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RESEARCH PAPER

The antipsychotic-like effects of positive allosteric modulators of metabotropic glutamate $mGlu_4$ receptors in rodents

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BACKGROUND AND PURPOSE

Because agonists at metabotropic glutamate receptors exert beneficial effects in schizophrenia, we have assessed the actions of Lu AF21934 and Lu AF32615, two chemically distinct, selective and brain-penetrant positive allosteric modulators (PAMs) of the $mGlu_4$ receptor, in several tests reflecting positive, negative and cognitive symptoms of schizophrenia in rodents.

EXPERIMENTAL APPROACH

Hyperactivity induced by MK-801 or amphetamine and head twitches induced by 2,5-dimethoxy-4-iodoamphetamine (DOI) in mice were used as models for positive symptoms. Disruption of social interaction and spatial delayed alternation tests induced by MK-801 in rats were used as models for negative and cognitive symptoms of schizophrenia, respectively.

KEY RESULTS

Lu AF21934 (0.1–5 $mg \cdot kg^{-1}$) and Lu AF32615 (2–10 $mg \cdot kg^{-1}$) dose-dependently inhibited hyperactivity induced by MK-801 or amphetamine. They also antagonized head twitches and increased frequency of spontaneous excitatory postsynaptic currents (EPSCs) in brain slices, induced by DOI. In mice lacking the $mGlu_4$ receptor ($mGlu_4^{-/-}$) mice, Lu AF21934 did not antagonize DOI-induced head twitches. MK-801-induced disruption in the social interaction test was decreased by Lu AF21934 at 0.5 $mg \cdot kg^{-1}$ and by Lu AF32615 at 10 $mg \cdot kg^{-1}$. In the delayed spatial alternation test, Lu AF21934 was active at 1 and 2 $mg \cdot kg^{-1}$, while Lu AF32615 was active at 10 $mg \cdot kg^{-1}$.

CONCLUSIONS AND IMPLICATIONS

We propose that activation by PAMs of the $mGlu_4$ receptor is a promising approach to the discovery of novel antipsychotic drugs.

Abbreviations

DAT, delayed alternation task; DOI, 2,5-dimethoxy-4-iodoamphetamine; EPSCs, excitatory postsynaptic currents; HPBCD, (2-hydropropyl)-β-cyclodextrin; NAM, negative allosteric modulator; PAM, positive allosteric modulator

Introduction

The glutamatergic theory of schizophrenia (Javitt, 1987) derives from the finding that NMDA receptor open channel blockers, such as phencyclidine (PCP), ketamine or dizocilpine (MK-801), potently induced a wide range of positive, negative and cognitive symptoms of psychosis in healthy volunteers. Therefore, pharmacological manipulation of this neurotransmitter may produce antipsychotic effects. The first clinical trials with agents acting on the glycine modulatory site on the NMDA receptor have been shown to improve cognition and decrease negative symptoms in schizophrenic subjects receiving standard antipsychotic therapy (Coyle and Tsai, 2004).

The identification of eight subtypes of metabotropic glutamate receptors ($mGlu_{1–8}$) divided into three groups (Pin and Duvoisin, 1995), provided a wide range of options to modulate the activity of glutamatergic neurotransmission, constituting a potentially safer alternative to NMDA activators (receptor nomenclature follows Alexander *et al.*, 2011). Support for the involvement of mGlu receptors in psychosis originated in the early clinical report of Patil *et al.* (2007) showing that treatment with LY2140023 ($mGlu_{2/3}$ receptor agonist) was safe and well-tolerated, and effective in reducing both positive and negative symptoms compared to placebo. Unfortunately, follow-up clinical studies did not confirm the drug's efficacy (Kinon *et al.*, 2011), and the trial was stopped recently (Lilly press release 08, 2012). However, despite this setback, ligands for the group II mGlu receptors provide a feasible new approach to antipsychotic therapy, with the particular feature of not targeting dopamine receptors (Weinberger, 2007). Indeed, the potential therapeutic efficacy in schizophrenia of ADX71149, a compound potentiating the $mGlu_2$ receptor, through an allosteric mechanism (*vide infra*), is currently being investigated in the clinic (Addex Therapeutics website).

From a drug design perspective, the architecture of cell membrane mGlu receptors provides two distinct mechanisms to potentiate receptor function (Conn *et al.*, 2009a). On the one hand, agonists acting at the orthosteric binding site compete with synaptic glutamate to effect receptor activation. In addition, in their transmembrane region, the mGlu receptors possess allosteric binding sites capable of interacting with lipophilic compounds. Following binding of ligands to the allosteric site, a change in receptor conformation is thought to lead to modification of receptor function (Sheffler *et al.*, 2011). Positive allosteric modulators (PAMs) enhance the receptor functional response, whereas negative allosteric modulators (NAMs) reduce it. While both strategies (orthosteric agonists and PAMs) provide activation of mGlu receptors, the pharmacology elicited by these different types of ligands is not necessarily identical. Theoretically, PAMs produce an augmentation of endogenous glutamate-driven effects, whereas orthosteric agonists, in general, lead to receptor activation dependent on the individual pharmacokinetics and concentration at the site of action (Melancon *et al.*, 2012).

Recent reports have demonstrated the preclinical antipsychotic-like activity of the non-selective orthosteric agonists of the group III mGlu receptors ($mGlu_{4/6/7/8}$), ACPT-I (Figure 1, **4**), and LSP1-2111 (Figure 1, **5**) (Acher *et al.*, 1997). Both compounds were shown to be active in animal models considered to be predictive of positive symptoms of schizophrenia (Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2012; 2013). However, these agonists lack subtype specificity, possibly activating more than one of the group III mGlu receptors, simultaneously. Therefore, in the present study, we evaluated the effects of two compounds selectively potenti-

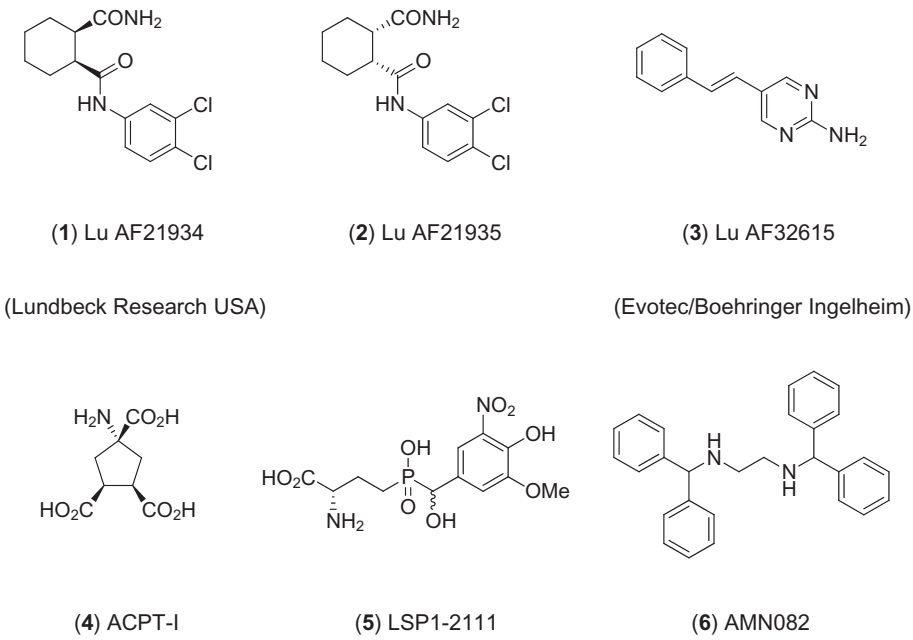

Figure 1

Chemical structures of $mGlu_4$ receptor PAMs used in this work and relevant group III mGlu receptor ligands.

ating the $mGlu_4$ receptor through allosteric mechanisms, Lu AF21934 (Figure 1, **1**), (1*S*,2*R*)-N^1-(3,4-dichlorophenyl) cyclohexane-1,2-dicarboxamide, Bennouar *et al.*, 2013) and Lu AF32615 (Figure 1, **3**), (East *et al.*, 2010, Evotec/Boehringer Ingelheim). These compounds are structurally different, selective and known to cross the blood brain barrier. The enantiomer of Lu AF21934 (Lu AF21935, Figure 1, **2**), inactive at the $mGlu_4$ receptor, was also used as a control to support conclusions invoking $mGlu_4$ receptor potentiation. We used a broad range of tests considered to be predictive of positive symptoms, such as MK-801- or amphetamine-induced hyperactivity, and head twitches induced by 2,5-dimethoxy-4-iodoamphetamine (DOI) (Geyer and Ellenbroek, 2003; Moghaddam and Jackson, 2003; Pałucha-Poniewiera *et al.*, 2008). The social interaction test was used as a model of negative symptoms and the delayed alternation test was used as a model of cognitive disturbances. Electrophysiological studies were also conducted in brain slices from mice. Our results show that Lu AF21934 and Lu AF32615 induced antipsychotic-like effects in all of the tests used, suggesting their potential as an alternative to the presently used antipsychotic therapy.

Methods

Animals and housing

All animal care and experimental procedures complied with the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the Ethics Committee of the Institute of Pharmacology, Polish Academy of Sciences in Krakow and Lundbeck Research USA. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 968 animals (618 mice and 350 rats) were used in the experiments described here.

Male Albino Swiss (20–25 g) mice (Charles River Laboratory, Sulzfeld, Germany) were used to assess MK-801- and amphetamine-induced hyperlocomotion and DOI-induced head twitches. Mice lacking the $mGlu_4$ receptor ($mGlu_4^{-/-}$) and wild type C57Bl/6J mice were used in DOI-induced head twitches. Heterozygous $mGlu_4^{-/+}$ mice were obtained from Abbot Laboratories (gift from Dr. Bespalov); $mGlu_4^{-/-}$ and $mGlu_4^{+/+}$ mice were bred in our institute. The genotypes of newborn mice were analysed by PCR. The person responsible for breeding $mGlu_4^{-/-}$ mice was M. Marciniak. Male Wistar rats weighing 250–300 g (Charles River Laboratory) were used in social interaction and spatial delayed alternation tests. All animals were kept under a 12:12 light–dark cycle at a room temperature of 19–21°C with free access to food and water. Each experimental group consisted of 8–10 animals per dose, and the animals were used only once in each test. All animals were experimentally naive prior to testing. The compounds were given in a volume of 10 mL·kg^{-1} (mice) or 1 mL·kg^{-1} (rats). All behavioural measurements were made by an observer unaware of the treatment.

Locomotor activity of habituated mice

The locomotor activity was recorded individually for each animal in OPTO-M3 locomotor activity cages (Columbus Instrument) linked online to a compatible PC. Each cage (13 cm × 23 cm × 15 cm) was surrounded with an array of photocell beams. Interruptions of these photobeams resulted in horizontal activity defined as ambulation scores. Mice were placed separately into activity cages for an acclimatization period of 30 min, then they were injected subcutaneously with Lu AF21934, Lu AF32615 or (2-hydropropyl)-β-cyclodextrin (HPBCD) and placed again into the same cages. After a further 60 min they were injected with saline (10 mL·kg^{-1}). From this point on, the ambulation scores were measured for 80 min.

MK-801- or amphetamine-induced hyperactivity

The locomotor activity was recorded for each animal in locomotor activity cages (according to Rorick-Kehn *et al.*, 2007a,b), with small modifications used in our previous studies (Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2012). The mice were placed individually into activity cages for an acclimatization period of 30 min; then they were injected s.c. with Lu AF21934, Lu AF32615 or HPBCD and placed again in the same cages. After 60 min all of the mice were injected i.p. with MK-801 at 0.3 mg·kg^{-1} or amphetamine at 3 mg·kg^{-1}, and once again returned to the same cage. From then on, the ambulation scores were counted for 80 min. All of the groups were compared with the MK-801 or amphetamine control group. The experiment also included a control group treated with neither MK-801 nor amphetamine.

Head twitch test

The experiment was performed according to Pałucha-Poniewiera *et al.* (2008) and Wierońska *et al.* (2011; 2012; 2013). In order to habituate mice to the experimental environment, each animal was transferred to a 12 (diameter) × 20 cm (height) glass cage, lined with sawdust, 30 min before the treatment. The head twitches of the mice were induced by DOI (2.5 mg·kg^{-1}, i.p). Immediately after the treatment, the number of head twitches was counted during a 20 min session. Lu AF21934 and Lu AF32615 were injected, 60 min before DOI; HPBCD was administered to the controls.

MK-801-induced deficits in social interaction test in rats

Social interaction tests were performed as described by Satow *et al.* (2009), using a circle made of wood, 90 cm in diameter divided into 10 × 10 cm squares by faint yellow lines. Each social interaction test between two rats was carried out during the light phase of the light/dark cycle. Rats were selected from separate housing cages to make a pair for the study. The body weights of the paired rats were matched, within 20 g. All rats were placed in an experimental room and the study was conducted 3.5 h after the s.c. injection of MK-801 (0.1 mg·kg^{-1}). Sixty minutes before the test, Lu AF21934 or Lu AF32615 was given; HPBCD was given as a vehicle. The test box was wiped clean between each trial. Social interaction between two rats was determined as the total time spent participating in social behaviour such as sniffing, genital investigation, chasing and fighting each other. The total number of social episodes mentioned above was also meas-

ured. In addition, control experiments in animals not receiving MK-801 were also conducted, in order to establish if the drugs had any influence on social behaviour when given alone.

Spatial delayed alternation test in rats

The animals, deprived of water overnight, were trained and tested in four wooden T-mazes, which consisted of white and black end-arms (33 cm × 22 cm × 25 cm) and a grey starting arm (15 cm × 20 cm × 25 cm). The end-arms were equipped with a spout bottle located 9 cm above the floor and containing a 10% sucrose solution. The three arms were separated from each other by guillotine doors.

Adaptation. On the first 3 days, the animals were allowed freely to explore the whole T-maze for 10 min. On the next 2 days, they were confined to either of the two end-arms and allowed to drink the sucrose solution there for 10 min twice daily.

Training. On the next 2 weeks, the animals received once daily training sessions. Each session consisted of one forced trial (i.e. when one of the end-arm was closed) followed by 10 free choice trials. For each free choice trial, the animals were placed in the starting arm, the guillotine doors were raised, and when the rat entered one of the end-arm, the guillotine door was closed and the rat was allowed to drink the sucrose solution there for 5 s. Then the rat was gently returned to the starting arm, where it stayed for 10 s (delayed interval). After that time, the guillotine door was raised and the rat was allowed to enter the end-arms. If the end-arm chosen was opposite to that visited on the previous trial (a correct response), the sucrose solution was provided, and the drinking was allowed for 5 s. If the end-arm chosen was the same as on the previous trial an incorrect response was scored and the animal gently returned to the starting arm for 10 s (delayed interval). This training was continued until the animals reach performance criterion, which was defined as at least seven correct responses in 10 trials for two consecutive daily sessions.

Testing. The animals were injected with a drug and the above procedure was repeated; the rat was placed in the starting arm, the guillotine doors raised and the rat was allowed to enter the end-arms. If it chose the correct end-arm (i.e. opposite to that visited on the previous trial), the sucrose solution was provided, 5 s later the rat was returned to the starting arm for 10 s (delayed interval). If the end-arm selected was incorrect (i.e. the same as on the previous trial), and the rat was returned to the starting arm for 10 s. Such testing sessions were carried out once a week and were preceded by two daily training sessions.

Electrophysiological studies

Albino Swiss mice were decapitated, their frontal cortices were dissected and cut into slices (420 μm thick) in the frontal plane using a vibrating microtome. Slices were kept submerged in the artificial cerebrospinal fluid (ACSF) consisting of (in mM): 126 NaCl, 4 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 KH_2PO_4, 26 $NaHCO_3$ and 10 glucose, bubbled with 95% O_2/5% CO_2, pH = 7.4. A single slice was transferred to the recording chamber (volume 1 mL) and superfused with warmed (32°C) ACSF at 2 mL·min^{-1}. Individual neurons were visualized using an upright microscope (Zeiss Axioskop 2FS) equipped with a long-range water immersion objective (40×) and an infrared camera. Recording micropipettes were pulled on a Flaming-Brown puller (P-87; Sutter Instruments, Novato, CA, USA) and had resistance 6–8 MΩ. Microelectrodes were filled with (in mM): 130 K-gluconate, 5 KCl, 0.3 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2-ATP, 0.4 Na-GTP, osmolarity 290 mOsm, pH = 7.2. Whole-cell recordings were made from layer V pyramidal cells. After confirming the electrophysiological characteristics of the neurons in the current-clamp mode, cells were voltage-clamped at −76 mV and spontaneous EPSCs were recorded. Signals were acquired using the SEC 05 L amplifier (NPI, Germany) and digitized using Digidata 1322 interface (Molecular Devices, Sunnyvale, CA, USA). Drugs kept as concentrated stocks were diluted in ACSF just before the experiment and applied in the superfusate. After stable baseline recording for at least 15 min, DOI (10 μM) was applied for 10 min and spontaneous excitatory postsynaptic currents (EPSCs) were recorded (10 min). Next DOI was applied concurrently with Lu AF21934 or Lu AF32615 for 15 min and again spontaneous EPSCs were recorded. The measured parameter was the frequency of spontaneous EPSCs. The data were analysed off-line, using Mini Analysis program (Synaptosoft Inc. ver.6.0.3).

Data analysis

The data are presented as the means ± SEM. Statistical analysis of the data was performed using the Statistica 10 package (StatSoft Inc., OK, USA). One-way ANOVA, followed by Dunnett's or Tukey's *post hoc* comparison test, was used in the analysis of the dose-dependent studies of Lu AF21934 and Lu AF32615. A P-value of <0.05 was considered as statistically significant.

Materials

The sources of the compounds used were as follows: Lu AF32615, Lu AF21934 ($mGlu_4$ receptor PAMs) and Lu AF21935 (as $mGlu_4$ receptor-inactive control) were provided by Lundbeck Research USA, and were characterized using H-1 and C-13 nuclear magnetic resonance spectroscopy and HPLC/mass-spectrometry methods. General *in silico* and *in vitro* properties of these compounds are shown in Table 1. The compounds were dosed using a suspension in 20% HPBCD and were injected s.c. 60 min before the tests. The administration schedules of Lu AF32615 and Lu AF21934 were planned based on brain, plasma and CSF concentration time course studies performed at Lundbeck Research USA (Bennouar *et al.*, 2013), as well as according to the behavioural results we observed in our previous studies on anxiety (Sławińska *et al.*, 2013) and in preliminary experiments. Brain, CSF and plasma exposures of Lu AF32615 were in agreement with literature reports (East *et al.*, 2010). The psychostimulants, amphetamine (3 mg·kg^{-1}), MK-801 (0.3 mg·kg^{-1}, Sigma-Aldrich, St. Louis, MO, USA) or DOI (2.5 mg·kg^{-1}) (4-iodo-2,5-dimethoxy-α-methylbenzene ethanamine hydrochloride) were dissolved in 0.9% NaCl, and the doses were selected on the basis of our previous work (Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2011;

Table 1
Characterization of Lu AF21934, Lu AF21935 and Lu AF32615

		Lu AF21934 (1)	Lu AF21935 (2)	Lu AF32615 (3)
Human $mGlu_4$ receptor PAM EC_{50}	nM	500	>10 000	910
%Modulation (E_{MAX})	%	120	18	170
Glutamate fold shift	–	5	–	10
Molecular weight	Amu	315.2	315.2	197.2
cLogP	–	3.3	3.3	2.4
$LogD_{7.4}$	–	3.3	3.3	n/a
tPSA	$Å^2$	72.2	72.2	51.8
Kinetic solubility pH 7.4	µM	120	120	170
Optical rotation	$[\alpha]^{21}{}_D$	+45[c]	−39[c]	Achiral
PAMPA P_{APP}	10^{-6} cm·s^{-1}	7	7	24
MDCK P_{APP}	A→B; B→A, Ratio	44; 30; 0.7	n/a	42; 28; 0.7
rPPB	%	95.6	87.6	88.3
Rat brain unbound fraction UB_{BR}	%	3.0	1.9	1.9
Rat CL_{int}	mL·min^{-1}	13	77	11
Brain	ng·g^{-1}	2422[b]	740[b]	822[d]
Plasma	ng·mL^{-1}	2763[b]	840[b]	350[d]
Brain/Plasma ratio	–	0.9 [b]	0.9[ab]	2.3[d]
CSF	ng·mL^{-1}	95[b]	42[b]	n/a
mGlu receptor selectivity	FLIPR screens at 10 µM	$mGlu_6$ receptor PAM; EC_{50} = 7 µM	No significant cross reactivity at 10 µM	Not active at $mGlu_{1,2,3,5,7}$ in PAM, NAM and agonist mode at 10 µM
Broad cross reactivity panel	Binding inhibition	A_{2A} antagonist K_i = 7 µM; 5-HT_{2B} antagonist K_i = 2 µM	No significant cross reactivity at 10 µM	No significant cross reactivity at 10 µM

a, Data for Lu AF21934 partly reported in Bennouar *et al.* (2013); *b*, Rat, SC dosing at 10 mg·kg^{-1}, 1 h post-dose, formulated in 20% aqueous HPBCD; *c*, MeOH/CH_2Cl_2 (1:1); 2 mg·mL^{-1}; *d*, PO, 10 mg·kg^{-1}, 1 h post-dose, same formulation as *b*.

2012) and that of the others (Geyer and Ellenbroek, 2003; Leite *et al.*, 2008; Satow *et al.*, 2009). Reference compounds clozapine (Tocris Biosciences, Bristol, UK) and risperidone (Tocris) were dispersed in 0.5% methylcellulose in 0.9% saline and haloperidol (5 mg·mL^{-1} ampoule; Warszawskie Zakłady Farmaceutyczne, Polfa) was diluted in 0.9% saline (which was used as the solvent in haloperidol ampoules). The reference compounds were injected i.p., 30 min before the test. All the solvents (including HPBCD) had no influence on the animals' behaviour, when given to appropriate controls.

Results

Effect of Lu AF21934 and Lu AF32615 on locomotor activity in mice habituated to activity cages

In mice adapted to activity cages for 30 min, neither compound tested at the doses that were active in MK-801- or amphetamine-induced hyperactivities (Lu AF21934, 0.5–5 mg·kg^{-1}; Lu AF32615, 2–10 mg·kg^{-1}) had any significant effect on the locomotor activity measured over 80 min, 60 min after the s.c. injection of the compounds (Figure 2A, B).

The effect of MK-801 on locomotor activity in mice

MK-801 (0.3 mg·kg^{-1}) increased [$F_{(1,35)}$ = 33.98, P < 0.0001] the ambulation scores within 80 min of the experimental session and this increase was reversed by the reference compounds, haloperidol (0.25 mg·kg^{-1}; P < 0.001) or clozapine (10 mg·kg^{-1}; P < 0.001) (Figure 3A). Lu AF21934 in the dose range of 0.5-2 mg·kg^{-1} reduced the MK-801-induced effect [$F_{(5,42)}$ = 6.458; P < 0.0002] and lower doses (0.1 mg·kg^{-1}) or higher doses (5 and 10 mg·kg^{-1}) were not effective (Figure 3B). Lu AF32615 reversed MK-801-induced locomotor activity [$F_{(3.28)}$ = 5.149, P = 0.005] only at the highest dose (10 mg·kg^{-1}; Figure 3C). The inactive enantiomer Lu AF21935 (0.1, 0.5 or 2 mg·kg^{-1}) did not influence the MK-801 induced effect, at any dose. (Figure 4).

Amphetamine-induced hyperactivity in mice

In the vehicle treated group, amphetamine (3 mg·kg^{-1}) produced a robust increase in the ambulation scores (Figure 5A,

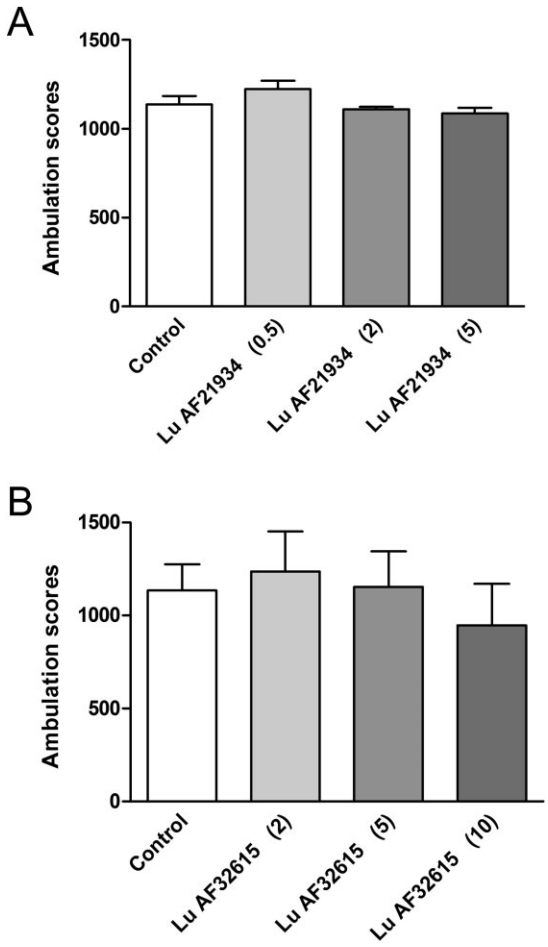

Figure 2

Effect of Lu AF21934 (A) and Lu AF32615 (B) on the locomotor activity of mice habituated to activity cages. The compounds were given s.c 60 min before the test. Locomotor activity was measured during 80 min time session. Data expressed as mean ± SEM were evaluated by one-way ANOVA. Values in parentheses represent the doses of the compounds in mg·kg^{-1}.

B, C). This effect of amphetamine was abolished by haloperidol (0.25 mg·kg^{-1}) and clozapine (10 mg·kg^{-1}), used as reference compounds [$F_{(1.35)}$ = 20.1, P < 0.001]. Both PAMs of the mGlu$_4$ receptor decreased the amphetamine-induced effect. Lu AF21934 was effective at 0.1, 0.5 and 2 mg·kg^{-1} [$F_{(5.,54)}$ = 8.275, P < 0.0001], but not at 5 and 10 mg·kg^{-1}. Lu AF32615 was effective only at 10 mg·kg^{-1} [$F_{(3.28)}$ = 5.922, P < 0.003].

DOI-induced head twitches in mice

Both clozapine and haloperidol diminished the number of head twitches induced by DOI [$F_{(2,21)}$ = 9.63, P < 0.01 and $F_{(2,21)}$ = 14.33, P < 0.01, respectively]. Lu AF21934 administered s.c. at 2, 5, 10 but not 0.5 mg·kg^{-1}, significantly decreased the number of DOI-induced head twitches in mice (45, 38.5 and 35%, respectively) [$F_{(4.38)}$ = 6.632, P = 0.0004]. Lu AF32615 also reduced number of head twitches at 5 and 10 mg·kg^{-1} (49,5% and 68%) [$F_{(3.36)}$ = 13.99, P < 0.0001], but the lower dose of this compound (2 mg·kg^{-1}) was not effective (Figure 6A, B, C).

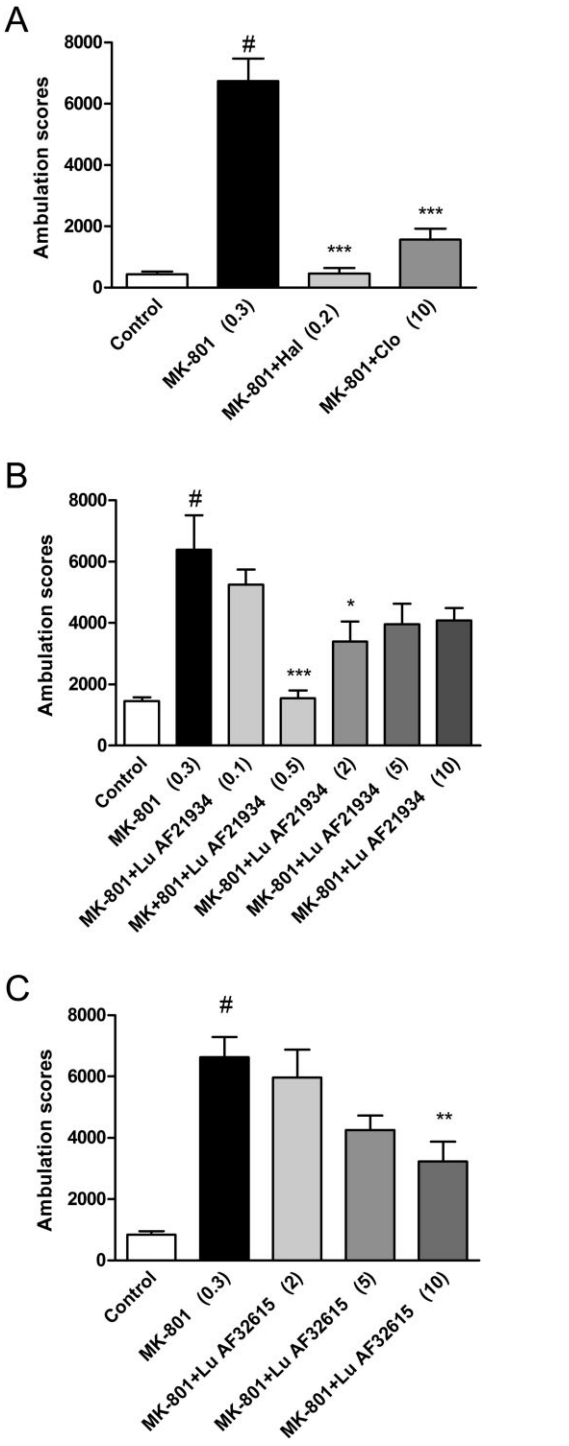

Figure 3

Effect of reference compounds haloperidol and clozapine (A), Lu AF21934 (B) and Lu AF32615 (C) on MK-801–induced hyperactivity in Albino Swiss mice. The reference compounds were given 30 min before the test. The test compounds were given 60 min before MK-801 administration. Locomotor activity was monitored over an 80 min session immediately following an injection of psychostimulant agent. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. The data are presented as mean ± SEM $^{\#}P$ < 0.001 versus control group and ***P < 0.001, **P < 0.01 and *P < 0.05 versus MK-801-treated group; one-way ANOVA and Dunnett's test.

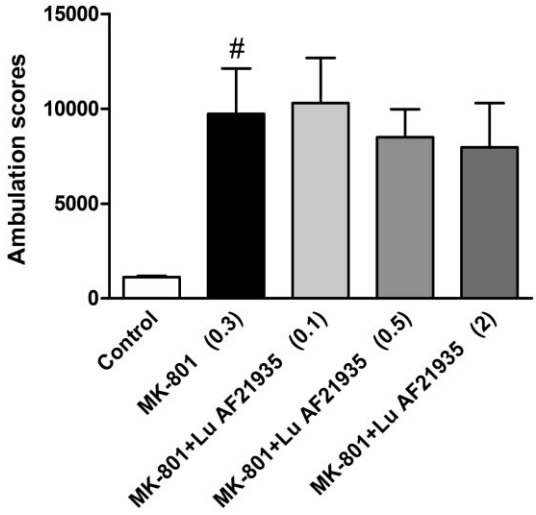

Figure 4

Effect of Lu AF21935, the enantiomer of Lu AF21934 devoid of measurable *in vitro* $mGlu_4$ receptor PAM activity, on MK-801–induced hyperactivity in Albino Swiss mice. The compound was given 60 min before MK-801 administration. Locomotor activity was monitored over an 80 min session immediately following an injection of MK-801. Values in parentheses represent the doses of the compounds in $mg \cdot kg^{-1}$. The data are presented as mean ± SEM $^{\#}P < 0.001$ versus control group; one-way ANOVA.

Lu AF21934 was not active when tested in $mGlu_4^{-/-}$ animals (Figure 7A). These mice showed a decrease in number of head twitches observed [$F_{(1.20)} = 12$; $P < 0.01$], relative to the wild type mice (Figure 7A, B). However, clozapine (5 $mg \cdot kg^{-1}$) used as the reference compound was active in both wild type and $mGlu_4^{-/-}$ animals [$F_{(1.20)} = 12.8$; $P < 0.01$] (Figure 7B).

DOI-induced spontaneous EPSCs

To investigate the effects of DOI on spontaneous excitatory postsynaptic currents (sEPSCs), voltage-clamp recordings were made from layer V cortical cells in the presence of picrotoxin (30 μM) to block $GABA_A$ receptor-mediated currents. All recorded cells (n = 69) had electrophysiological characteristics of regular spiking pyramidal neurons (tested in current clamp; McCormick *et al.*, 1985). Their mean resting membrane potential (RMP) was –74 ± 5 mV (±SEM) and the mean input resistance (R_{in}) was 252 ± 27 MΩ (±SEM). The mean basal frequency of spontaneous synaptic activity ranged between 2.9 and 7.5 Hz (±SEM; 4.9 ± 0.3 Hz) and its mean amplitude was 9.77 ± 0.3 pA (±SEM). Spontaneous postsynaptic currents were blocked by the non-NMDA glutamatergic receptor antagonist CNQX (5 μM; n = 5, data not shown), indicating that they represented excitatory currents. The application of DOI (10 μM) increased the mean sEPSCs frequency to: 147 ± 5% (± SEM) of baseline. The measurements performed on a separate group of five neurons demonstrated that the effect of DOI on sEPSCs did not desensitize over a 40 min continuous application of DOI (Figure 8).

Lu AF21934, when applied concurrently with DOI, reversibly suppressed DOI-induced increase in the frequency, but did not affect the mean amplitude of sEPSCs. The dose

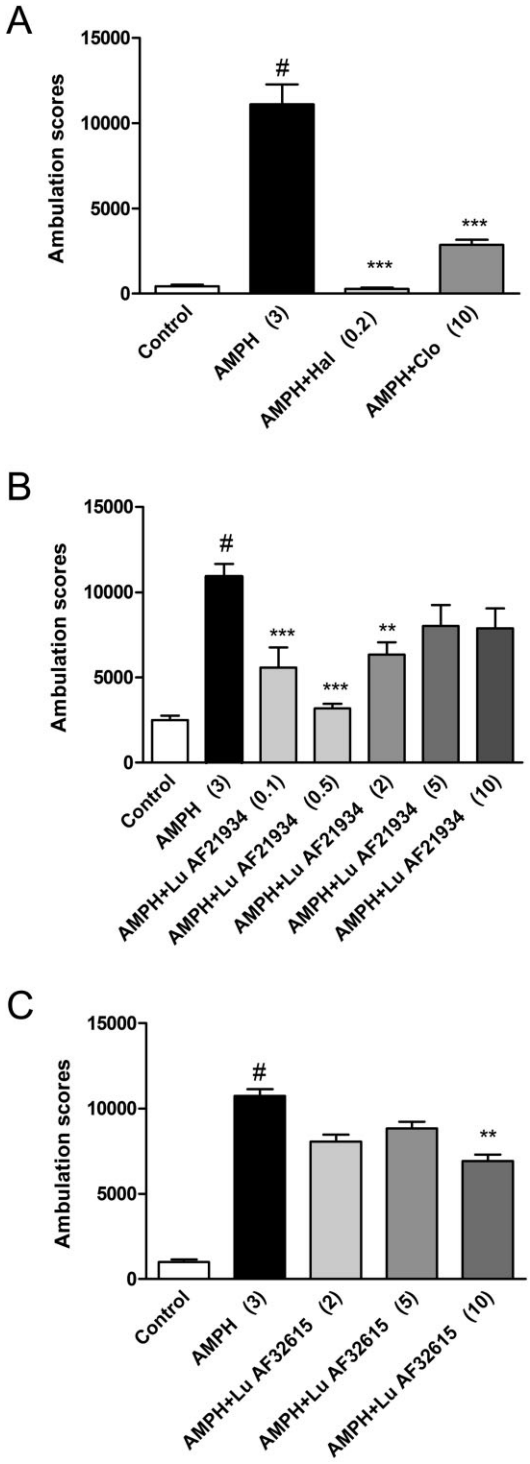

Figure 5

Effect of reference compounds haloperidol and clozapine (A), Lu AF21934 (B) and Lu AF32615 (C) on amphetamine–induced hyperactivity in Albino Swiss mice. The compounds were given 30 or 60 min before amphetamine administration. Locomotor activity was monitored over an 80 min session immediately following an injection of amphetamine. Values in parentheses represent the doses of the compounds in $mg \cdot kg^{-1}$.The data are presented as mean ± SEM. $^{\#}P <$ 0.001 versus control group and $^{***}P < 0.001$, $^{**}P < 0.01$ versus amphetamine-treated group; one-way ANOVA and Dunnett's test.

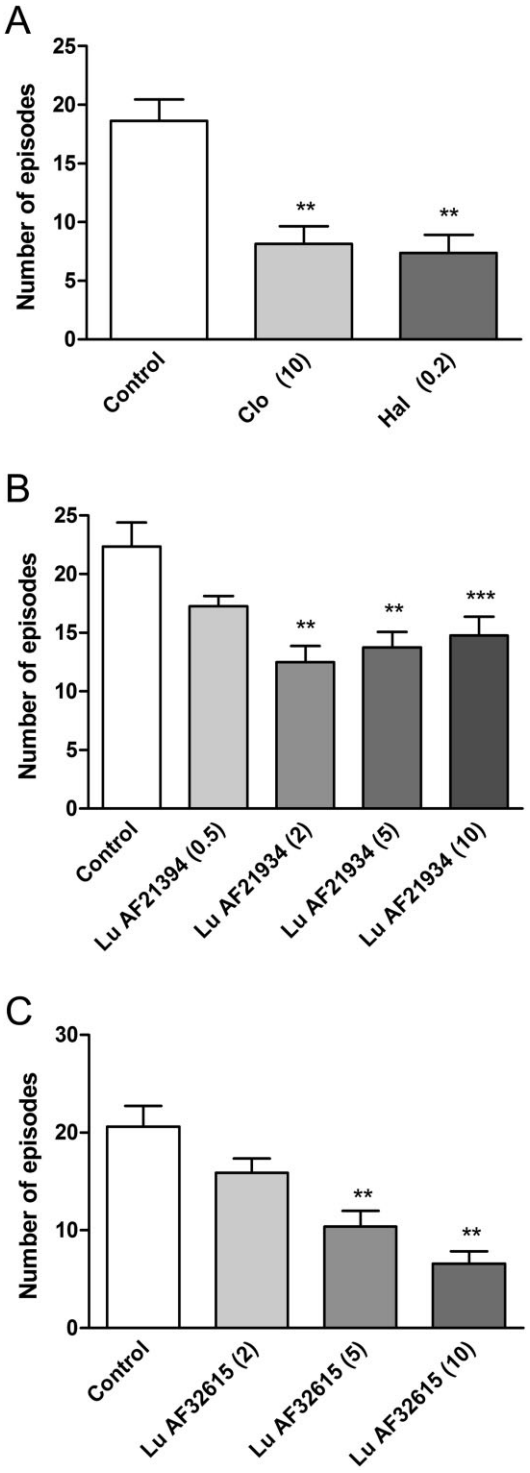

Figure 6

The suppression of DOI-induced head twitches by reference compounds haloperidol and clozapine (A), Lu AF21934 (B) and Lu AF32615 (C). The compounds were given 30 (A) or 60 min before DOI (2.5 mg·kg^{-1}) administration, and immediately after DOI the test begun. The values represent the number of head twitches (mean ± SEM) during a 20-min session. Values in parentheses represent the doses of the compounds in mg·kg^{-1}.The data are presented as means ± SEM. ***P < 0.001 and **P < 0.01 versus DOI-treated group; one-way ANOVA and Dunnett's test.

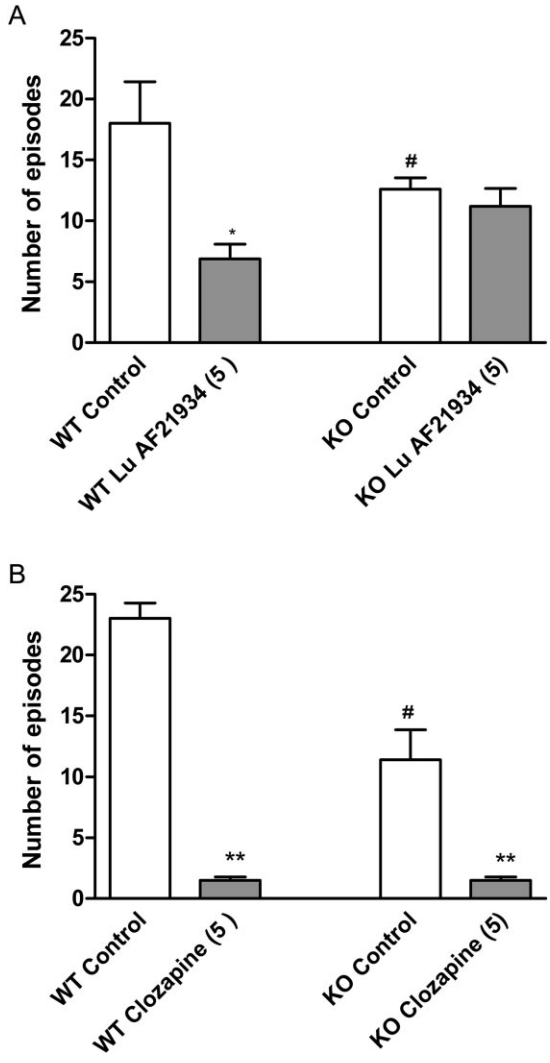

Figure 7

The suppression of DOI-induced head twitches by Lu AF21934 (A) and reference compound clozapine (B) in wild type (WT) and mGlu$_4^{-/-}$ (KO) mice. The compounds were given 60 min before DOI (2.5 mg·kg^{-1}) administration, and immediately after DOI the test begun. The values represent the number of head twitches (mean ± SEM) during a 20-min session. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. The data is presented as means ± SEM. #P < 0.05 versus DOI-treated WT mice, *P < 0.05 versus DOI-treated WT mice and **P < 0.01 versus DOI-treated WT group; one-way ANOVA and Dunnett's test.

dependency of Lu AF21934 effect was U shaped with the highest decrease of sEPSCs frequency observed at 1 μM (P < 0.05) (Figure 9A). The effects of the second compound, Lu AF32615, was concentration-dependent from 2.5 to 10 μM (Figure 9B).

Social interaction test

MK-801 (0.1 mg·kg^{-1}) disrupted both the number of social episodes between rats and the total time of interaction, decreasing these behaviours to 25.8% and to 22.4% of

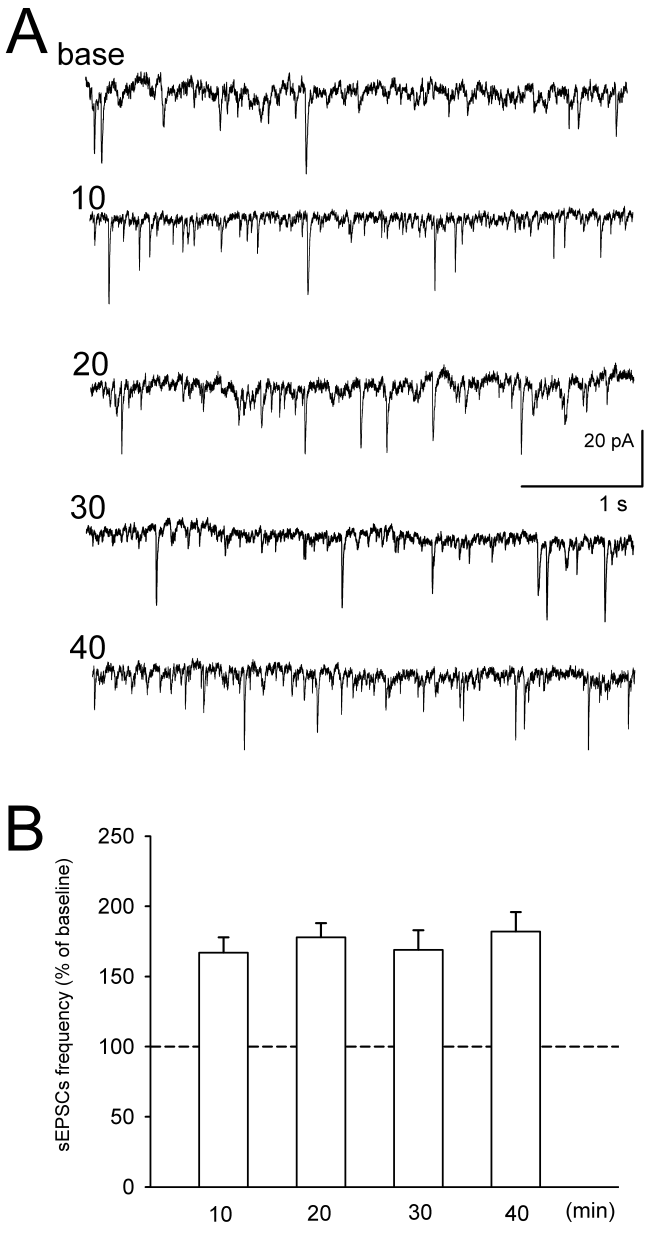

Figure 8

(A). Examples of sEPSCs recordings from brain slices. Base – recording before DOI application. Numbers denote time after the beginning of DOI application (minutes). (B) Mean (± SEM) frequency of sEPSCs over 5 min periods beginning at indicated time points. Data are normalized to baseline recording obtained before 10 uM DOI application (n = 4).

control level, respectively. The reference compound, clozapine (7.5 mg·kg^{-1}, i.p), increased the number of social episodes [$F_{(2.24)}$ = 22.97; P < 0.05] and duration of contact [$F_{(2.24)}$ = 27.23; P < 0.01], impaired by MK-801 (Figure 10), having no effect by itself (data not shown). Similar attenuation of the number of episodes and time of interactions were observed after Lu AF21934 administration at 0.5 mg·kg^{-1} [$F_{(4.40)}$ = 5.44; P < 0.05 and $F_{(4.40)}$ = 5.66; P < 0.01, respectively] but not at the two other doses (0.2 and 1 mg·kg^{-1})

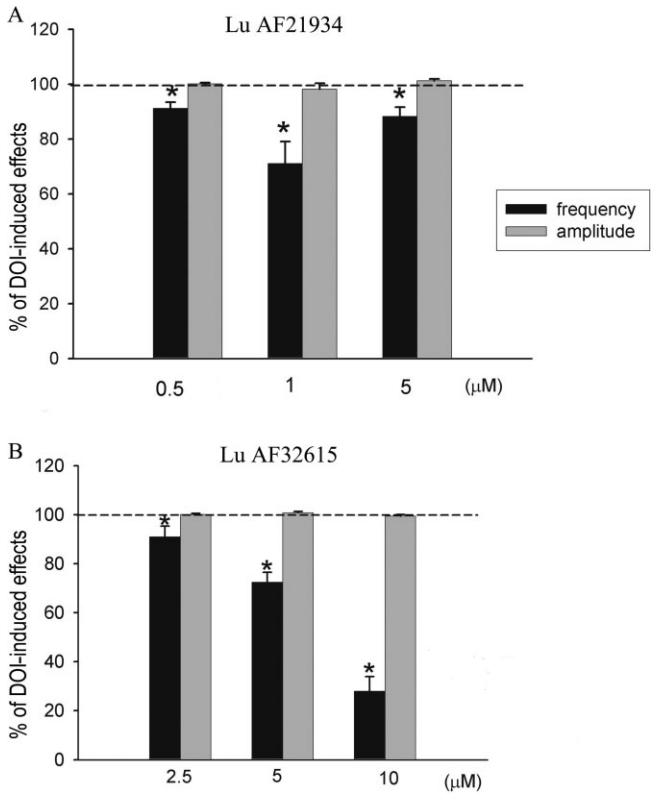

Figure 9

Suppression by Lu AF21934 (A) or Lu AF32615 (B) of the increase in sEPSCs activity induced by DOI recording after 10 min incubation with DOI and after 10 min incubation with Lu AF21934 or Lu AF32615 in the continuous presence of DOI. *P < 0.05 versus DOI effect; paired t-test.

(Figure 11A, B). Control experiments revealed that the PAM did not change the behaviour of rats when administered alone (Figure 11C, D).

The second compound, Lu AF32615, was effective at 10 mg·kg^{-1}, but not at 2 and 5 mg·kg^{-1}, increasing the time of interaction [$F_{(4.43)}$ = 6.55; P < 0.01], and the number of episodes [$F_{(4.43)}$ = 7.19; P < 0.01] (Figure 12A, B). Control experiments revealed that the drug had no influence on the social behaviour of animals when given alone (Figure 12C, D).

Delayed spatial alternation task

MK-801 (0.1 mg·kg^{-1}) impaired this behaviour, that is, it decreased the number of alternations, an effect reversed by the reference compound, risperidone [$F_{(2.24)}$ = 20.17; P < 0.01] (Figure 13). Lu AF21934 was effective only at 1 or 2 mg·kg^{-1} [$F_{(4.42)}$ = 6.7; P < 0.01] (Figure 14A). The second compound, Lu AF32615 was tested over a higher dose range (5–15 mg·kg^{-1}) and was effective only at 10 mg·kg^{-1} [$F_{(3.35)}$ = 6.17; P < 0.05] (Figure 14B). Control experiments revealed that neither risperidone (not shown) nor the PAMs had any effect on the behaviour of animals when given alone (Figure 14C, D).

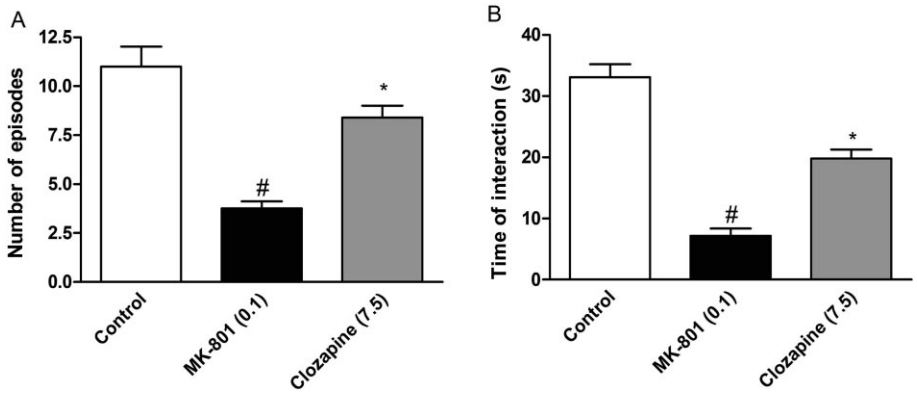

Figure 10

Effects of reference compound, clozapine, on the MK-801-induced deficits in social interaction test. The graphs represent the disruption in the number of social episodes (A) and in the total duration of social episodes (B). Clozapine was given 60 min before the test, and MK-801 was administered 3.5 h before the test. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. Data are presented as means ± SEM. $^{\#}P < 0.01$ versus control group $^{*}P < 0.05$, and $^{**}P < 0.01$ versus MK-801-treated group; one-way ANOVA and Tukey's *post hoc* test.

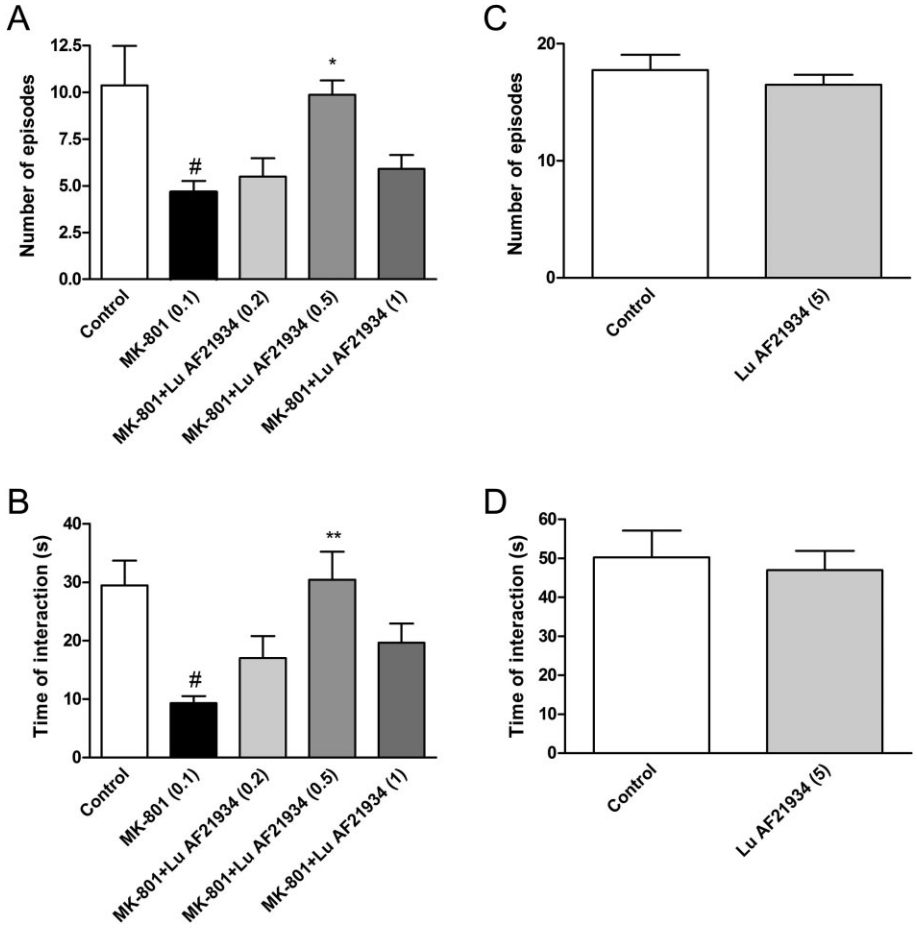

Figure 11

Effects of Lu AF21934 on the MK-801-induced deficits in social interaction test. The graphs represent the disruption in the number of social episodes (A) and in the total duration of social episodes (B). Lu AF21934 was given 60 min before the test, and MK-801 was administered 3.5 h before the test. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. Data are presented as mean ± SEM. $^{\#}P < 0.01$ versus control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus MK-801-treated group; one-way ANOVA analysis followed by Tukey's *post hoc* tests. The control experiments without MK-801 administration are represented by the panels C and D. The control experiments without MK-801 administration are represented by the panels C and D.

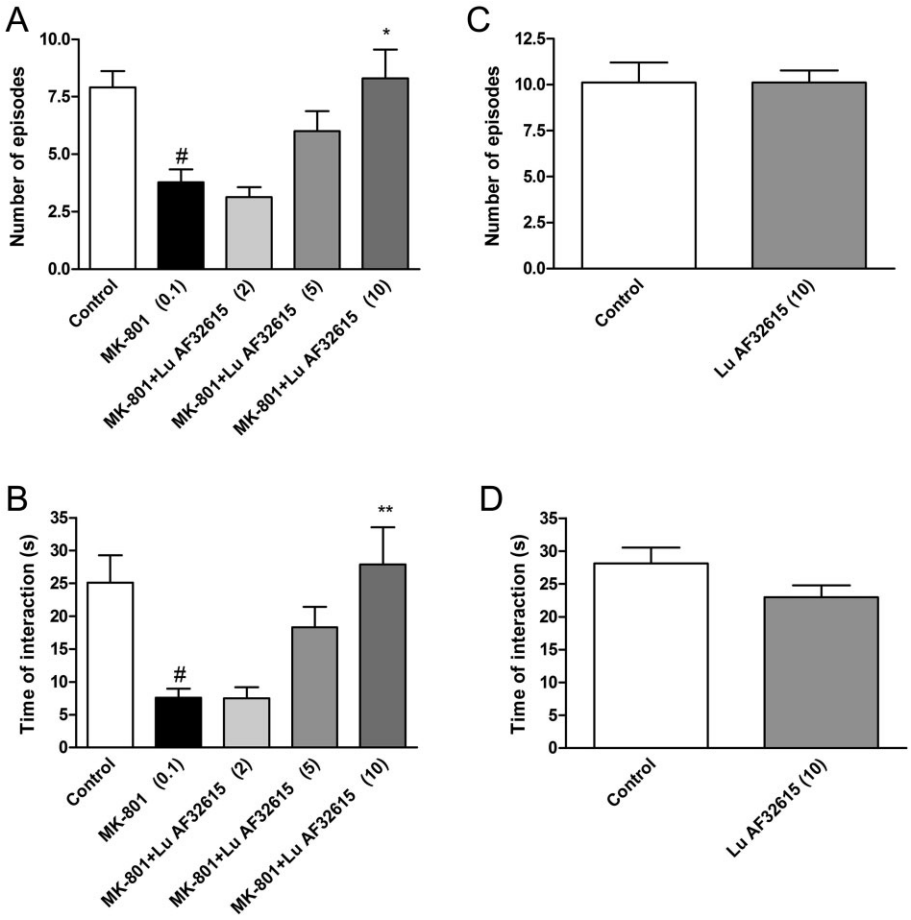

Figure 12

Effects of Lu AF32615 on MK-801-induced deficits in social interaction test. The graphs represent the disruption in the number of social episodes (A) and in the total duration of social episodes (B). Lu AF32615 was given 60 min before the test, and MK-801 was given 3.5 h before the test. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. Data are presented as mean ± SEM. $^{\#}P < 0.01$ versus control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ versus MK-801-treated group; one-way ANOVA analysis followed by Tukey's *post hoc* test. The control experiments without MK-801 administration are represented by the panels C and D.

Discussion

In the present work, we explored the antipsychotic-like activity of Lu AF21934 and Lu AF32615, two selective, structurally distinct PAMs of the $mGlu_4$ receptor, using *in vivo* behavioural and *ex vivo* electrophysiological assessments. These tool compounds have been thoroughly characterized in terms of physicochemical and *in vitro* pharmacological properties, as well as brain penetration in rat (East *et al.*, 2010; Bennouar *et al.*, 2013). Their properties are presented in Table 1. While Lu AF21934 shows very weak activity at the $mGlu_6$ receptor (PAM EC_{50} = 7 μM, 14-fold selectivity vs. $mGlu_4$ receptors), the narrow and extra-CNS localization of this receptor suggests that this cross-reactivity will not interfere with effects of action at sites within the CNS (Nakanishi *et al.*, 1998). The antipsychotic-like activity of these compounds was evaluated with respect to the positive, negative and cognitive symptoms of schizophrenia using well-characterized preclinical tests. The experimental schedules used in the present study were adapted from previous reports from our group (Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2012; 2013).

The reversal of MK-801- or amphetamine-induced hyperlocomotion are widely used animal models for detecting the effectiveness of drugs in reversing positive symptoms of schizophrenia (Geyer and Ellenbroek, 2003; Jones *et al.*, 2011a,b). Lu AF21934 reversed MK-801- and amphetamine-induced hyperlocomotions with a U-shaped dose-responsive effect, while the action of Lu AF32615 was monotonic, within the restricted dose range studied (up to 10 mg·kg^{-1}). Moreover, the actions of Lu AF21934 were consistently seen at *ca.* 10-fold lower doses than for Lu AF32615, which is in agreement with its improved *in vitro* potency (twofold), brain free fraction (1.6-fold) and absolute brain concentrations (threefold, see Table 1) (Hammarlund-Udenaes, 2010). In addition, its enantiomer lacking $mGlu_4$ receptor PAM activity, Lu AF21935, was inactive in MK-801-induced hyperlocomotion when tested at comparable doses. Lu AF21934 was effective at doses similar to that described earlier when its anxiolytic-like properties were demonstrated (Sławińska *et al.*, 2013).

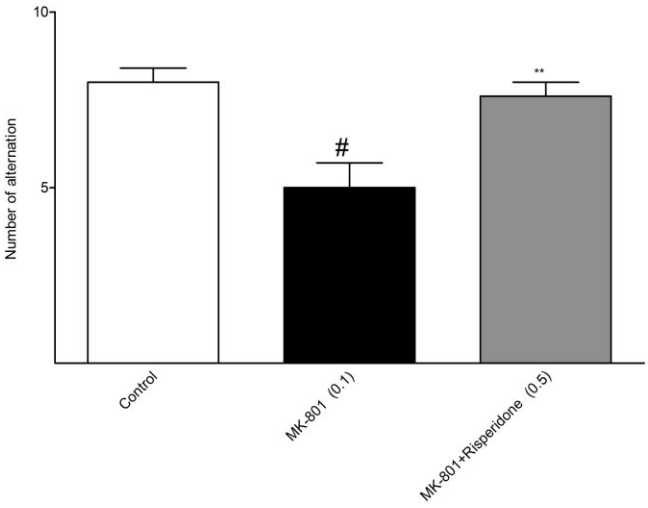

Figure 13

Effects of reference compound risperidone on MK-801-induced deficits in delayed spatial alternation task. Risperidone was given 30 min before the test. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. Data are presented as mean ± SEM. $^{\#}P < 0.01$ versus control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ versus MK-801-treated group; one-way ANOVA analysis followed by Tukey's *post hoc* test.

The biphasic dose-behavioural response relationship observed here for Lu AF21934 was demonstrated earlier for other $mGlu_4$ receptor agonists in a variety of experimental settings. Thus, a number of group III mGlu receptor agonists, such as APCPr, ACPT-I, LSP1-2111, exhibited biphasic dose-response relationships when tested *in vivo* in the reversal of haloperidol-induced catalepsy test (Sibille *et al.*, 2007; Beurrier *et al.*, 2009; Lopez *et al.*, 2012). The group III-selective agonist L-AP4 also generated biphasic concentration-response curves during whole cell patch-clamp recordings from neurons in the globus pallidus (Valenti *et al.*, 2003). Thus, our observations are consistent with previous observations on the pharmacology of $mGlu_4$ receptor activation. Two hypotheses have been put forward to explain these biphasic responses: opposing pharmacology caused by the activation of $mGlu_7$ or $mGlu_8$ receptors at high agonist concentrations or agonist-driven $mGlu_4$ receptor desensitization. The latter is the prevailing explanation at this time (Duty, 2010).

To further investigate the hypothesis of antipsychotic activity of $mGlu_4$ receptor PAMs in models predictive of positive symptoms of schizophrenia, we used the 5-HT receptor agonist, DOI. In humans, activation of the 5-HT_{2A} receptors (with LSD for example) induces hallucinogenic effects (Jacobs and Trulson, 1979). In rodents, an injection of DOI (a 5-HT_{2A} receptor agonist) induces so-called head twitches (Vickers *et al.*, 2001), that are reversed by compounds with both typical and atypical neuroleptic efficacy in the clinic (see Results). We found that both Lu AF21934 and Lu AF32615 attenuated this DOI-induced effect, as did agonists of group III metabotropic receptors (Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2012), and again the action of Lu AF21934 was more evident at low doses. The compound was also not active in $mGlu_4^{-/-}$ animals, although the reference compound clozapine showed strong significant activity, confirming the specificity of Lu AF21934 for the $mGlu_4$ receptor. However, due to limited number of available $mGlu_4^{-/-}$ mice, Lu AF32615 was not investigated. Moreover, DOI effects appeared to be reduced in $mGlu_4^{-/-}$ animals, and it does not align well with the effects of $mGlu_4$ receptor activators in this model, and may be a result of compensatory mechanisms. Analogous effects were observed previously for $mGlu_7$ knockout mice, which showed antidepressant-like phenotype, and similar antidepressant-like efficacy was observed for a $mGlu_7$ PAM, AMN082 (see Pałucha-Poniewiera *et al.*, 2008). There are other studies showing that knockout animals may behave differently from WT animals treated with antagonists. For example, mice that lack the 5-HT transporter exhibit increased anxio-depressive-like behaviour, although blockade of that transporter is currently the most common way to treat depression (Holmes *et al.*, 2003; Lira *et al.*, 2003).

A prominent negative feature of schizophrenia is social withdrawal, manifested as reduced social behaviour that often precedes the onset of the first psychotic episode and is strongly associated with poor psychosocial function (Cramer *et al.*, 1992; Mueser and McGurk, 2004). Generally, in rodents social withdrawal can be obtained by the administration of NMDA antagonists, but not through activation of the dopaminergic system (Sams-Dodd, 1997). This effect can be reversed after administration of atypical, but not typical neuroleptics (Corbett *et al.*, 1993; 1995); therefore clozapine was selected as a reference compound. The social withdrawal in the social interaction paradigm was induced by MK-801 and the number of social episodes and time of interaction between two rats was investigated in a novel environment, such as open field apparatus (Sams-Dodd, 1995; 1996). We adapted the modification of the method from the work of Satow *et al.*, 2009, in which the positive effect of CFMTI, an $mGlu_1$ receptor antagonist, was established. Both compounds investigated in this report reversed MK-801-induced social withdrawal and, as observed in earlier experiments, the action of Lu AF21934 was U-shaped and the activity of Lu AF32615 was evident in a linear manner within the dose range tested. Control experiments revealed that the drugs did not influence the social behaviour of rats when given alone.

In the last part of our investigations, we used the spatial delayed alternation task (DAT) as a model predictive of cognitive disturbances. Similarly to earlier described negative symptoms, efficacy of orthosteric agonists of the group III mGlu receptors towards cognitive disturbances had not been investigated previously. The DAT is based on the tendency of rodents to choose alternative maze arms or locations when they are re-exposed to an apparatus (Dudchenko, 2004) and is considered as working memory task because the animals must remember their initial response in order to select an alternative response. In our experimental schedule, we used 10% sucrose solution which helped the rats to make the resolution. A number of earlier studies established that NMDA receptor antagonists (PCP, MK-801, ketamine) resulted in chance-level performance in the DAT (Verma and Moghaddam, 1996; Wedzony *et al.*, 2000; Aultman and Moghaddam, 2001). In our studies, we used risperidone as a reference compound, as haloperidol is ineffective in this paradigm and the effectiveness of clozapine is not evident. Both

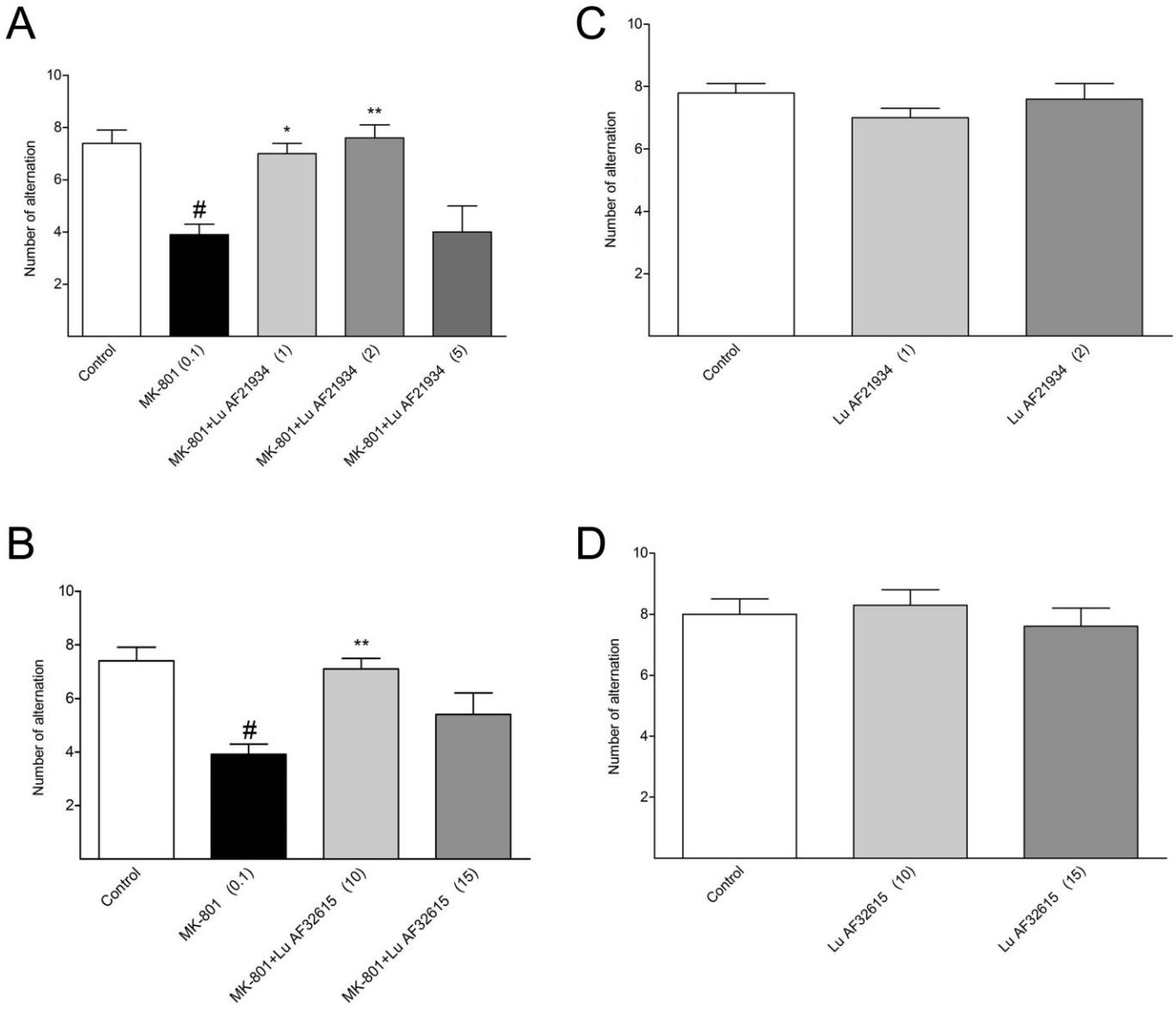

Figure 14

Effects of Lu AF21934 (A) and Lu AF32615 (B) on MK-801-induced deficits in delayed spatial alternation task. Compounds were given 60 min before the test. Values in parentheses represent the doses of the compounds in mg·kg^{-1}. Data are presented as mean ± SEM. $^{\#}P < 0.01$ versus control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ versus MK-801-treated group; one-way ANOVA analysis followed by Tukey's *post hoc* test. The control experiments without MK-801 administration are represented by the panels C and D.

mGlu$_4$ receptor PAMs reversed the MK-801 induced deficit in the dose ranges similar to those effective in previous behavioural models. The results again confirm that the mGlu$_4$ receptor activation may constitute a promising alternative to currently used antipsychotic therapy. This aspect of our work seems to be of particular interest. The efficacy of the compounds towards not only positive, but also negative and cognitive symptoms of schizophrenia may constitute an advantage of the activators of the mGlu$_4$ receptors over presently used antipsychotic therapy.

Considering the mechanisms of the behavioural effects described here, we would cite the hypothesis of Conn *et al.* (2009b). The psychotic symptoms evoked by MK-801 antagonists are thought to result from an over-activation of thalamocortical glutamatergic neurons that provide excitatory input to pyramidal neurons in the prefrontal cortex, leading to an enhanced glutamate release and the excessive activation of postsynaptic structures in the prefrontal cortex. The mGlu$_4$ receptors, localized on glutamatergic terminals in the prefrontal and pyriform cortex (Benítez *et al.*, 2000; Wierońska *et al.*, 2007), as well as in thalamocortical circuitry (Wang *et al.*, 2005) regulate glutamate release. The stimulation of those presynaptic mGlu$_4$ receptors may counteract the MK-801-induced dysfunctions, by inhibiting the abnormal glutamate efflux (Figure 15). Mechanistic support for this line of thinking was further gained through electrophysiological

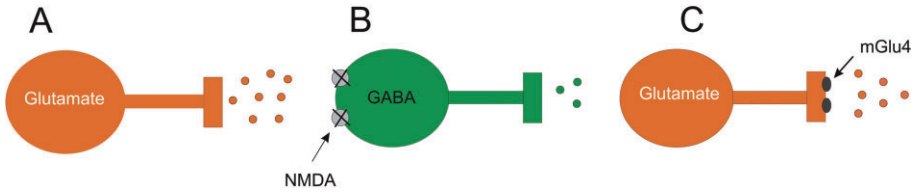

Figure 15

Schematic representation of the possible mechanisms by which $mGlu_4$ receptor PAMs exert their antipsychotic action. Blockade of the NMDA receptors on midbrain inhibitory GABAergic neurons, which are normally under excitatory control from glutamatergic afferents (A), results in decreased excitation of GABAergic inhibitory neurons (B) and the loss of inhibitory control on excitatory glutamatergic thalamocortical neurons (C) that project to pyramidal neurons in the prefrontal cortex. This decreased activity of GABAergic inhibitory neurons leads to overactivation of thalamocortical glutamatergic neurons that normally provide excitatory input to pyramidal neurons in the prefrontal cortex.. This results in an enhanced release of glutamate and the excessive activation in postsynaptic structures in the prefrontal cortex, including pyramidal neurons. The $mGlu_4$ receptors are expressed presynaptically at the thalamocortical pyramidal neurons and their activation may inhibit this abnormal glutamate release (see Conn *et al.*, 2009b).

experiments performed in this studies, in which exposure to DOI increased the frequency and the amplitude of spontaneous EPSCs in layer V pyramidal neurons in the mice cortical slices, confirming earlier data (Kłodzinska *et al.*, 2002; Pałucha-Poniewiera *et al.*, 2008; Wierońska *et al.*, 2012). Lu AF21934 and Lu AF32615 suppressed only the frequency, and not the amplitude of the spontaneous EPSCs, suggesting that their actions could be attributed to a presynaptic, rather than a postsynaptic, site (van der Kloot, 1991). The modulation by $mGlu_4$ receptors of synaptic currents induced by $5\text{-}HT_{2A}$ receptor activation in the prefrontal cortex/neocortex mimics the effects of the selective $mGlu_{2/3}$ receptor agonists LY354740 and LY379268 (Marek *et al.*, 2000; Schoepp and Marek, 2002; Wischhof *et al.*, 2012). Therefore, this may constitute a common neuronal mechanism of antipsychotic effects of those compounds, which is also evident for atypical neuroleptics, such as clozapine (Schoepp and Marek, 2002).

The simultaneous anti-Parkinsonian effectiveness of $mGlu_4$ receptor PAMs may be puzzling, as both of these compounds have shown dose-dependent efficacy reversing haloperidol-induced catalepsy (East *et al.*, 2010; Bennouar *et al.*, 2013). The efficacy of Lu AF21934 potentiating the positive motor effects of L-DOPA in the 6-OHDA rat model has also been documented (Bennouar *et al.*, 2013). The symptomatic effects in Parkinson's disease are derived from a re-balancing of the direct and indirect striatopallidal pathways (Lindsley and Hopkins, 2012). The key GABAergic nucleus in the basal ganglia is the globus pallidus, which receives an inhibitory striatal projection (see Valenti *et al.*, 2003). Modulation of this striatopallidal synapse by $mGlu_4$ receptors expressed on GABAergic terminals (Corti *et al.*, 2002) could decrease the excessive inhibition within the globus pallidus that has been proposed in Parkinson's disease (Valenti *et al.*, 2003; Marino *et al.*, 2006). Therefore, the anti-Parkinsonian, as distinct from the antipsychotic, activity of $mGlu_4$ receptor PAMs may be related to the different mechanisms and aetiologies of these diseases and the receptor localization in different brain regions. The efficacy of $mGlu_4$ receptor PAMs in schizophrenia is thought to derive from a reduction of the excessive glutamatergic tone within thalamocortical circuits that characterizes this disease, while the anti-Parkinsonian effects would be a result of decreasing abnormal GABA efflux in the globus pallidus (GP).

In summary, the results of the present study constitute another step in the progress of ligands for metabotropic glutamate receptors from drug design to clinical efficacy. These initial results show that the effectiveness of $mGlu_4$ receptor PAMs was evident in a broad spectrum of animal models of schizophrenia, thought to be predictive of positive, negative and cognitive disturbances. The current working hypothesis is based on the inhibition of glutamate release due to stimulation of presynaptic receptors localized on glutamatergic axons. The exact mechanism of action of these ligands is still under investigation.

Acknowledgements

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Conflict of interest

DD is an employee of Lundbeck.

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