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$mGlu_5$-$GABA_B$ interplay in animal models of positive, negative and cognitive symptoms of schizophrenia

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ABSTRACT

Diverse preclinical studies exploiting the modulation of the GABAergic and/or glutamatergic system in brain via metabotropic receptors suggest their potential therapeutic utility. GS39783 and CDPPB, positive allosteric modulators of $GABA_B$ and $mGlu_5$ receptors, were previously shown to reverse behavioral phenotypes in animal models to mimic selected (predominantly positive) symptoms of schizophrenia. In the present study we investigated the activity of selected $GABA_B$ (GS39783 and CGP7930) and $mGlu_5$ (CDPPB) positive allosteric modulators. We focused mainly on the aspects of their efficacy in the models of negative and cognitive symptoms of schizophrenia. We used modified swim test, social interactions (models of negative symptoms) and novel object recognition (model of cognitive disturbances). The activity of the compounds was also tested in haloperidol-induced catalepsy test. The mutual interaction between $GABA_B$/$mGlu_5$ ligands was investigated as well. In the second part of the study, DHPG-induced PI hydrolysis in the presence of $GABA_B$ receptor antagonist (SKF97541), and SKF97541-induced inhibition of cAMP formation in the presence of DHPG, was performed. Both $mGlu_5$ and $GABA_B$ receptor modulators effectively reversed MK-801-induced deficits in behavioral models of schizophrenia. Moreover, the concomitant administration of sub-effective doses of CDPPB and GS39783 induced a clear antipsychotic-like effect in all the procedures used, except DOI-induced head twitches. The concomitant administration of group I mGlu and $GABA_B$ agonists did not displayed any synergistic effects in vitro. Summing up, an activation of both types of receptor may be a promising mechanism for the development of novel antipsychotic drugs, efficacious toward positive, negative and cognitive symptoms.

1. Introduction

The clinical picture of schizophrenia is complicated and involves many different symptoms. Those symptoms are divided into positive, negative and cognitive. Positive symptoms involve feelings and behaviors that most individuals do not normally experience, but are present in schizophrenia patients. They include delusions, disordered thoughts and speech, and hallucinations. Positive symptoms generally respond well to medication. Negative and cognitive symptoms impoverish normal responses, and commonly include flat or blunted affect and emotion, poverty of speech, anhedonia, associality, lack of motivation, disturbances in working memory, significant impartment of intelligence, etc. (Rössler et al., 2005). Negative and cognitive symptoms worsen significantly the quality of life and are the main causes of functional disability. People with prominent negative and cognitive symptoms often have a history of poor adjustment before the onset of illness, and their response to medication is often limited (Jung et al., 2014).

The presently used antipsychotic treatment is based predominantly on the blockade of D_2 receptors. The antipsychotic drugs of the new generations possess the affinity toward other types of receptors, such as serotonergic, histaminergic or muscarinic receptors (Meltzer, 2013). It makes them more efficient and better tolerated. The risk of inducing adverse effects that still exist and the need of prolonged administration constitute a serious limitation of their use. Therefore, the search for novel drugs is highly needed (Meltzer, 2013).

According to the glutamatergic theory of schizophrenia, proposed by Javitt (1987, 2010) and developed later on by Conn et al. (2009) it seems that the ligands of the metabotropic glutamate receptors may constitute a good alternative for presently used neuroleptic therapy. The antipsychotic-like effects were observed for a variety of ligands in preclinical studies. The most widely described are positive modulators of $mGlu_{2/3}$ and $mGlu_5$ receptors (Fell

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et al., 2012; Kanuma et al., 2010). Recently, some reports concerning the activity of group III mGlu ligands in animal models of psychosis were released from our laboratory. The antipsychotic-like activity was showed for mixed orthosteric agonists of $mGlu_4$/$mGlu_8$ receptors and for positive modulators of $mGlu_4$ receptors (Pałucha-Poniewiera et al., 2008; Sławińska et al., 2013; Wierońska et al., 2013). Thus, the ligands of mGlu receptors became an important part of modern psychopharmacology, as, on the basics of preclinical studies, they may be considered as a future of antipsychotic treatment. The efficacy toward not only positive, but also negative or cognitive disturbances, may constitute their advantage over presently used antipsychotic therapy. The clinical trials that were performed with $mGlu_{2/3}$ PAMs, modulators of NMDA receptor and GlyT1 inhibitors partially confirm this hypothesis (Bugarski-Kirola et al., 2014). Some of them were discouraging (Adams et al., 2010; Kinon et al., 2011), but recently released by Addex positive results concerning activity of $mGlu_{2/3}$ PAMs in the clinic indicate, that further works are needed to improve the compounds, to ameliorate their bioavailability and to minimize the risk of adverse effects they can induce.

Next to glutamatergic hypothesis, the impartment of GABAergic transmission was described as one of the cues in schizophrenia pathophysiology (Fatemi et al., 2009; Guidotti et al., 2000). Several papers, including our study, showed that the activators of GABAergic system, especially $GABA_B$ receptor modulators and orthosteric agonists, may exert antipsychotic-like action in animal models (Arai et al., 2008, 2009; Frau et al., 2014; Mizoguchi and Yamada, 2011; Wierońska et al., 2011). Such an activity was shown for GS39783 and CGP44532 (Wierońska et al., 2011). However, the papers were focused predominantly on the efficacy of those compounds in the models of positive symptoms of schizophrenia. Their efficacy in haloperidol-induced catalepsy was also reported (Wierońska et al., 2011).

The present set of experiments was focused on the activity of the positive allosteric modulators (PAM) of $mGlu_5$ and $GABA_B$ receptors in the animal models of schizophrenia. We used 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl) benzamide, CDPPB (Lindsley et al., 2004), a $mGlu_5$ receptor PAM, and two $GABA_B$ receptor PAMs, *N*,*N*′-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine, GS39783 (Urwyler et al., 2003) and 3,5-*bis*(1,1-dimethylethyl)-4-hydroxy-β,β-dimethyl-benzenepropanol, CGP7930 (Urwyler et al., 2001). All the compounds were investigated in the forced swim test, social interaction test and novel object recognition test, considered as the models of negative and cognitive symptoms. Although the paper was focused predominantly on the aspects of social and cognitive impartments, we used also DOI-induced head twitches as the models of positive symptoms. Haloperidol-induced catalepsy was performed to establish the anti-cataleptic activity of CDPPB. $GABA_B$ receptor ligands were previously shown to possess anti-cataleptic activity (Wierońska et al., 2011). The mutual interaction between $mGlu_5$ and $GABA_B$ receptors was also investigated in our studies. This aspect of antipsychotic treatment was not touched so far, and can bring a new life in schizophrenia research, as the mechanism of action of majority of neuroleptics involves serotonergic and/or dopaminergic systems.

In the second part of our studies the levels of second messengers were determined. We examined DHPG-stimulated hydrolysis in the presence of GABAB agonist, SKF97541, and SKF97541-induced inhibition of cAMP formation in the presence of group I agonist, DHPG.

2. Materials and methods

2.1. Animals and housing

Male Albino Swiss mice (20–25 g, Charles River, Germany) were used for the DOI-induced head twitches, modified swim test and haloperidol-induced catalepsy. Male Wistar rats (200–220 g, Charles River, Germany) were used in the social interaction and novel object recognition tests. 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 8–10 animals/dose, and the animals were used only once in each test. All of the animals were experimentally naive prior to testing. For all the experiments in mice, the compounds were injected at a volume of 10 ml/kg and in rats at a volume of 1 ml/kg. The experiments were performed by an observer blind to the treatment and were conducted according to the procedures approved by the Animal Care and Use Committee at the Institute of Pharmacology, Polish Academy of Sciences in Krakow.

2.2. Drugs

GS39783, CGP7930 and CDPPB were obtained from Tocris Biosciences (Tocris House, IO Centre Moorend Farm Ave, Bristol, UK). GS39783 and CGP7930 were dissolved in small amount of EtOH and then were filled up with 0.2% Tween 80. CDPPB was dissolved in 0.5% methylcellulose. All drugs were administered i.p. 30 min before the test.

The psychostimulants (MK-801 and DOI) were dissolved in saline and administered at the same doses and schedule as in our previous studies (Pałucha-Poniewiera et al., 2008; Wierońska et al., 2011, 2012, 2013). The schedule of administration will be described later for each test separately. The administration schedule of the compounds was not only adapted from the other studies, but also was based on our long-lasting experience with the ligands used. The doses and routes of administration of $GABA_B$ ligands (GS39783, CGP7930) were described earlier (Slattery et al., 2005; Wierońska et al., 2011). The doses and timing of CDPPB were taken from the studies of Pałucha-Poniewiera and Pilc (2012), Uslaner et al. (2009), Vardigan et al. (2010) and Horio et al. (2013). In the interaction studies, all animals received the same number of injections: when one of the drugs was omitted, an appropriate vehicle was given instead. The control animals received injections of the appropriate vehicles.

2.3. Modified swim test

The MK-801-induced immobility in the forced swim test was performed according to the model of Langen et al. (2012) with small modifications, and was based on the PCP model of Noda et al. who proposed it as a model of the negative symptoms of schizophrenia (Corbett et al., 1999; Noda et al., 1995, 1997).

For the forced swim test, a glass cylinder (height, 20 cm; internal diameter, 15 cm) containing 11 cm water maintained at 23–26 °C was used. On day 0 of the experiment, the mice were forced to swim in the water for 3 min, and the immobility time was recorded during the whole 3-min period. Afterwards, animals were removed from the water and dried with a paper towel and put into the home cage.

Starting the following day, mice were treated with 0.4 mg/kg MK-801 i.p. once daily for 13 days. The control animals were treated with saline. After the 13th administration, the animals had a 1-day washout period. On day 15 of the experiment, a second swim test was performed according to the procedure described earlier. Thirty minutes before this second swim test, compound or vehicle were administered. CDPPB (0.1, 0.5, and 2 mg/kg), CGP7930 (0.5, 1 and 2 mg/kg), and GS39783 (0.1, 1, 2.5 i 5 mg/kg) were administered 30 min before the social interaction test. Non-effective doses of GS39783 (0.05 mg/kg) and CDPPB (0.1 mg/kg) were administered at the same time 30 min before the test.

2.4. Locomotor activity in 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 the photobeams resulted in horizontal activity expressed as horizontal activity counts.

The locomotor activity was measured as the control experiment for the modified swim test. Briefly, mice were treated with MK-801 (0.4 mg/kg), once daily for 13 days. Concomitantly, one group of mice received saline injections. After the 1 day break the locomotor activity was measured during the 5 min test. Three groups of mice receiving MK-801 were additionally treated with the best active doses of the compounds used in the modified swim test, e.g. CGP7930 (5 mg/kg), GS39783 (1 mg/kg and 0.05 mg/kg), CDPPB (1 mg/kg and 0.5 mg/kg), CDPPB (0.1 mg/kg) + GS39783 (0.05 mg/kg). The compounds were given 30 min before measurement.

2.5. MK-801-induced deficits in social interaction in rats

Social interaction tests were performed according to the method 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. The 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 of variance. The study was conducted 3.5 h after the subcutaneous, acute administration of MK-801 at 0.1 mg/kg, s.c. Each pair of rats was diagonally placed in opposite corners of the box so that they faced away from each other. Saline was administered as the vehicle. CDPPB (0.25, 0.5, and 1 mg/kg), CGP7930 (0.5, 1 and 2 mg/kg), and GS39783 (1, 2.5 and 5 mg/kg) were administered 30 min before the social interaction test. Non-effective doses of GS39783 (0.1 mg/kg) and CDPPB (0.1 mg/kg) were administered at the same time 30 min before the test. The behavior of the animals was monitored and recorded on a video recorder located outside the box over a 10-min period. The test box was wiped clean between each trial. The social interaction between two rats was determined as the total time spent participating in social behavior such as sniffing, genital investigation, chasing and fighting each other. The number of episodes was counted as a separate paradigm. The treatment groups included 8–10 animals.

2.6. Novel object recognition (NOR)

The method was adapted from Horiguchi et al. (2011a, 2011b) and Dere et al. (2012). The animals were trained and tested in a black wooden circular open field (100 cm in diameter, 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 with the tested drugs, placed in the apparatus and allowed to explore two identical objects (cylinder-shaped 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 1 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 rats were returned to the open field for 5 min, and the duration of exploration (ie. sitting in close proximity to the objects, sniffing or touching them) of each object was measured separately by a trained observer. All drugs were administered before the training (T_1) session. MK-801 (0.1 mg/kg, s.c) was given once, 30 min before the session. CDPPB (1, 2, and 5 mg/kg), CGP7930 (0.5, 1 and 2 mg/kg), and GS39783 (1, 2.5 i 5 mg/kg) were administered 30 min before MK-801 administration. Non-effective doses of GS39783 (1 mg/kg) and CDPPB (0.1 mg/kg) were administered at the same time 30 min before the test.

2.7. Head twitch test

The experiments were performed according to our previous studies (Pałucha-Poniewiera et al., 2008; Wierońska et al., 2011, 2012). Briefly, in order to habituate mice to the experimental environment, each animal was transferred to a 12 cm (diameter) x 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. CDPPB (0.1, 0.5, and 2 mg/kg), CGP7930 (0.5, 1 and 2 mg/kg), and GS39783 (1, 2.5 and 5 mg/kg) were administered 30 min before MK-801 administration. The treatment groups included 8–10 animals.

2.8. Haloperidol-induced catalepsy

The catalepsy test was performed according to Siuciak et al. (2007), with small modifications. The catalepsy response of one mouse placed in an observation box was measured from the duration of an abnormal posture in which the forelimbs of the mouse were placed on a horizontal 0.2-mm-diameter wire bar suspended 7.5 cm above a platform. The catalepsy test ended when the forelimbs touched the bottom or the wall of the box or when the mouse climbed onto the bar. CDPPB (0.25, 0.5, and 1 mg/kg) and GS39783 (0.1 mg/kg) were administered alone and 30 min prior haloperidol (0.1 mg/kg). The catalepsy was measured 45 min after haloperidol administration. All the experiments were performed by the observer blind to the treatment.

2.9. IP1 determination

The method was adapted from Pilc et al., 1998 and Chruścicka et al., 2015 with small modifications. Rats were decapitated and frontal cortices were dissected. Isolated cortex was cross chopped into slices of 350 × 350 µm with the McIlwain tissue chopper. The sections were then transferred into 25 ml of oxygenated Krebs–Henseleit'a buffer (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4 \times 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.7 mM glucose; pH 7.4; 37 °C), and washed three times during 5 min of incubation in a water bath, with gentle shaking and oxygenation (37 °C). Thereafter, four additional incubation steps in fresh Krebs–Henseleit'a buffer for 20 min with oxygenation and gently shaking were performed. After that, slices were concentrated by gravity, and 50 µl aliquots were placed into polyethylene tubes, containing Krebs–Henseleit'a buffer supplemented with 10 mM LiCl, and incubated in 37 °C for 5 min. Following 5 min preincubation with group I mGlu agonist, (S)-3,5-DHPG (Tocris, House, IO Centre Moorend Farm Ave, Bristol, UK), 25 µl of $GABA_B$ agonist, SKF97541 (30 or 100 µM) was added, to a final volume 500 µl. Tubes were oxygenated, capped, and incubated for 60 min in water bath (37 °C) with gentle shaking. The reactions were stopped by adding 200 µl of lysis buffer (buffer included in IP-One HTRF® assay kit from Cisbio). The samples were sonicated two times for 30 s and centrifuged at 10 000 × *g*, for 10 min. The supernatants were used for IP1 determination. The level of IP1 was measured with IP-ONE HTRF bioassay kit (Cisbio) using time-resolved measurements of fluorescence technique (TR-FRET). This method is a competitive immunoassay between endogenous IP1 produced by cells and dye labeled IP1 conjugated with d2 day (modified allophycocyanin). Ten microliters of supernatants was transferred to a 384-well plate with 5 µl of IP-one-d2 conjugate in lysis buffer

by means of an automated pipetting system (Tecan Evo 200; Tecan, Mannedorf, Switzerland). Then, 5 µl antibodies conjugated with cryptate against IP1 were mixed with sample on orbital shaker (800 rpm/min.). After 1 h incubation in RT the fluorescence signal at 620 nm and 665 nm was read (Tecan Infinity M1000). The results were calculated as the 665 nm/620 nm ratio multiplied by 10^4. The specific signal was inversely proportional to the concentration of IP1 in the sample. Sample IP1 concentration was read from the standard curve as M concentration of IP1.

2.10. cAMP determination

The method was adapted from Wierońska et al., 2007 and Chruścicka et al., 2015 with small modifications. The samples for cAMP determination were prepared as described earlier for (IP) determination with small modification. After concentration of slices by gravity, Krebs–Henseleit'a buffer was not supplemented with 10 mM LiCl, and incubation in 37 °C lasted 15 min. Following 5 min preincubation with 25 µl of $GABA_B$ agonist (30 or 100 µM) and 25 µl forskolin (30 µM), 25 µl (S)-3,5-DHPG (100 µM), was added, to a final volume 500 µl. Tubes were oxygenated, capped, and incubated for 60 min in water bath (37 °C) with gentle shaking. The reactions were stopped by adding 200 µl of lysis buffer (buffer included in HTRF® cAMP dynamic 2 assay kit from Cisbio). The samples were sonicated two times for 30 s and centrifuged at 10 000 × *g*, for 10 min. The supernatants were used for cAMP determination. The level of cAMP was measured with bioassay kit (Cisbio) using time-resolved measurements of fluorescence technique (TR-FRET). This method is a competitive immunoassay between endogenous cAMP produced by cells and dye labeled cAMP conjugated with d2 day (modified allophycocyanin). Ten microliters of supernatants was transferred to a 384-well plate with 5 µl of cAMP-d2 conjugate in lysis buffer by means of an automated pipetting system (Tecan Evo 200; Tecan, Mannedorf, Switzerland). Then, 5 µl antibodies conjugated with cryptate against cAMP were mixed with sample on orbital shaker (800 rpm/min.). After 1 h incubation in RT the fluorescence signal at 620 nm and 665 nm was read (Tecan Infinity M1000). The results were calculated as the 665 nm/620 nm ratio multiplied by 10^4.

Samples cAMP concentration was read from the standard curve as M concentration of cAMP.

2.11. Statistical analysis

The data are presented as the means ± S.E.M. Statistical analysis of the data was performed using the Graph Pad Prism Software, ver. 5.0, and Statistica 10 package (StatSoft Inc., OK, USA). A one-way ANOVA followed by the Neuman–Keuls post-hoc comparison was used in the dose-dependence studies, while a two-way ANOVA, followed by Newman–Keuls post hoc comparison test, was used in the interaction studies. The *P* value of at least $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The effects of $GABA_B$ modulators in the modified forced swim test in mice

Repeated administration of MK-801 according to the scheme described in Section 2 induced a significant increase in the immobility time in the forced swim test (Fig. 1A–C).

CGP7930 was given at the doses 1, 2.5 and 5 mg/kg. The compound abolished the MK-801-induced effect only at the higher dose (5 mg/kg); however, it did not reached the statistical significance when calculated with one-way ANOVA. Only t-test revealed statistical significance of the compound when compared with MK-801-treated group.

One-way ANOVA followed by Newman–Keuls multiple comparison test revealed that GS39783, given in doses 0.5, 1 and 2 mg/kg, dose-dependently reduced the immobility time induced by MK-801 administration $F_{(4.31)} = 6.512$, $P < 0.0006$. The effects of doses of 0.5 and 1 mg/kg were shown to be significant, $P < 0.05$ (Fig. 1A).

3.2. The effects of $mGlu_5$ modulator, CDPPB, in the modified forced swim test in mice

The effect of $mGlu_5$ modulator, CDPPB, was shown to be also significant. One-way ANOVA followed by Newman–Keuls multiple comparison test revealed that CDPPB, given in doses 0.5, 1 and 2 mg/kg reduced the immobility time induced by chronic MK-801 administration $F_{(4.41)} = 4.849$, $P < 0.002$ (Fig. 1C). The dose of 0.1 mg/kg was ineffective.

3.3. The effect of concomitant administration of CDPPB and GS39783 on the deficits induced by acute MK-801 administration in the social interaction test

CDPPB was given at a subeffective dose of 0.1 mg/kg, i.p., 30 min before the test, and GS39783 was given also at a subeffective dose of 0.05 mg/kg, i.p. together with CDPPB administration. Concomitant administration of both compounds induced clear antipsychotic effect, shortening MK-801-induced increase in the immobility time. Two-way ANOVA followed by Newman–Keuls multiple comparison test revealed the statistical effects, $F_{(1.33)} = 7.42$; $P < 0.01$ (Fig. 2A).

3.4. Locomotor activity in mice

During the 5 min session, there were no changes in spontaneous locomotor activity observed between saline and mice chronically treated with MK-801. Neither of the compounds tested changed the activity of MK-801 treated animals in a statistically significant way (Figs. 1D–F, 2B).

3.5. The effect of $GABA_B$ modulators on the MK-801-induced deficits in the social interaction test

Subcutaneous, acute administration of MK-801 at 0.1 mg/kg significantly decreased the total duration of the social interaction between two naive rats and the total number of social episodes when compared to the vehicle-treated group. CGP7930 administered 30 min before the test significantly improved social withdrawal induced by MK-801 (Fig. 3A,B), without affecting the behavior of the animals when administered on its own.

One-way ANOVA followed by Neuman–Keuls post hoc comparison revealed that CGP7930 at the doses of 1 and 2 mg/kg reversed MK-801-induced deficits in the social interaction, increasing both the number of episodes [$F_{(4.18)} = 38.63$; $P < 0.0001$] and total duration of interactions [$F_{(4.18)} = 33.93$; $P < 0.0001$]. The lowest dose (0.5 mg/kg) was ineffective.

GS39783 administered 30 min before the test also significantly improved social withdrawal induced by MK-801 (Fig. 3C,D), without affecting the behavior of the animals when administered on its own.

One-way ANOVA followed by Neuman–Keuls post hoc comparison revealed that GS 39783 at the doses of 1, 2.5 and 5 mg/kg reversed deficits induced by acute MK-801 administration in the social interaction, increasing both the number of episodes [$F_{(4.20)} = 8.77$; $P < 0.0003$] and total duration of interactions [$F_{(4.20)} = 8.88$; $P < 0.0003$].

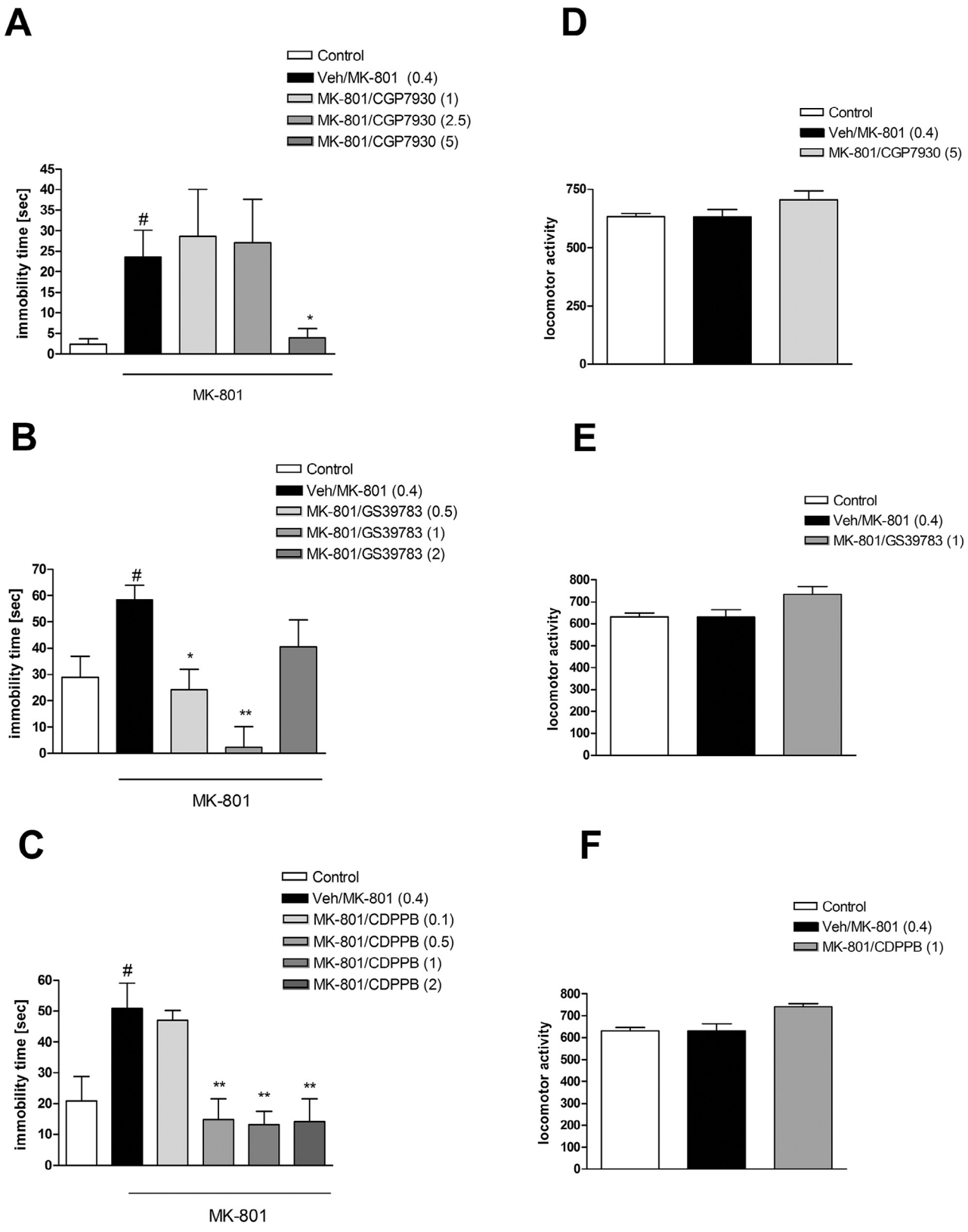

Fig. 1. Effect of repeated MK-801 administration on immobility in the forced swim test and on locomotor activity. Data are shown as mean ± SEM. Saline or 0.4 mg/kg MK-801 i.p. was administered for 13 days. Forced swim test was performed before starting repeated treatment (=day 0) and after 1 day of washout (=day 15). Maximal immobility time in the swim test was 180 s. Locomotor activity was recorded 1 day after the forced swim test (day 16). Activity was recorded for 5 min. $^{\#}$At least $P < 0.01$ vs saline-treated group, $^{*}P < 0.01$ and $^{**}P < 0.001$ vs MK-801-treated group. $n = 8–10$ mice/group.

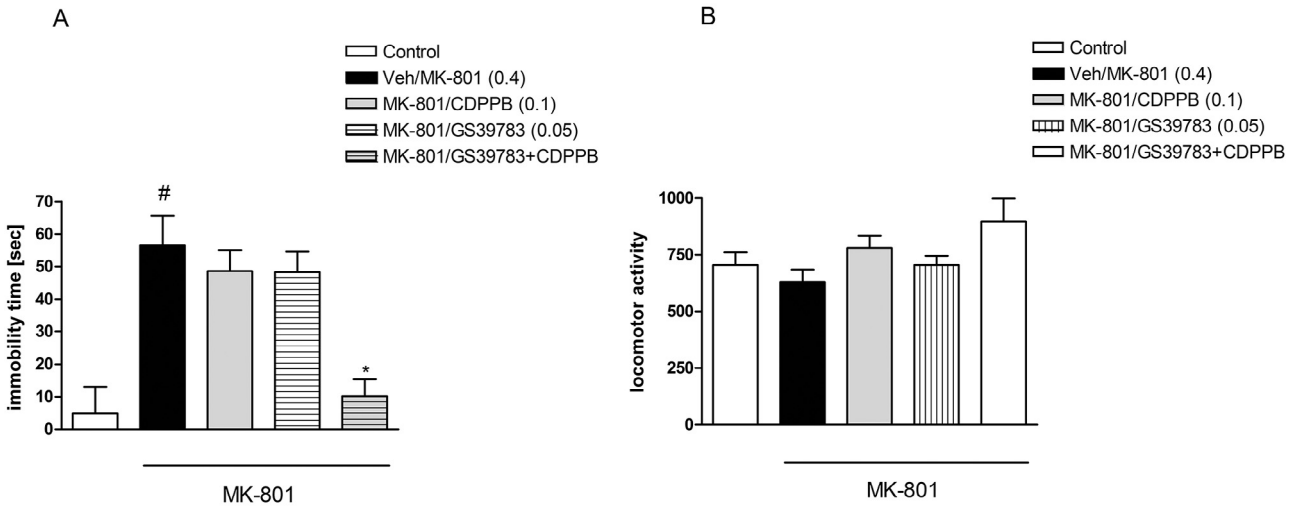

Fig. 2. The results of the concomitant administration of $mGlu_5$ and $GABA_B$ PAMs in the modified forced swim test (A) and locomotor activity (B). Data are shown as mean ± SEM. Saline or 0.4 mg/kg MK-801 i.p. was administered for 13 days. Forced swim test was performed before starting repeated treatment (=day 0) and after 1 day of washout (=day 15). #At least $P < 0.01$ vs saline-treated group, *$P < 0.01$ vs MK-801-treated group. $n = 8–10$ mice/group.

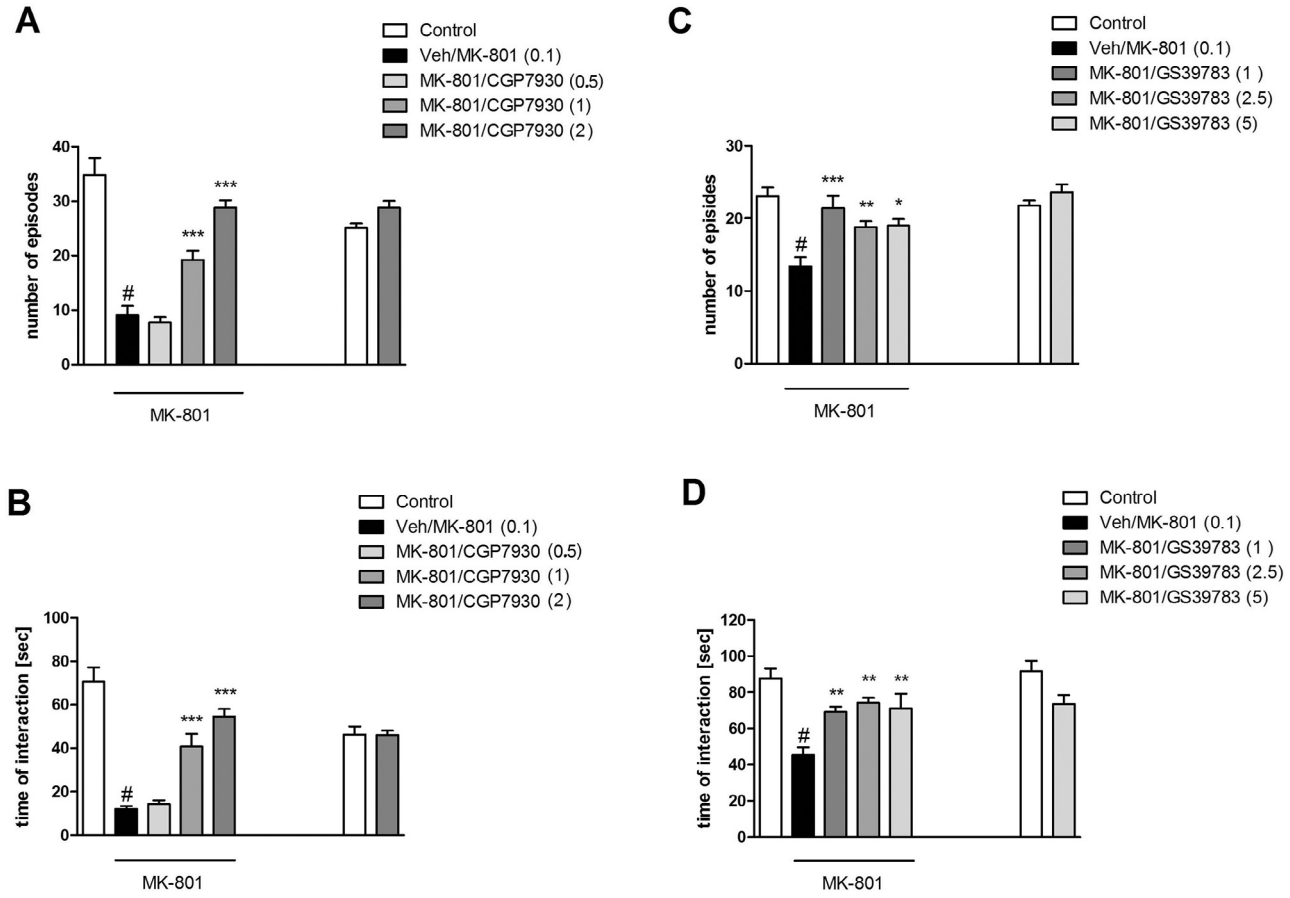

Fig. 3. Effects on MK-801-induced deficits in social interaction. Number of episodes of social contacts and time of social interactions were measured. The dose-dependent effect of CGP7930 (A, B) and GS39783 (C, D). Data are presented as means ± SEM. Doses in mg/kg are indicated in parentheses. At least #$P < 0.01$ vs controls, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs MK-801–treated group. The graphs also include control experiments. $n = 6$ pairs of rats/group.

3.6. The effect of CDPPB on the deficits induced by acute MK-801 administration in the social interaction test

CDPPB was given at doses of 0.25, 0.5 and 1 mg/kg, i.p., 30 min before the test. The effect was statistically significant when measured with one-way ANOVA followed by Neuman–Keuls post-hoc comparison. The statistical analysis for the number of episodes were: $F_{(4.20)} = 6.99$, $P < 0.001$, and for the time of interaction $F_{(4.20)} = 24.23$; $P < 0.0001$. (Fig. 4A,B). The compound had no effect when given alone.

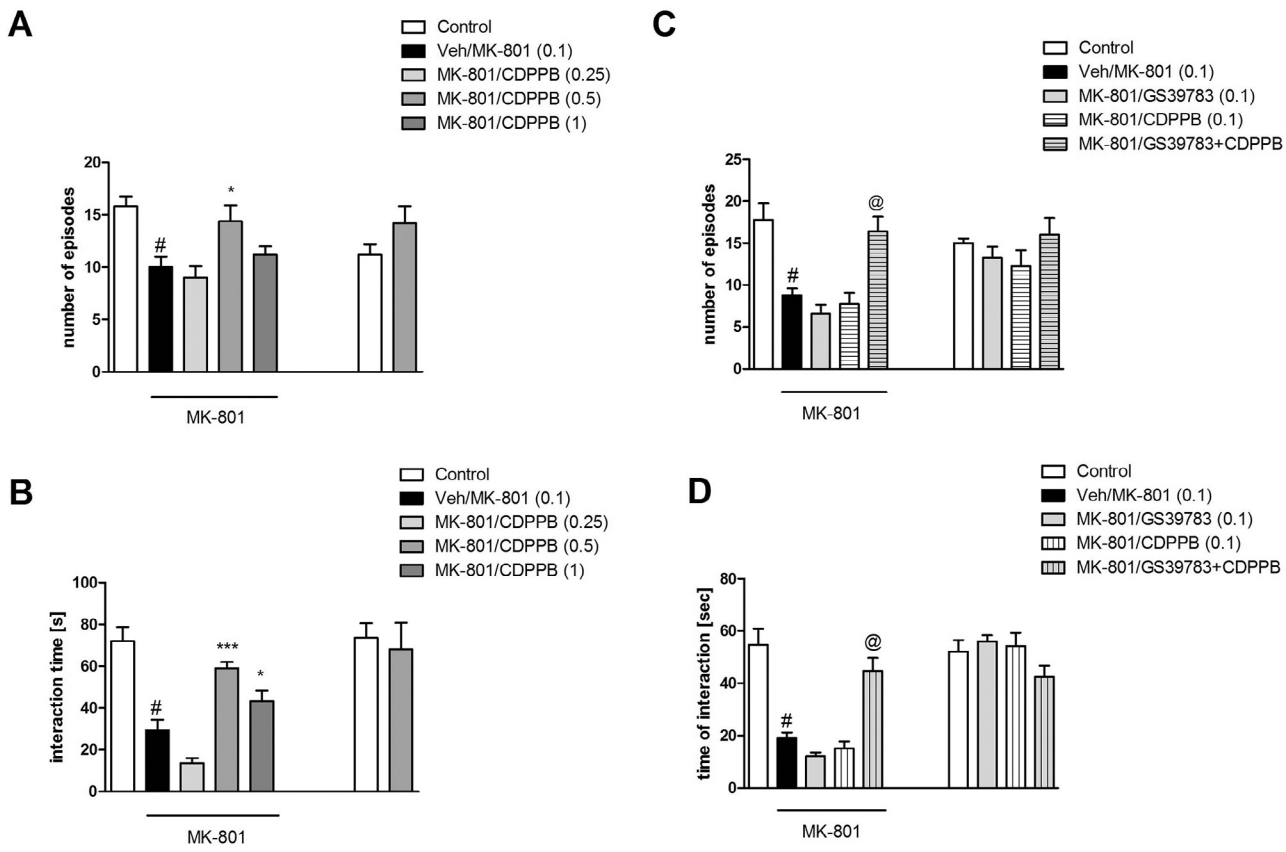

Fig. 4. Effects on MK-801-induced deficits in social interaction. Number of episodes of social contacts and time of social interactions were measured. The dose-dependent effect of CDPPB (A, B) and the effect of combined administration of sub effective doses of CDPPB and GS39783 (C, D). Data are presented as means ± SEM. Doses in mg/kg are indicated in parentheses. At least $^{\#}P < 0.01$ vs controls, $^{*}P < 0.05$, $^{***}P < 0.001$ and $^{@}P < 0.03$ vs MK-801–treated groups. The graphs also include control experiments. $n = 6$ pairs of rats/group.

3.7. The effect of concomitant administration of CDPPB and GS39783 on the deficits induced by acute MK-801 administration in the social interaction test

CDPPB was given at a subeffective dose of 0.1 mg/kg, i.p., 30 min before the test, and GS39783 was given also at a subeffective dose of 0.1 mg/kg, i.p. together with CDPPB administration. Concomitant administration of both compounds induced clear antipsychotic effect, increasing both time of interaction and number of episodes Two-way ANOVA followed by Newman–Keuls multiple comparison test revealed the statistical effects, $F_{(1.34)} = 4.59$; $P < 0.03$ and $F_{(1.34)} = 77.53$, $P < 0.0001$, respectively (Fig. 4C,D).

The control experiment with the groups of CDPPB+ GS39783 revealed that neither of the group had any behavioral effect when given alone.

3.8. The effect of $GABA_B$ modulators on the deficits induced by MK-801 administration in the novel object recognition test

Subcutaneous administration of MK-801 at 0.1 mg/kg significantly decreased the recognition index when compared with the vehicle-treated group. Both CGP7930 and GS 39783 were given i.p. 30 min before MK-801 administration, and dose-dependently reversed MK-801-induced deficits. The effect of CGP7930 was observed at the dose of 1 mg/kg $F_{(6.63)} = 3.8$; $P < 0.002$ (Fig. 5A,B). The lower doses (0.1 and 0.5 mg/kg) and the higher doses (2 and 5 mg/kg) were not effective.

GS39783 was effective at the doses of 1 mg/kg $F_{(6.63)} = 3.06$, $P < 0.01$. The lower and the higher doses were ineffective. The compounds had no their own effects (Fig. 5B).

3.9. The effect of CDPPB on the MK-801-induced deficits in the novel object recognition test

CDPPB was given at doses of 1, 2 and 5 mg/kg, and dose-dependently inhibited MK-801 induced distributions, $F_{(4.44)} = 5.01$; $P < 0.002$ (Fig. 5C). The compound had no own effect when given alone.

3.10. The effect of concomitant administration of CDPPB and GS39783 on the deficits induced by MK-801 administration in the novel object recognition test

CDPPB was administered at a non-effective dose of 1 mg/kg, and GS39783 was administered also in sub effective dose 0.1 mg/kg. Both compounds were administered 30 min before the acute MK-801 administration.

The co-administration of those two compounds induced clear antipsychotic-like effect in the NOR paradigm. The statistical significance of the interaction was observed $F_{(1.34)} = 39.25$; $P < 0.00001$ (Fig. 5D). Any of the treatment had no effect on the animal's behavior.

3.11. The effects of CDPPB and $GABA_B$ ligands in DOI-induced head twitches

One-way ANOVA revealed that CDPPB (1, 5 and 10 mg/kg) given 30 min before the test, i.p., abolished DOI-induced head twitches in the statistically significant way. The effect was observed at the doses 1–10 mg/kg, but not at the dose of 1 mg/kg (6A), $F_{(4.29)} = 14.19$, $P < 0.0001$.

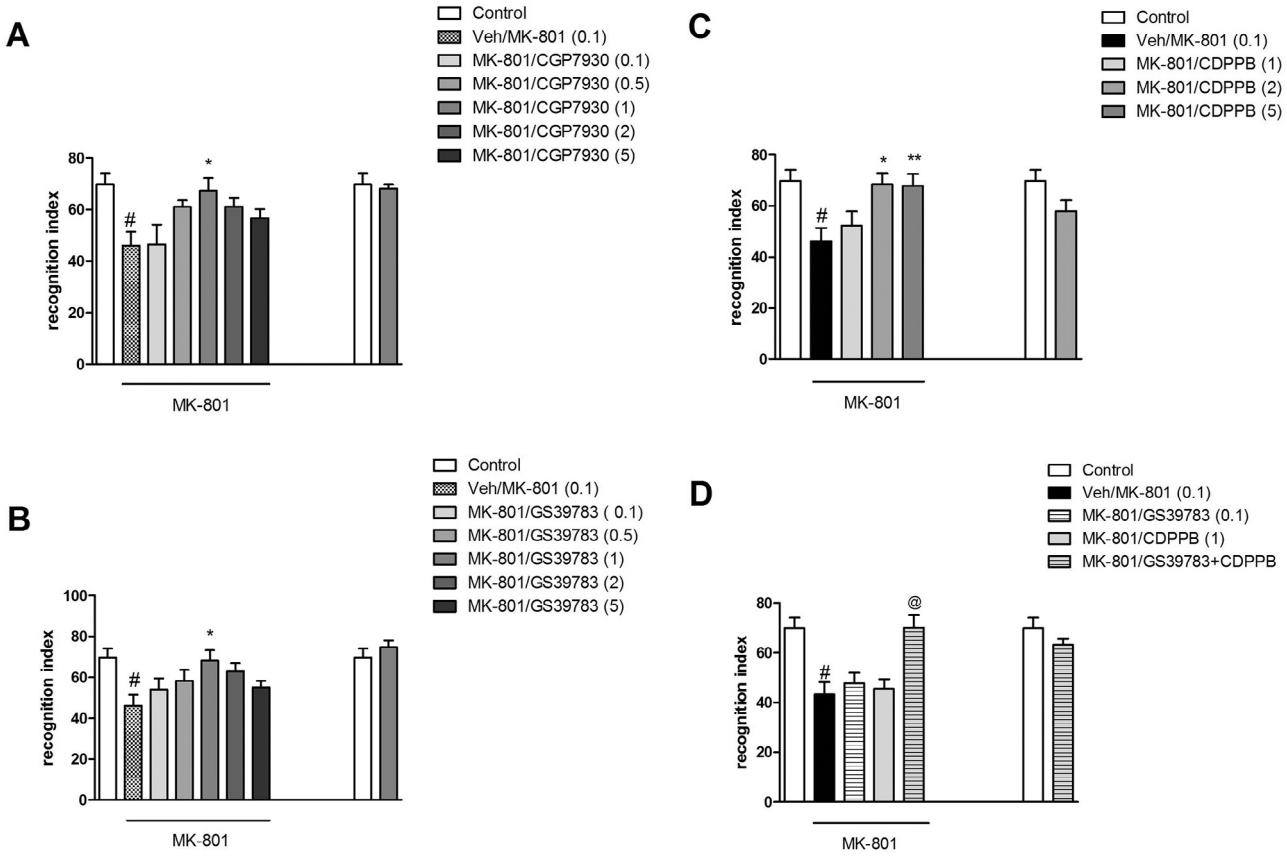

Fig. 5. Effects on MK-801-induced deficits in novel object recognition test. The dose-dependent effect of CGP7930 (A), GS39783 (B), CDPPB (C) and the concomitant administration of subeffective doses of GS39783 and CDPPB (D) is shown. Data are presented as means ± SEM. Doses in mg/kg are indicated in parentheses. At least $^{\#}P < 0.01$ vs controls, $^{*}P < 0.05$, $^{**}P < 0.001$ and $^{@}P < 0.0001$ vs MK-801–treated groups. $n = 8–10$ rats/group.

CGP7930 was administered at the doses 0.5, 1 and 2 mg/kg. The effect was observed at the dose 2 mg/kg, but not at the lower doses $F_{(3.18)} = 5.7$; $P < 0.006$ (6B). The co-administration of sub effective doses of CDPPB (0.5 mg/kg) and GS39783 (0.1 mg/kg) or CGP7930 (10 mg/kg) was not effective in this test (Fig. 6C,D).

3.12. Effect of CDPPB on haloperidol-induced catalepsy

CDPPB given at the doses of 0.25, 0.5, 1 and 2 mg/kg dose-dependently inhibited haloperidol-induced catalepsy (Fig. 7A), $F_{(4.34)} = 3.315$, $P < 0.02$. The compound did not induce any effect when given alone. Co-administration of CGPPB with GS39783 had no effect in haloperidol-induced catalepsy (Fig. 7B).

3.13. (S)-3.5-DHPG -stimulated IP hydrolysis in the presence of SKF97541

One-way ANOVA followed by Tukey's post hoc comparison revealed that (S)-3.5-DHPG (100 μM) stimulated IP hydrolysis in a statistically significant way ($F_{(5.30)} = 4.68$). Such an effect was not observed for both doses of SKF97541 (30 and 100 μM). The concomitant administration of both compounds e.g. (S)-3.5-DHPG (100 μM) and SKF97541 (30 μM) slightly increased IP1 concentration $P < 0.05$. Such an effect was not observed when the higher dose of SKF97541 was applied (Fig. 8A).

3.14. SKF97541-induced inhibition of cAMP formation in the presence of (S)-3.5-DHPG

A 30 μM forskolin stimulated cAMP accumulation up to 653% of control level. One-way ANOVA followed by Tukey's post-hoc comparison revealed $GABA_B$ agonist SKF97541 given at doses 30 and 100 μM inhibited the forskolin-induced cAMP accumulation up to 50% and 69%, respectively ($F_{(3.8)} = 36.53$; $P < 0.001$). (S)-3.5-DHPG (100 μM) also decreased forskolin-induced cAMP accumulation, but to a lesser extend (22%), $P < 0.05$. The concomitant administration of (S)-3.5-DHPG with SKF97541 (30 and 100 μM) inhibited forskolin-stimulated cAMP administration of about a similar way as SKF97541 ($F(3.8) = 21.74$; $P < 0.001$ and $P < 0.01$) (Fig. 8B).

4. Discussion

The present study was focused on the two aspects of antipsychotic treatment.

Firstly, we investigated the activity of $GABA_B$ and $mGlu_5$ receptor positive allosteric modulators in the variety of animal models of schizophrenia. Modified forced swim test and social interactions were used as the models of negative disturbances, while novel object recognition was used as the model of cognitive symptoms of schizophrenia. DOI-induced head twitches in mice reflected human hallucinations. The activity of the compounds was also tested in haloperidol-induced catalepsy test. All of the tests we used are widely accepted, and were described earlier in our papers (see: Sławińska et al., 2013; Wierońska et al., 2012, 2013, 2015), except modified forced swim test (FST) that we used for the first time. Enhancement of immobility in the modified FST induced by chronic MK-801 treatment was proposed in 1995, and was supposed to reflect depressive-like negative symptoms of schizophrenia (Noda et al., 1995). The test differs from the forced swim test typically used to detect antidepressants and has different faces and construct validities (see Section 2). Atypical antipsychotics (clozapine, risperidone

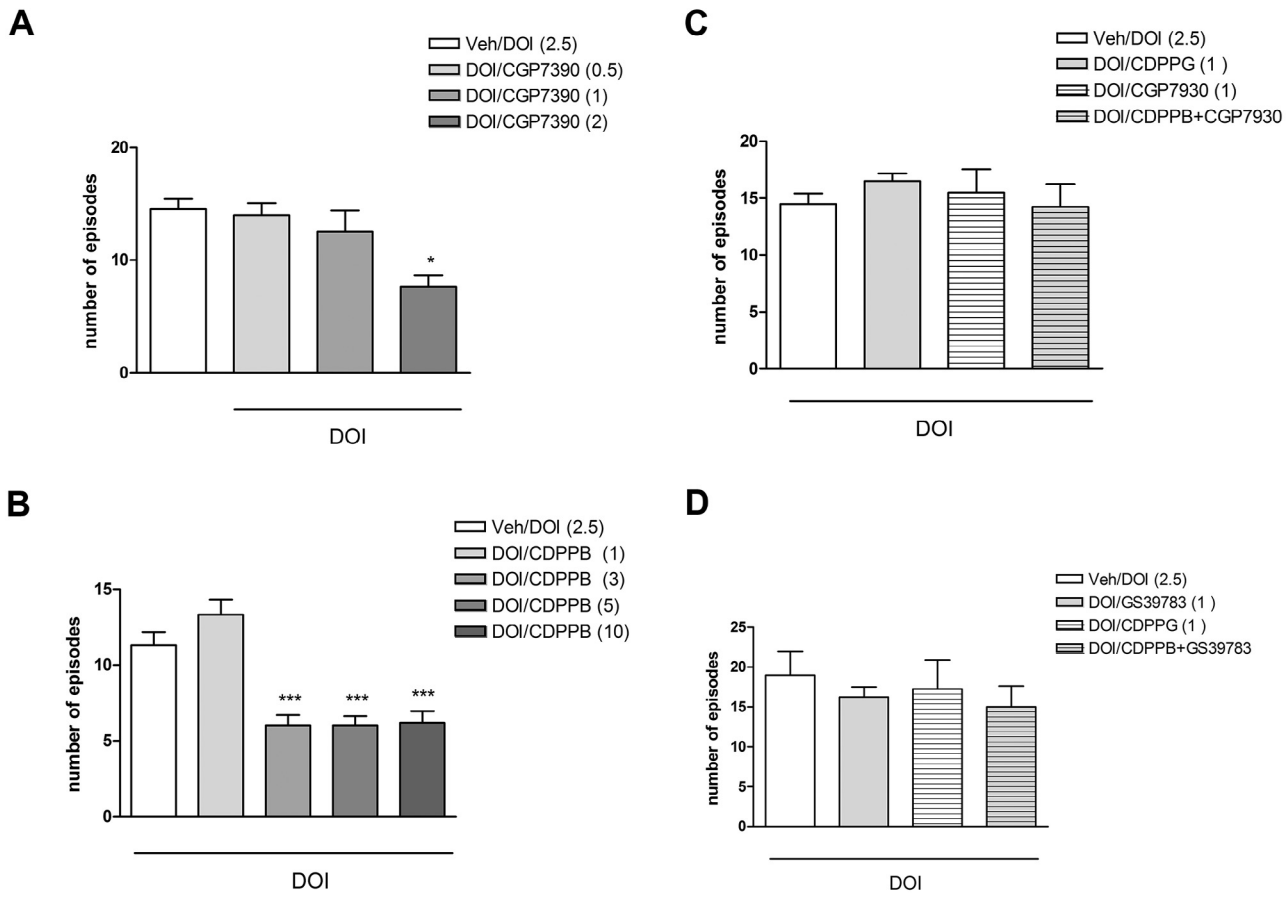

Fig. 6. Effects on DOI induced head twitches. The dose-dependent study of CGP7930 (A) and CDPPB (B), and the administration of the sub effective doses of GABAB PAMs with CDPPB (C, D). Data are presented as means ± SEM. Doses in mg/kg are indicated in parentheses. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs DOI-treated group. $n = 8–10$ mice/group.

or olanzapine) reverse the MK-801-induced increase of immobility (Noda et al., 2000).

In the second part of the behavioral studies, the interrelationship between $GABA_B$ and $mGlu_5$ receptors was investigated. We used the models mentioned earlier to see if the $GABA_B$ and $mGlu_5$ receptor ligands may exert mutual action.

Metabotropic $GABA_B$ receptor for the principal inhibitory neurotransmitter GABA, and the large group of metabotropic glutamate receptors (mGlu) belong to the ‹‹class C›› of G-protein coupled receptors (GPCRs) (Bockaert et al., 1993). They are widely expressed and distributed in the central nervous system. Their role was implicated in a variety of neurodegenerative and mental disorders including epilepsy, chronic pain, depression, drug addiction and schizophrenia (Flor and Acher, 2012; Fuxe et al., 2012; Rominger et al., 2014). Several earlier papers indicate that $GABA_B$ and mGlu activators may exert antipsychotic-like action in the animal models of schizophrenia. The action of baclofen or CGP44532, orthosteric agonists of $GABA_B$ receptor, was described in the methamphetamine-induced cognitive impartments, prepulse inhibition, or in the MK-801-induced hyperactivity and DOI-induced head twitches (Arai et al., 2008, 2009; Mizoguchi and Yamada, 2011; Wierońska et al., 2011). The putative therapeutic potential of $GABA_B$ agonists, however, is limited due to their neuromuscular adverse effects. Allosteric positive modulators seem to constitute an excellent alternative for orthosteric agonist. The antipsychotic-like activity of some $GABA_B$ PAMs (racBHFF or GS39783) was described in a few experiments, such as PPI, MK-801-induced hyperactivity and DOI-induced head twitches (Frau et al., 2014; Wierońska et al., 2011).

The antipsychotic activity of mGlu receptor ligands is much better documented (Wierońska et al., 2009). $mGlu_5$ receptor, a member of the I group of mGlu receptors, is linked through Homer and Shank proteins to NMDA receptor and regulates its function (Tu et al., 1999). Variety of $mGlu_5$ PAMs were investigated in the models of positive symptoms (hyperactivity test), or in some aspects of cognitive symptoms of schizophrenia. These were such compounds as: ADX47273, LSN2463359, VU0360172, CPPZ and CDPPB (Darrah et al., 2008; Gastambide et al., 2012; Kinney et al., 2005; Liu et al., 2008; Rodriguez et al., 2010; Spear et al., 2011; Uslaner et al., 2009; Vardigan et al., 2010). CDPPB was also investigated in an animal model of anhedonia (Vardigan et al., 2010).

The excitement around development of $mGlu_5$ positive allosteric modulators for treatment of schizophrenia has dampen after the discovery of neurotoxicity associated with activation of $mGlu_5$/NMDA receptor complex. However, recent studies revealed that stimulation of $mGlu_5$ receptor subtype not necessarily must be accompanied with NMDA activation. Besides, the neurotoxicity is observed after administration of relatively high doses of the compounds (Parmentier-Batteur et al., 2014; Rook et al., 2013).

In the present studies we focused on the action of $GABA_B$/$mGlu_5$ PAMs in animal models of negative and cognitive symptoms of schizophrenia, resistant to presently used antipsychotic treatment. We used two $GABA_B$ positive modulators, GS39783 and CGP7930, and $mGlu_5$ PAM, CDPPB. GS39783 potentiates the effects of GABA on [^{35}S]GTPγS binding at recombinant and native $GABA_B$ receptors (EC_{50} values are 2.1 and 3.1 μM respectively) (Cryan et al., 2004; Mombereau et al., 2007; Urwyler et al., 2003). CGP7930 also

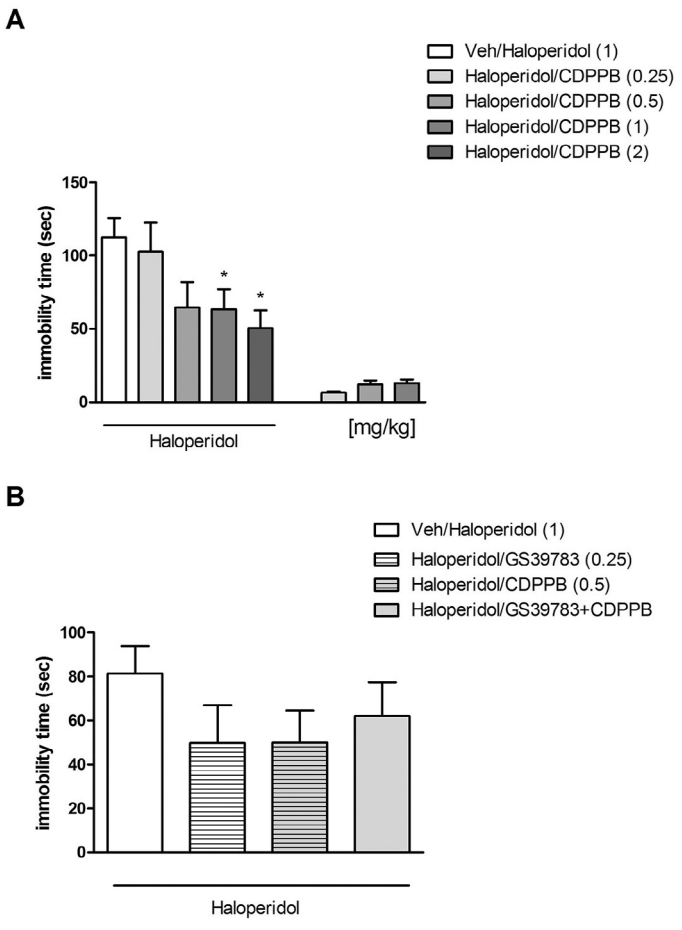

Fig. 7. Effects on haloperidol-induced catalepsy in mice. The dose-dependent effect of CDPPB (A) and the combined administration of sub effective doses of CDPPB and GS39783 (B). Data are presented as means ± SEM. Doses in mg/kg are indicated in parentheses. $^{*}P < 0.05$ vs haloperidol-treated group. $n = 8–10$ mice/group.

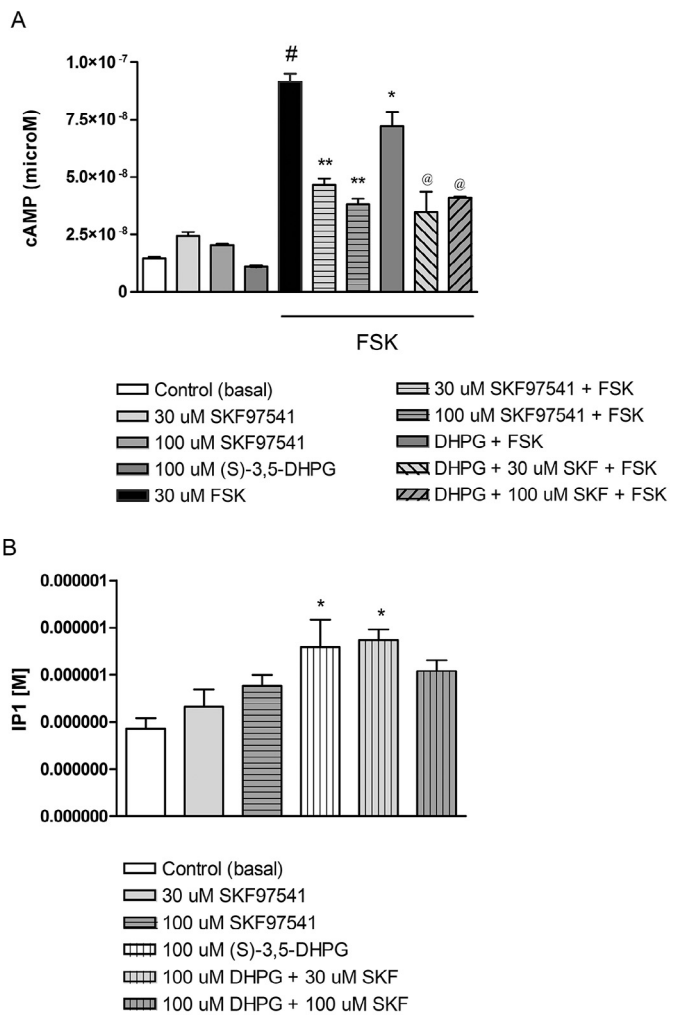

Fig. 8. (S)-3-DHPG-stimulated PI hydrolysis in the presence of SKF97541 (A) and SKF97541-induced inhibition of cAMP formation in the presence of group I mGlu agonist, DHPG (B). All the results are shown in M. Data are presented as means ± SEM. $^{\#}P < 0.0001$ vs control, $^{**}P < 0.001$ vs forskolin (30 μM), $^{*}P < 0.05$ and vs forskolin (30 μM) and $^{@}P < 0.01$ vs forskolin (30 μM).

increases the potency and efficacy of GABA at $GABA_B$ receptors (EC_{50} values are 5.37 and 4.60 μM respectively) and enhances the inhibitory effect of the agonist L-baclofen in cultured cortical neurons (Chen et al., 2006; Urwyler et al., 2001).

In our studies both $GABA_B$ compounds (CGP7930, GS39783) and CDPPB were active in the modified FST. However, the activity of CGP7930 was observed only at the highest dose. Contrary, the effect of GS39783 was observed in relatively lower doses, 0.5 and 1 mg/kg. The effect of 2 mg/kg dose was not statistically significant, although a slight reversal of MK-801-induced effect was observed. This lack of statistically significant effect of the compound could be caused by off-target effects or receptor desensitization. The other possibility is that at the lower doses the drug acts more preferentially at the postsynaptic sites (Hirono et al., 2001; Tabata and Kano, 2010).

Investigated PAMs effectively reversed MK-801-induced impartments in the social interaction test. The action of $GABA_B$ receptor PAMs was dose-dependent and their activity was observed in relatively low doses, from 1 mg/kg up to 5 mg/kg. The $mGlu_5$ PAM, CDPPB, was active in an inverse U-shaped manner, and a statistically significant result was observed at the 0.5 and 1 mg/kg doses. The lower and higher doses were ineffective.

In the further part of the study we concentrated on the selected aspects of cognition, using MK-801-induced disruption in the novel object recognition test. The activity of both $GABA_B$ activators was observed in an inverse U-shaped manner, and both compounds were active at a 1 mg/kg dose. On the other hand, the action of CDPPB was observed at a dose-dependent manner and the effective doses were 2 and 5 mg/kg. The action of the compound was described earlier for higher doses (10 mg/kg and above) (Horio et al., 2013; Reichel et al., 2011; Uslaner et al., 2009). However, the schedule of the experiments and the way of recognition interruption used in those studies were significantly different than in our experiments.

To establish if there is a mutual interaction between those two receptors, we used the sub effective doses of GS39783 ($GABA_B$ PAM) and CDPPB ($mGlu_5$ PAM). The concomitant administration of compounds reversed the dysfunction evoked by MK-801 administration in all the procedures. The combinations did not induce any own effects.

The aspect of positive symptoms of psychosis was not the main stream of the present study, as presently available drugs seem to be effective enough toward this group of disturbances. However, to extend the picture of our story we used the DOI-induced head twitches as the model of human hallucinations (Sadzot et al., 1989; Scruggs et al., 2003). The mechanism of DOI-induced action is known and well investigated. The compound activates $5\text{-}HT_{2A}$ receptor expressed on glutamatergic terminals of the prefrontal cortex. In consequence it leads to the enhancement of glutamate release, the effect responsible for head twitches. The counteraction of this

glutamate efflux can be achieve through the stimulation of presynaptic receptors, such as $GABA_B$ or mGlu (Sławińska et al., 2013; Wierońska et al., 2011). GS39783 was investigated earlier in this test (Wierońska et al., 2011), in contrast to CGP7930 and CDPPB. Our results show that the compounds in the dose-dependent manner reversed DOI-induced effects. However, no interplay between $GABA_B$ and $mGlu_5$ receptors was observed in this test.

As the allosteric positive modulators of $GABA_B/mGlu_5$ receptors may constitute a novel promising target for antipsychotic treatment, their ability to reverse adverse effects typical for presently used D_2 receptor blockers would be a great benefit. Previously it was shown that $GABA_B$ activators may weaken the catalepsy induced by haloperidol administration (Wierońska et al., 2011). Here we show that such an activity characterizes also the $mGlu_5$ PAM, CDPPB. However, no synergistic interaction between $mGlu_5$ and $GABA_B$ receptors PAMs was observed in that respect.

This result requires some commentary, as majority of available data shows that rather $mGlu_5$ NAMs and not PAMs displayed anticataleptic activity in haloperidol-induced catalepsy (Ossowska et al., 2005, 2006). In contrast, there are no data concerning anti-cataleptic activity of $mGlu_5$ PAMs. The only available study was made with ADX47273 compound, showing its putative cataleptogenic potency in very high doses (300 mg/kg) (Liu et al., 2008). Therefore, our study is the first to show anti-cataleptic activity of $mGlu_5$ PAM in haloperidol-induced catalepsy test. It seems plausible that CDPPB activates direct pathway of the basal ganglia motor circuit, in contrast to $mGlu_5$ NAMs, that inhibit the indirect pathways (Cannella et al., 2015). This problem is open for further investigations.

Communication between cells requires special signaling, and the large number of GPCRs constitute a fundamental element of this signaling, activating multiple pathways that are integrated via mechanisms still not well understood. The receptors of the 3rd group of GPCRs have been shown to functionally crosstalk leading to synergistic or new signaling responses (Flor and Acher, 2012; Fuxe et al., 2012; Rominger et al., 2014). This crosstalk may result not only from receptor oligomerization, but also from signaling crossroads or synergistic regulation independent of oligomerization.

The detailed biochemical and histochemical data concerning the expression of $mGlu_5/GABA_B$ receptors are still limited; it seems that such an interplay may exist between those two kinds of receptors. Although the G(i/o) protein-coupled $GABA_B$ receptor (mainly the $GABA_{B1a}$ subtype) is mainly regarded as presynaptic autoheteroreceptor (Craig et al., 2013), exposed to the direct regulation of GABA or glutamate from the terminals of inhibitory or pyramidal neurons (Bräuner-Osborne and Krogsgaard-Larsen, 1999; Wierońska et al., 2011), it may also mediate some postsynaptic effects (Craig et al., 2013), and sense a low concentration of GABA and Ca^{2+} usually contained in the extracellular fluid and GABA split over from the neighboring inhibitory synapses (Hirono et al., 2001; Tabata and Kano, 2010). The $GABA_B$ receptor mediated potentiation of the $mGlu_1$ receptor signaling was also reported, and the mechanism of this interaction, to all the possibility, was not based on the physical interaction between those receptors, but rather on the more general mechanism in which beta-gamma subunits which built the Gi-coupled $GABA_B$ receptor enhanced the mGlu-mediated Gq response (Rives et al., 2009; Tabata and Kano, 2006, 2010).

Such a mechanism of action may underlay for the other specific properties of cells expressing two different Gi- and Gq-coupled receptors, including $mGlu_5$ receptor, that is the close relative of $mGlu_1$, belonging to the same 1st group of mGlu receptors.

In the biochemical part of the study we determined the level of the second messengers, measuring DHPG-stimulated hydrolysis in the presence of $GABA_B$ agonist, SKF97541, and SKF97541-induced inhibition of cAMP formation in the presence of group I agonist, DHPG. The levels of second messengers, IP1 and cAMP, were estimated. Due to some methodological limitations, $GABA_B/mGlu_5$ agonists, and not PAMs were used. The experiments were performed in the cortical slices of the rat brain, and no any synergism between compounds was observed.

A lack of synergistic action of $GABA_B/mGlu_5$ PAMs in DOI-induced head twitches, as well as the lack of interaction between $GABA_B/mGlu_5$ agonists in IP1/cAMP determination studies may indirectly support the hypothesis that the augmentation of DOI-induced head twitches are mediated through two independent $mGlu_5$ and $GABA_B$-mediated mechanisms that do not meet at the level of neuronal signaling. Contrary, neuronal pathways underlying the reversal of selected negative/cognitive dysfunctions include $GABA_B/mGlu_5$ receptors, but it requires more complex studies to explain the mechanism underlying that interaction.

Taken together we propose the novel mechanism of action for antipsychotic drugs, based on the concomitant activation of $GABA_B$ and $mGlu_5$ receptors, that could especially be dedicated for the patients with predominant negative and cognitive dysfunctions.

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