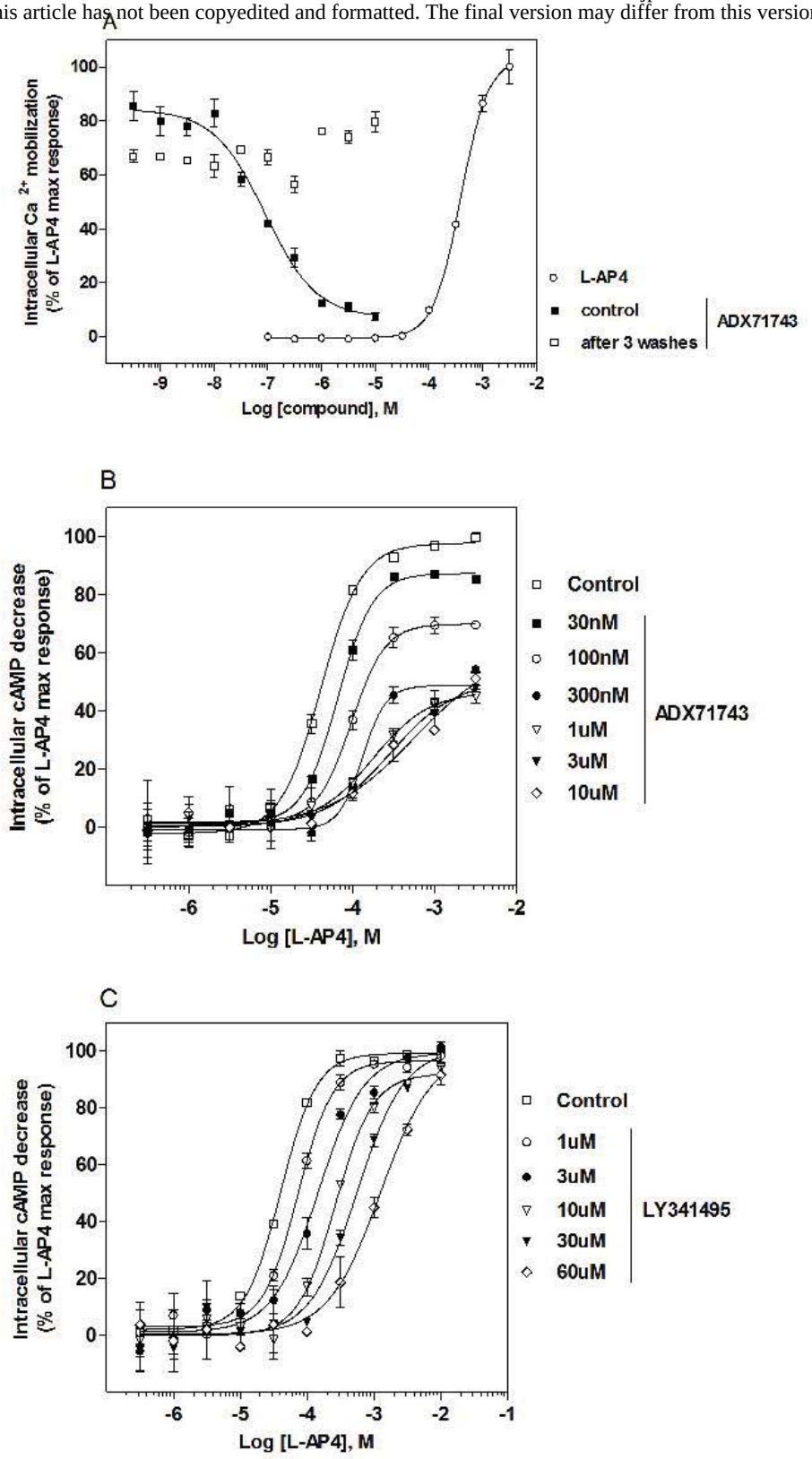

Figure 2

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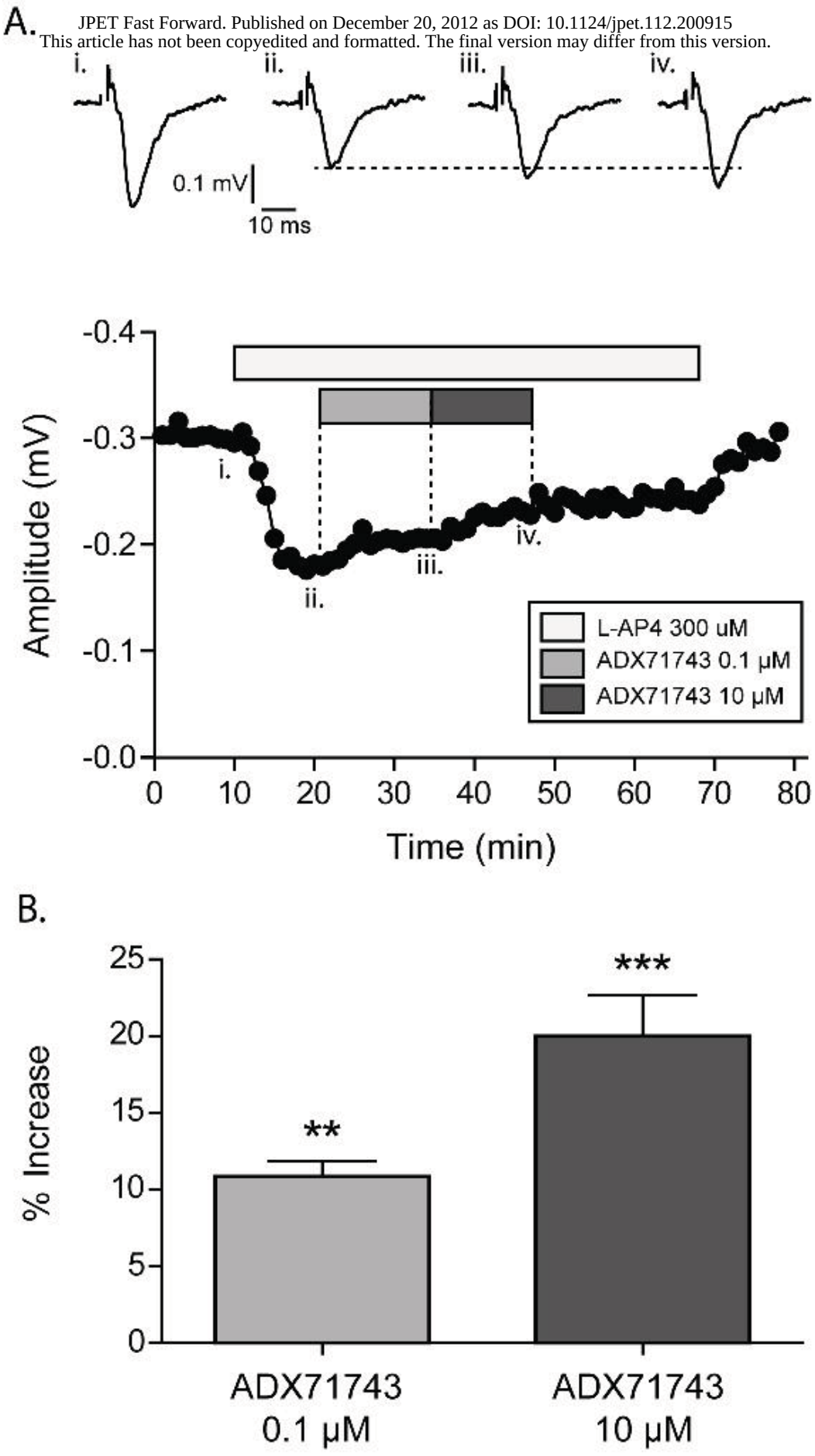

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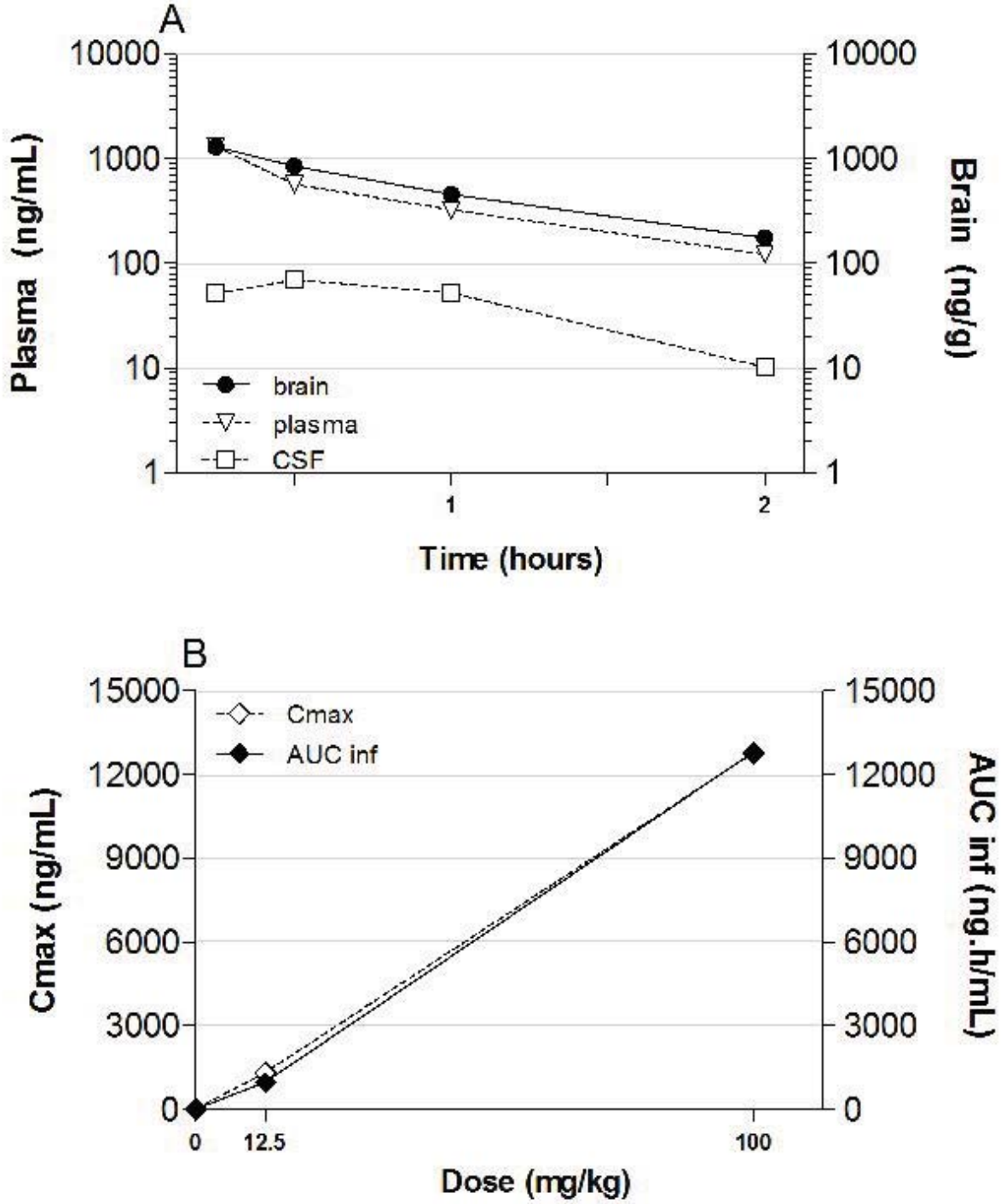

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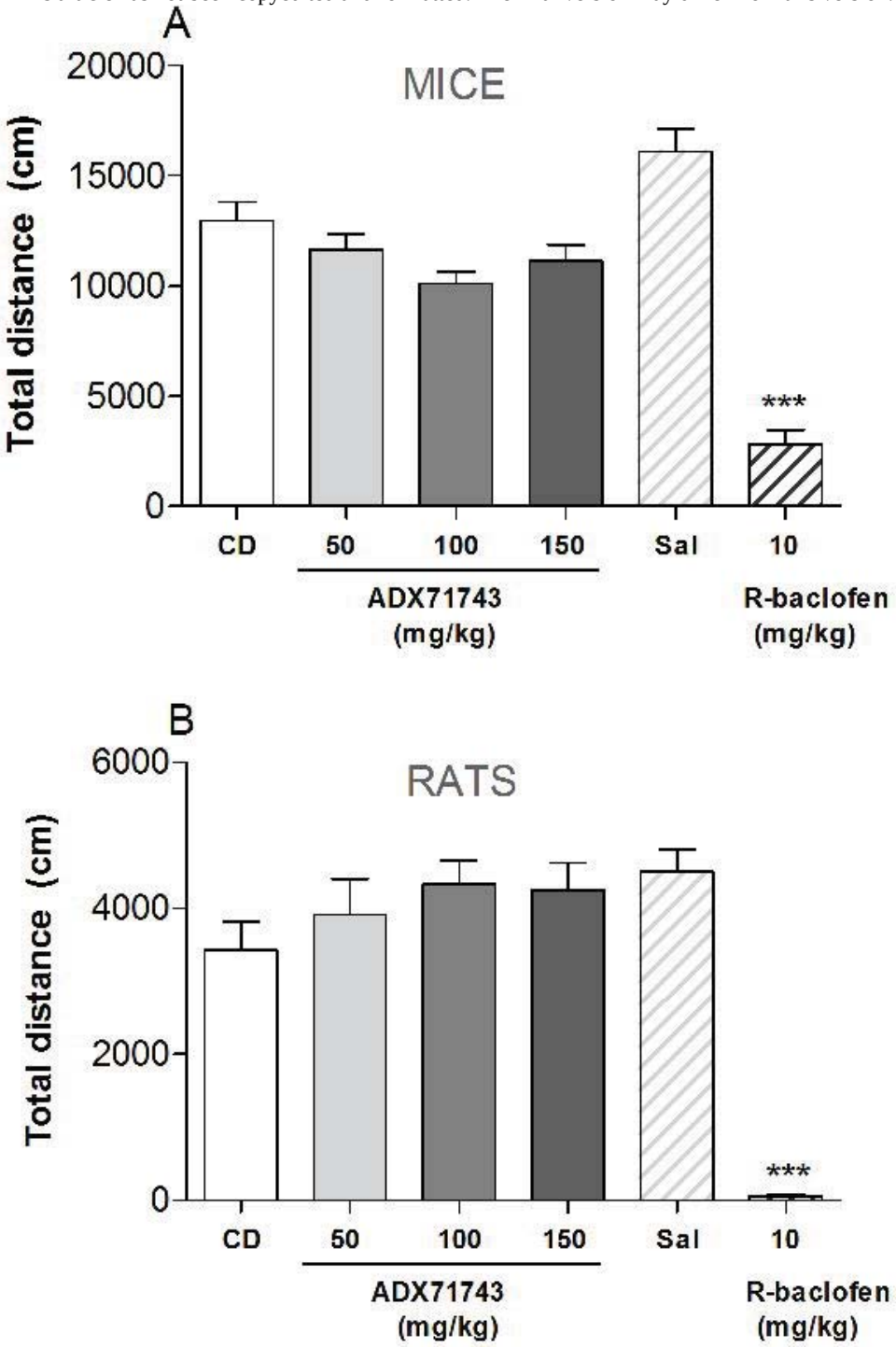

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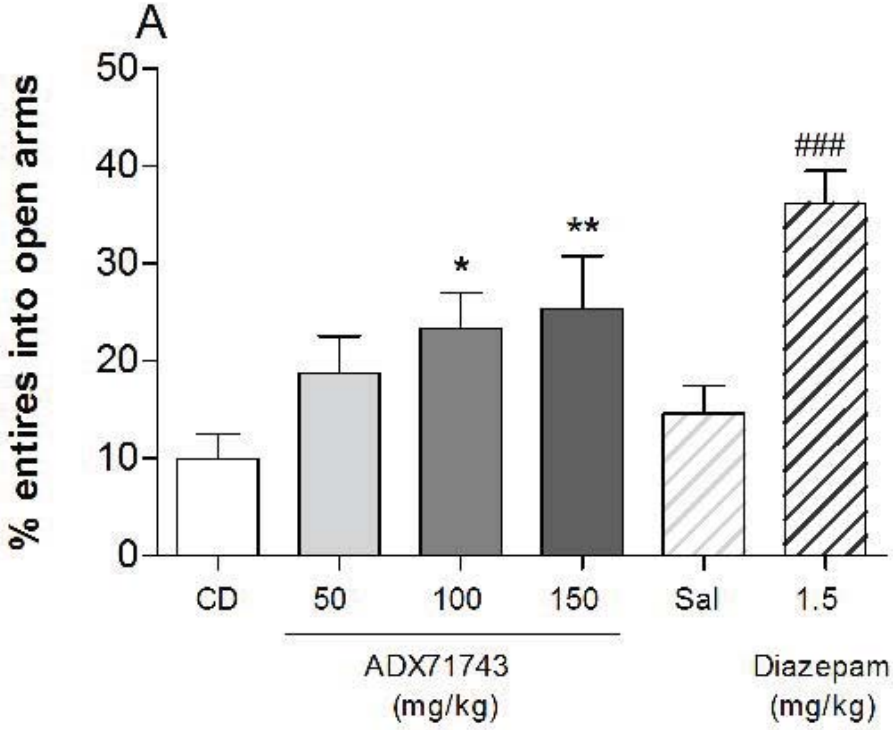

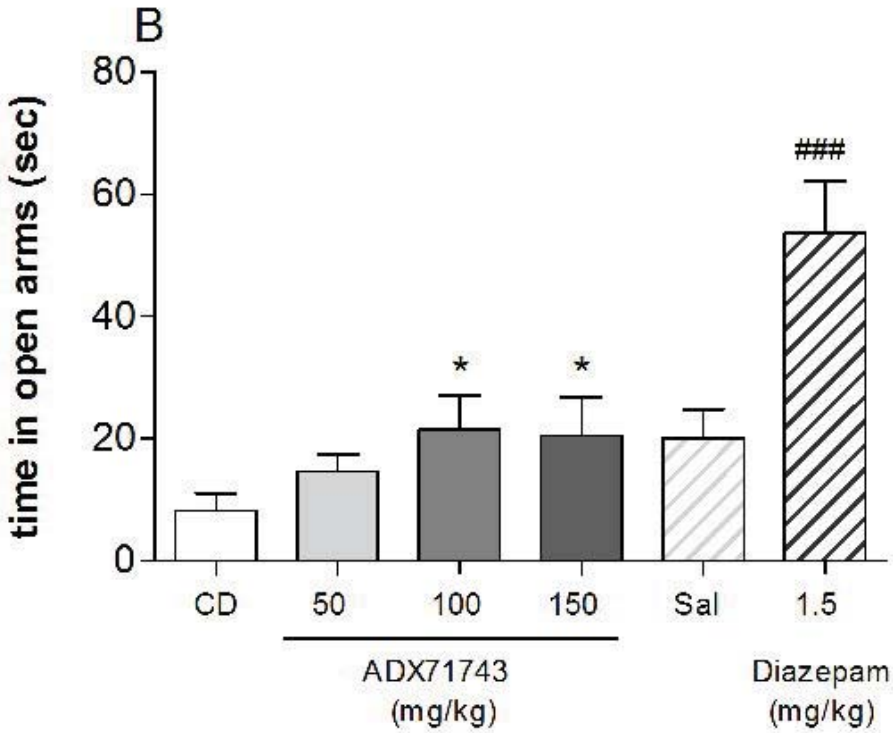

Figure 6

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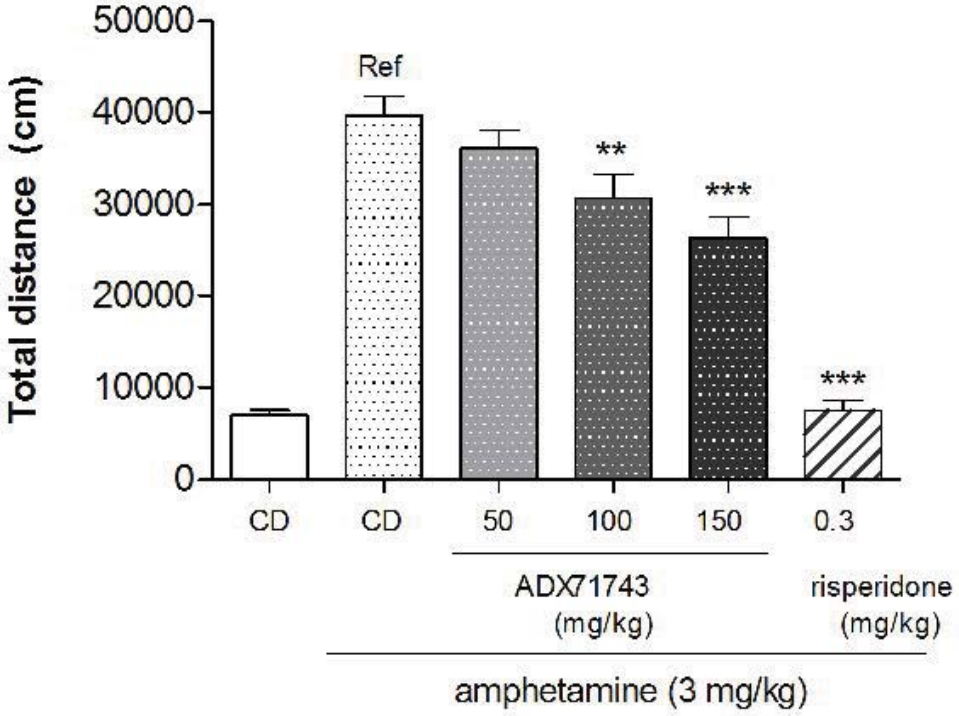

Figure 7

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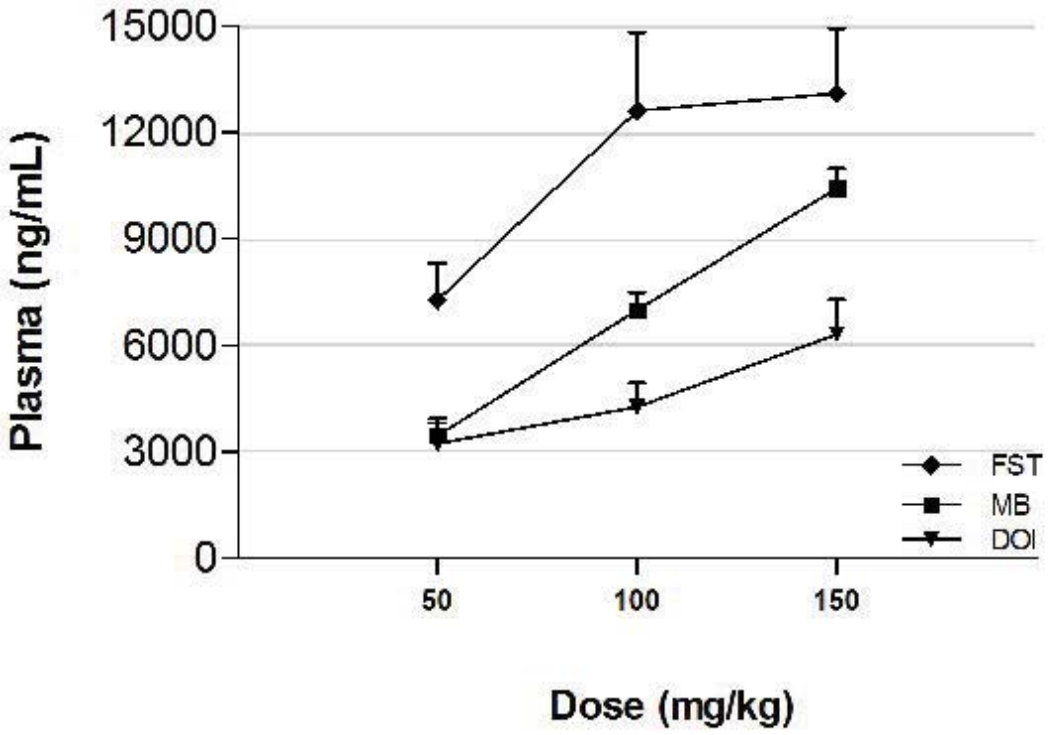

Figure 8