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Neurochemical and behavioral studies on the 5-HT_{1A}-dependent antipsychotic action of the $mGlu_4$ receptor agonist LSP4-2022

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ABSTRACT

LSP4-2022 is a novel, orthosteric agonist of $mGlu_4$ receptor that induces antipsychotic-like activity in animal studies. In the present study, the involvement of 5-HT_{1A} receptors in LSP4-2022-induced antipsychotic actions and the neurochemical background of that interaction were investigated. In several behavioral tests the actions of effective doses of the compound (0.5–2 mg/kg) were antagonized via the administration of the 5-HT_{1A} antagonist WAY100635 (0.1 mg/kg). The co-administration of sub-effective dose of the 5-HT_{1A} agonist (R)-(S)-8-OH-DPAT (0.01 mg/kg) intensified the activity of ineffective doses of LSP4-2022, having no influence on the efficacy of the active doses. The co-administration of effective doses of both compounds did not intensify each other's action.

In the microdialysis *in vivo* tests, MK-801 (0.6 mg/kg) induced an enhancement of the release of dopamine, serotonin, glutamate and GABA in the prefrontal cortex. Administration of LSP4-2022 (2 mg/kg) abolished this MK-801-induced effect on neurotransmitter release. Co-administration with WAY100635 (0.1 mg/kg), a 5-HT_{1A} antagonist, completely (dopamine, serotonin) or partially (glutamate, GABA) counteracted this LSP4-2022-induced effect. Subsequently, the patch-clamp recordings of spontaneous EPSCs were performed. sEPSCs were evoked in slices from the mouse prefrontal cortex by DOI (10 μM). LSP4-2022 (2.5; 5 and 10 μm) reversed DOI-induced changes in both the frequency and amplitude of the sEPSCs, but the more robust effect on the frequency was observed. The administration of WAY100635 had no effect on the LSP4-2022-induced effects on sEPSCs, indicating that the $mGlu_4$-5-HT_{1A} interaction does not occur via single-neuron signaling but involves neuronal circuits that regulate neurotransmitter release.

This article is part of the Special Issue entitled 'Metabotropic Glutamate Receptors, 5 years on'.

1. Introduction

Our previous research was focused on the role of the glutamatergic system in the pathophysiology and treatment of severe mental disorders, concentrating mainly on the role of metabotropic glutamatergic (mGlu) receptors that are linked with G-proteins and mediate slow synaptic currents (Pin and Duvoisin, 1995). These receptors are divided into three groups and further divided into eight subtypes based on sequence homology, pharmacology and the second messenger system they activate (Pin and Duvoisin, 1995). In preclinical and some clinical trials, it has been shown that mGlu receptors constitute a promising target for the treatment of a variety of CNS diseases (e.g., Conn et al., 2009; Wieronska and Pilc, 2009) due, to some extent, to their ability to regulate the release of glutamate and/or GABA, which are two main amino-acid neurotransmitters in the CNS that ensure homeostasis in the brain via the maintenance of an excitatory/inhibitory balance (Linden and Schoepp, 2006). Because of the therapeutic potential of mGlu receptors as putative drug targets, an intensive search has been performed investigating the efficacy of selective ligands of those receptors in animal models of CNS disorders, such as models of depression, anxiety, schizophrenia and other neurological diseases (Amalric et al., 2013; Conn, 2003; Niswender et al., 2005; Pilc et al.,

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2013; Wieronska et al., 2010; Wieronska et al., 2015), where selective positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), as well as orthosteric ligands have been investigated. Among all mGlu receptors ligands, the third group of these receptors is of special interest as this is not only the largest group of mGlu receptors, with many splice variants of each subtype, but also the group that is the least investigated, partially because of a lack of selective ligands for these receptors. The group consists of $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors, with the distribution of the $mGlu_6$ receptor limited to the retina (Pin and Duvoisin, 1995; Conn and Pin, 1997). The $mGlu_{4/7/8}$ receptors have been indicated as important factors involved in the regulation of glutamate release (Cartmell and Schoepp, 2000; Mercier and Lodge, 2014; Schoepp, 2001). All of the subtypes are negatively linked to adenylyl cyclase activity, and their activation inhibits glutamate release (Linden and Schoepp, 2006; Conn and Pin, 1997). Data on the antipsychotic-like activity of the ligands of these receptors were published by Palucha-Poniewiera et al. (2008) for a non-selective agonist of these receptors, ACPT-1 (Acher et al., 1997). These data were concerned mainly with its activity in the tests for positive symptoms of schizophrenia. Subsequently, more selective compounds were synthesized, such as LSP1-2111, which preferentially activates the $mGlu_4$ receptor and has a 30-fold higher selectivity towards that subtype than towards $mGlu_7$/$mGlu_8$ receptors (Beurrier et al., 2009). This compound was active in animal models of schizophrenia, including those of positive (Wieronska et al., 2012), negative and cognitive symptoms (Wierońska et al., 2013). Similar results were obtained in the study of the effects of two selective positive allosteric modulators of $mGlu_4$ receptors, Lu AF21934 and Lu AF32615 (Slawinska et al., 2013). It has been shown that both of these PAMs induce a dose-dependent reversal in the deficits observed in preclinical models that mimic positive, negative and cognitive symptoms of schizophrenia (Slawinska et al., 2013; Wieronska et al., 2015). Simultaneously, the involvement of the $mGlu_7$ subtype was excluded as a potential target as the $mGlu_7$ receptor PAM, AMN082, was inactive in animal models of schizophrenia (Wieronska et al., 2012).

In our subsequent studies it was established that the antipsychotic-like activity of $mGlu_4$ orthosteric agonists and PAMs was dependent on serotonergic signaling via 5-HT_{1A} receptors (Wierońska et al., 2013, 2015).

In the present study we used the recently developed, selective $mGlu_4$ receptor orthosteric agonist LSP4-2022. LSP4-2022 is the first selective orthosteric agonist of $mGlu_4$ receptors, and has an $EC_{50} = 0.11\ \mu M \pm 0.2$. The affinity of the compound to other group III mGlu receptor subtypes is 100–300 times lower, with the EC_{50} values $= 11.6\ \mu M \pm 1.9$ or $29.2\ \mu M \pm 4.2$ for $mGlu_7$ and $mGlu_8$ receptors, respectively (Goudet et al., 2012; Flor and Acher, 2012). No activity on the group I and II mGlu receptors at 100 μM was noticed. This compound was shown to possess antiparkinsonian properties after central or systemic administration in a haloperidol-induced catalepsy test (Goudet et al., 2012). It has been shown previously that this compound also has antipsychotic-like activities in a variety of animal models of schizophrenia, such as hyperactivity, DOI-induced head twitches, social interaction, a modified forced-swim test, and novel object recognition (Wozniak et al., 2016), and in selected procedures its actions were $GABA_B$-receptor dependent. Additionally, its pro-depressant rather than antidepressant activity has been proposed (Podkowa et al., 2015).

In the present study we focused on the interactions between the $mGlu_4$ and the 5-HT_{1A} receptors, applying in addition to LSP4-2022, the selective 5-HT_{1A} receptor antagonist WAY100635 (Fletcher et al., 1996; Routledge et al., 1993) and the selective agonist (*R*)-(+)-8-hydroxy-DPAT (Cornfield et al., 1991; Hjorth and Magnusson, 1988) as tool compounds. Moreover, we investigated the mechanism of these interactions using electrophysiology and *in vivo* microdialysis techniques.

2. Materials and methods

2.1. Animals and housing

Male Albino Swiss (20–25 g) mice were used in behavioral tests and for electrophysiology. Male Wistar rats (250–300 g) were used in the microdialysis experiments. The 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 eight to ten animals, and the animals were used only once in each test. All compounds were administered in a volume of 10 ml/kg when given to mice and 1 ml/kg when injected into rats. All behavioral measurements were made by an observer blinded to the treatment. All procedures were conducted according to 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.

2.2. Drugs

The following drugs used are describe below. LSP4-2022 ($mGlu_4$ receptor agonist), synthesized in Francine Acher's lab and characterized using H-1 and C-13 nuclear magnetic resonance spectroscopy, HPLC/mass-spectrometry methods, and X-ray crystallography. The compound was dissolved in saline and was administered intraperitoneally (i.p.) 45 min before the tests. The administration schedule for LSP4-2022 was planned based on our previous studies (Wozniak et al., 2016). MK-801 (0.35 mg/kg, Sigma-Aldrich, St. Louis, USA) was dissolved in 0.9% NaCl, and the doses were consistent with our previous work (Wieronska et al., 2012, 2013) and the works of the others (Geyer and Ellenbroek, 2003; Leite et al., 2007; Satow et al., 2009). WAY100635 and (*R*)-(+)-8-hydroxy-DPAT (Tocris Bioscience, Bristol, United Kingdom) were dissolved in 0.9% saline and were administered as in our previous studies and the studies of the others (Wierońska et al., 2013; Wedzony et al., 2000).

2.3. Locomotor activity of habituated mice

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 beams resulted in a record of horizontal activity, which was defined by ambulation scores. Mice were placed separately into activity cages for an acclimatization period of 30 min. They were then injected with LSP4-2022, WAY100635, (*R*)-(+)-8-hydroxy-DPAT or a combination of drugs (the timing and doses of the administration were similar to those described below for MK-801-induced hyperactivity). From this point on, the ambulation scores were measured for 60 min.

2.4. MK-801-induced hyperactivity

The locomotor activity was recorded for each animal in locomotor activity cages (according to Rorick-Kehn et al., 2007), with small modifications used in our previous studies (Wieronska et al., 2012, 2013). The mice were placed individually into actometers for an acclimatization period of 30 min. Then, they were administered the most active dose of LSP4-2022 (2 mg/kg) and WAY100635 (45 min before MK-801, 0.1 mg/kg, i.p), the sub effective dose of the LSP4-2022 (0.1 mg/kg) co-administered with (*R*)-(+)-8-hydroxy-

DPAT (15 min before MK-801, 0.01 mg/kg, s.c.), or vehicle and placed back in the same cages. After the proper time all of the mice were intraperitoneally administered MK-801 at a dose of 0.35 mg/kg and once again returned to the same cage. From then on, the ambulation scores were counted for 60 min. All groups were compared with the MK-801 control group. The experiment also included a control group not treated with MK-801.

2.5. Head twitch test

The experiment was performed according to Wieronska et al. (2012, 2013). 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, i.p.). Immediately after the treatment, the number of head twitches was counted during a 20 min session. The most active dose of LSP4-2022 (2 mg/kg) was co-administered with WAY100635 and the sub-effective dose with (*R*)-(+)-8-hydroxy-DPAT.

In parallel, the activity of LSP4-2022 was investigated in this test after chronic administration (10 days) at three doses: 0.5, 1 and 2 mg/kg.

2.6. Tests for MK-801-induced deficits in social interaction in mice

Social interaction tests were performed according to the method described by the others (Oh et al., 2013; de Moura Linck et al., 2008). Each social interaction test between two mice was carried out during the light phase of the light/dark cycle. Mice were selected from separate housing cages to make a pair for the study. The body weights of the paired mice were matched to within 10% difference. All mice were placed in an experimental room and the study was conducted in dark, plastic boxes 50 × 30 × 35 cm, 30 min after the subcutaneous administration of MK-801 at a dose of 0.3 mg/kg. The most active dose of LSP4-2022 (1 mg/kg) was co-administered with WAY100635, and all doses (0.1, 0.5 and 1 mg/kg) of the compound were co-administered with subeffective dose of (*R*)-(+)-8-hydroxy-DPAT (0.01 mg/kg). Additionally, both compounds were also tested after co-administration of the active doses (LSP4-2022 at 1 mg/kg and (RS)-8-OH-DPAT at 0.025 mg/kg).

The test box was wiped clean between each trial. Social interactions between two mice were determined based on the total time spent participating in social behaviors such as sniffing, genital investigation, chasing and fighting each other. The total number of social episodes was also measured. In addition, control experiments with animals not receiving MK-801 were conducted to determine whether the drugs had any influence on social behavior when given alone.

2.7. Novel object recognition (NOR)

The method was adapted from Nilsson et al. (2007). The animals were trained and tested in a black, plastic, open field (50 × 30 cm, 35 cm high) with the floor divided into 20-cm square sections. The open field was in a dark room illuminated only by a 25 W bulb. On the first day (adaptation) the animals were allowed to explore the open field for 10 min. On the next day (training, T_1) the animals were administered the tested drugs, placed in the apparatus and allowed to explore two identical objects (cylindrical objects with walls painted white, 7 cm in diameter, 11 cm high) for the time required to complete 15 s of exploration of either object. For the retention trial (T_2) conducted one h later, one of the objects presented in T_1 was replaced with a novel object (a prism-shaped object with walls painted black, 5 cm wide, 14 cm high). The mice were returned to the open field for 5 min, and the duration of exploration (i.e., sitting in close proximity to the objects or sniffing or touching them) of each object was video-recorded and then measured separately by a trained observer. All drugs were administered before the training (T_1) session. MK-801 (0.3 mg/kg, i.p.) was given 30 min before the session. The most active dose of LSP4-2022 (2 mg/kg) was co-administered with WAY100635 and all the doses (0.5, 1 and 2 mg/kg) were co-administered with (*R*)-(+)-8-hydroxy-DPAT (0.01 mg/kg). Additionally, both compounds were also tested after administration of the active doses (LSP4-2022 1 mg/kg and (RS)-8-OH-DPAT 0.025 mg/kg). All injections were given at a volume of 10 ml/kg of body weight. The treatment groups included 8 animals.

2.8. In vivo microdialysis

Rats were anaesthetized with ketamine (75 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Their skulls were exposed, and small holes were drilled for the insertion of microdialysis probes into the brain structures using appropriate coordinates (Paxinos and Watson, 2007). Vertical microdialysis probes were constructed as described in detail elsewhere (Golembiowska and Dziubina, 2012). Twenty-four hours after the surgery, probe inlets were connected to a syringe pump (BAS, IN, USA) that delivered an artificial CSF composed of the following compounds [mM]: NaCl 147, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 2.2; pH = 7.4, delivered at a flow rate of 2.0 μl/min. Baseline samples were collected every 20 min for 2 h after the washout period to obtain a stable extracellular neurotransmitter level. Then, the tested drugs were injected and the subsequent fractions of dialysates were collected for 4 h. At the end of the experiment, the rats were sacrificed and their brains were examined histologically to validate probe placement.

DA and 5-HT were analyzed via high performance liquid chromatography (HPLC) using coulochemical detection. Chromatography was performed using an Ultimate 3000 System (Dionex, USA) and a Coulochem III detector (model 5300, ESA, USA) with a 5020 guard cell, 5014B microdialysis cell and a Hypersil Gold-C_{18} analytical column (3 × 100 mm). The mobile phase was composed of 0.1 M potassium phosphate buffer adjusted to pH 3.8, 0.5 mM EDTA, 96 mg/L 1-octanesulfonic acid sodium salt, and 2% methanol. The flow rate during analysis was set at 0.7 ml/min. The applied potential of a guard cell was +600 mV, whereas those of microdialysis cells were as follows: $E_1 = -50$ mV and $E2 = +300$ mV, with a sensitivity set at 50 nA/V. The chromatographic data were processed by Chromeleon v. 6.80 (Dionex, USA) software run on a PC computer.

GABA and glutamate in the extracellular fluid were measured electrochemically after derivatization with an OPA/sulfite reagent to form an isoindole-sulfonate GABA-derivative. Chromatography was performed using an LC-10 AD pump (Shimadzu Europa GmbH, Warsaw, Poland) and an LC-4B amperometric detector with a cross-flow detector cell (BAS, IN, ISA), and an HR-80 column (80 × 4.6 mm, 3 μm; ESA, Inc. USA). The mobile phase consisted of 100 mM monosodium orthophosphate and 25% methanol at pH 4.6. The flow rate was 0.9 ml/min, and the applied potential of a 3-mm glassy carbon electrode was +600 mV at a sensitivity of 5 nA/V. GABA and glutamate-derivative peaks were compared with their respective standards and were processed using Chromax 2005 (Pol-Lab, Warsaw, Poland) software on a personal computer.

The obtained values were not corrected for in vitro probe recovery, which was approximately 10%.

2.9. Electrophysiology studies

Albino Swiss mice were decapitated, and their frontal cortices were dissected out and cut into slices (420 μm thick) in the frontal plane using a vibrating microtome. Slices were kept submerged in 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 a flow rate of 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 a resistance of 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, and 0.4 Na-GTP at 290 mOsm and pH = 7.2. Whole-cell recordings were made from layerV 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 a Digidata 1440 interface (Molecular Devices, USA). Drugs from concentrated stocks were diluted in ACSF just before the experiment and applied in the superfusate. After stable baseline recordings were observed for at least 15 min, DOI (10 μM) was applied for 10 min and spontaneous EPSCs were recorded (10 min). Next, DOI was applied concurrent with LSP4-2022 for 15 min and again spontaneous EPSCs were recorded. The measured parameter was the frequency of spontaneous EPSCs. The data were analyzed off-line using the Mini Analysis program (Synaptosoft Inc. ver. 6.0.3).

2.10. Statistical analysis

The data are presented as the means ± S.E.M. Statistical analyses of the data were performed using the Statistica 10 package (StatSoft Inc., OK, USA). A two-way ANOVA followed by Newman-Keuls *post hoc* comparison test was used in the interaction studies. Repeated-measures ANOVA followed by Tukey's *post hoc* comparison test was used in *in vivo* microdialysis studies. P values < 0.05 were considered statistically significant.

3. Results

3.1. MK-801-induced hyperactivity

3.1.1. The effects of LSP4-2022, WAY100635 and (R)-(+)-8-hydroxy-DPAT on locomotor activity in mice habituated to activity meters

Two-way ANOVA revealed that LSP4-2022 in combination with the selective agonist of 5-HT_{1A} receptors (*R*)-(+)-8-hydroxy-DPAT hydrobromide (0.01 mg/kg, 15 min before the test) or with the selective antagonist of 5-HT_{1A} receptors WAY100635 (0.1 mg/kg, 45 min before the test) did not change the locomotor activity of mice adapted to activity meters for 30 min. No statistically significant effects from the co-administration of LSP4-2022 with (*R*)-(+)-8-hydroxy-DPAT hydrobromide or LSP4-2022 (1 mg/kg) with WAY100635 were observed (Fig. 1A, B).

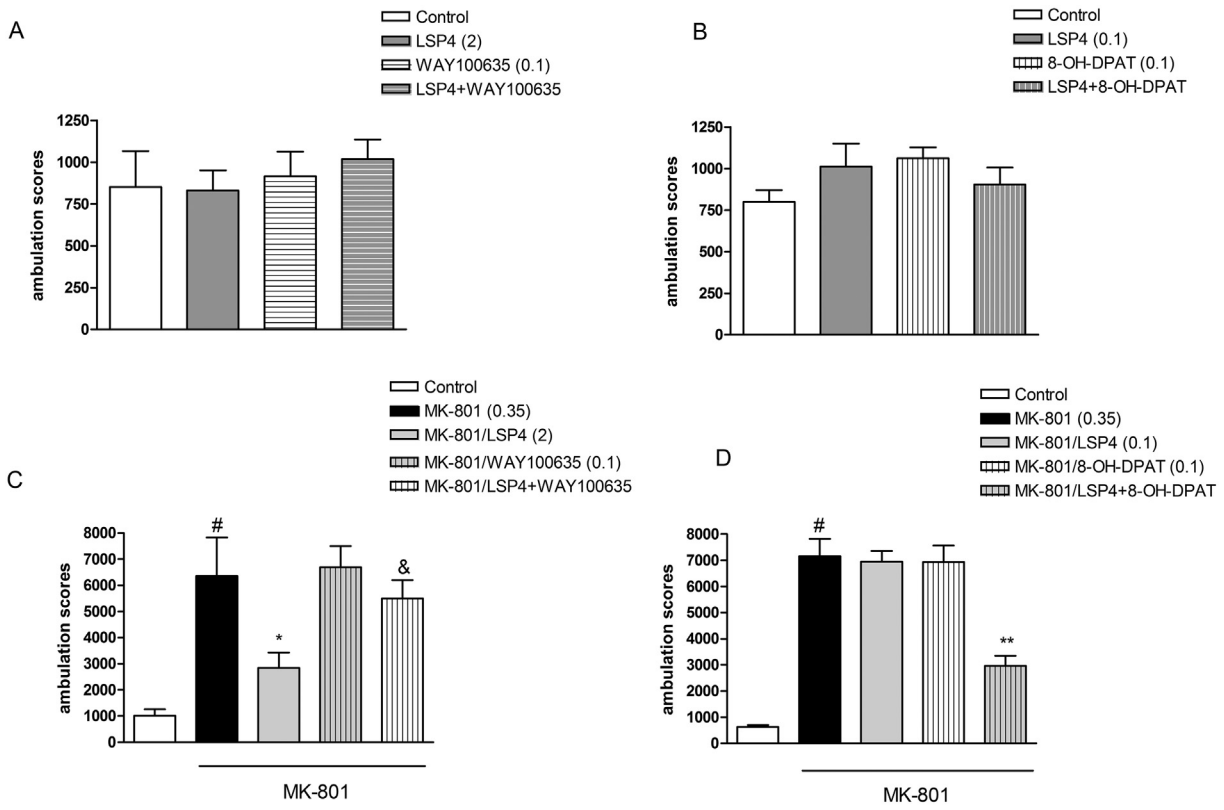

Fig. 1. Effects of LSP4-2022 and 5-HT_{1A} ligands on spontaneous (A, B) and MK-801-induced (C, D) locomotor activity. The combined administration of LSP4-2022 with WAY100635 (A, C), and the co-administration of LSP4-2022 with (R)-(S)-8-OH-DPAT (8-OH-DPAT) (B, D) are presented. The control experiments without MK-801 administration are shown on panels C and D. Data are presented as the means ± SEM. Doses (mg/kg) are indicated in parentheses. #P < 0.001 versus the control, *P < 0.001 and **P < 0.0001 versus the MK-801–treated group, and @ P < 0.04 versus the LSP4-2022 -treated group.

3.1.2. The effects of the combined administration of WAY100635 and LSP4-2022 on MK-801-induced hyperactivity in mice

The NMDA receptor antagonist MK-801 induced a profound increase in the ambulation scores (Fig. 1C, D). LSP4-2022 administered at a dose of 2 mg/kg reversed the MK-801-induced hyperactivity ($P < 0.001$). WAY100635, administered at a dose of 0.1 mg/kg, i.p did not have any effect on its own. However, when co-administered with LSP4-2022, it reversed the inhibitory action of LSP4-2022. The two-way ANOVA analysis revealed a significant effect of the LSP4-2022×WAY100635 interaction [$F_{(1.36)} = 4.13$; $P < 0.04$] (Fig. 1C).

3.1.3. The effects of the combined administration of sub-effective doses of (R)-(+)-8-hydroxy-DPAT hydrobromide and LSP4-2022 on MK-801-induced hyperactivity in mice

LSP4-2022 was administered at a low dose, 0.1 mg/kg, and (*R*)-(+)-8-hydroxy-DPAT hydrobromide was given at a dose of 0.01 mg/kg. Neither compound had any effect when administered separately. Co-administration of both compounds induced a clear reversal of hyperactivity. Two-way ANOVA of the main effects revealed a significant effect of the (*R*)-(+)-8-hydroxy-DPAT and LSP4-2022 interaction [$F_{(1.32)} = 5.11$, $P < 0.002$]. Post-hoc Newman-Keuls analysis revealed a significant effect of the (*R*)-(+)-8-hydroxy-DPAT×LSP4-2022 interaction compared with the effects in the MK-801-treated animals, $P < 0.0001$ (Fig. 1D).

3.2. DOI-induced head twitches

3.2.1. The effects of the combined administration of WAY100635 and LSP4-2022 on DOI-induced head twitches in mice

LSP4-2022 administered at a dose of 2 mg/kg significantly, by approximately 60%, reduced the number of DOI-induced head twitches ($P < 0.01$). WAY100635 administered at a dose of 0.1 mg/kg did not have any effect on its own. Co-administration of LSP4-2022 and WAY100635 resulted in the inhibition of the LSP4-2022-induced effect [$F_{(1.35)} = 4.34$, $P < 0.04$]. Post hoc Newman-Keuls analysis revealed a significant LSP4-2022×WAY100635 interaction compared with the effects in the LSP4-2022 treated group, $P < 0.002$ (Fig. 2A).

3.2.2. The effect of the combined administration of (R)-(+)-8-Hydroxy-DPAT hydrobromide and a subeffective dose of LSP4-2022 on DOI-induced head twitches in mice

LSP4-2022 was administered at a dose of 0.1 mg/kg, and (*R*)-(+)-8-hydroxy-DPAT hydrobromide was given at a dose of 0.01 mg/kg. Neither drug had effects when administered alone. Co-administration of subeffective doses of the 5-HT_{1A} receptor agonist and the $mGlu_4$ receptor agonist induced a clear reduction in the number of DOI-induced head twitches. Two-way ANOVA of the main effects revealed a significant effect of the LSP4-2022×(*R*)-(+)-8-hydroxy-DPAT hydrobromide interaction [$F_{(1.29)} = 4.66$, $P < 0.03$]. Post-hoc Newman-Keuls analysis revealed a significant effect of the LSP4-2022×(*R*)-(+)-8-hydroxy-DPAT hydrobromide interaction compared with the effects in the DOI-treated animals, $P < 0.001$ (Fig. 2B).

3.2.3. The effect of the chronic administration of LSP4-2022 in DOI-induced head twitches

LSP4-2022 at a single doses of 0.5–2 mg/kg i.p. in our earlier studies induced a clear antipsychotic-like effect in decreasing the number of DOI-induced head twitches (Woźniak et al., 2015). Eight injections of LSP4-2022 (once daily for 8 days) significantly decreased the number of head twitches in this test in a similar manner as observed with the acute treatment $F_{(3.36)} = 6.91$; $P < 0.001$ (Fig. 2C).

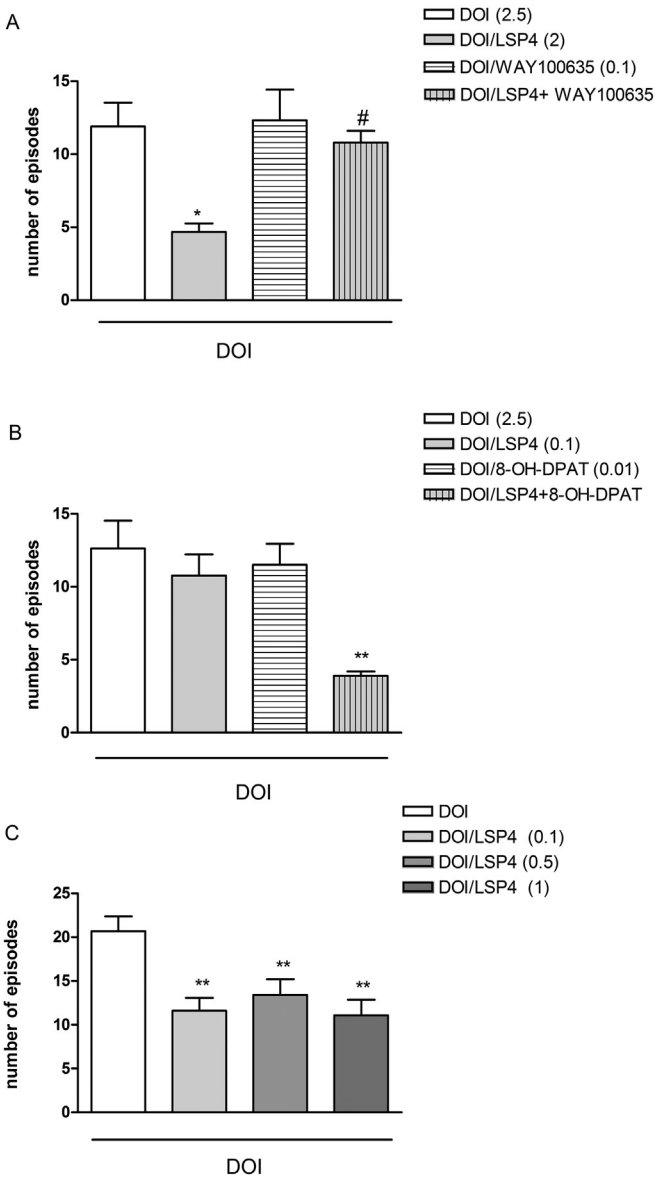

Fig. 2. Effects of LSP4-2022 and 5-HT_{1A} ligands on DOI-induced head twitches. The combined administration of LSP4-2022 with WAY100635 (A) and the co-administration of LSP4-2022 with (R)-(S)-8-OH-DPAT (8-OH-DPAT) (B), as well as the results of chronic (8 days) LSP4-2022 administration (C), are presented. Data are presented as the means ± SEM. Doses (mg/kg) are indicated in parentheses. *$P < 0.01$ and **$P < 0.001$ versus the DOI–treated group, and #$P < 0.05$ versus the LSP4-2022-treated group.

3.3. Social interactions

3.3.1. The effect of LSP4-2022 alone and in combined administration with WAY100635 in the social interaction test in mice

MK-801 induced profound social deficits both in the time of interaction, and the numer of episodes ($P < 0.0001$) (Fig. 3 A–D). The three doses of LSP4-2022 (0.1, 0.5 and 1 mg/kg, i.p, 45 min before MK-801 administration) were given to investigate if the compound reverses MK-801-induced deficits. The compound was active in the highest investigated dose 1 mg/kg both in the time of interaction and in the number of episodes. One-way ANOVA analysis revealed statistical significance, $F_{(3.16)} = 4.74$ and $F_{(3.16)} = 5.44$,

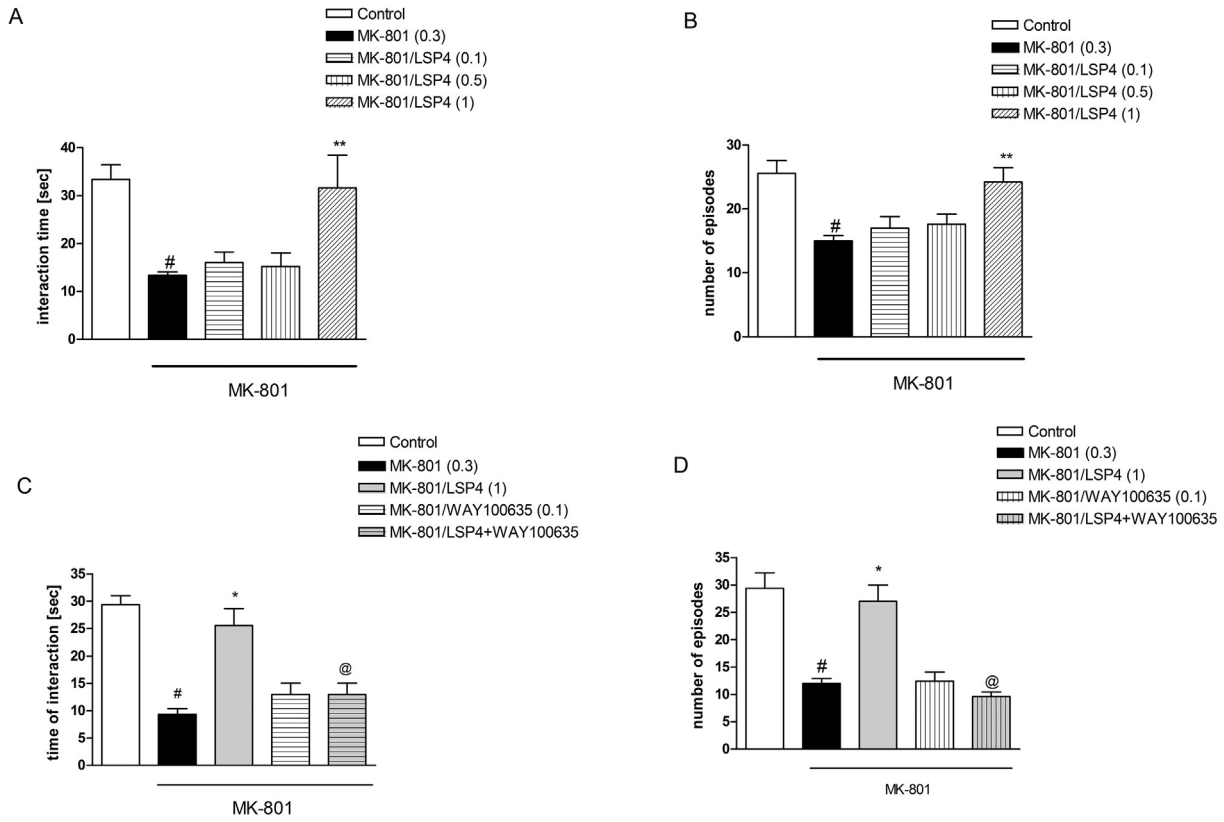

Fig. 3. Effects of LSP4-2022 (LSP4) alone and in combination with 5-HT_{1A} antagonist WAY100635 on MK-801-induced deficits in social interactions. The time of social interactions and number of episodes of social contacts were measured. (A, B) effects of LSP4-2022 and (C, D) effects of the combined administration of LSP4-2022 and WAY100635 in MK-801-treated mice. Data are presented as the means ± SEM. Doses (mg/kg) are indicated in parentheses. $^{\#}P < 0.0001$ versus the controls, $^{**}P < 0.001$ or $^{*}P < 0.01$ versus the MK-801−treated group, and $^{@}P < 0.01$ versus the LSP4-2022-treated group.

respectively, $P < 0.01$. The other investigated doses, 0.1 and 0.5 mg/kg were ineffective (Fig. 3A,B). Subsequently, the active dose of LSP4-2022 was given together with WAY100635 administered at a dose of 0.1 mg/kg (which itself had no effect). Co-administration of LSP4-2022 with WAY100635 resulted in the inhibition of the LSP4-induced effects. Two-way ANOVA analysis of the number of episodes revealed a significant effect of the LSP4-2022×WAY100635 interaction [$F_{(1.34)} = 56.02$, $P < 0.001$]. Post-hoc Newman-Keuls analysis revealed a significant LSP4-2022×WAY100635 interaction, $P < 0.0001$, compared with effects in the LSP4-2022 treated group. Two-way ANOVA of the time of interaction also revealed a significant effect of the LSP4-2022×WAY100635 interaction [$F_{(1.34)} = 48.6$; $P < 0.0001$], and the post-hoc Newman-Keuls analysis revealed a significant effect of the LSP4-2022×WAY100635 interaction, $P < 0.0001$, compared with the effects in the LSP4-2022- treated rats (Fig. 3C, D).

The two-way ANOVA revealed no changes between the particular groups not injected with MK-801 (Table 1).

3.3.2. The effect of the combined administration of (R)-(+)-8-hydroxy-DPAT hydrobromide and LSP4-2022 in the social interaction test in mice

Three doses of LSP4-2022 (0.1, 0.5 and 1 mg/kg) were administered together with (*R*)-(+)-8-hydroxy-DPAT hydrobromide at a dose of 0.01 mg/kg (15 min before the test, s.c), that itself had no effect. Simultaneous administration of sub-effective dose of the 5-HT_{1A} receptor agonist with three doses of $mGlu_4$ receptor agonist induced clear antipsychotic-like effects, as measured in two parameters. The effects of all combinations were compared to the MK-801. Two-way ANOVA revealed that (*R*)-(+)-8-hydroxy-DPAT

Table 1
The control experiments for the groups not treated with MK-801 (for social interaction and novel object recognition studies).

	Social interaction (time of interaction)	Novel object recognition
Control	31 ± 1.78 *n.s*	0.48 ± 0.05 *n.s*
LSP4-2022 (1) or (2)	29 ± 2.73 *n.s*	0.50 ± 0.04 *n.s*
WAY100635 (0.1)	28 ± 2.08 *n.s*	0.52 ± 0.07 *n.s*
LSP4-2022 (1)+WAY100635 (0.1)	33 ± 1.12 *n.s*	0.46 ± 0.06 *n.s*
8-OH-DPAT (0.1)	26 ± 3.06 *n.s*	0.45 ± 0.05 *n.s*
LSP4-2022 (0.1)+8-OH-DPAT (0.01)	35 ± 2.11 *n.s*	0.53 ± 0.03 *n.s*
LSP4-2022 (0.5)+8-OH-DPAT (0.01)	34 ± 1.02 *n.s*	0.55 ± 0.04 *n.s*
LSP4-2022 (1)+8-OH-DPAT (0.01)	28 ± 1.45 *n.s*	0.46 ± 0.01 *n.s*
8-OH-DPAT (0.025)	28 ± 3.7 *n.s*	0.41 ± 0.02 *n.s*
LSP4-2022 (1)+8-OH-DPAT (0.025)	30.8 ± 3.5 *n.s*	0.39 ± 0.06 *n.s*

hydrobromide significantly intensified the effect of the lowest dose of LSP4-2022 (0.1 mg/kg) both in the time of interaction [$F_{(1.30)} = 10.99$; $P < 0.002$] and in the number of episodes [$F_{(1.30)} = 12.5$ $P < 0.001$]. The compound slightly intensified the effect of LSP4-2022 when administered at a dose of 0.5 mg/kg, but this effect did not reach the statistical significance, at least with two-way ANOVA analysis, and it had no influence on the action of LSP4-2022 at the highest dose, 1 mg/kg (Fig. 4 A, B).

The active dose of LSP4-2022 (1 mg/kg) was co-administered with the active dose of (*R*)-(+)-8-hydroxy-DPAT hydrobromide (0.025 mg/kg). Statistical analysis revealed the significant effect of both compounds in the time of interaction [$F_{(1.29)} = 4.5$ and $F_{(1.29)} = 21.4$, $P < 0.0001$], and in the number of episodes [$F_{(1.29)} = 5.3$ and $F_{(1.29)} = 26.3$, $P < 0.0001$]. No any intensification of the co-administration of the compounds was observed ($P < 0.1$) (Fig. 4C, D). The control experiments with all the groups investigated above, but not injected with MK-801 revealed that neither of the combinations had any effect on the animals' behavior (Table 1).

3.4. Novel object recognition

3.4.1. The effect of the LSP4-2022 alone and in combined administration with WAY100635 in the novel object recognition test in mice

MK-801 induced a profound decrease in the NOR test (Fig. 5, A–D) in the time of interaction, and the number of episodes. LSP4-2022 which was administered at a doses of 1, 2 and 4 mg/kg 45 min before the test, increased the recognition index that was disturbed by MK-801 administration ($P < 0.005$) at the dose of 2 mg/kg [$F_{(3.24)} = 6.27$, $P < 0.005$] displaying an inverted U-shaped profile, while the other investigated doses were ineffective (Fig. 5A). WAY100635 given at a dose of 0.1 mg/kg 45 min before the test did not have any effect on the action of MK-801, while when dosed with LSP4-2022 (2 mg/kg), antagonized the LSP4-2022-induced effect in the NOR test (Fig. 5B). Two-way ANOVA and post-hoc Newman-Keuls comparison revealed a significant decrease in the recognition index ($F_{(1.36)} = 9.6$; $P < 0.003$) compared to effects in the LSP4-2022-treated animals (Fig. 5B). The analysis of the control experiment with the groups treated with LSP4-2022, WAY100635 or LSP4-2022 + WAY100635 revealed that the combination had no influence on the recognition index (Table 1).

3.4.2. The effect of the combined administration of (R)-(+)-8-hydroxy-DPAT hydrobromide (0.01 mg/kg) and three doses of LSP4-2022 in the novel object recognition test in mice

LSP4-2022 was given at three doses (1, 2, 4 mg/kg) 45 min before the test, and (*R*)-(+)-8-hydroxy-DPAT hydrobromide was given at a dose of 0.01 mg/kg 15 min before the test. Neither drug had an effect when administered alone (Table 1). When LSP4-2022 was given to MK-801 treated mice together with low ineffective dose of 8-hydroxy-DPAT the two-way ANOVA revealed that (*R*)-(+)-8-hydroxy-DPAT hydrobromide significantly intensified the

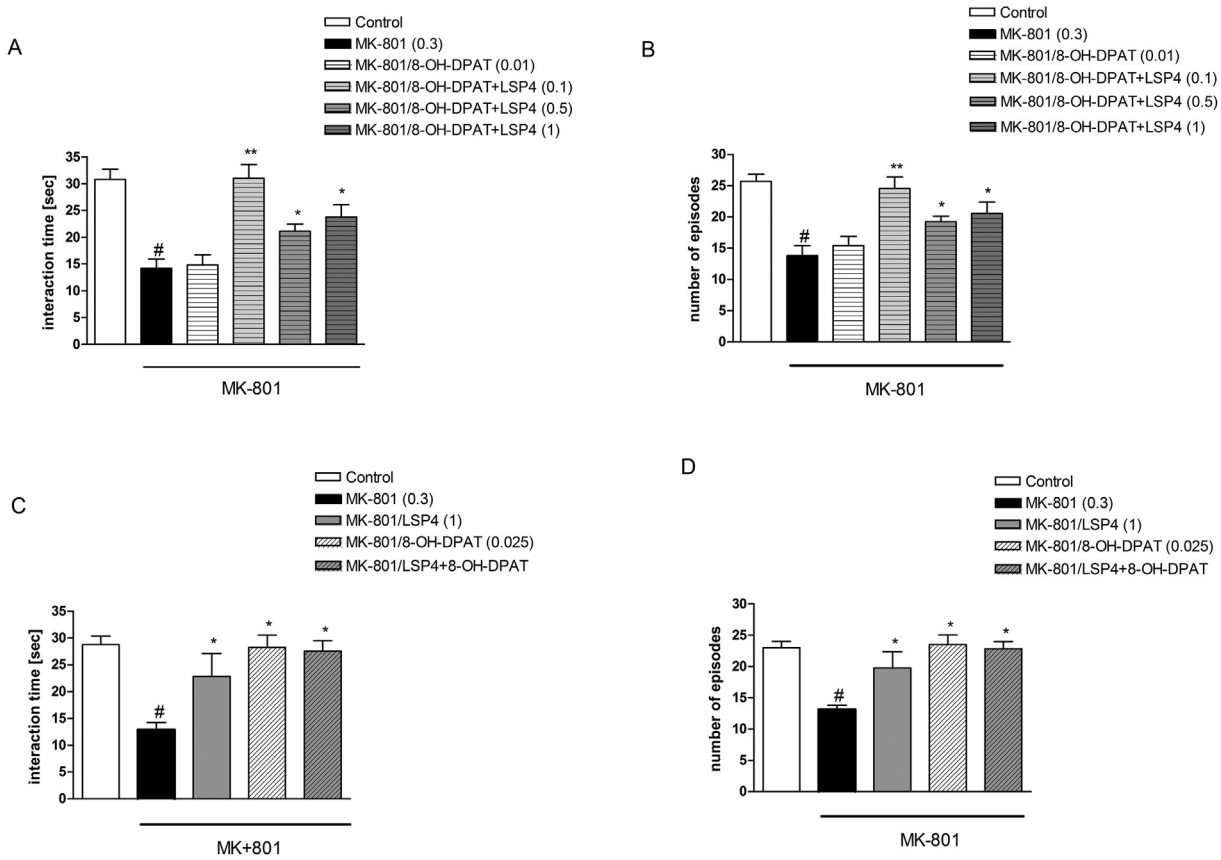

Fig. 4. Effects of LSP4-2022 (LSP4) and 5-HT_{1A} agonist (R)-(S)-8-OH-DPAT (8-OH-DPAT) on MK-801-induced deficits in social interaction. Number of episodes of social contact and the time of social interactions were measured. (A, B) effects of the combined administration of three doses of LSP4-2022 and low dose of (R)-(S)-8-OH-DPAT (0.01 mg/kg) in MK-801-treated mice and (C, D) effects of the combined administration of the effective dose of LSP4-2022 (1 mg/kg) and effective dose of (R)-(S)-8-OH-DPAT (0.025 mg/kg) in MK-801-treated mice. Data are presented as the means ± SEM. Doses (mg/kg) are indicated in parentheses. #P < 0.0001 versus the controls, and * or ** when at least P < 0.01 versus the MK-801–treated group.

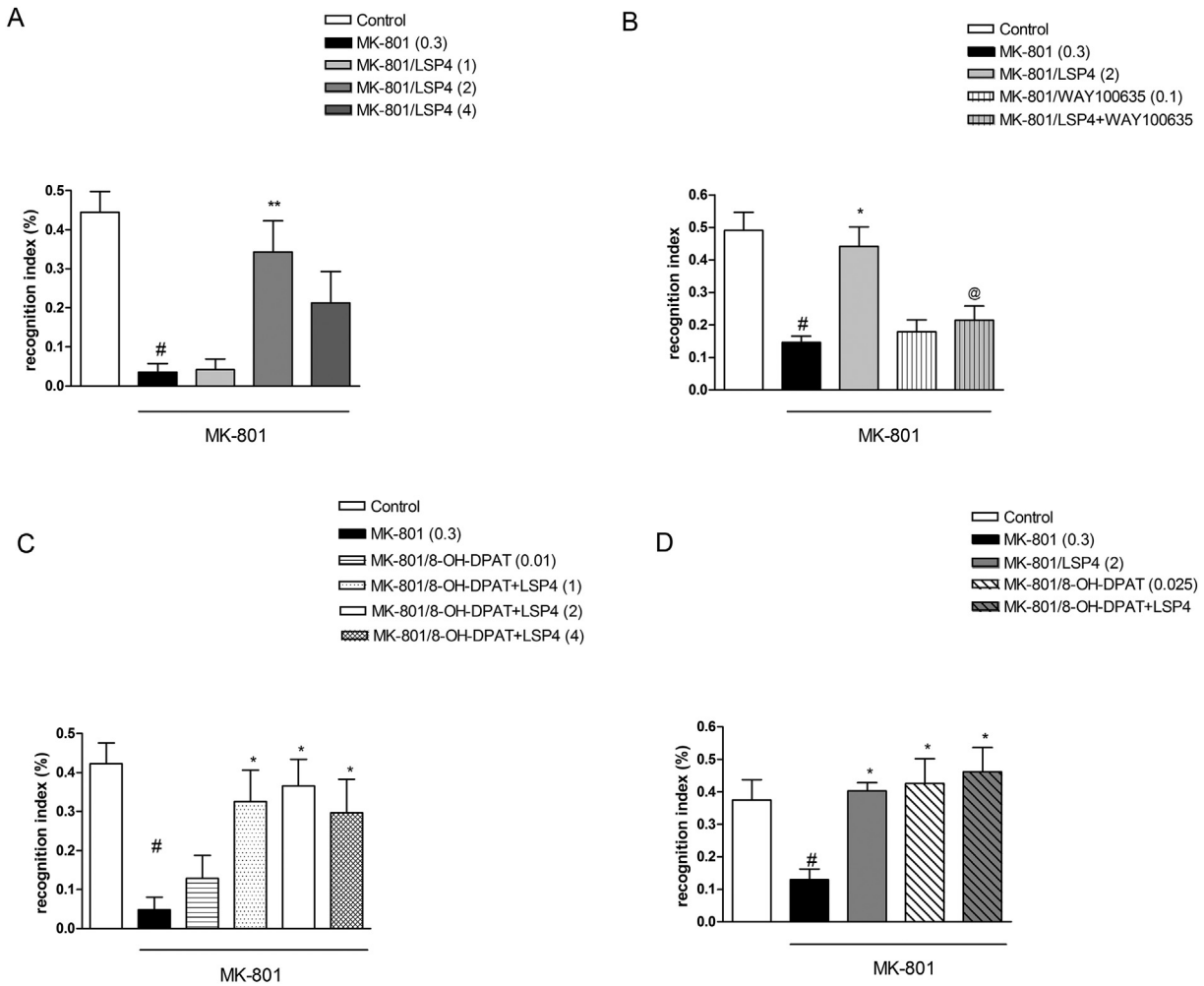

Fig. 5. Effects of LSP4-2022 (LSP4) alone and in combination with 5-HT_{1A} antagonist WAY100635 or 5-HT_{1A} agonist (R)-(S)-8-OH-DPAT on MK-801-induced deficits in NOR. LSP4-2022 was given in three doses (A) and the combined administration of effective dose of LSP4-2022 (2 mg/kg) with WAY100635 (B), are presented. (C) effects of the combined administration of three doses of LSP4-2022 and low dose of (R)-(S)-8-OH-DPAT (0.01 mg/kg) in MK-801-treated mice and (D) effects of the combined administration of the effective dose of LSP4-2022 (2 mg/kg) and effective dose of (R)-(S)-8-OH-DPAT (0.025 mg/kg) in MK-801-treated mice. Data are presented as the means ± SEM. Doses (mg/kg) are indicated in parentheses. #P < 0.01 versus the controls, *P < 0.05 or **P < 0.01 versus the MK-801–treated group, and @P < 0.001 versus the LSP4-2022-treated group.

effect of all three doses of LSP4-2022 [$F_{(1.27)} = 4.35$; $P < 0.04$] (Fig. 5 C).

However as the dose of 1 mg/kg of LSP4 was not effective in reversing the action of MK-801 (Fig 5A) and the dose of 2 mg/kg was active, we can talk about synergistic interaction between the dose of 0.01 mg/kg of 8-hydroxy-DPAT and the dose of 1 mg/kg of LSP4 (Fig. 5C).

The control experiments with all the groups investigated above, but not injected with MK-801 revealed that neither of the combinations had any effect on the animals' behavior (Table 1).

3.4.3. *The effect of the combined administration of (R)-(+)-8-hydroxy-DPAT hydrobromide (0.025 mg/kg) and active dose of LSP4-2022 (1 mg/kg) in the social interaction test in mice*

The active dose of LSP4-2022 (2 mg/kg) in reversing the effect of MK-801 (Fig 5A) was co-administered with the active dose of (*R*)-(+)-8-hydroxy-DPAT hydrobromide (0.025 mg/kg) (Fig. 5D). The statistical analysis revealed the significant effect of both compounds and the interaction [$F_{(1.28)} = 14.5$, $F_{(1.28)} = 10.67$ and $F_{(1.28)} = 6.27$, respectively, $P < 0.01$], however no intensification when the compounds were co-administered was observed ($P = 0.5$) (Fig. 5 D). The control experiments of animals not treated with MK-801 are presented in Table 1.

3.5. *In vivo microdialysis*

3.5.1. *The release of dopamine in the rat frontal cortex*

The extracellular DA level in the rat frontal cortex was significantly increased after administration of LSP4-2022 (2 mg/kg) and WAY100635 (0.2 mg/kg) to ca. 350% of baseline starting from 20 min until 240 min of fractions collection (Fig. 6A).

MK-801 at a dose of 0.6 mg/kg significantly increased cortical DA levels, reaching a maximal effect between 80 and 120 min after administration (Fig. 7). WAY100635 enhanced and LSP4-2022 attenuated this MK-801-induced increase in DA release (Fig. 7). The co-administration of WAY100635 and LSP4-2022 counteracted each other's actions on the DA release induced by MK-801. The results of the repeated measures ANOVA and Tukey's *post hoc* comparisons are presented in Table 2 A, B.

3.5.2. *The release of serotonin in the rat frontal cortex*

Both LSP4-2022 (2 mg/kg) and WAY100635 (0.2 mg/kg) given

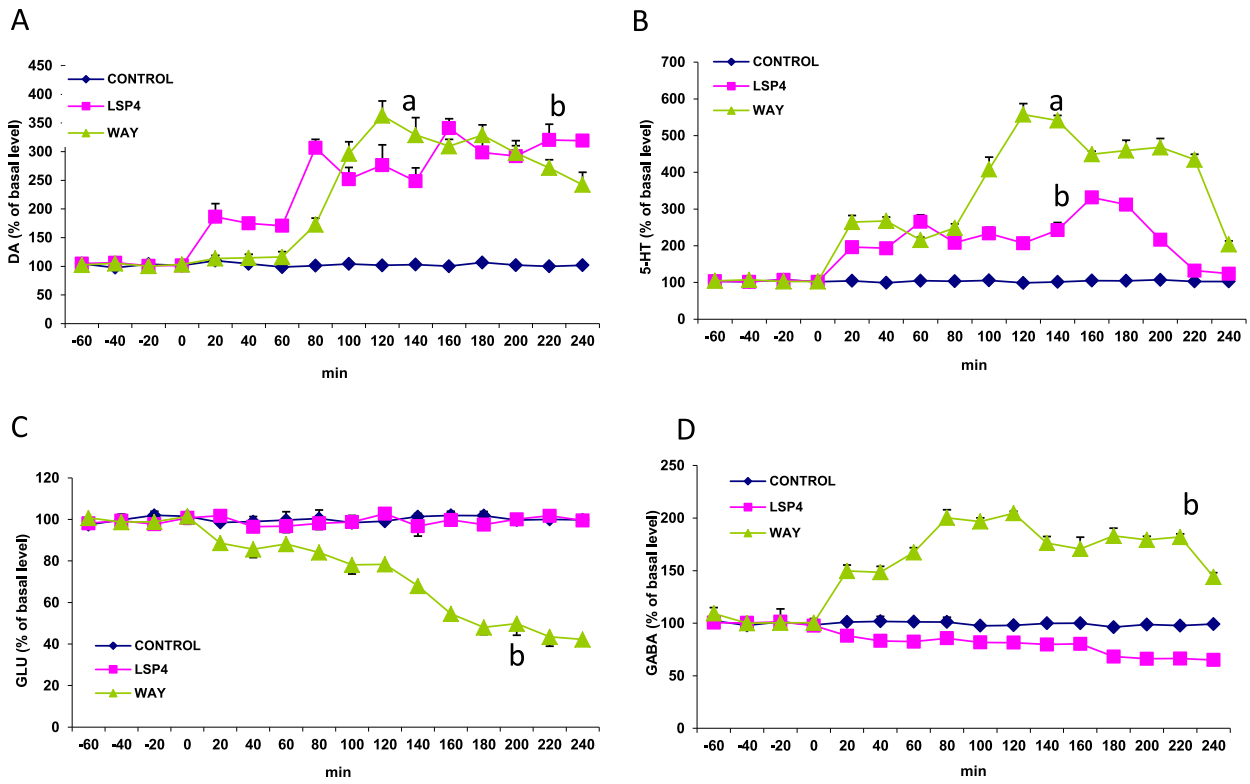

Fig. 6. Extracellular concentration of dopamine (DA) (A), serotonin (5-HT) (B), glutamate (Glu) (C) and GABA (D) in the rat frontal cortex of the rat brain after administration of LSP4-2022 (LSP4) and WAY100635 (WAY). The detailed statistical analysis can be found in Table 2.

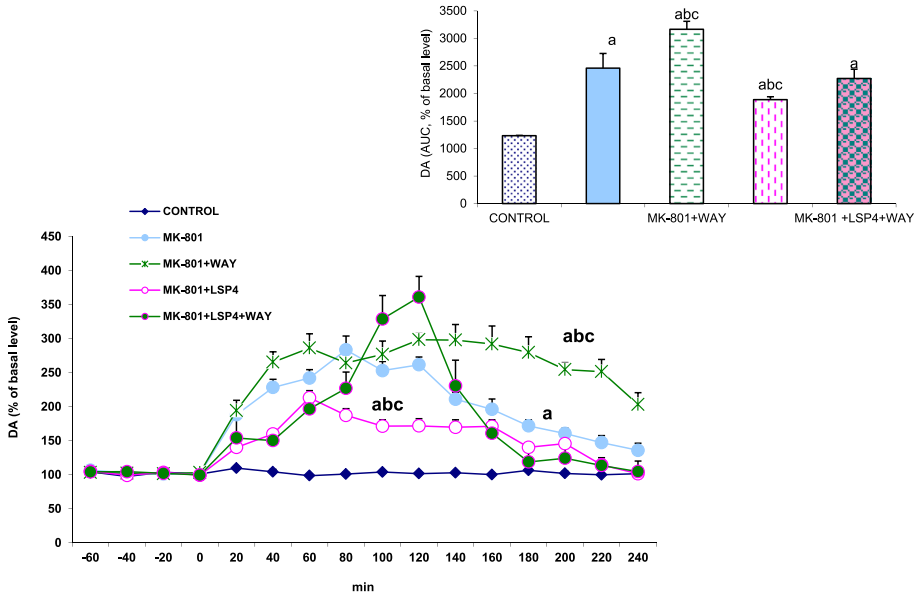

Fig. 7. Effect of LSP4-2022 (LSP4) and WAY100635 (WAY) on MK-801-induced enhancement of DA release. The figure shows the time-course of the changes in DA level between 20 and 240 min of the sample collection period. Inset shows the total effect expressed as the area under the curve (AUC) of the all data points represented in the curves. Values are the mean ± SEM, $n = 12–13$ rats. The basal extracellular level of DA in dialysates from rat frontal cortices was 1.53 ± 0.16 pg/10 μl of the fraction, and no differences between experimental groups were found. Details of the statistical analysis are presented in Table 2.

alone significantly increased extracellular level of 5-HT to ca. 300 and 600% of baseline, respectively (Fig. 6B).

MK-801 induced an increase in the release of serotonin (Fig. 8). The increase in 5-HT induced by MK-801 (0.6 mg/kg) was significantly decreased by LSP4-2022 and WAY100635. The combined treatment with LSP4-2022 and WAY100635 had no effect on the MK-801-induced effect on 5-HT release (Fig. 8). The results of the repeated measures ANOVA and Tukey's *post hoc* comparisons are presented in Table 2.

Table 2
Repeated measures ANOVA followed by TUK post hoc analysis for *in vivo* microdialysis studies (treatment, time and treatment × time interaction).

Part A			
DOPAMINE	Repeated measures ANOVA		
	Treatment	F(2.273) = 290	*P < 0.0001**
	Time	F(12.273) = 28.6	*P < 0.0001**
	Treatment × time	F(24.273) = 16.9	*P < 0.0001**
	TUK post-hoc analysis		
	Control vs LSP4-2022	*P* < 0.001*	
	Control vs WAY100635	*P* < 0.001*	
SEROTONINE	Repeated measures ANOVA		
	Treatment	F(2.273) = 73.19	*P < 0.0001**
	Time	F(12.273) = 12.8	*P < 0.0001**
	Treatment × time	F(24.273) = 42.29	*P < 0.0001**
	TUK post-hoc analysis		
	Control vs LSP4-2022	*P* < 0.001*	
	Control vs WAY100635	*P* < 0.001*	
GLUTAMATE	Repeated measures ANOVA		
	Treatment	F(2.273) = 440	*P < 0.0001**
	Time	F(12.273) = 14.82	*P < 0.0001**
	Treatment × time	F(24.273) = 16.01	*P < 0.0001**
	TUK post-hoc analysis		
	Control vs LSP4-2022	*P < 0.5*	ns
	Control vs WAY100635	*P* < 0.05*	
GABA	Repeated measures ANOVA		
	treatment	F(2.273) = 1831	*P < 0.0001**
	Time	F(12.273) = 14.61	*P < 0.0001**
	Treatment × time	F(24.273) = 21.19	*P < 0.0001**
	TUK post-hoc analysis		
	Control vs LSP4-2022	*P < 0.5*	ns
	Control vs WAY100635	*P* < 0.01*	
Part B			
DOPAMINE	Repeated measures ANOVA		
	Treatment	F(6.27) = 45.4	*P = 0***
	Time	F(11.297) = 42.8	*P = 0***
	Treatment × time	F(66.297) = 16.7	*P = 0***
	TUK post-hoc analysis		
	MK-801 vs	MK-801/LSP4-2022	*P < 0.0018***
		MK-801/WAY100635	*P < 0.0004***
		MK-801/LSP4-2022/WAY100635	P < 0.68
	MK-801/LSP4-2022/WAY100635	MK-801/LSP4-2022	*P < 0.00015 ***
		MK-801/WAY100635	*P < 0.0017***
SEROTONIN	Repeated measures ANOVA		
	Treatment	F(6.28) = 123	*P = 0***
	Time	F(11.308) = 82.4	*P = 0***
	Treatment × time	F(66.308) = 18.7	*P = 0***
	TUK post-hoc analysis		
	MK-801 vs	MK-801/LSP4-2022	*P < 0.0001***
		MK-801/WAY100635	P < 0.062
		MK-801/LSP4-2022/WAY100635	*P < 0.0008**
	MK-801/LSP4-2022/WAY100635	MK-801/LSP4-2022	*P < 0.0004***
		MK-801/WAY100635	P < 0.65
GLUTAMATE	Repeated measures ANOVA		
	Treatment	F(6.26) = 554	*P = 0***
	Time	F(11.286) = 39.15	*P = 0***
	Treatment × time	F(66.286) = 14.21	*P = 0***
	TUK post-hoc analysis		
	MK-801 vs	MK-801/LSP4-2022	*P < 0.0001***
		MK-801/WAY100635	*P < 0.0001***
		MK-801/LSP4-2022/WAY100635	*P < 0.0001***
	MK-801/LSP4-2022/WAY100635	MK-801/LSP4-2022	*P < 0.0001***
		MK-801/WAY100635	*P < 0.001***
GABA	Repeated measures ANOVA		
	Treatment	F(6.27) = 313	*P = 0***
	Time	F(11.297) = 112	*P = 0***
	Treatment × time	F(66.297) = 37.3	*P = 0***
	TUK post-hoc analysis		
	MK-801 vs	MK-801/LSP4-2022	*P < 0.00014***
		MK-801/WAY100635	*P < 0.00014***
		MK-801/LSP4-2022/WAY100635	*P < 0.00014***
	MK-801/LSP4-2022/WAY100635	MK-801/LSP4-2022	*P < 0.0024***
		MK-801/WAY100635	P < 0.1142

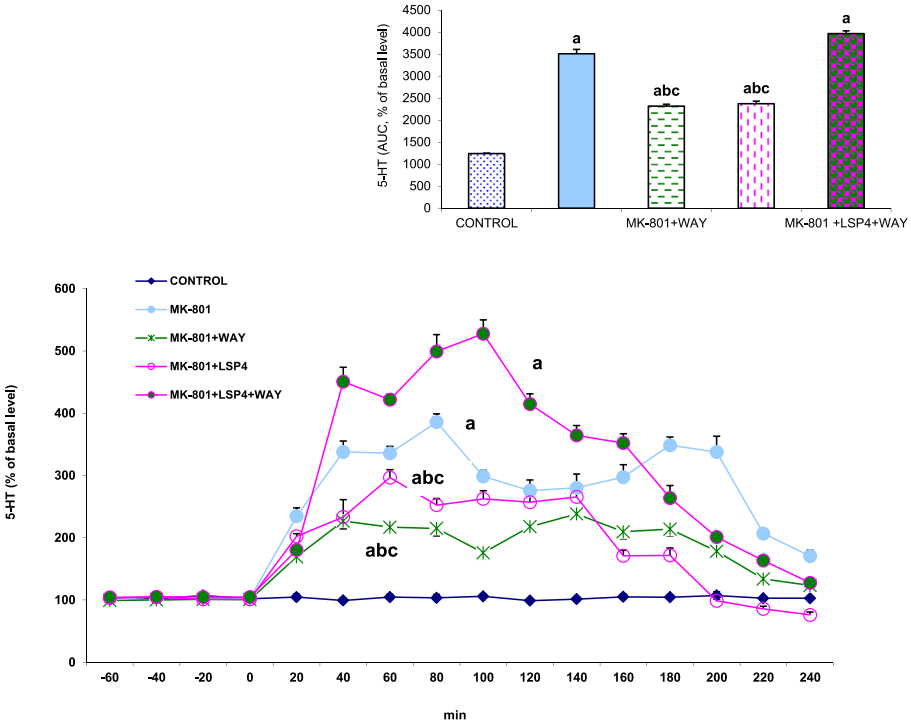

Fig. 8. Effect of LSP4-2022 (LSP4) and WAY100635 (WAY) on MK-801-induced enhancement of 5-HT release. The figure shows the time-course of the changes in 5-HT level between 20 and 240 min of the sample collection period. Inset shows the total effect expressed as the area under the curve (AUC) of the all data points represented in the curves. Values are the mean ± SEM, $n = 12–13$ rats. The basal extracellular level of 5-HT in dialysates from rat frontal cortices was 0.36 ± 0.07 pg/10 μl of the fraction, and no differences between experimental groups were found. Details of the statistical analysis are presented in Table 2.

3.5.3. *The release of glutamate*

The extracellular glutamate level was not changed by LSP4-2022 (2 mg/kg), but WAY100635 (0.2 mg/kg) decreased glutamate release to ca. 40% of the basal level (Fig. 6C).

The MK-801 (0.6 mg/kg)-induced increase in the extracellular levels of glutamate was markedly reversed by WAY100635 and LSP4-2022 (Fig. 9). The combination of both drugs also effectively decreased the effect of MK-801 but to a lesser degree than that observed when the drugs were administered with MK-801 separately (Fig. 9). The results of the repeated measures ANOVA and Tukey's *post hoc* comparisons are presented in Table 2.

3.5.4. *The release of GABA in the rat frontal cortex*

LSP4-2022 (2 mg/kg) decreased GABA extracellular level to ca. 65% of baseline while WAY100635 (0.2 mg/kg) increased it to maximum 200% of the basal level between 80 and 120 min after administration (Fig. 6D).

The rise in GABA induced by MK-801 (0.6 mg/kg) was followed by a decline in GABA levels from 160 min until the end of the fraction collection period (Fig. 10). The enhancement in GABA release induced by MK-801 was inhibited by LSP4-2022 and WAY100635. The effect of co-administration of LSP4-2022 and WAY100635 along with MK-801 was minimal and was significantly weaker than the effect in the group treated with MK-801 and LSP4-2022, and it did not differ from the effect observed in the group treated with WAY100635 and MK-801 (Fig. 10). The results of the repeated measures ANOVA and Tukey's *post hoc* comparisons are presented in Table 2.

3.6. *Electrophysiological studies*

To investigate the effects of LSP4-2022 on spontaneous excitatory postsynaptic currents (sEPSCs), voltage-clamp recordings were made from layerV cortical cells in the presence of DOI (10 μM). All recorded cells ($n = 37$) showed electrophysiological characteristics of regular-spiking pyramidal neurons (tested in a current clamp; McCormick et al., 1985). Their mean resting membrane potential (RMP) was −73 ± 5 mV (±SEM), and the mean input resistance (R_{in}) was 268 ± 28 MΩ (±SEM). The mean basal frequency of spontaneous synaptic activity was 2.7663 ± 0.3 Hz (±SEM), and its mean amplitude was 9.4259 ± 0.72 pA (±SEM). Spontaneous postsynaptic currents were blocked by the non-NMDA glutamatergic receptor antagonist CNQX (5 μM; $n = 4$, data not shown), indicating that they represented excitatory currents.

The application of DOI (10 μM) increased the mean sEPSCs frequency to 146 ± 5% (±SEM) of baseline (Fig. 11 A,B) but did not affect the mean amplitude of the sEPSCs (Fig. 11 A,B).

LSP4-2022, when applied concurrently with DOI, reversibly suppressed the DOI-induced increase in the frequency. This effect was concentration-dependent from 2.5 to 10 μM. LSP4-2022 also slightly decreased the mean amplitude of the sEPSCs (2.5 and 10 μM) (Fig. 11 B).

The 5-HT_{1A} receptor antagonist WAY100635 did not antagonize the suppressing effect of LSP4-2022 on the DOI-induced increase in sEPSCs frequency. WAY100635 also did not affect the mean amplitude of the sEPSCs (Fig. 11 C). The results of the statistical analysis are presented in Table 3.

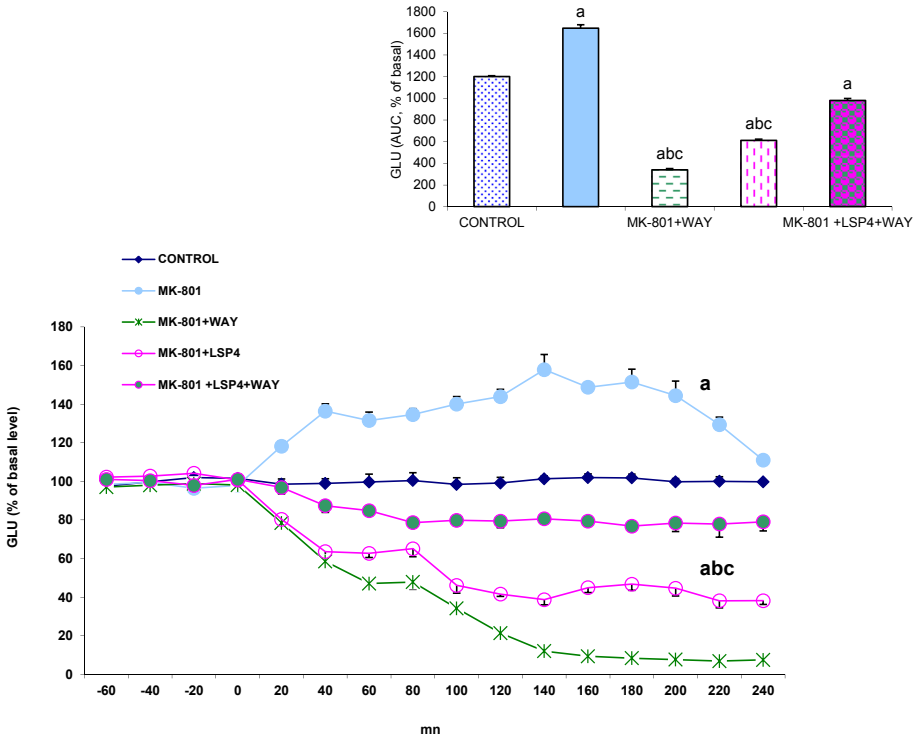

Fig. 9. Effect of LSP4-2022 (LSP4) and WAY100635 (WAY) on MK-801-induced enhancement of GLU release. The figure shows the time-course of the changes in GLU level between 20 and 240 min of the sample collection period. Inset shows the total effect expressed as the area under the curve (AUC) of the all data points represented in the curves. Values are the mean ± SEM, $n = 12–13$ rats. The basal extracellular level of GLU in dialysates from rat frontal cortices was 5.93 ± 0.59 ng/10 μl of the fraction, and no differences between experimental groups were found. Details of the statistical analysis are presented in Table 2.

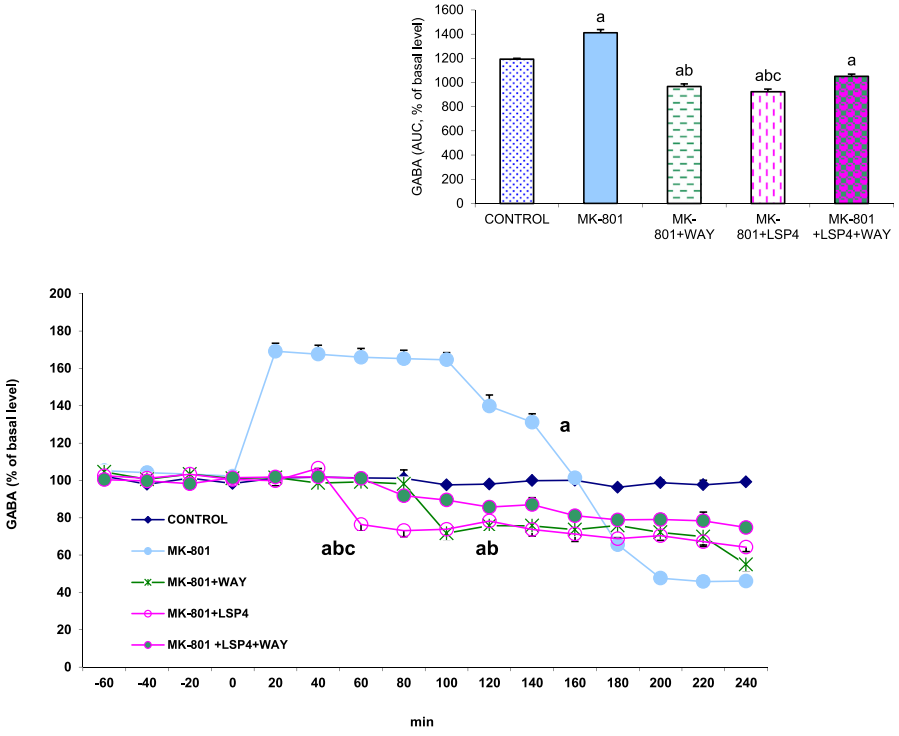

Fig. 10. Effect of LSP4-2022 (LSP4) and WAY100635 (WAY) on MK-801-induced enhancement of GABA release. The figure shows the time-course of changes in GABA level between 20 and 240 min of the sample collection period. Inset shows the total effect expressed as the area under the curve (AUC) of the all data points represented in the curves. Values are the mean ± SEM, $n = 12–13$ rats. The basal extracellular level of GABA in dialysates from rat frontal cortices was 0.32 ± 0.03 ng/10 μl of the fraction, and no differences between experimental groups were found. Details of the statistical analysis are presented in Table 2.

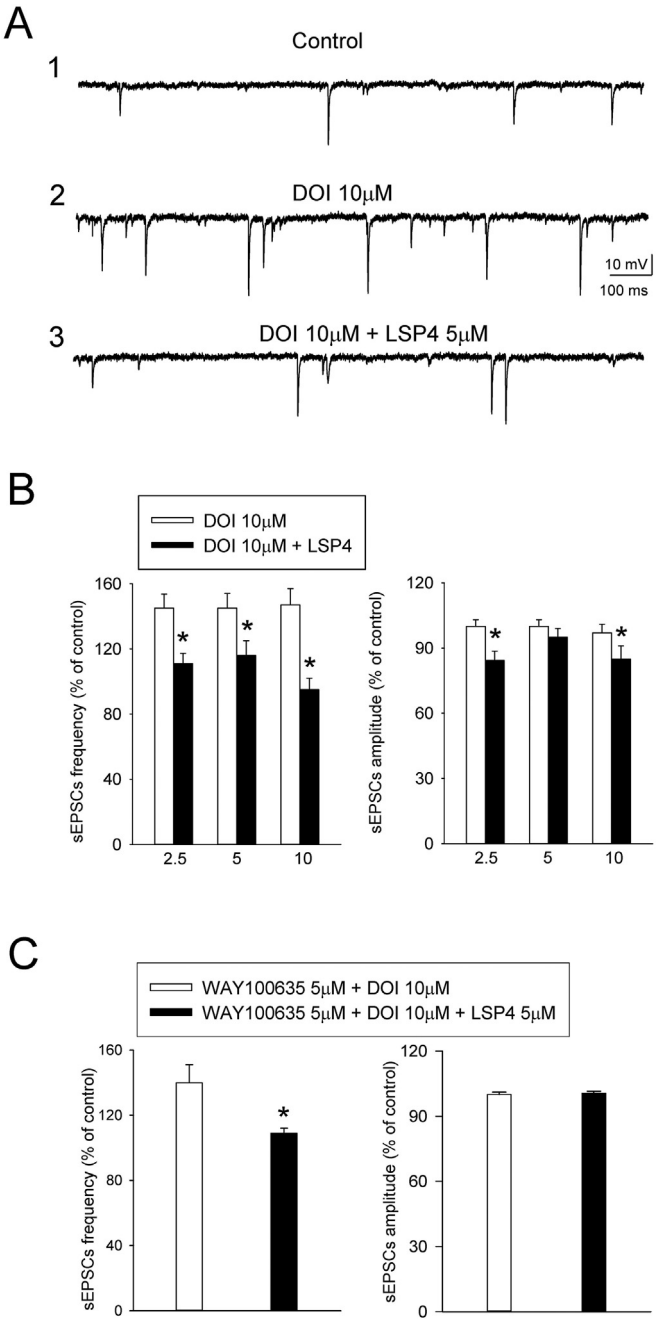

Fig. 11. Suppression of the excitatory effect of (±)1-(2.5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (10 μM) on the frequency and amplitude of spontaneous EPSCs (sEPSCs) by LSP4-2022. (A) Examples of recordings from representative neurons. (1) Baseline activity, (2) a recording after a 10-min incubation with DOI, and (3) a recording after a 10-min incubation with LSP4-2022 (5 μM) in the presence of DOI. (B) Dose-dependent suppression of the effect of 10 μM DOI on the mean frequency and amplitude (±SEM) of spontaneous EPSCs by LSP4-2022 and (C) recordings after simultaneous administration of LSP4-2022 + WAY100635 in the continuous presence of DOI. *$P < 0.05$ vs the DOI effect in the paired *t*-test. For detailed statistical results, please see Table 3.

4. Discussion

The present research confirmed and extended our earlier findings showing that LSP4-2022, the first selective orthosteric agonist of $mGlu_4$ receptors (Goudet et al., 2012), exerted antipsychotic-like activity in animals (Wozniak et al., 2016). In this study, we confirmed its antipsychotic efficacy. Moreover, we demonstrate here that chronic (8 days) LSP4-2022 administration can also produce antipsychotic-like effects, as demonstrated by the inhibition of DOI-induced head twitches, indicating not only the lack of a development of tolerance to the effect of the drug but also its ability to induce antipsychotic effects after doses as low as 0.1 mg/kg, which are not effective in acute dosing.

It has previously been shown that the mechanism of action of LSP4-2022 involves $GABA_B$ signaling, at least in the context of positive symptoms of schizophrenia (Wozniak et al., 2015). In the present paper we focused on the involvement of 5-HT_{1A} receptors in the mechanism of action of the compound. The dose-dependency studies of LSP4-2022 actions in the social interactions and novel object recognition tests were repeated in mice, as in our previous studies those experiments were performed only on rats. Subsequently, the 5-HT_{1A} antagonist WAY100635 was administered together with LSP4-2022, and the compound reversed LSP4-2022-induced effects on MK-801-induced hyperactivity, DOI-induced head twitches, MK-801-induced disruptions of social interactions and novel object recognition. At the same time the intensification of the LSP4-2022-induced actions via the simultaneous administration of sub-effective doses of the 5-HT_{1A} agonist (R)-(S)-8-OH-DPAT along with several doses of LSP4-2022 was also observed. The simultaneous administration of sub-effective doses of (R)-(S)-OH-DPAT and LSP4-2022 intensified each other's action and induced clear antipsychotic effect in all tests. However, as observed in the social interaction and novel object recognition tests, the effects of effective doses of LSP4 were not intensified with the administration of sub-effective dose of (R)-(S)-OH-DPAT. Similarly, no intensification of effective doses of LSP4-2022 was observed when the compound was administered together with the effective dose of (R)-(S)-OH-DPAT. Therefore the co-administration of the low doses of both compounds seems to have the greatest impact in the behavioral tests, and the decreasing of dosing may be less burdened with adverse effects development.

To study the neurochemical and physiological mechanisms underlying the 5-HT_{1A}-dependent antipsychotic action of LSP4-2022, we performed *in vivo* microdialysis and patch-clamp recordings. In the series of *in vivo* microdialysis experiments, the effects of the intraperitoneal administration of LSP4-2022 and WAY100635 alone and on the MK-801-induced release of such neurotransmitters such as dopamine, serotonin, glutamate and GABA were investigated. LSP4-2022 and WAY100635 act upon the receptors ($mGlu_4$ and 5-HT_{1A}, respectively) that are crucial in the regulation of the release of neurotransmitters (Cartmell and Schoepp, 2000; Mercier and Lodge, 2014; Schoepp, 2001; Sharp and Hjorth, 1990; Kreiss and Lucki, 1994). In our studies we observed that both compounds had their own effects on the release of investigated neurotransmitters. The drug of the interest, LSP4-2022, increased both dopamine and serotonin efflux and had no effect on the release of glutamate or GABA.

The administration of MK-801, which builds a neurochemical model of schizophrenia, produced a marked increase in the release of dopamine, serotonin and glutamate in the prefrontal cortex, as has been demonstrated in several other studies (Yonezawa et al., 1998; Castane et al., 2008; Etou et al., 1998; Wieronska et al., 2015; Zuo et al., 2006; Lopez-Gil et al., 2007, 2009). An elevated level of GABA release was also observed in our study, but it is difficult to compare this result with other reports as the available data concerning the influence of peripheral MK-801 administration on GABA release is rather scarce. For example, it has been shown that a local infusion of NMDA antagonists (MK-801 or PCP) to frontal cortex decreased GABA release in this structure (Yonezawa et al., 1998).

We assume that the neurotransmitter efflux observed after MK-

Table 3
Paired-t test analysis of the results of the patch-clamp recordings.

sEPSCs frequency	
DOI 10 μM + LSP4-2022 2.5 μM	n = 9, t = 5.57, df = 8, P < 0.001**
DOI 10 μM + LSP4-2022 5.0 μM	n = 11, t = 2.81, df = 10, P = 0.018**
DOI 10 μM + LSP4-2022 10.0 μM	n = 9, t = 5.96, df = 8, P = 0.00017**
DOI 10 μM + WAY100635 5 μM + LSP4-2022 5 μM	n = 5, t = 3.83, df = 4, P = 0.019**
sEPSCs amplitude	
DOI 10 μM + LSP4-2022 2.5 μM	n = 9, t = 3.23, df = 8, P = 0.012**
DOI 10 μM + LSP4-2022 5.0 μM	n = 11, t = −2.1, df = 10, P = 0.063
DOI 10 μM + LSP4-2022 10.0 μM	n = 9, t = −2.7, df = 8, P = 0.0135**
DOI 10 μM + WAY100635 5 μM + LSP4-2022 5 μM	n = 9,5, t = 0.99, df = 4, P = 0.375

801 administration is a result of a cascade of events within neuronal loops as described in the glutamatergic theory of schizophrenia suggested by several independent groups (Conn et al., 2009; Krystal et al., 2002; Javitt and Zukin, 1991; Javitt et al., 2004; Moghaddam and Jackson, 2003). The main assumption of this theory states that after the administration of NMDA receptor antagonists, the hypofunctional NMDA receptors on GABAergic interneurons attenuate the activity of subcortical GABAergic interneurons, which subsequently innervate pyramidal thalamo-cortical neurons. The hyperactivity of these glutamatergic neurons leads to an increased glutamate release (confirmed by the present results), which is suggested as being responsible for the symptomatology of schizophrenia (Conn et al., 2009). Based on this theory, the loss of inhibitory control over several CNS neuronal pathways and/or their overstimulation resulting from excessive glutamate release may be responsible for the extended GABA, 5-HT and DA release observed is the present study (also see Wieronska et al., 2015) (Fig. 12).

The administration of LSP4-2022 reversed the MK-801-induced increases in dopamine, serotonin, glutamate and GABA releases, which is consistent with the notion that LSP4-2022 activates the presynaptic autoreceptors and heteroreceptors that are inhibitory in nature (Cartmell and Schoepp, 2000; Schigemoto et al., 1997). The result is especially interesting and important in the context of observed increased in dopamine or serotonin efflux induced after administration of LSP4-2022 alone and supports the hypothesis, that the action of the drug may restore the disrupted balance within the CNS. Therefore, hypothetically thinking the drug may be used in the population of so-called normodopaminergic schizophrenic patients, and in hyperdopaminergic patients with concomitant increase in glutamate release (Howes and Kapur, 2014). The ability to decrease the dose of LSP4-2022 and to supplement its action with a 5-HT_{1A} agonist may give potential new therapeutic options to avoid any unwanted effects in hyperdopaminergic patients.

The co-administration of WAY100635 with LSP4-2022 abolished/attenuated the LSP4-2022-induced attenuation of DA and 5-HT_{1A} release, indicating that LSP4-2022 action is 5-HT_{1A}-receptor dependent. Regarding the effect of the drugs on GABA release, all treatments attenuated the MK-801-induced GABA release, and no interactions between $mGlu_4$ and 5-HT_{1A} receptors were observed in this case.

Thus, the action of the $mGlu_4$ receptor agonist on DA, 5-HT and glutamate (but not on GABA) release in the rat frontal cortex seems to be 5-HT_{1A}-receptor dependent. According to the theory proposed by Conn et al. (2009), the $mGlu_4$-mediated antipsychotic-like action is mediated via a presynaptic mechanism that regulates (decreases) the release of glutamate from glutamatergic terminals in frontal cortex (Fig. 12). The contribution of 5-HT_{1A} receptors to this presynaptic inhibition of neurotransmitter release by $mGlu_4$ receptors is a rather complex mechanism that may involve intercellular loops and/or may occur at the level of a single neuron. To investigate this, patch-clamp recordings were performed in which we used DOI (a 5-HT_{2A} agonist) to induce spontaneous excitatory postsynaptic currents (sEPSCs). The stimulation of 5-HT_{2A} receptors with DOI induced increases in both the amplitude and frequency of the spontaneous EPSCs. These effects were attenuated by the administration of LSP4-2022 at three doses (2.5, 5 and 10 μm). The influence of LSP4-2022 on both the frequency and the amplitude of sEPSCs indicates a pre- and postsynaptic effect of the compound, respectively, but the presynaptic effect was more prominent and was observed at all three concentrations of LSP4-2022 used. The observed results may be due to the fact that $mGlu_4$ receptors are predominantly expressed presynaptically (Shigemoto et al., 1997; Bradley et al., 1996, 1999), although weak dendritic labelling is also occasionally observed (Benítez et al., 2000). Therefore, it may be the case that the neuron sampled in the patch-clamp recordings is one of those expressing dendritic $mGlu_4$ receptors. In our earlier electrophysiological tests, $mGlu_4$ PAMs (Lu AF21934 and Lu AF32615) decreased only the frequency and not the amplitude of sEPSCs, indicating a clear presynaptic mechanism of their action (Slawińska et al., 2013). A presynaptic mechanism of action of LSP4-2022 has been observed in electrophysiological studies of EPSCs in cerebellar slices (Goudet et al., 2012). Moreover, in 2007 Zhang and Marek also showed, that group III mGlu receptor agonists (non-selective $mGlu_4$ and $mGlu_8$ agonists) suppressed the frequency of 5-HT-induced EPSCs in the mPFC. They also showed that the group III mGlu receptor agonists, in contrast to mGlu II agonists, appear to have relatively minimal effects on glutamate released by sources other than thalamocortical afferents, supporting the mechanism of action of the agonists of these receptors raised by Conn et al. (Conn et al., 2009).

Our additional experiments with the co-administration of the 5-HT_{1A} antagonist WAY100635 (10 μm) with LSP4-2022 at a dose of 5 μm had no effect on the LSP4-2022-induced attenuation of the DOI-induced increase in the frequency of spontaneous EPSCs. This result indicates that the inhibition of 5-HT_{1A} receptors does not influence the action of LSP4-2022. It should be noted that 5-HT_{1A} receptors are expressed postsynaptically on pyramidal neurons in the prefrontal cortex (Palchaudhuri and Flugge, 2005; Amargós-Bosch et al., 2004), therefore their presynaptic co-localization (with $mGlu_4$ receptors?) is rather doubtful.

Taken together, our present results confirm once again, that the $mGlu_4$ receptor is a promising target for antipsychotic drug discovery. Targeting this receptor may be more efficient and may result in a lower risk of inducing adverse effects than presently used neuroleptics for various reasons (e.g., low risk of inducing parkinsonian-like syndromes). Whether the treatment with $mGlu_4$ receptor agonist could result in an improved clinical profile

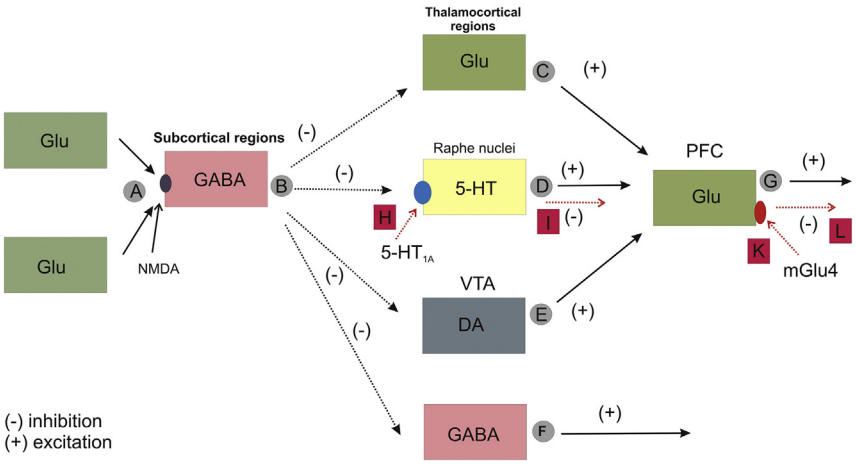

Fig. 12. The schematic representation how the interaction between $mGlu_4$-5-HT_{1A} may work in the raphe-thalamo-cortical loops. Adapted partially from Conn et al., 2009.

compared to the disappointing clinical results with the $mGlu_{2/3}$ receptor agonist prodrug pomaglumetad methionil remains an open question, and only the clinical trials can give a credible answer. Whether the heterocomplexes with 5-HT_{2A} receptors formed by $mGlu_2$ receptors (González-Maeso et al., 2008; Fuxe et al., 2009) have an impact on the action of antipsychotics still remains not fully clear. The important thing that we established is that the action of $mGlu_4$ activators can be intensified via the simultaneous stimulation of 5-HT_{1A} receptors, and similar effect was not observed for $mGlu_{2/3}$ receptors ligands (Wierońska et al., 2013). It is not clear if the $mGlu_4$ and 5-HT_{1A} receptors form heterocomplexes or not. The immunoreactivity of $mGlu_4$ receptors in the prefrontal cortex and in the other structures important in antipsychotic treatments is moderate to low (Bradley et al., 1999). Our theory assumes that the antipsychotic-like effect is a result of a cascade of events that occur between several parts of the subcortical and cortical structures, and it involves different types of nerve endings that innervate postsynaptic neurons, which differentially regulate transmitter release. The schematic explanation of how this interaction may function is schematically represented in Fig. 12.

In subcortical regions, glutamatergic neurons innervate the subpopulation of GABA interneurons expressing dysfunctional NMDA receptors (i.e impaired by MK-801 binding) (A) and, as a result, produce less of GABA (B) that controls the activity of thalamocortical glutamatergic neurons (C), raphe serotonergic neurons (D), dopaminergic neurons in VTA (E), and some populations of interneurons (F). Disinhibited neurons are over-activated and further stimulate cortical pyramidal neurons, which produce an excess of glutamate (G).

The putative targets of a $mGlu_4$-5-HT_{1A}-based antipsychotic treatment are indicated as red pathway. 5-HT_{1A} receptors may be expressed in some populations of pyramidal neurons in the cortex (Wedzony et al., 2008; Palchaudhuri and Flugge, 2005) and may act as inhibitory receptors at these sites after low-dose stimulation. Much larger pools of 5-HT_{1A} receptors are expressed in raphe nuclei (Beer et al., 1990; Chalmers and Watson, 1991; Hjorth and Sharp, 1991; Chilmonczyk et al., 2015). The administration of low doses of 5-HT_{1A} agonists preferentially stimulate somatodendritic receptors in raphe nuclei (H) (Ago et al., 2003; Bubeníková-Valesová et al., 2007; Sakaue et al., 2000), that leads to a decrease in serotonin release from serotonergic terminals innervating the prefrontal cortex or thalamocortical regions (I). Activation of presynaptic $mGlu_4$ receptors (K) inhibits the release of glutamate (L).

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