ADX71743, a Potent and Selective Negative Allosteric Modulator of Metabotropic Glutamate Receptor 7: In Vitro and In Vivo Characterization

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ABSTRACT

Metabotropic glutamate receptor 7 (mGlu7) has been suggested to be a promising novel target for treatment of a range of disorders, including anxiety, post-traumatic stress disorder, depression, drug abuse, and schizophrenia. Here we characterized a potent and selective mGlu7 negative allosteric modulator (NAM) (+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzof[oxazol-4(5H)]-one (ADX71743). In vitro, Schild plot analysis and reversibility tests at the target confirmed the NAM properties of the compound and attenuation of L-([+]-2-amino-4-phosphonobutyric acid)-induced synaptic depression confirmed activity at the native receptor. The pharmacokinetic analysis of ADX71743 in mice and rats revealed that it is bioavailable after s.c. administration and is brain penetrant (cerebrospinal fluid concentration/total plasma concentration ratio at C\text{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. Whereas ADX71743 caused a small reduction in amphetamine-induced hyperactivity in mice, it was inactive in the mouse 2,5-dimethoxy-4-iodoamphetamine-induced head twitch and the rat conditioned avoidance response tests. In addition, the compound was inactive in the mouse forced swim test. These data suggest that ADX71743 is a suitable compound to help unravel the physiologic role of mGlu7 and to better understand its implication in central nervous system diseases. Our in vivo tests using ADX71743, reported here, suggest that pharmacological inhibition of mGlu7 is a valid approach for developing novel pharmacotherapies to treat anxiety disorders, but may not be suitable for treatment of depression or psychosis.

Development in understanding the function of mGlu7 and its use as a potential target for drug discovery has been hampered by the lack of bioavailable 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 the possible role of mGlu7 in the CNS largely came from a series of studies involving mGlu7 knock-out (KO) mice and those implementing gene silencing techniques (Masugi et al., 1999; Sansig et al., 2001; Cryan et al., 2003). Compared with wild-type controls, mGlu7 KO mice showed consistent reductions in anxiety- and depression-like responses in a variety of behavioral tests (Cryan et al., 2003; Callaerts-Vegh et al., 2006). In addition, mGlu7 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 (Masugi et al., 1999; Callaerts-Vegh et al., 2006).

The discovery of \( N,N' \)-dibenzhydrylthene-1,2-diamine dihydrochloride (AMN082), presumably the first orally active and brain-penetrant mGlu7 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 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 adrenocorticotropic hormone 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 and four-plate tests (Stachowicz et al., 2008) and an antidepressant-like profile in the mouse forced swim test (FST) and tail suspension test (Palucha et al., 2007), an outcome that was not expected from the mGlu7 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, dopamine, and norepinephrine transporters (Sukoff Rizzo et al., 2011).

Recent discovery of the systemically active negative allosteric modulator (NAM) of mGlu7 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP) with inverse agonist activity was another step toward uncovering the role of this receptor (Suzuki et al., 2007; Nakamura et al., 2010). 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 revealed that these compounds show a context-dependent activity when expressed in recombinant cell lines, but were found inactive in a physiologic 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 (+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one (ADX71743), a potent, selective, and brain-penetrant mGlu7 NAM (Fig. 1A). It was developed through chemical lead optimization of a hit compound (Tang et al., manuscript 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 Schiold 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 and rats after 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, such as the mouse marble burying (MB) and EPM tests; depression, such as the mouse FST; and psychosis, such as the amphetamine-induced hyperactivity 2,5-dimethoxy-
4-idoamphetamine (DOI)–induced head twitch tests in mice and the conditioned avoidance response (CAR) test in rats.

Materials and Methods

Stable Cell Lines. The cDNAs encoding the human or the rat mGlu7 (hmGlu7; and rmGlu7, respectively) were subcloned into an expression vector also containing the hygromycin resistance gene. For intracellular calcium flux measurement, the cDNAs encoding a chimeric Go protein allowing redirection of the activation signal to intracellular calcium flux was subcloned into a different expression vector also containing the puromycin resistance gene, and both of these vectors were co-transfected into human embryonic kidney (HEK)293 cells with PolyFeet 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 hmGlu7-containing expression vector was transfected into HEK293 cells expressing Phoenix, a cAMP biosensor allowing a dynamic real-time cAMP measurement in live cells described previously (Lütjens et al., 2010). Positive functional cellular clones expressing hmGlu7 were identified based on using the reference group III mGluR agonist 1-[(+)-2-amino-4-phosphonobutyric acid (L-AP4) and the nonselective mGlu orthosteric antagonist (2S)-2-amino-2-[[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)-propanoic acid (LY341495). HEK293 cells expressing rat or human mGlu7 were maintained in media containing Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (Geneticin) (100 μg/ml), hygromycin B (40 μg/ml), and puromycin (1 μg/ml) at 37°C with 5% CO2 in a humidified atmosphere.

Fluorescent Cell-Based Ca2+ 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 MgSO4, 1 mM CaCl2, 0.125 mM sulfipyrazone, and 0.1% glucose. Twenty-four hours before the pharmacological experiment, hmGlu7- or rmGlu7–transfected HEK293 cells were plated out at a density of 2.5×104 cells/well in black-well/clear-bottomed and poly(1-ornithine)–coated 384-well plates in DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, supplemented with 1 μg/ml doxycycline. Cells were incubated overnight at 37°C with 5% CO2 in a humidified atmosphere. On the day of the assay, the cells were loaded with a 3-μM dye solution of Fluo-4-AM (Invitrogen, Lucerne, Switzerland) in assay buffer containing 0.03% pluronic acid. After 1 hour at 37°C with 5% CO2 in a humidified atmosphere, the extracellular dye was removed by washing the cell plate three times with 1× phosphate-buffered saline (Invitrogen, Lucerne, Switzerland). Assay buffer was added to cells and calcium flux was measured using a fluorometric imaging plate reader (FLPR) (Molecular Devices, Sunnyvale, CA). After 10 seconds of basal fluorescence recording, compounds to be tested were added toells in a concentration-dependent manner, and left for incubation on the cells for 95 seconds. The cells were then costimulated by 10 μM forskolin (Sigma-Aldrich, Buchs, Switzerland), and glutamate or L-AP4 EC80 (concentration giving 80% of the maximal agonist response) for an additional 10 minutes 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 an agonist, positive allosteric modulator, or NAM of other rat or human members of the mGlu family (mGlu1, mGlu2, mGlu3, mGlu4, mGlu5, mGlu6, mGlu8) using the above-described fluorescent cell-based Ca2+ mobilization assay. In addition, ADX71743 was tested in agonist and antagonism mode in the Cerep P27 cellular functional G-protein coupled receptor (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, Gelles Ferry, CT). This reusable 96-well plate is assembled so that each well is divided vertically in two parts by a dialysis membrane. Molecular weight cut-off regenerated dialysis cellulose membranes (Dialysis Membrane Strips; HT Dialysis) with a molecular weight cut-off 12,000 Da were conditioned according to the manufacturer and used for all experiments. One microliter of a 1 mg/ml dimethylsulfoxide (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 were added to one side of the membrane and the pH 7.4 phosphate buffer solutions was added to the other side of the well. The addition in the two compartments was 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 they were immediately stored at 4°C after 6 and 18 hours. The serum and buffer samples were subsequently analyzed by a specific liquid chromatography–mass spectrometry (LC-MS) method to measure ADX71743 concentrations. The measured concentration of ADX71743 in serum and buffer was determined. The portion of bound ADX71743 in serum was calculated according to the following equation (Wright et al., 1996):

\[
\% \text{ new chemical entities (NCE) bound} = \frac{C_{\text{serum}} - C_{\text{buffer}}}{C_{\text{serum}}} \times 100
\]

where \( C_{\text{serum}} \) is the total (bound + free) concentration of ADX71743 in serum after equilibrium is reached and \( C_{\text{buffer}} \) 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 was reached after 5–6 hours.

Animals. Unless otherwise specified below, the studies used adult male C57Bl/6J mice (24–30 g) and Sprague-Dawley rats (250–350 g). Charles River, L’Arbresle, France). Upon arrival to the animal facility, mice were group-housed (n = 5 per cage) in type II cages (16 × 22 × 24 cm), whereas rats were group-housed (n = 2 per cage) in type III cages (22 × 37 × 18 cm). Animals were maintained on a 12-hour light/dark cycle (lights on from 07:00 to 19:00 h) under constant temperature (22°C ± 2°C) and humidity (>45%) conditions. Standard
15% ethylenediaminetetraacetic acid (EDTA) solution and immediately placed on ice. Samples were centrifuged at 4°C for 12 minutes at 5900g for 3 minutes (equivalent to 8000g 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 tubes. For sample preparation, CSF samples were checked carefully for blood contamination and transferred into Eppendorf tubes containing 20-μl 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).

**Hippocampal Electrophysiology.** Adult (≥8 weeks) female C57Bl6J mice (Harlan, Bicester, UK) were killed by decapitation and the brain was removed and placed into ice-cold oxygenated sucrose Krebs medium containing 202 mM sucrose, 2 mM KCl, 1.25 mM KH2PO4, 1 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose. The brain was hemisected along the midline and 300-μm 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 with 124 mM NaCl, 2 mM KCl, 1.25 mM KH2PO4, 1 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose. After at least 1 hour of recovery, individual slices were transferred to an interface recording chamber where they were perfused with Krebs solution (21.5°C–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 (5 kHz) 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 (0.1-ms pulses applied every 10 seconds; 3.2–4.5 V 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 six consecutive trials with a steady baseline for at least 0.1%. For the electrophysiological studies the average peak amplitude of the fEPSPs was measured over six consecutive trials with a steady baseline for at least 0.1%. For the electrophysiological studies the average peak amplitude of the fEPSPs was measured over six consecutive trials with a steady baseline for at least 0.1%.

**Pharmacokinetic Studies.** Pharmacokinetic studies after s.c. administration of ADX71743 to mice and rats were performed with a suspension of the drug in a 50% water solution of -cyclodextrin (CD). The volume of administration for the compound in plasma were 1.57 and 0.6 ng/ml at 12.5 and 100 mg/kg doses, respectively.

The time spent on rotarod (seconds) was analyzed by the Kruskal Wallis test followed by Dunn’s multiple comparisons.

**Rotarod Test in Mice.** A mouse rotarod apparatus (MED Associates, St. Albans, VT) 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 (saline) and were tested 60 and 120 minutes after treatment. Two additional groups of mice (n = 10/group) were treated via oral gavage (p.o.) with either (R)-4-amino-3-(4-chloro-phenyl)-butanoic acid ((R)-baclofen) (10 mg/kg) or its vehicle (saline) and were tested 60 and 120 minutes after treatment. The time spent on rotarod (seconds) was analyzed by the Kruskal-Wallis test followed by Dunn’s multiple comparisons.

**Spontaneous Locomotor Activity Test in Mice.** Spontaneous locomotor activity, assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011). Spontaneous locomotor activity was assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (35 × 55 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint, Lyon, France) as described previously (Campo et al., 2011).

**Characterization of an mGlu7 Negative Modulator ADX71743.** Table 1 summarizes the functional potency of reference agonists L-AP4 and glutamate (EC50 values), and of ADX71743 and LY341495 (IC50 values) on recombinant human mGlu7 receptors. EC50 values (μM) and IC50 values (nM) are expressed as mean ± S.E.M. from at least five independent experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human mGlu7 Recombinant</th>
<th>Rat mGlu7 Recombinant</th>
<th>Phoenix cAMP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-AP4 EC50</td>
<td>630 ± 15</td>
<td>645 ± 61</td>
<td>133 ± 8</td>
</tr>
<tr>
<td>Glutamate EC50</td>
<td>Not saturating</td>
<td>Not saturating</td>
<td>264 ± 37</td>
</tr>
<tr>
<td>ADX71743 IC50 vs EC50 of L-AP4</td>
<td>63 ± 2</td>
<td>85 ± 9</td>
<td>125 ± 17</td>
</tr>
<tr>
<td>ADX71743 IC50 vs EC50 of glutamate</td>
<td>22 ± 4</td>
<td>449 ± 42</td>
<td>2262 ± 268</td>
</tr>
<tr>
<td>LY341495 IC50</td>
<td>345 ± 9.5</td>
<td>449 ± 42</td>
<td>2262 ± 268</td>
</tr>
</tbody>
</table>

**Summary of functional potency of reference agonists L-AP4 and glutamate (EC50 values), and of ADX71743 and LY341495 (IC50 values) on recombinant human mGlu7 receptors.** Ec50 values (μM) and IC50 values (nM) are expressed as mean ± S.E.M. from at least five independent experiments performed in duplicate.
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 60 minutes after administration of ADX71743 and (R)-baclofen, respectively, mice were individually placed into arenas and their locomotor activity was monitored for 60 minutes. The total distance traveled during the test (centimeters) was analyzed by one-way analysis of variance (ANOVA) followed by planned comparisons.

**Spontaneous Locomotor Activity Test in Rats.** Spontaneous locomotor activity, assessed as horizontal distance traveled (centimeters), was monitored using Plexiglas arenas (50 × 50 × 50 cm) in conjunction with video tracking and computerized analysis systems (Viewpoint). 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 60 minutes after administration of ADX71743 and (R)-baclofen, respectively, mice were individually placed into arenas and their locomotor activity was monitored for 60 minutes. The total distance traveled during the test (centimeters) was analyzed by one-way ANOVA followed by planned comparisons.

**MB 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 10 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 60 minutes after administration of ADX71743 or chlordiazepoxide, respectively, animals were individually placed in experimental cages and were left undisturbed for 30 minutes. 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 two-thirds 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 analyzed as described for the pharmacokinetic studies. The number of buried marbles was analyzed by the Kruskal–Wallis test followed by Dunn’s multiple comparisons.

**EPM Test in Mice.** The EPM was made of opaque plastic and consisted of four arms of equal lengths and widths (15 × 4 cm) arranged in the form of a plus sign. Two opposite arms, referred to as closed arms, were enclosed by walls 12 cm high, 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 center of the maze facing one of the closed arms and was left to explore the maze for 5 minutes. The arms were cleaned with 35% ethanol between each test session. The experiment was performed under dim light conditions (approximately 70 Lux). The numbers 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 60 minutes after administration of ADX71743 and chlordiazepoxide, respectively, animals were individually placed on the maze and left to explore it for 5 minutes. Percept entries into open arms, as well as time (seconds) spent on open and closed arms of the maze, was analyzed by one-way ANOVA followed by planned comparisons.

**FST in Mice.** The procedure was performed as described previously by Campo et al. (2011). Briefly, 1 day before the experiment, mice were pre-exposed to the swim session using individual glass covers) used in the experiment contained extra amounts (5 cm high) of sawdust bedding and had 10 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 treated p.o. with either (R)-baclofen (10 mg/kg) or its vehicle (saline). Thirty and 60 minutes after administration of ADX71743 and (R)-baclofen, respectively, mice were individually placed into arenas and their locomotor activity was monitored for 60 minutes. The total distance traveled during the test (centimeters) was analyzed by one-way analysis of variance (ANOVA) followed by planned comparisons.

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**MB 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 10 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 60 minutes after administration of ADX71743 or chlordiazepoxide, respectively, animals were individually placed in experimental cages and were left undisturbed for 30 minutes. 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 two-thirds 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 analyzed as described for the pharmacokinetic studies. The number of buried marbles was analyzed by the Kruskal–Wallis test followed by Dunn’s multiple comparisons.

**EPM Test in Mice.** The EPM was made of opaque plastic and consisted of four arms of equal lengths and widths (15 × 4 cm) arranged in the form of a plus sign. Two opposite arms, referred to as closed arms, were enclosed by walls 12 cm high, 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 center of the maze facing one of the closed arms and was left to explore the maze for 5 minutes. The arms were cleaned with 35% ethanol between each test session. The experiment was performed under dim light conditions (approximately 70 Lux). The numbers 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 60 minutes after administration of ADX71743 and chlordiazepoxide, respectively, animals were individually placed on the maze and left to explore it for 5 minutes. Percept entries into open arms, as well as time (seconds) spent on open and closed arms of the maze, was analyzed by one-way ANOVA followed by planned comparisons.

**FST in Mice.** The procedure was performed as described previously by Campo et al. (2011). Briefly, 1 day before the experiment, mice were pre-exposed to the swim session using individual glass cylinders (height: 25 cm; diameter: 10 cm) containing water 10 cm deep at 24°C ± 1°C for 15 minutes. On the test day, 24 hours later, animals were treated s.c. with ADX71743 (50, 100, 150 mg/kg) or its vehicle (CD); n = 10/group). Two additional groups (n = 10/group) were treated i.p. with imipramine (30 mg/kg) or its vehicle (saline). Thirty or 60 minutes after administration of ADX71743 or imipramine, respectively, animals were exposed to the test swim session for 6 minutes 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 behavioral measures scored included the duration of immobility exhibited during the last 4 minutes of the 6-minute 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 analyzed as described for the pharmacokinetic studies. The time spent immobile (seconds) in the FST was analyzed 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%
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 minutes 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 minutes. The total distance traveled during the test (centimeters) was analyzed by one-way ANOVA followed by planned comparisons.

**DOI-Induced Head Twitches in Mice.** Adult male C57Bl6J 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 minutes (ADX71743 or CD) or 30 minutes (olanzapine) 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 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 analyzed as described for the pharmacokinetic studies. The number of DOI-induced head twitches was analyzed by one-way ANOVA followed by planned comparisons.

**CAR Test in Rats.** Adult male Wistar rats were purchased from Charles River and group-housed under standard laboratory conditions at RenaSci Ltd. Conditioned avoidance behavior was assessed using automated shuttle boxes (42 × 16 × 20 cm; MED Associates), 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 10 seconds upon administration of the conditioned stimulus (tone and light), to avoid exposure to the unconditioned stimulus (footshock, 0.5 mA for a maximum duration of 10 seconds) via the grid floor. In the first phase of training, each animal underwent 30 trials in a 30-minute test session with a variable intertrial interval of 20–30 seconds. 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-minute session with a variable intertrial interval of 20–30 seconds. This was continued until 80% (i.e., eight correct trials) avoidance responses were obtained for the group. A baseline (pretest) session was run the day before the experiment. In this session, all animals were dosed with vehicle 60 minutes before the test. Animals then underwent the test protocol (10 trials in a 10-minute session with a variable intertrial interval of 20–30 seconds). Animals that exhibited stable performance (>80% avoidance responses for the last three drug-free CAR sessions) 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-dependently inhibited avoidance response and increased escape responses (unpublished data). One week after the last validation experiment with an antipsychotic drug, animals were tested with a vehicle (CD) and the day after received ADX71743 (10, 30, 100 mg/kg s.c.), its vehicle (CD s.c.), or olanzapine (0.1 mg/kg p.o.) 60 minutes before being evaluated in the above-described test protocol. At the end of the experiment blood from all ADX71743-treated animals was collected via tail vein and plasma was analyzed as described for the pharmacokinetic studies.

**Drugs.** L-AP4 (suspended in 0.1 N NaOH) was purchased from AbCam Biochemicals (Cambridge, UK). Glutamate, or L-glutamic acid hydrochloride ((S)-2-aminoglutaric acid, (S)-2-amino-3-phosphonopropionic acid ammonium salt), (R)-baclofen, D-amphetamine, chlordiazepoxide, imipramine, olanzapine and risperidone were purchased from Sigma-Aldrich.

![Image](https://example.com/image.png)

**Fig. 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 = 3–4. Error bars represent S.E.M. **P < 0.01; ***P < 0.001.

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

<table>
<thead>
<tr>
<th>Route</th>
<th>Mice</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>12.5</td>
<td>100</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>0.68</td>
<td>0.40</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>1308</td>
<td>12,766</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>CSF concentration/plasma concentration (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Brain/plasma&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> At Cmax.

LY341495 (mGlu2/3 orthosteric antagonist; Kingston et al., 1998) was purchased from Tocris Bioscience (Bristol, UK). DOI was purchased from Steroplast Ltd. (Manchester, UK).

ADX71743 was synthesized at Addex Therapeutics. The compound was suspended in water containing CD. The suspensions were homogenized with stainless steel balls for 30 minutes at 30 Hz in a 2-ml Eppendorf tube, and then vortexed and sonicated for 10 minutes. D-amphetamine was dissolved in saline and administered i.p. at 3 mg/kg.
(R)-Baclofen was dissolved in saline and administered 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 Receptors. After a high-throughput screening campaign of the Addex corporate library using a HEK293 cell line stably coexpressing mGlu7 with a chimeric Ga protein, several compounds inhibiting the calcium flux induced by L-AP4 were identified. After a hit confirmation and lead optimization processes, ADX71743 was identified (Tang et al., manuscript submitted; Fig. 1A). This compound tested in cell lines expressing hmGlu7 or rmGlu7 together with a chimeric Gα protein, allowing redirection of receptor activation onto calcium signaling, was found to fully inhibit an EC80 of L-AP4 (2 mM) with full efficacy and an IC50 of 63 ± 62 nM and 88 ± 9 nM, respectively (Fig. 1B; Table 1). In comparison, the IC50 of reference compound LY341495, a nonselective orthosteric mGlu2/3 antagonist was 345 ± 69.5 nM and 449 ± 42 nM for the hmGlu7 and rmGlu7 clones, respectively (Fig. 1B; Table 1), in agreement with values previously reported (Kingston et al., 1998).

mGlu7 is naturally coupled to the adenylate cyclase through G/0. Therefore, to demonstrate activity of ADX71743 on the physiologic signaling of mGlu7, the compound was tested on cells coexpressing hmGlu7 and a cAMP biosensor Phoenyx (Lütjens et al., 2010). In this assay, a full concentration-response curve with glutamate, the natural ligand of the mGlu7 receptor, saturates, allowing calculation of an EC50 of 264 ± 37 μM (Table 1). ADX71743 could therefore be tested against an EC80 of glutamate (IC50 of 22 ± 4 nM) as well as against an EC80 of L-AP4 (IC50 of 125 ± 17 nM) (Table 1; Fig. 1C). The IC50 of LY341495 when tested against L-AP4 was 2262 ± 268 nM (Fig 1C; Table 1).

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 EC50 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 (Fig. 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 (Fig. 2C; calculated pA2 = 5.92, slope = 0.83). To test the reversibility of
Characterization of an mGlu7 Negative Modulator ADX71743

ADX71743 activity, experiments were performed in the calcium assay using the hmGlu2 clone. Cells were either washed three times or were kept without washing after addition of ADX71743, followed by measurement of calcium levels. ADX71743 was found active on an EC50 of 1.74 μM as expected in nonwashed cells, whereas the inhibitory effect was not observed in washed cells, demonstrating the reversible effect of the compound on mGlu7 (Fig. 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 hmGlu3, hmGlu4, rmGlu5, hmGlu6, and hmGlu8. A negligible inhibition of rmGlu1 (32% at 30 μM) and a weak positive allosteric modulator effect on hmGlu2 (EC50 of 11 μM) was measured. When further tested in a functional GPCR screen against 27 targets (Cerep profile P27, excluding muscarinic M2 and M4 receptors, unavailable at the time of the test) in agonist and antagonist modes, no stimulation or inhibition above 27% was observed (unpublished data).

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. Coapplication 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) (Fig. 3A and 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 pharmacokinetic profile (Table 2). After s.c. administration of 12.5 mg/kg ADX71743 in mice, plasma reached high Cmax between 0.25 and 0.5 hours, and then declined rapidly, after a half-life of approximately 0.5 hours (Fig. 4A; Table 2). After s.c. administration of 12.5 mg/kg, CSF and brain concentrations of ADX71743 also reached Cmax rapidly. Thereafter, brain concentrations declined rapidly, whereas the CSF concentrations were sustained for a slightly longer period (Fig. 4A; Table 2). The ratio between the CSF and plasma concentration at Cmax 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 area under the curve and Cmax values of plasma exposure show linear and similar increases in relation to the dose after s.c. administration of 12.5 and 100 mg/kg ADX71743 in mice (Fig. 4B).

Spontaneous Locomotor Activity Test in Mice and Rats. ADX71743 (50, 100, 150 mg/kg) had no effect on spontaneous locomotor activity in mice, whereas baclofen markedly suppressed (80%; P < 0.001) the total distance traveled by animals compared with vehicle treatment (Fig. 5A). ADX71743 (50, 100, 150 mg/kg) also had no effect on spontaneous locomotor activity in rats, whereas baclofen resulted in a virtually full (P < 0.001) suppression of activity (Fig. 5B).

Rotarod Test in Mice. ADX71743 (50, 100, 150 mg/kg) had no effect on latencies to fall from rotarod (seconds) when animals were tested 30 and 90 minutes after 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 minutes after administration (Table 3).

MB Test in Mice. ADX71743 resulted in an approximately 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 (approximately 75%; P < 0.001) at 150 mg/kg compared with its vehicle (Table 4). The corresponding concentrations of ADX71743 in plasma in animals treated at

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose</th>
<th>Animal</th>
<th>First Session</th>
<th>Second Session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/kg</td>
<td>n</td>
<td>mean ± S.E.M.</td>
<td>% change</td>
</tr>
<tr>
<td>CD</td>
<td>s.c.</td>
<td>10</td>
<td>178 ± 2.3</td>
<td>0</td>
<td>176 ± 3.8</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>50</td>
<td>10</td>
<td>173 ± 6.7</td>
<td>−2.5</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>100</td>
<td>10</td>
<td>174 ± 4.9</td>
<td>−1.9</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>150</td>
<td>10</td>
<td>159 ± 11.4</td>
<td>−10.6</td>
</tr>
<tr>
<td>Saline</td>
<td>p.o.</td>
<td>10</td>
<td>180 ± 0.0</td>
<td>0</td>
<td>180 ± 0.0</td>
</tr>
<tr>
<td>Baclofen</td>
<td>p.o.</td>
<td>10</td>
<td>47 ± 6.7***</td>
<td>−74.1</td>
<td>43 ± 3.4***</td>
</tr>
</tbody>
</table>

* ***P < 0.001 compared with corresponding vehicle.

### Table 4
Activity of ADX71743 in the mouse MB test and measured plasma, CSF concentrations, and CSF concentration/IC50 (in vitro) of ADX71743 in mice at the end of the MB experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose</th>
<th>Animal</th>
<th>Marbles Buried</th>
<th>Change</th>
<th>Plasma Exposure</th>
<th>CSF Exposure</th>
<th>CSF/EC50 (in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/kg</td>
<td>n</td>
<td>mean ± S.E.M.</td>
<td>%</td>
<td>ng/ml</td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>CD</td>
<td>s.c.</td>
<td>10</td>
<td>6.3 ± 0.5</td>
<td>0</td>
<td>62</td>
<td>3451</td>
<td>12,814</td>
<td>152</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>50</td>
<td>2.4 ± 0.6**</td>
<td>−62</td>
<td>57</td>
<td>6990</td>
<td>25,958</td>
<td>308</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>100</td>
<td>2.7 ± 0.8**</td>
<td>−57</td>
<td>73</td>
<td>10,430</td>
<td>38,732</td>
<td>459</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>150</td>
<td>1.7 ± 0.7***</td>
<td>−73</td>
<td>57</td>
<td>6990</td>
<td>25,958</td>
<td>308</td>
</tr>
<tr>
<td>Saline</td>
<td>p.o.</td>
<td>10</td>
<td>6.3 ± 0.9</td>
<td>0</td>
<td>62</td>
<td>3451</td>
<td>12,814</td>
<td>152</td>
</tr>
<tr>
<td>Baclofen</td>
<td>p.o.</td>
<td>10</td>
<td>2.3 ± 0.6**</td>
<td>−63</td>
<td>57</td>
<td>6990</td>
<td>25,958</td>
<td>308</td>
</tr>
</tbody>
</table>

* P < 0.01; **P < 0.05; and ***P < 0.001 compared with corresponding vehicle.
resulted in 2.3-fold ($P < 0.05$) and 2.5-fold ($P < 0.01$) increases in open arm entries compared with corresponding vehicle-treated controls (Fig. 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-fold and 2.5-fold (both $P < 0.05$) increases in time spent on open arms compared with corresponding vehicle-treated controls (Fig. 6B). 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 with vehicle-treated control (Fig. 6, A and B). There was no effect of treatment on the number of closed arm entries (unpublished data).

**FST in Mice.** ADX71743 (50, 100, 150 mg/kg) had no effect on the time animals spent in immobility, whereas 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, 12,607, and 13,107 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 [$F(5, 95)=46.2; P < 0.001$; Fig. 7]. Specifically, at 100 and 150 mg/kg, there were approximately 20% ($P < 0.01$) and 30% ($P < 0.001$) reductions in hyperactivity compared with corresponding vehicle-pretreated controls (Fig. 7). Risperidone-treated animals exhibited robust reduction in amphetamine-induced hyperactivity (approximately 80%; $P < 0.001$; Fig. 7).

**DOI-Induced Head Twitches in Mice.** ADX71743 (50, 100, 150 mg/kg) had no effect on the number of DOI-induced head twitches, whereas 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 4371 ng/ml, respectively. These plasma concentrations resulted in CSF/IC$_{50}$ values of 4, 6, and 8, respectively (Table 6).

**Conditioned Avoidance 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 an approximately 60% ($P < 0.01$) 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

![Fig. 6. Percent entries into open arms (A) and time spent (seconds) on open arms (B) by male C57Bl6/J mice (n = 10/group) during 5 minutes of the EPM test. Animals were pretreated with ADX71743 (50, 100, 150 mg/kg s.c.), CD (s.c.), diazepam (1.5 mg/kg p.o.), or saline (p.o.). ADX71743 and its vehicle were administered 60 minutes before testing. Each point represents the observed mean (+S.E.M.). *$P < 0.01$; **$P < 0.001$ compared with saline (Sal).](image)

### Table 5

Activity of ADX71743 in the FST and measured plasma, CSF concentrations, and CSF concentration/IC$_{50}$ (in vitro) of ADX71743 in mice at the end of the experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose</th>
<th>Animal</th>
<th>Immobility</th>
<th>Plasma Exposure</th>
<th>CSF Exposure</th>
<th>CSF/EC$_{50}$ (in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>n</td>
<td>mean ± S.E.M.</td>
<td>ng/ml</td>
<td>nM</td>
<td>ng/ml</td>
<td>nM</td>
</tr>
<tr>
<td>CD</td>
<td>s.c.</td>
<td>10</td>
<td>164 ± 10</td>
<td>7265</td>
<td>26,977</td>
<td>320</td>
<td>1187</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>50</td>
<td>155 ± 11</td>
<td>12,607</td>
<td>46,813</td>
<td>555</td>
<td>2060</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>100</td>
<td>145 ± 10</td>
<td>13,107</td>
<td>48,671</td>
<td>577</td>
<td>2142</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>150</td>
<td>159 ± 11</td>
<td>7265</td>
<td>26,977</td>
<td>320</td>
<td>1187</td>
</tr>
<tr>
<td>Saline</td>
<td>i.p.</td>
<td>10</td>
<td>137 ± 15</td>
<td>12,607</td>
<td>46,813</td>
<td>555</td>
<td>2060</td>
</tr>
<tr>
<td>Imipramine</td>
<td>i.p.</td>
<td>30</td>
<td>63 ± 14**</td>
<td>7265</td>
<td>26,977</td>
<td>320</td>
<td>1187</td>
</tr>
</tbody>
</table>

**$P < 0.01$ compared with corresponding vehicle.**
of the mGlu7 receptor provided important first evidence of accumulating. Although initial studies using inactivation (Fig. 8).

Plasma Concentration of ADX71743 in In Vivo Studies. Concentrations of ADX71743 in plasma after 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 Fig. 8. Plasma concentrations of ADX71743, measured in the FST study, were higher than those measured in the DOI and MB studies, probably due to different postdosing sampling time (30 versus 60 minutes) and the rapid clearance of the compound (Fig. 8).

Discussion

Over the last decade, data supporting the hypothesis that the mGlu7 receptor plays a pivotal role in the CNS have been accumulating. Although initial studies using inactivation of the mGlu7 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.

Table 6

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose</th>
<th>Route</th>
<th>Animal</th>
<th>DOI-Induced Twitches</th>
<th>Plasma Exposure</th>
<th>CSF Exposure</th>
<th>CSF/EC50 (In Vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD/saline</td>
<td>0/0</td>
<td>s.c./p.</td>
<td>CD</td>
<td>8</td>
<td>0.7 ± 0.3</td>
<td>3233</td>
<td>12,007</td>
</tr>
<tr>
<td>CD/DOI</td>
<td>0.3</td>
<td>s.c./p.</td>
<td>8</td>
<td>23.1 ± 1.8</td>
<td>18.1 ± 1.6</td>
<td>2111</td>
<td>15,865</td>
</tr>
<tr>
<td>ADX71743/DOI</td>
<td>0.3</td>
<td>s.c./p.</td>
<td>8</td>
<td>21.1 ± 1.5</td>
<td>21.1 ± 1.5</td>
<td>21.1 ± 1.5</td>
<td>21.1 ± 1.5</td>
</tr>
<tr>
<td>ADX71743/DOI</td>
<td>0.3</td>
<td>s.c./p.</td>
<td>8</td>
<td>21.8 ± 2.0</td>
<td>21.8 ± 2.0</td>
<td>21.8 ± 2.0</td>
<td>21.8 ± 2.0</td>
</tr>
<tr>
<td>Olanzapine/DOI</td>
<td>0.3</td>
<td>p.o./p.</td>
<td>8</td>
<td>5.0 ± 0.4***</td>
<td>5.0 ± 0.4**</td>
<td>5.0 ± 0.4***</td>
<td>5.0 ± 0.4***</td>
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</tbody>
</table>

**P < 0.001 compared with corresponding vehicle.
ADX71743 showed an anxiolytic-like profile in the MB and the EPM tests in mice. Both tests are known to be sensitive to typical anxiolytic drugs (Pellow et al., 1985; Lister, 1990; Nicolas et al., 2006), the latter also being relevant to obsessive compulsive disorder (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 an approximately 60% reduction in the number of buried marbles corresponded to CSF/IC50 values of 4–9 (at 50 and 100 mg/kg), followed by further reductions (73%) at CSF/IC50 = 14 (at 150 mg/kg). In accordance with our findings, mGlu7 KO mice exhibited an approximately 60% reduction in the number of buried marbles compared with 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 nonspecific changes in activity on closed arms. In accord with our findings, mGlu7 KO mice showed increases in open arm entries and time spent on these arms compared with their wild-type controls (Callaerts-Vegh et al., 2006).

At this point, we can only speculate on how the reduction in activity of the mGlu7 receptor can lead to reduced anxiety-like reactivity. The mGlu7 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 the mGlu7 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). Although an mGlu7 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 mGlu7 receptor. In addition, there is a possibility that reduced activity of mGlu7 will impact anxiety-like reactivity via modulating several downstream targets, especially those involved in reactivity to stress. According to Mitsukawa et al. (2006), the mGlu7 KO mice exhibit signs of HPA axis dysregulation, including upregulation of glucocorticoid (GR) and 5-HT1A receptors in the hippocampus, increased sensitivity to GR-mediated negative feedback, and increases in brain-derived neurotrophic factor protein in the hippocampus (Mitsukawa et al., 2006). These changes correlate well with reduced anxiety- and depression-like reactivity of mGlu7 KO mice (Cryan et al., 2003). Whether inhibition of mGlu7 with a NAM can lead to alterations in HPA axis seen in mGlu7 KO animals remains to be investigated.

ADX71743 failed to show an antidepressant-like profile in the mouse FST despite reaching CSF/IC50 values of 9, 16, and 17 at 50, 100, and 150 mg/kg doses, respectively. In addition, 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 et al., 1993; Wettstein et al., 1999; Wadenberg, 2010). The rationale for testing ADX71743 in models of psychosis, in part, came from several independent studies showing a link between polymorphisms in mGlu7 and schizophrenia (Ohtsuki et al., 2008; Ganda et al., 2009; Shibata et al., 2009). ADX71743 resulted in a 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 mGlu7 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 mGlu7 in mice and rats, which is virtually identical in these species (Kinoshita et al., 1998). In fact, only in the medial habenula, mGlu7 is present in high abundance in rats, while being absent in mice, whereas the opposite trend is seen in the cerebellar nuclei.

**TABLE 7**

Activity of ADX71743 in the CAR test together with the measured plasma, CSF concentrations, and CSF concentration/IC50 (in vitro) of ADX71743 in rats at the end of the experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose (mg/kg)</th>
<th>Animal</th>
<th>Avoidances</th>
<th>Escapes</th>
<th>Plasma Exposure</th>
<th>CSF Exposure</th>
<th>CSF/EC50 (In Vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>s.c.</td>
<td>8</td>
<td>8</td>
<td>8.1 ± 1.0</td>
<td>1.9 ± 1.0</td>
<td>1188</td>
<td>4411</td>
<td>37</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>10</td>
<td>8</td>
<td>9.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>3351</td>
<td>12,444</td>
<td>104</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>30</td>
<td>8</td>
<td>9.1 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>8900</td>
<td>36,391</td>
<td>304</td>
</tr>
<tr>
<td>ADX71743</td>
<td>s.c.</td>
<td>100</td>
<td>8</td>
<td>9.3 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>1.0</td>
<td>1.9</td>
<td>1128</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>p.o.</td>
<td>3</td>
<td>8</td>
<td>3.0 ± 1.0**</td>
<td>6.5 ± 1.0**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**P < 0.01 compared with corresponding vehicle.

**Fig. 8.** Plasma concentration (ng/ml) of ADX71743 after s.c. administration at 50, 100, and 150 mg/kg in mice across three experiments including DOI-induced head twitches, MB, and FST. Samples were taken approximately 30 minutes (FST) or 60 minutes (DOI and MB) after administration of the compound.
(Kinoshita et al., 1998). One explanation of in vivo results is involvement of mGlu7 in anxiety-like reactivity, but not in depression or psychosis. Future studies involving more disease-relevant animal 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 mitoxin methyloxazyme-anal. tent 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 mGlu7 concentration in a specific neural circuit recruited in the in vivo test. Such possibility is unlikely, since both ampheta-me induced hyperactivity (where a weak effect was seen) and the CAR test (where there was no effect) rely on the nucleus accumbens and the ventral tegmental area (Geyer and Wadenberg, 2010), anatomic regions that have similar low concentration of mGlu7 (Kinoshita et al., 1998).

In conclusion, we present in vitro and in vivo characterization of ADX71743 as a centrally active compound suitable for investigation of the role of mGlu7 receptor. This compound shows potent NAM activity at the mGlu7 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 mGlu7 activity via the NAM mechanism is more relevant for anxiety than depression. The compound also was largely inactive in models predictive of antipsychotic-like activity. Further optimization of this and other series of mGlu7 NAMs is underway to provide even more potent and better exposed compounds for future studies. In short, these data suggest that mGlu7 inhibition merits further study as a novel approach for the treatment of obsessive compulsive disorder and other anxiety disorders.

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Participated in research design: Kalinichev, Girard, Charvin, Campo, Le Poul, Mutel, Poli, Neale, Salt, Lütjens.
Conducted experiments: Rouillier, Girard, Royer-Urios, Bournique, Finn, Neale.
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Wrote or contributed to the writing of the manuscript: Kalinichev, Rouillier, Poli, Neale, Salt, Lütjens.

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