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Research report

Simultaneous activation of muscarinic and $GABA_B$ receptors as a bidirectional target for novel antipsychotics

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ABSTRACT

Recent preclinical studies point to muscarinic and $GABA_B$ receptors as novel therapeutic targets for the treatment of schizophrenia. This study was aimed to assess the role of muscarinic and $GABA_B$ receptor interactions in animal models of schizophrenia, using positive allosteric modulators (PAMs) of $GABA_B$ receptor (GS39783), muscarinic M_4 (VU0152100) and M_5 (VU0238429) receptor, and partial allosteric agonist of M_1 receptor (VU0357017).

DOI-induced head twitches, social interaction and novel object recognition tests were used as the models of schizophrenia. Analyses of DOI-induced increases in sEPSCs (spontaneous excitatory postsynaptic currents) were performed as complementary experiments to the DOI-induced head twitch studies. Haloperidol-induced catalepsy and the rotarod test were used to examine the adverse effects of the drugs.

All three activators of muscarinic receptors were active in DOI-induced head twitches. When administered together with GS39783 in subeffective doses, only the co-administration of VU0152100 and GS39783 was effective. The combination also reduced the frequency but not the amplitude of DOI-induced sEPSCs. Neither VU0357017 nor VU0238429 were active in social interaction test when given alone, and also the combination of VU0152100 and GS39783 failed to reverse MK-801-induced deficits observed in this test. All muscarinic activators when administered alone or in combination with GS39783 reversed the MK-801-induced disruption of memory in the novel object recognition test, and their actions were blocked by specific antagonists. None of the tested compounds or their combinations influenced the motor coordination of the animals. The compounds had no effect on haloperidol-induced catalepsy and did not induce catalepsy when administered alone. Pharmacokinetic analysis confirmed lack of possible drug-drug interactions after combined administration of GS39783 with VU0357017 or VU0152100; however, when the drug was co-administered with VU0238429 its ability to pass the blood-brain barrier slightly decreased, suggesting potential drug-drug interactions.

Our data show that modulation of cholinergic and GABAergic systems can potentially be beneficial in the treatment of the positive and cognitive symptoms of schizophrenia without inducing the adverse effects typical for presently used antipsychotics.

1. Introduction

The exact etiology and pathogenesis of schizophrenia are still not well understood as schizophrenia is a complex and heterogeneous disease in which the intensity and prevalence of positive, negative and cognitive symptoms vary among patients and may result in different responses to treatment [1]. Schizophrenia has a huge impact on the mental abilities, social life and lifespan of patients, and, as a result, approximately 80–90% of schizophrenic patients are unemployed [1].

The mechanism of action of currently prescribed antipsychotics is predominantly based on the blockade of dopaminergic D_2 receptors. Although the drugs are quite effective in reducing positive symptoms of

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Table 1
Data concerning the basic in vitro potency, selectivity, and central penetrance of the compounds used in the present studies. n/a = not available.

		In vitro potency	selectivity	central penetrance
VU0357017	M_1 allosteric agonist	overall efficacy measured by Ca^{2+} mobilization assay: % CChmax = 41.7 ± 2.37 [34] EC50 = 198 ± 13.2 nM and AChmax = 80.52% ± 7.67% max [56]	very small responses at D_4 dopamine receptors and β_3-adrenergic receptors [34]	readily cleared from the CNS, but displays good pharmacokinetic properties with a log BB = 1.46 and a 4:1 brain/plasma ratio (4338.90 ± 1071.20 ng/mL to 1053.49 ± 276.03 ng/mL) [56]
VU0152100	M_4 positive allosteric modulator	EC_{50} = 380 ± 93 nM (in presence of an Ach ED20) [9]	devoid of significant activity at all targets included in LeadProfilingScreen® by MDS Pharma [9]	log P = 3.6; AUC brain/AUC plasma ratio = 0.86 ± 0.08; half-life = 1.12 ± 0.01 h [9]
VU0238429	M_5 positive allosteric modulator	EC50 = 4.9 μM and 80% ACh maximum response [33] 14-fold leftward shift of Ach CRC; 10-fold increase in ACh affinity for M5 at 30 μM [33]	mAChR selectivity ≫30 μM vs. M1–M4 [33]	maximal plasma concentration = 161.7 ng/mL (achieved within 1 h); elimination half-life = 4.7 h; AUCbrain/ AUCplasma value of 0.25 [33]
GS39783	$GABA_B$ positive allosteric modulator	at 30 μM increased of both the potency (maximally about an 8-fold increase) as well as the maximal intrinsic efficacy (about a 2.2-fold increase) of GABA [57]	No CEREP or LeadProfilingScreen® by MDS Pharma data	n/a
CGP7930	$GABA_B$ positive allosteric modulator	at 30 and 100 μM, increased the potency of (−)-baclofen by 2.2- and 3.0-fold, respectively (pEC50: 4.75 ± 0.05, and 4.87 ± 0.09, respectively) [58]	partial $mGlu_5$ agonists for IP_1 accumulation, but not iCa^{2+} mobilization [63]	n/a
VU0255035	M_1 antagonist	IC50 = 309.1 ± 100.5 nM or IC50 of 132.6 ± 28.5 nM at M1 [60]	VU0255035 Ki - nM M1: 14.87 ± 0.66; M2: 661.33 ± 57.64; M3: 876.93 ± 151.44; M4: 1177.67 ± 124.42; M5: 2362.33 ± 577.49 [59] devoid of significant activity at all targets included in LeadProfilingScreen [59]	Tmax = 0.5 h; brain/plasma (AUC for the 8-h dosage) = 0.48; Cmax = 1307.89 ± 327.69 ng/ml for plasma and 251.32 168.32 ng/g for the brain; elimination half-life (T1/2) 1.29 h for the plasma and 2.58 h for the brain [59]
Tropicamide	M_4-preferring antagonist	Small degree of M4 selectivity [60]	pKi (nM): M1: 7.08 ± 0.04; M2: 7.19 ± 0.10; M3: 6.99 ± 0.07; M4: 6.86 ± 0.12; M5: 6.42 ± 0.14 [61]	n/a
VU6008667	M_5 negative allosteric modulator	human M5 IC50 = 1.2 μM, pIC50 = 5.93 ± 0.02, 2.3 ± 0.03 % ACh min and rat M5 IC50 = 1.6 μM, pIC50 = 5.78 ± 0.02, 2.6 ± 0.03 % ACh min [62]	selective for M_5 over M_{1-4} no CEREP or LeadProfilingScreen® by MDS Pharma data	elimination half-life (t1/2 = 2.3 h) volume (Vss = 7.4 L/kg) and higher clearance (CLp = 82 mL/min/kg), oral bioavailability (17% F) [62]

schizophrenia, their efficacy towards negative and cognitive symptoms is still not satisfactory. Moreover, chronic treatment may induce a wide range of adverse effects, such as extrapyramidal motor effects or elevated prolactin levels, which result from D_2 receptor inhibition in the striatum and hypothalamus [2,3]. Hence, there is a need to develop a new therapeutic strategy that would effectively ameliorate schizophrenia symptoms without exerting adverse effects that contribute to treatment cessation.

A growing body of evidence suggests that the stimulation of metabotropic receptors for glutamate, acetylcholine or γ-aminobutyric acid (GABA) may be considered as promising targets in antipsychotic drug discovery [4,5]. Xanomeline, a nonselective, M_1/M_4-preferring muscarinic receptor orthosteric agonist, has been shown to improve positive, negative and cognitive symptoms in schizophrenic patients just after one week of treatment [6]. However, due to gastrointestinal side effects, the study has been discontinued [7]. This finding proves that targeting muscarinic receptors might be beneficial in treating schizophrenia but also highlights the need to develop receptor-specific compounds or to use lower doses.

Recently, a number of selective muscarinic ligands were synthetized, of which those targeting the M_1, M_4 and M_5 receptor seem to be the most important for the treatment of various CNS disorders, including schizophrenia. Various modulators of the M_4 receptor have been shown to reverse amphetamine-induced hyperlocomotion, reduce the conditioned avoidance response and reverse the suppression of the acoustic startle response [8–11]. In our previous experiments, VU0152100 (PAM of M_4 receptor) was shown to reverse several schizophrenia-related changes in animals [12]. Furthermore, simultaneous stimulation of $mGlu_4$ and M_4 receptors were shown to potentially have an additive effect [12].

The current study is a follow-up study concerning the putative synergistic action of the ligands that activate receptors that regulate the neuronal network disrupted in schizophrenia [12–15]. Our investigations are based on the glutamatergic theory of schizophrenia, which assumes that excess glutamate is responsible for its development [16,17]. Therefore, treatments aimed at reducing the release of excess glutamate may be considered to be effective antipsychotic formulations. $GABA_B$ and muscarinic receptors have been shown to be expressed on the axon terminals of glutamatergic neurons and to be involved in the regulation of glutamate release and inhibition of glutamate efflux after stimulation [4,18–22]. Therefore, we hypothesized that the simultaneous administration of $GABA_B$ and mACh receptor activators may have an additive antipsychotic effect, which will allow for the doses and use of each compound to be minimized, thus reducing the risk of the development of putative adverse effects.

Here, several combinations of $GABA_B$ and mACh receptor ligands were investigated in various behavioral models of schizophrenia and electrophysiological studies. The rotarod test and haloperidol-induced catalepsy were used to assess the influence of drugs on the typical adverse effects observed after antipsychotic treatment. Also, to exclude the possibility that the observed effects could be due to simple drug-drug interactions resulting in altered metabolism of one or both administered compounds, pharmacokinetic analysis were also performed.

2. Materials and methods

2.1. Animals and housing

Male Albino Swiss mice (20–25 g, Charles River, Germany) were used in all experiments. The animals were kept in a room with a 12:12-h light-dark cycle at a temperature of 21–22 °C with free access to food and water. The experimental groups consisted of 5–10 animals. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. All procedures were conducted according to the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the II Local Ethics Committee by the Institute of Pharmacology, Polish Academy of Sciences in Krakow. The results were calculated by an experimenter blinded to the treatment.

2.2. Drugs

All drugs used, their in vitro potency, selectivity and brain penetration were collected in Table 1. VU0152100, VU0357017, VU0238429, GS39783, CGP7930, VU0255035, tropicamide, DOI and MK-801 were purchased from Tocris Bioscience, Bristol, United Kingdom. VU06008667 was purchased from Axon Medchem (Groningen, The Netherlands). Haloperidol was purchased from WZF Polfa S.A. VU0152100 was dissolved in 10% Tween 80, VU0357017, MK-801, and DOI were dissolved in 0.9% NaCl. Haloperidol and risperidone were dissolved in 0.2% Tween 80. GS39783 and CGP7930 were dissolved in a small amount of EtOH (final concentration 1%) and then titrated in 0.2% Tween 80. VU0255035, tropicamide, VU06008667 were dissolved in small amount of DMSO (final concentration of DMSO was 2%) and then were adjusted with 10% Tween 80 to proper volume. Animals not treated with drugs (control groups) received appropriate vehicles. Doses used in all behavioral experiments were based on our previous studies, for $GABA_B$ ligands please see [4,13,15], for VU0152100 [9,12,23]. The doses of VU0357017 and VU0238429 were based upon pilot studies.

2.3. Head-twitch test

The experiment was performed according to the methods described by Wierońska et al. [24,25]. For habituation to the experimental environment, the animals were transferred to a 12 (diameter) × 20-cm (height) glass cylinder lined with sawdust 30 min before the experiment. Head twitches were induced by i.p. injection of DOI (2.5 mg/kg). Immediately after the treatment, the number of head twitches was counted for 20 min. VU0152100 was administered at a dose of 0.2 mg/kg and GS39783 at a dose of 0.1 mg/kg 30 min before DOI administration.

2.4. DOI-induced EPSCs

To investigate the effects of GS39783/VU0152100 on spontaneous excitatory postsynaptic currents (sEPSCs), voltage-clamp recordings were acquired from layer 5 cortical cells in the presence of 10 μM DOI. All recorded cells (n = 11) had electrophysiological characteristics of regular spiking pyramidal neurons (tested in current clamp) [26]. Their mean resting membrane potential (RMP) was −73 ± 5 mV, and the mean input resistance (R_{in}) was 268 ± 28 MΩ. The mean basal frequency of spontaneous synaptic activity was 2.7663 ± 0.3 Hz, and its

Table 2
Mass spectrometric parameters for multiple reaction monitoring. Q1 quadrupole 1, Q3 quadrupole 3, DP- declastering potencial, CE - Collision energy, CXP - Collision exit potential.

Compound	Q1 mass (Da) Precursor ion	Q3 mass (Da) Product ion	DP (volts)	CE (volts)	CXP (volts)
GS 39783	338.09	202.00*	146	37	18
GS 39783	338.09	185.10	146	47	16
IS GS 39783	307.16	154.00	91	47	14
IS GS 39783	307.16	171.00	91	33	16
VU 0152100	342.92	206.00*	106	33	18
VU 0152100	342.92	205.00	106	33	16
IS VU0152100	446.78	310.00	121	31	28
IS VU0152100	446.78	162.00	121	47	14
VU0238429	351.62	121.00*	26	21	10
IS VU0238429	367.02	121.00	91	33	10
IS VU0238429	367.02	78.00	91	91	8
VU0357017	334.06	162.10*	131	29	14
VU0357017	334.06	91.00	131	73	8

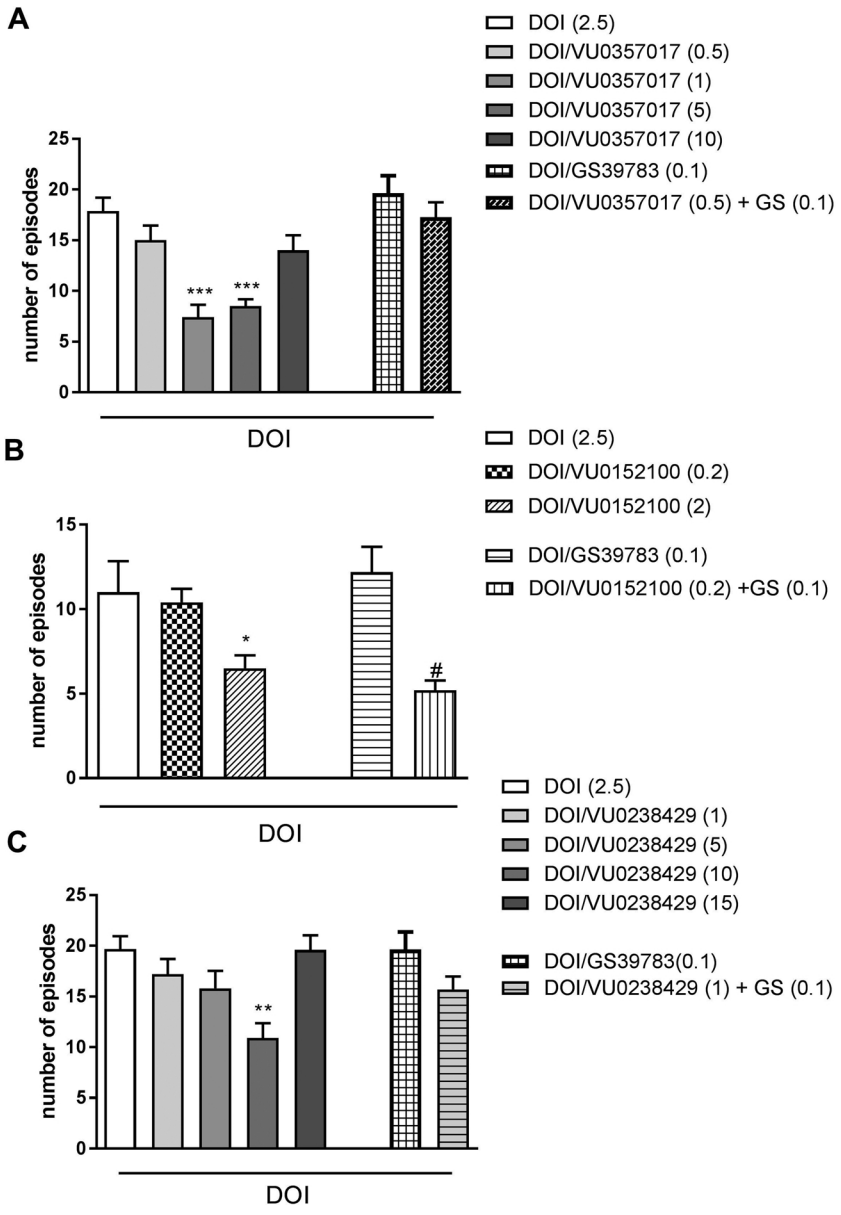

Fig. 1. DOI-induced head twitches in mice. The effects of VU0357017 (A), VU0152100 (B) and VU0238429 (C) alone or in combination with GS39783 on DOI-iduced head twitches. The compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. $^{\#}$p and *p < 0.05 **p < 0.01 and ***p < 0.001 vs DOI-treated group. N = 8–10/group.

mean amplitude was 9.4259 ± 0.72 pA. Spontaneous postsynaptic currents were blocked by the non-NMDA glutamatergic receptor antagonist CNQX (5 μM; n = 6, data not shown), indicating that these spontaneous currents represented excitatory currents. Drugs kept as concentrated stocks were diluted in ACSF just before the experiment and applied in the superfusate. After a stable baseline was recorded for at least 15 min, DOI (10 μM) was applied for 15 min, and sEPSCs were recorded (8 min). Next, DOI was applied concurrently with VU0152100 and GS39783 for 15 min, and sEPSCs were recorded again. The frequency and amplitude of sEPSCs were measured. The data were analyzed off-line using the Mini Analysis program (Synaptosoft Inc. ver. 6.0.3).

2.5. Social interaction test

The method was adapted from de Moura Linck et al. and Woźniak et al. [13,27]. Body weight-matched (± 10%) mice from separate housing cages were paired for the study. The animals were habituated and tested in a dark room in a black plastic rectangular open field (40 × 30 × 35 cm) illuminated with the light intensity of 335 lx. For the habituation trial, each mouse was placed individually in the open field and was allowed to explore the environment for 10 min per day for 2 days. In the test trial, each mouse pair was placed in the open field for 5 min. The social interactions between the two mice were analyzed based on the total time spent participating in social behavior such as genital investigation, sniffing, chasing and fighting each other. The total number of social episodes was also measured. The test was video-recorded and viewed by a trained observer blinded to the treatment. VU0152100 (0.5 mg/kg, i.p.), GS39783 (0.1 mg/kg, i.p.), CGP7930 (0.5 mg.kg, i.p.), VU0357017 (1, 5 and 10 mg/kg; i.p.), VU0238429 (1, 5 and 10 mg/kg; i.p.) and/or risperidone (0.1 mg/kg, i.p.) were administered 30 min before MK-801 (0.3 mg/kg, i.p.), which was administered 30 min before the test.

2.6. Novel object recognition test

The experiment was performed according to Nilsson et al. [28] with minor modifications [14]. The mice were habituated, trained and tested in a dark room in a black plastic rectangular open field (40 × 30 x 35 cm) illuminated with the light intensity of 335 lx. For the habituation trial, each mouse was placed individually in the open field in the absence of objects and was allowed to explore the environment for 10 min per day for 2 days. A training trial and test trials were conducted 24 h after the habituation trial. In the training trial (T_1), mice were allowed to explore two identical objects (red glass cylinder; 6.5 cm in diameter and 4.5 cm high) for 5 min. One hour later, a test trial (T_2) was conducted, where one of the familiar objects was replaced by a novel object (a transparent glass elongated sphere-like object with an orange cap; 5.5 cm in diameter and 8.5 cm high). The animals were allowed to explore the objects for 5 min. VU0152100 (0.25; 0.5 mg/kg, i.p.), GS39783 (0.1 mg/kg, i.p.), CGP7930 (0.1 mg/kg, i.p.), VU0357017 (1, 5 and 10 mg/kg; i.p.), VU0238429 (5, 10 and 20 mg/kg, i.p.) and/or risperidone (0.1 mg/kg; i.p.) were administered 30 min before MK-801 (0.3 mg/kg, i.p.), which was administered 30 min before T_1. Appropriate antagonists were administered 15 min before the activator (45 min before MK-801). The time spent exploring (i.e., sniffing or touching) the familiar ($T_{familiar}$) or novel object (T_{novel}) was measured by a trained observer, and then the recognition index [%] was calculated for each mouse $[(T_{novel} - T_{familiar})/(T_{familiar} + T_{novel})]*100$.

2.7. Rotarod test

The rotarod test was performed as described by Sałat et al. [29] with small modifications. The animals were trained on the rotarod at 18 rpm in one 3-min session per day for 3 consecutive days. If a mouse fell during the habituation period, it was placed back on the apparatus. On the following day, the test trial was performed. After the mice were placed on the apparatus (Mouse Rota-Rod NG, UGO BASILE S.R.L.) moving at the speed of 12 rpm, the accelerating mode was started (maximum speed: 24 rpm). The latency to fall was measured during a 3-min test session. Mice were injected with the investigated compounds 30 min before the test trial.

2.8. Haloperidol-induced catalepsy

The experiment was performed according to the methods described by Wierońska et al. [15]. All groups received haloperidol (1 mg/kg, i.p). VU0152100 (0.5 mg/kg), GS39783 (0.1 mg/kg) or risperidone (0.1 mg/kg) were administered 30 min before haloperidol (1 mg/kg). Catalepsy was measured 45 min and 90 min after haloperidol administration. For each measure of cataleptic state, animals were gently placed with their forepaws on a metal rod suspended 5.5 cm above ground, and the time elapsed before the mice climbed down from the bar was recorded in seconds (3 trials with a cutoff time of 180 s).

2.9. Statistical analysis

Statistical analysis was performed using Statistica 12 package (StatSoft Inc., OK, USA). A two-way ANOVA followed by Newman-Keuls *post hoc* test was used to determine the significance of the behavioral test results, and Student's *t*-test was used in the analysis of the electrophysiological data. Data are presented as the mean ± SEM.

2.10. Internal standards synthesis (IS)

All substrates used were purchased in Tocris a Bio-Techne brand. ^{1}H NMR spectra were recorded on a Varian Unity Plus 500 apparatus. MS analyses were performed on a QTRAP 4500 (AB SCIEX, USA) in positive ion mode with electrospray ionization. Reaction courses and purity of products were monitored by thin-layer chromatography (TLC) on Merck Kieselgel 60 F254 plates and visualized with UV.

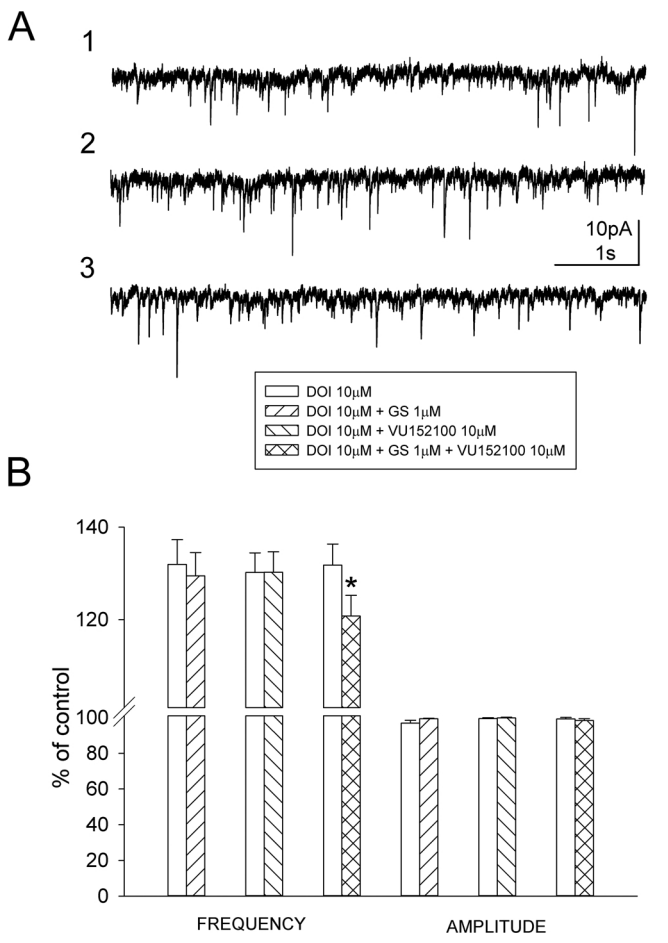

Fig. 2. Effect of combined administration of VU0152100 and GS39783 on DOI-induced spontaneous EPSCs. (A) Examples of recordings from a representative neuron: (1) baseline activity, (2) recording obtained after a 10 min incubation with DOI, and (3) recording obtained after a 10 min incubation with VU152100/GS39783 in the presence of DOI (10 μM). Effects of GS39783 (1 μM) with VU0152100 (10 μM) administration on DOI-induced increase in sEPSCs frequency and amplitude. Data are presented as the means ± SEM. *p < 0.0001 vs. the DOI-incubated group. N = 11.

2.10.1. 3-Benzamido-N-(4-methoxybenzyl)-4,6-dimethylthieno[2,3-b] pyridine-2-carboxamide (IS VU0152100)

Title compound was synthesized according to the method previously described [30] with modifications. Briefly, to a solution of 3-amino-*N*-(4-methoxybenzyl)-4,6-dimethylthieno[2,3-*b*]pyridine-2-carboxamide (49.8 μmol, 17 mg) in dry tetrahydrofuran (0.45 mL) and dry triethylamine (104 μmol, 14.5 μL), benzoyl chloride (81 μmol, 9.4 μL) was added, and reaction mixture was stirred for 5 h at 70 °C. The reaction mixture was left in refrigerator for 2 days. The solid was collected by filtration, washed with tetrahydrofuran (2 × 0.25 mL) and dried. The product was crystallized from ethanol giving the title compound (11.9 mg, 54%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.50 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 3.68 (s, 3H, CH_3), 4.36 (d, J = 5.8 Hz, 2H, CH_2), 6.70 (d, J = 8.5 Hz, 2H, arom.), 7.12 (d, J = 8.8 Hz, 2H, arom.), 7.15 (s, 1H, arom.), 7.54 (t, J = 7.7 Hz, 2H, arom.), 7.64 (d, J = 7.3 Hz, 1H, arom.), 7.93 (d, J = 7.4 Hz, 2H, arom.), 8.49 (t, J = 5.4 Hz, 1H, NH), 10.29 (s, 1H, NH) ppm; MS: calcd. ($C_{25}H_{23}N_3O_3S$) $[M+H]^+$ 446.15, found 446.78.

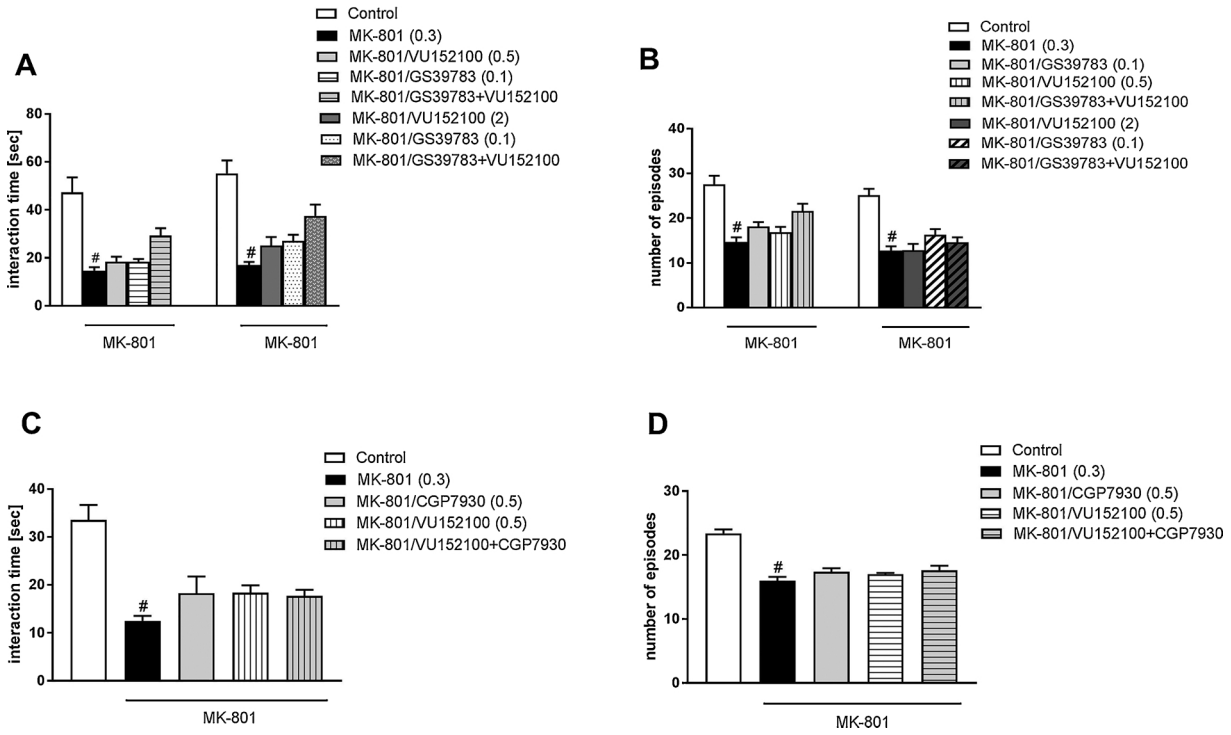

Fig. 3. Effect of the combined administration of VU0152100 and GS39783 (A, B) or CGP7930 (C, D) on MK-801-induced deficits in social interaction as measured by social interaction duration and number of episodes. The compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. $^{\#}p < 0.05$ vs. the control group. N = 8–10/group.

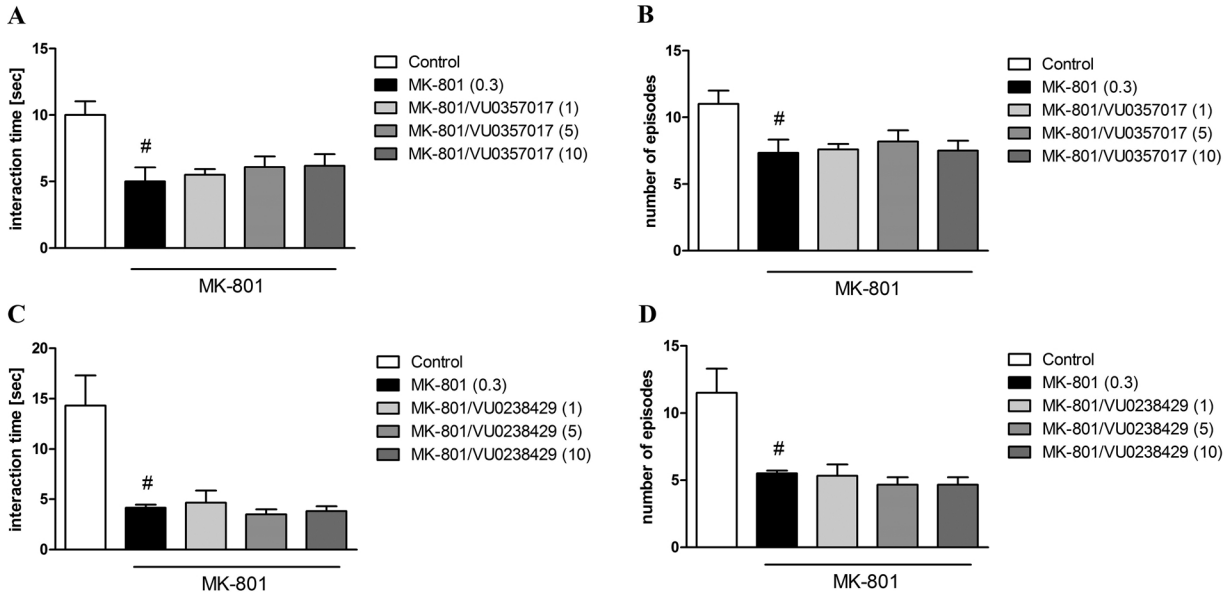

Fig. 4. Effect of VU0357017 (A, B) and VU0238429 (C, D) on MK-801-induced deficits in social interaction as measured by social interaction duration and number of episodes. Both compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the mean ± SEM. $^{\#}p < 0.05$ vs. the control group. N = 8–10/group (A, B); N = 6/group (C, D).

2.10.2. N^4,N^6-dicyclopentyl-5-nitropyrimidine-2,4,6-triamine (IS GS39783)

A solution of N^4,N^6-dicyclopentyl-2-(methylthio)-5-nitropyrimidine-4,6-diamine (74 µmol, 25 mg) in a mixture of *p*-dioxane (3 mL) and NH_4OH (10 mL, 30%) was heated in a pressure tube at 110 °C for 50 h. The solid thus formed after cooling to room temperature was separated by filtration, washed with water (2 x 1 mL) and dried. Synthesis was performed based on previously described method [31] with modifications. The product was crystallized from ethanol giving the title compound (9.5 mg, 42%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.43–1.50 (m, 2H, CH_2), 1.53–1.60 (m, 2H, CH_2), 1.64–1.72 (m, 2H, CH_2), 1.94–2.00 (m, 2H, CH_2), 4.38–4.44 (m, 1H, CH), 7.09 (s, 1H, NH), 9.40 (d, 1H, NH) ppm; MS calcd. ($C_{14}H_{22}N_6O_2$) $[M+H]^+$ 307.18, found. 307.16.

2.10.3. 3-(Hydroxyimino)-1-(4-methoxybenzyl)-5-(trifluoromethoxy) indolin-2-one (IS VU0238429)

Compound was synthesized based on previously described method [32] with modifications. Briefly, a solution of 1-(4-methoxybenzyl)-5-

(trifluoromethoxy)indoline-2,3-dione (71 μmol, 25 mg), hydroxylamine hydrochloride (100 μmol, 6.9 mg) and sodium acetate (89 μmol, 12 mg) in a mixture of ethanol (0.5 mL) and water (0.5 mL) was stirred at room temperature for 18 h. The reaction mixture was left in refrigerator overnight, then a precipitate was collected by filtration and dried. In this manner pure title compound (22.1 mg, 81%) was obtained: ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.70 (s, 3H, CH_3), 4.88 (s, 2H, CH_2), 6.88 (d, J = 8.6 Hz, 2H, arom.), 7.10 (d, J = 8.8 Hz, 1H, arom.), 7.28 (d, J = 8.7 Hz, 2H, arom.), 7.42 (d, J = 8.5 Hz, 1H, arom.), 7.89 (s, 1H, arom.), 13.87 (s, 1H, OH) ppm; MS calcd. ($C_{17}H_{13}F_3N_2O_4$) $[M+H]^+$ 367.09, found 367.02.

2.11. Evaluation of drug concentration in plasma and brain

The experiment was performed according to the methods described by Bridges et al. [33].

Each compound was administered alone at the highest dose tested and in combination with GS39783. Animals were decapitated, the brains were removed, thoroughly washed in 0.9% saline and immediately frozen on dry ice. Blood was collected in 5% EDTA and plasma was separated by centrifugation at 3500 rpm for 15 min at 4 °C and stored at −80 °C until analysis. Seven to ten animals were used for each group.

On the day of analysis, frozen whole-mouse brains were weighed and homogenized in 1:2 (w/v) volumes of ice-cold acetonitrile containing 0.1% FA and internal standard mix. Sample were homogenized using a Bio-Gen PRO200 homogenizer 220v at min speed for 1 min, vortexed, kept on ice for 20 min and followed by centrifugation at 14,000 rpm for 20 min at 4 °C. Finally 50 μl of supernatant was diluted with 0.1% FA in water (1:1, v/v) and was analyzed by means of HPLC/MS/MS.

The sample extraction of plasma (20 μl) was performed after spiking with internal standard mix by a method of protein precipitation, using three volumes of ice-cold acetonitrile containing 0.1% formic acid. Extract was vortex mixed, kept on ice for 20 min and followed by centrifugation at 14,000 rpm for 20 min at 4 °C. The supernatant was diluted with 0.1% FA in water (1:1, v/v) and analyzed by means of HPLC/MS/MS using a QTRAP 4500 (AB Sciex) mass spectrometer in positive ion mode in Multiple Reaction Monitoring (MRM screening) coupled with UHPLC (NEXERA XR, Shimadzu). The chromatographic separation was achieved on Synergi 4 μm Fusion-RP 80 A 50 x 2 mm (Phenomenex) column at flow rate of 0.2 ml/min. The gradient program was as follows: 20% B (0.3 min), 20–100% B (1.7 min), 100% B (3 min), 100 – 20% B (0.1 min), 20% B (2 min), solvent A (95: 5: 0.1% formic acid in water: metanol) and solvent B (acetonitrile with 0.1% formic acid). The column temperature was set at 40 °C. The software Analyst was used to control the instrument and collect data. The electrospray ionization source was fitted with a stainless steel capillary (100 μm i.d.). The ion transfer tube temperature was 300 °C. The spray voltage, collision energy, declastering potential and gas parameters were optimized to achieve maximal response using the test compounds. Selected reaction monitoring was carried out using the transitions parameters presented in Table 2. For all compounds two transition ion were selected: prime (*) was used for quantification and second for confirmation, only for VU0238429 we were not able to detect second ion at any concentration level, because of his low ionization proficiency. For VU0357017, IS VU0152100 was used as internal standard. Sample concentration of Internal standard was 1 ng/ml for IS GS39783, 100 ng/ml for IS VU0238429 and 50 ng/ml for IS VU0152100. The calibration curves were constructed and linear response was obtained in the range of 0.25–500 ng/ml (VU0152100 and VU357017), 0.15–300 ng/ml (GS39783) and 1.25–2500 ng/ml (VU0238429) by spiking known amounts of each compound in blank brain homogenates and plasma.

3. Results

3.1. Head-twitch test

All muscarinic receptors PAMs reversed DOI-induced head twitches. VU0152100 was active at the dose of 2 mg/kg ($F_{(2.13)}$ = 4.41; $p < 0.03$), VU0357017 was active at the doses 1 and 5 mg/kg ($F_{(4.47)}$ = 12.74; $p < 0.0001$) and VU0238429 was active at the dose 10 mg/kg ($F_{(4.29)}$ = 5.44; $p < 0.002$) (Fig. 1A–C). VU0152100 or GS39783 administered at the doses of 0.2 and 0.1 mg/kg, respectively, had no effect on DOI-induced head twitches when given alone (Fig. 1B). However, co-administration of both substances resulted in a reduction in DOI-induced head twitches by approximately 50% ($F_{(1.16)}$ = 6.16; $p < 0.05$) (Fig. 1B). The combinations of VU0357017 or VU0238429 administered at sub-effective doses (0.5 and 1 mg/kg) with GS39783 failed to reverse DOI-induced heat twitches (Fig. 1A and C).

3.2. DOI-induced EPSCs

The application of DOI (10 μM) increased the mean sEPSC frequency to about 136 of baseline but did not affect the mean sEPSC amplitude. GS39783 (1 μM) and VU0152100 (10 μM) were applied concurrently with DOI (10 μM). The compounds had no effect when applied alone (GS39783: n = 9, t = 1.714; df = 8; p = 0.125 and VU0152100: n = 9; t = -0.0455; df = 8; p = 0.965) (Fig. 2A). The mix of compounds reversibly suppressed the DOI-induced increase in frequency but did not affect the mean amplitude of the sEPSCs (n = 11; t = 6.461; df = 10; $p < 0.0001$) (Fig. 2B).

3.3. Social interaction test

MK-801 at a dose of 0.3 mg/kg significantly decreased both the duration of social interactions and the number of episodes. VU0152100 administered at the doses of 0.5 or 2 mg/kg in combination with GS39783 administered at a dose of 0.1 mg/kg, failed to reverse the MK-

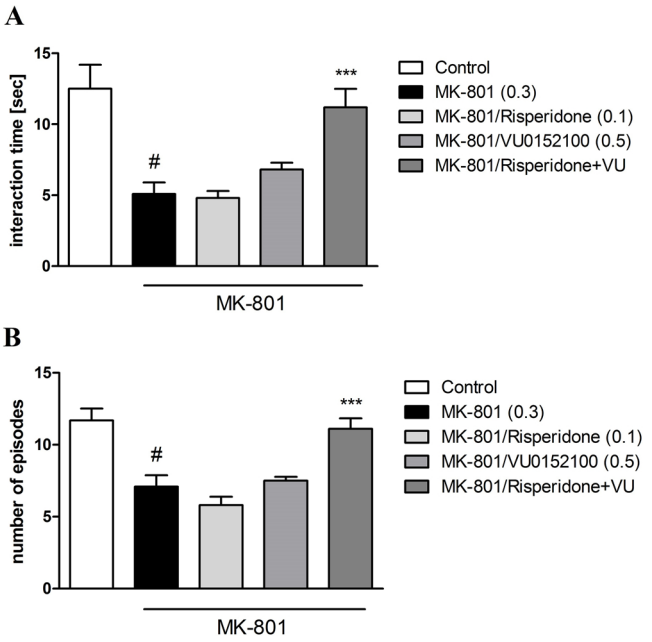

Fig. 5. Effect of the combined administration of VU0152100 and risperidone on MK-801-induced deficits in social interaction as measured by social interaction duration (A) and number of episodes (B). Both compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. $^{\#}p < 0.05$ vs. the control group; *** $p < 0.001$ vs. the MK-801-treated group. N = 8–10/group.

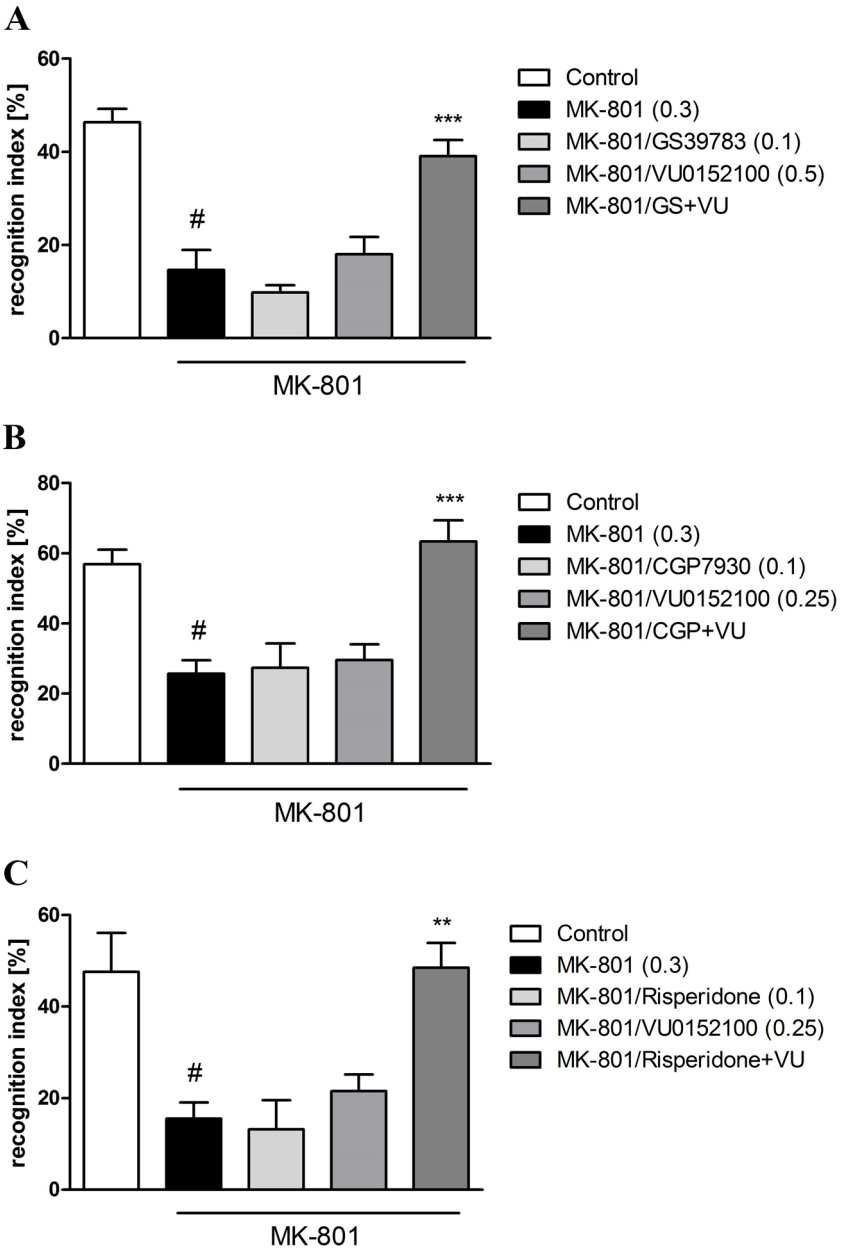

Fig. 6. Effect of the combined administration of VU0152100 and GS39783 (A) or CGP7930 (B) or risperidone (C) on MK-801-induced deficits in novel object recognition. The compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. # $p < 0.05$ vs. the control group; *** $p < 0.001$ and **$p < 0.01$ vs. the control group. N = 8–10/group.

801-induced deficits in both measured parameters ($F_{(1.35)} = 2.92$; $F_{(1.35)} = 0.26$) (Fig. 3A, B). The combination of VU0152100 with another $GABA_B$ receptor PAM, CGP7930, also failed to reverse the MK-801-induced disruptions in social interactions ($F_{(1.30)} = 0.22$ and $F_{(1.30)} = 0.04$) (Fig. 3C, D).

Allosteric activators of M_1 and M_5 receptors (VU0357017 and VU0238429) had no effect on the MK-801-induced disruptions in the duration and number of social interactions (VU0357017: time of interaction $F_{(3.35)} = 0.46$ and number of episodes $F_{(3.35)} = 0.24$; VU0238429: interaction duration $F_{(3.20)} = 0.49$ and number of episodes $F_{(3.20)} = 0.55$) (Fig. 4A–D).

The concomitant administration of ineffective doses of VU0152100 (0.5 mg/kg) and risperidone (0.1 mg/kg) potentiated each other's action (interaction duration $F_{(1.31)} = 5.7813$, $p < 0.001$; number of episodes $F_{(1.31)} = 12.71$, $p < 0.001$) (Fig. 5A, B).

3.4. Novel object recognition test

MK-801 induced a disruption in novel object recognition, measured as the recognition index. VU0152100 or GS39783 administered at the ineffective doses of 0.25 and 0.1 mg/kg, respectively, failed to reverse the MK-801-induced deficits in memory. Concomitant administration of VU0152100 and GS39783 significantly reversed the MK-801-induced effect ($F_{(1.36)} = 14.32$; $p < 0.001$) (Fig. 6A). The second $GABA_B$ receptor PAM, CGP7930, also reversed the novel object recognition deficits caused by MK-801 ($F_{(1.28)} = 9.0547$; $p < 0.001$) (Fig. 6B).

VU0357017, a muscarinic M_1 receptor partial allosteric agonist, dose-dependently reversed the novel object recognition deficit induced by MK-801 ($F_{(3.35)} = 40.13$; $p < 0.001$), and its concomitant administration at a sub-effective dose with GS39783 also had a positive effect ($F_{(1.36)} = 8.27$; $p < 0.001$) (Fig. 7A, B).

VU0238429 reversed the MK-801 induced disruptions in memory at

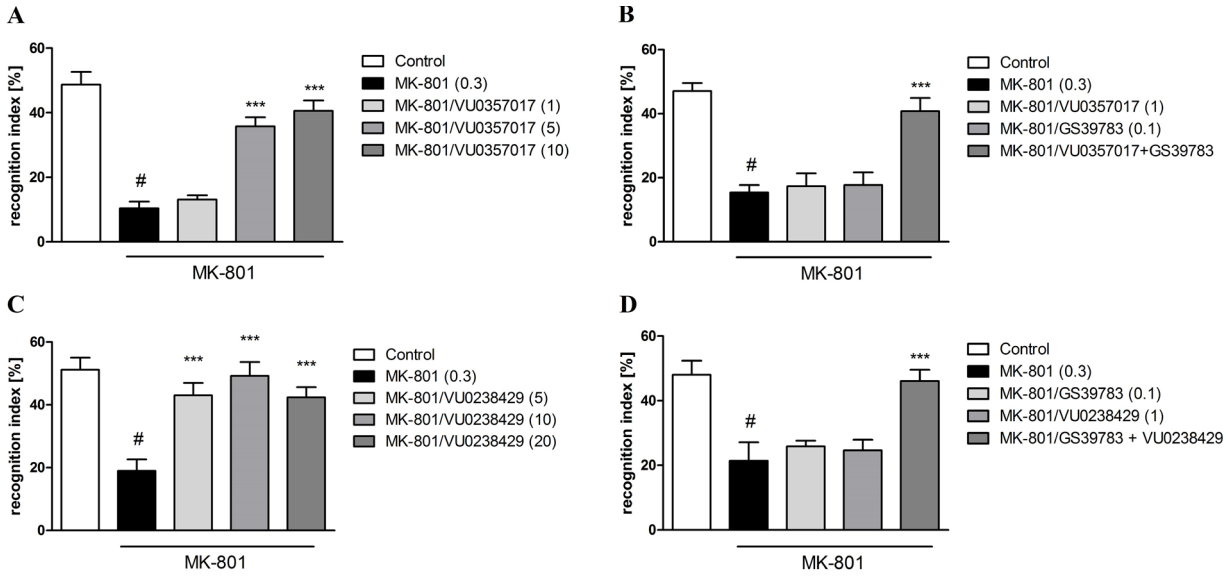

Fig. 7. Effect of VU0357017 and VU0238429 when administered alone (A, C) or concomitantly with GS39783 (B, D) on MK-801-induced deficits in novel object recognition. The compounds were administered 30 min before MK-801 injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. # $p < 0.05$ vs. the control group; ***$p < 0.001$ vs. the control group. N = 10/group (A–C); N = 8–10/group (D).

all tested doses ($F_{(3.36)} = 12.09$; $p < 0.001$), and the same effect was observed when a sub-effective dose was administered concomitantly with GS39783 ($F_{(1.34)} = 4.47$; $p < 0.001$) (Fig. 7C, D).

The administration of ineffective doses of VU0152100 and risperidone also reversed the MK-801-induced disruption in novel object recognition ($F_{(1.20)} = 6.33$; $p < 0.01$) (Fig. 7C).

The action of all three activators (VU0357017, VU0152100, VU0238429) was blocked the antagonists of particular receptors (VU0255035, M_1 antagonist), tropicamide (M_4 antagonist) and VU06008667 (M_5 negative allosteric modulator), which had no effect when given alone (Fig. 8A–C). Two-way ANOVA followed by Neuman-Keuls *post hoc* comparison revealed the significant effect of VU0357017xVU0255035 interaction [$F_{(1.34)} = 5.62$, $p < 0.02$], VU0152100xTropicamide interaction [$F_{(1.29)} = 10.33$, $p < 0.003$] and VU0238429xVU06008667 interaction [$F_{(1.27)} = 21.61$, $p < 0.0001$].

3.5. Motor coordination

In the rotarod test, the combination of VU0238429 with risperidone (0.1 mg/kg), and the combination of VU0357017 with haloperidol (0.2 mg/kg) induced detectable motor impairments when compared to control treatment ($F_{(4.35)} = 3.62$; $p < 0.05$ for all) (Fig. 9B). The other drugs and their combinations had no effect on rotarod performance (Fig. 9A, C).

3.6. Haloperidol-induced catalepsy

Neither the M_1 nor M_5 muscarinic receptor ligand had any effect on haloperidol-induced catalepsy measured 45 min or 90 min after haloperidol administration (VU0357017: 45 min $F_{(2.19)} = 2.25$ and 90 min $F_{(2.19)} = 2.41$; VU0238429: 45 min $F_{(2.19)} = 0.72$ and 90 min $F_{(2.19)} = 2.14$) (Fig. 10A–D). VU0152100 (0.5 mg/kg) when administered concomitantly with GS39783 (0.1 mg/kg) also had no effect on haloperidol-induced catalepsy, neither 45 min ($F_{(2.17)} = 0.40$) nor 90 min ($F_{(2.17)} = 0.48$) after haloperidol injection (Fig. 11A, B).

3.7. Evaluation of drug concentration in plasma and brain

We evaluated drug exposure levels in plasma and brain for each compound separately at the highest dose used and in combination with GS39783 at sub-effective dose. The highest level of brain penetration was observed for GS39783 (5 mg/kg), where brain concentration was 7.3 times higher than in plasma. VU0357017 (10 mg/kg) showed similar brain and plasma exposure levels, suggesting good blood-brain barrier penetration. Both, VU0152100 (1 mg/kg) and VU0238429 (20 mg/kg) showed poor brain accumulation, giving values 8–12% of plasma concentration (Fig. 12A). GS39783 (0.1 mg/kg) retained his high brain penetration capacity in combination with VU0152100 (0.5 mg/kg) and VU0357017 (1 mg/kg). However, when combined with VU0238429 (1 mg/kg) lost 50% of this ability, without improving M_5 agonist brain penetration. VU0152100 retained his properties of brain penetration when administered concomitantly with GS39783 (0.1 mg/kg) and VU0357017 (1 mg/kg) in combination with $GABA_B$ receptor shown slightly less brain accumulation than administrated independent (Fig. 12B).

4. Discussion

In the present studies, we demonstrated that the simultaneous administration of sub-effective doses of allosteric ligands of muscarinic and $GABA_B$ receptors effectively reversed MK-801-induced disruptions in some schizophrenia-related behaviors without inducing adverse effects typical for currently used antipsychotics. This is a follow-up study of several earlier publications that have been released by our group that focused on investigations on whether the simultaneous administration of sub-effective doses of selected compounds would exert similar antipsychotic-like activity as the administration of each compound alone. The compounds that were investigated regulate glutamate release via presynaptic mechanisms and, therefore, may act in concert to normalize the glutamate efflux that is disrupted in the schizophrenic brain. A necessary condition for the synergistic action of the ligands is the sufficient expression of the receptors that they activate in the structures responsible for particular disturbances.

Here, two $GABA_B$ PAMs were investigated, GS39783 and CGP7930, which have been previously shown to have antipsychotic-like effects in animals [13,15]. Sub-effective doses of these drugs were co-administered with sub-effective doses of several activators of mACh receptors, such as VU0357017 (partial allosteric agonist of M_1 receptor), VU0152100 (positive allosteric modulator of M_4 receptor) and VU0238429 (positive allosteric modulator of M_5 receptor).

First, the activity of VU0357017 and VU0238429 was investigated in DOI-induced head twitches, which is the model of positive symptoms

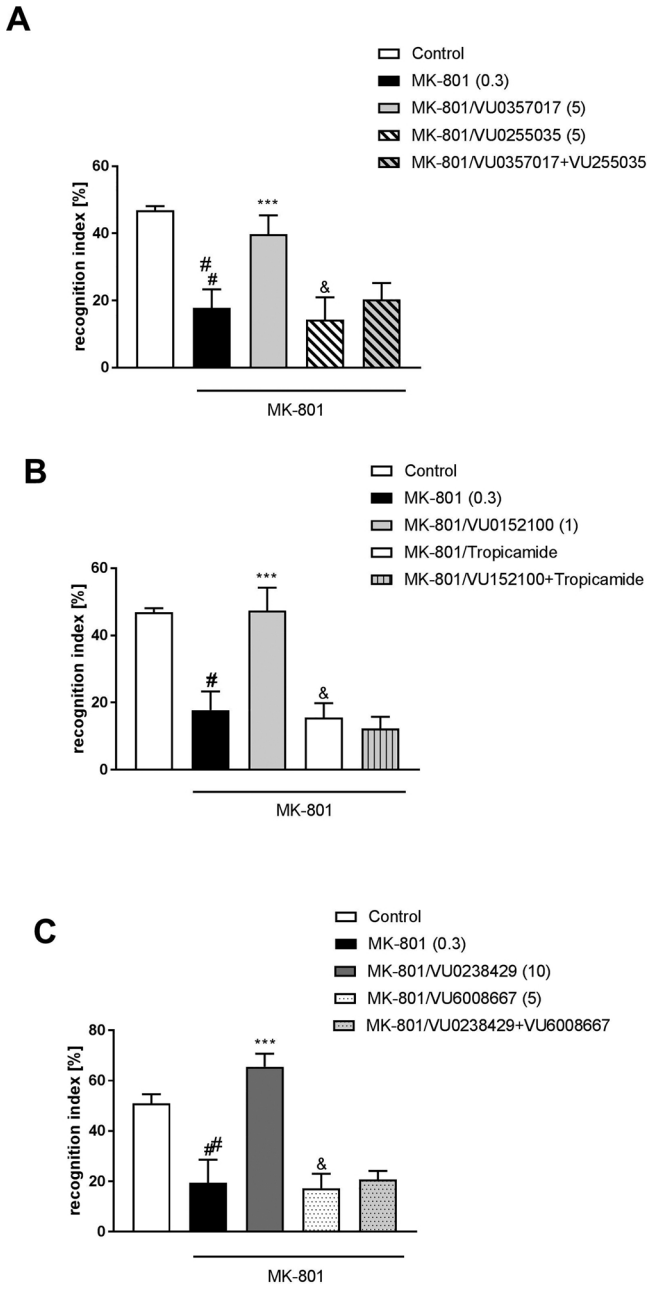

Fig. 8. The blockade of the activity of muscarinic ligands with selective antagonists. (A) VU0357017 (M_1 partial allosteric agonist) (B) VU0152100 (positive allosteric modulator of M_4 receptor) and (C) VU0238429 (positive allosteric modulator of M_5 receptor). The activators were administered 30 min before MK-801 injection, while antagonists were administered 15 min prior activator. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. # $p < 0.01$ vs. the control group; ***$p < 0.001$ vs. the MK-801 group and $^{\&}p < 0.001$ vs. VUs-treated groups N = 810/group.

of schizophrenia. The activity of VU0152100 was reported previously [12]. Both compounds induced clear inhibition of DOI-induced effect, with inverted U shaped dose-related effects. At least in the case of VU0357017 this effect may be partially explained by the fact that the degree of agonist activity for the compound and subsequent downstream effects have been reported to vary based on factors of inherent stimulus-bias at M_1 and/or regional difference in receptor reserve, therefore the compound may not be capable of fully activating some responses, even in systems in which the receptor is highly expressed [34]. The combinations of sub-effective dose of GS39783 with sub-effective doses of VU0357017, VU0152100 and VU0238429 were investigated and a reversal of the schizophrenia-related deficits was observed only for VU0152100 with GS39783. Subsequently, electrophysiological experiments were performed, and the effects of the combinations of these drugs (VU0152100 with GS39783) on the DOI-induced increase in spontaneous EPSC frequency in mouse prefrontal cortex slices were examined. Previously, both $GABA_B$ and M_4 activators were shown to dose-dependently inhibit the frequency of DOI-induced spontaneous EPSCs, confirming the presynaptic action of these compounds [4,12]. In the present studies, a similar effect was observed after simultaneous administration of sub-effective doses of GS39783 and VU0152100, confirming the results of behavioral studies and indicating that both receptors may act in tandem to regulate glutamate release.

Investigations of the efficient treatment of the negative and cognitive schizophrenia symptoms are of more importance than the investigations of the positive symptoms, as the negative symptoms affect daily functioning and are more resistant to the presently used neuroleptics. Earlier, the M_4 PAM VU0152100 and $GABA_B$ PAMs were shown to exert antipsychotic-like action in social interaction test, animal model of negative symptoms of schizophrenia [12,15]. However, the combination of M_4 and $GABA_B$ modulators in low, non-effective doses did not exert any activity in this test, despite the use of two different PAMs of the $GABA_B$ receptor in more than one dose. The co-administration of the M_4 PAM with a low dose of the atypical neuroleptic, risperidone, induced a clear reversal of the MK-801-induced deficits.

In the social interactions test neither M_1 partial allosteric agonist nor PAM of M_5 receptor reversed the MK-801-induced disruption in social behavior, suggesting that those receptors are not important in the development and treatment of negative symptoms of schizophrenia. In that case, further experiments with the concomitant administration of sub-effective doses of those compounds with $GABA_B$ ligands were not justified. However, this is the first report showing the lack of antipsychotic-like activity of M_1 and M_5 PAMs in the social interaction test.

Novel object recognition is a well-known test used to assess working memory, in which the animal must discriminate between a known, previously introduced object and a novel one [35].

Previously, the M_4 PAM, VU0152100, was shown to reverse the MK-801-induced deficit in this test [12]. Here, we investigated the efficacy of M_1 and M_5 activators as well, and both activators dose-dependently reversed the MK-801-induced deficits. This is the first report showing the activity of partial allosteric agonist of M_1 and PAM of M_5 receptor in novel object recognition, and the result is consistent with earlier studies suggesting that the receptors may have important roles in cognition [36,37].

Comparable effects were observed when sub-effective doses of M_1 or M_5 activators were co-administered with GS39783, a $GABA_B$ PAM. The co-administration of one of the mACh receptor PAM, VU0152100, with risperidone in a sub-effective dose also enhanced the activity of the atypical antipsychotic drug.

All ligands that were used in our studies activate G protein-coupled receptors (GPCRs) that regulate slow synaptic currents and mediate modulatory action in the CNS. Metabotropic receptors are the most extensively investigated drug targets, and approximately 34% of all recently approved drugs act at 108 GPCRs [38]. More than 300 other compounds have undergone clinical trials, mostly for indications towards obesity, diabetes and Alzheimer's disease. However, the treatment of central nervous system disorders also involves GPCRs. Both typical and atypical neuroleptics act through GPCRs (dopaminergic, serotonergic or adrenergic receptors). Unfortunately, compounds targeting those receptors are not optimal for efficient and safe antipsychotic treatment due to the insufficient efficacy and adverse effects that they can induce. The simultaneous activation of $GABA_B$ and muscarinic receptors that is proposed here may be a more efficient and safer treatment method.

Activation of $GABA_B$ receptor may be associated with a risk of inducing adverse effects, such as myorelaxation or sedation; however, those effects are predominantly observed after administration of

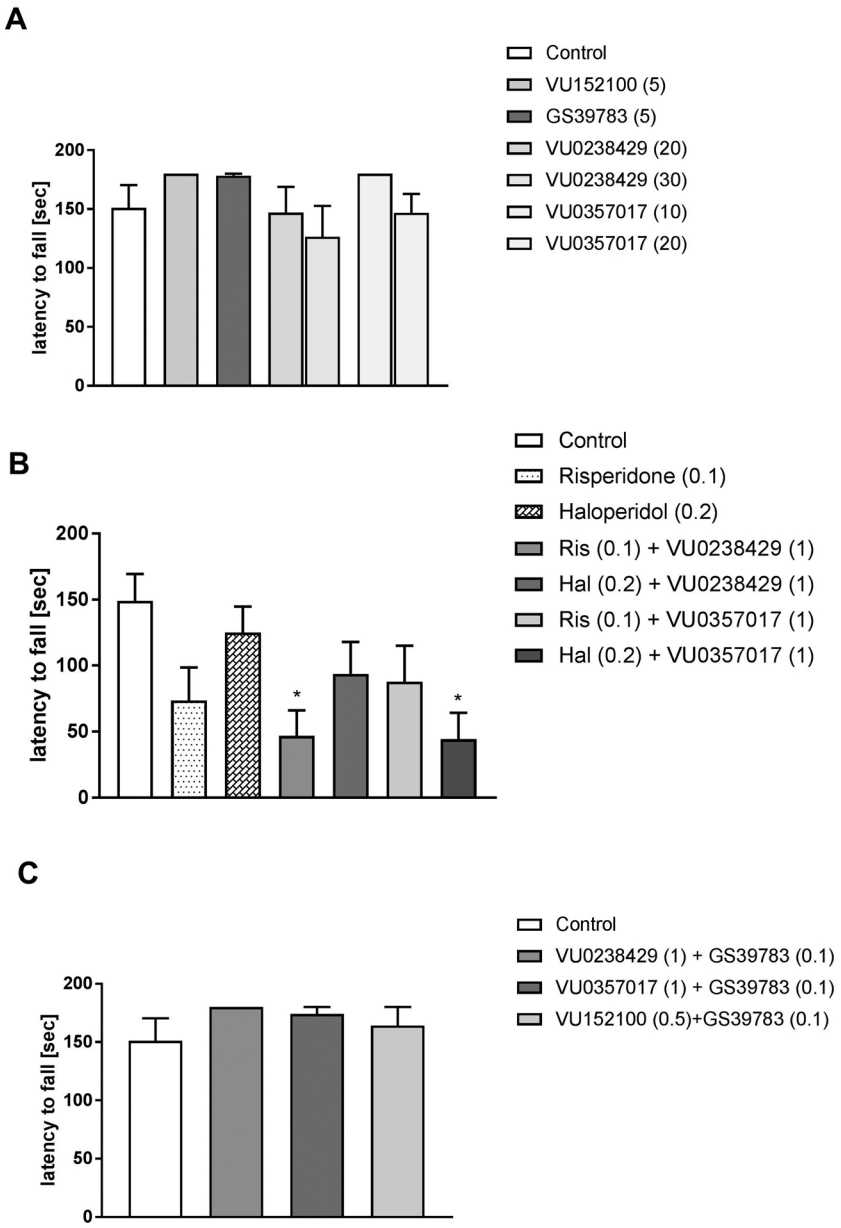

Fig. 9. Effect of VU0152100, VU0357017 and VU0238429 when administered alone (A) or concomitantly with GS39783 (C), risperidone (B) or haloperidol (B) on motor coordination measured in the rotarod test. The compounds were administered 30 min before the test. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. *p < 0.05 vs. the control group. N = 8–10/group.

orthosteric agonists (e.g., baclofen) [39,40]. The use of positive allosteric modulators of the receptor proposed here does not appear to induce such effects [4,41]. Additionally, their administration in low doses in combination with low doses of mACh partial allosteric agonist or PAMs may exert antipsychotic-like efficacy without or with minimal risk of the adverse effects that could develop when both ligands are taken in high doses.

Here, we show that neither of the mACh activators nor their combinations with a PAM of the $GABA_B$ receptor influenced motor coordination, which is often affected by standard antipsychotic treatment. Moreover, the co-administration of standard neuroleptics (such as risperidone) in subthreshold doses with muscarinic compounds still impairs motor coordination [12]. The drugs had no influence on haloperidol-induced catalepsy and did not induce catalepsy by themselves. This lack of an effect was shown earlier for VU0152100 [23], and here, we demonstrated it for two other activators of M_1 and M_5 receptors.

In our earlier reports it was shown that subthreshold doses of $GABA_B$ PAMs, when co-administered with allosteric modulators of metabotropic glutamatergic receptors (mGlu) may exert mutual action. For example, $mGlu_4$-$GABA_B$ ligands were active only in the models of positive symptoms [13], while $mGlu_5$-$GABA_B$ PAMs were active in the models of negative and positive symptoms [15]. Here, the mACh-$GABA_B$ combination may be proposed for the treatment of positive and cognitive disturbances, but not negative symptoms.

Pharmacokinetic experiments that were performed confirmed high penetration capacity for GS39783 when administered independently at a high dose and in combination with activators of muscarinic M_1, M_4, M_5 receptors according to Sturchler et al. [42]. Subsequently, we did not observe any brain penetration decrease for VU0152100 or VU0357017 when administered concomitantly with GS39783, which probably excludes the possibility of drug-drug interaction and confirms their independent transport though the Blood Brain Barrier. However, the combined administration of GS39783 with VU0238429 interfered its brain penetration, suggesting the possibility of some drug-drug

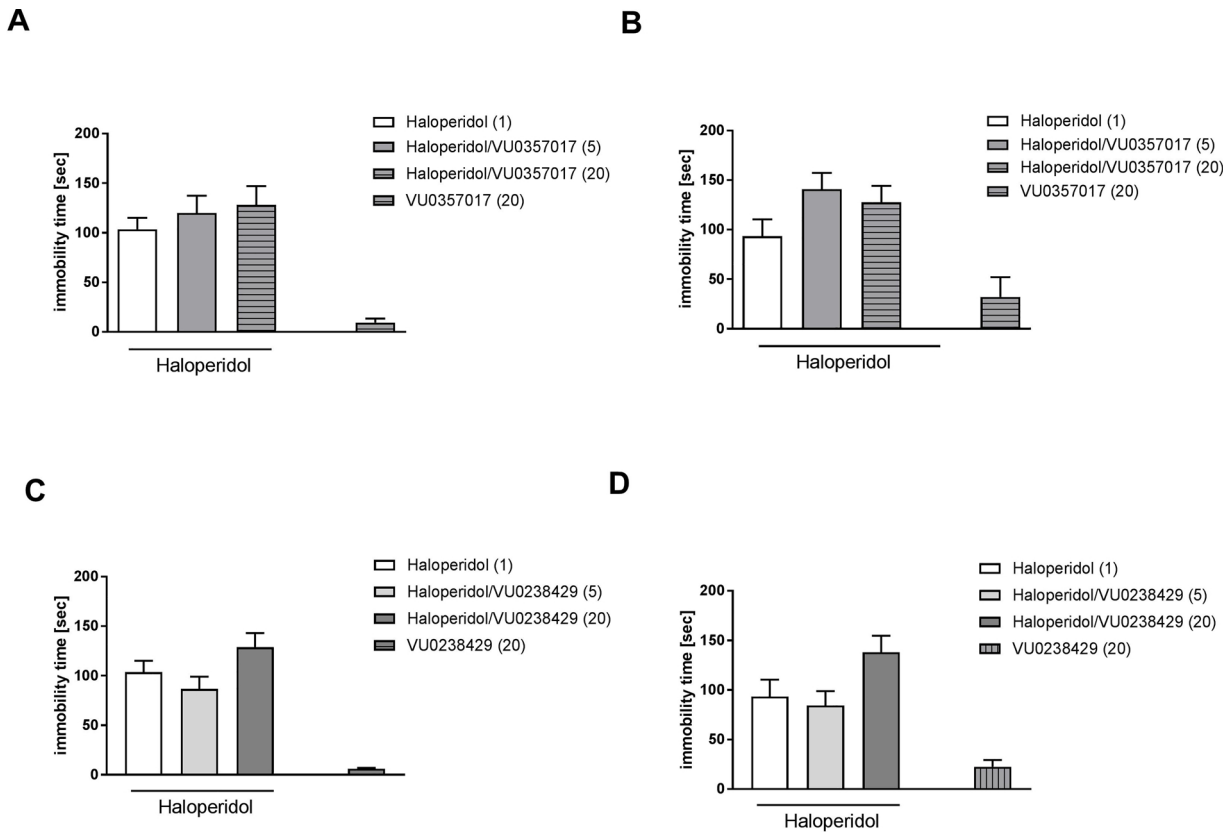

Fig. 10. Effect of VU0357017 (A, B) and VU0238429 (C, D) on haloperidol-induced catalepsy measured 45 min (A, C) and 90 min (B, D) after haloperidol administration. The compounds were administered 30 min before haloperidol injection. Doses in mg/kg are indicated in parentheses. Data are presented as the mean ± SEM. N = 6–10/group (A–D).

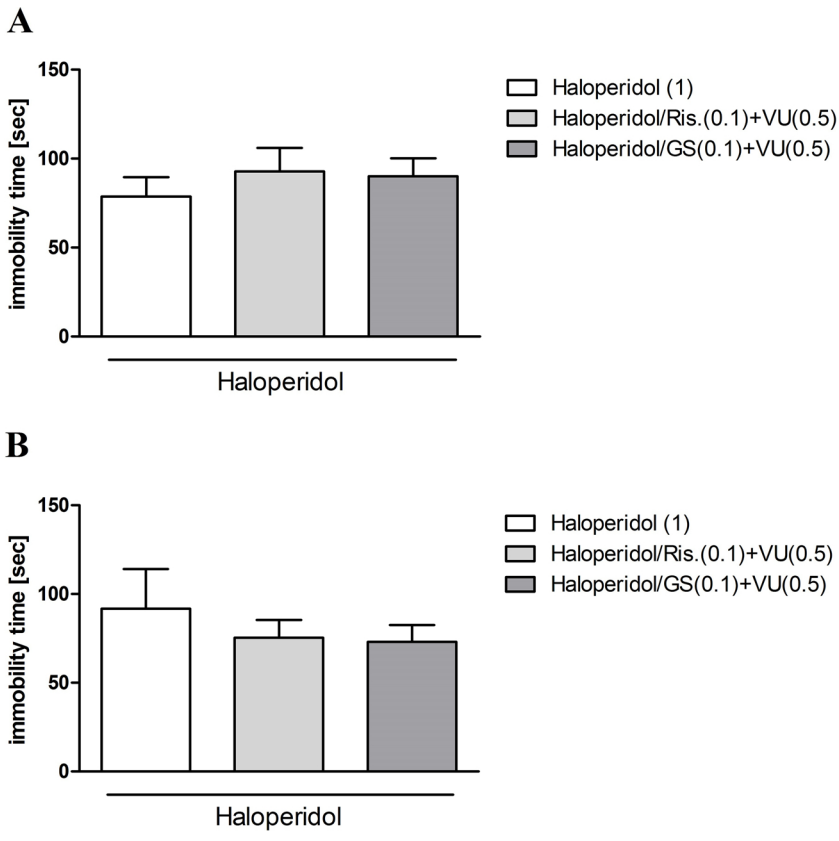

Fig. 11. Effect of combined administration of VU0152100 and GS39783 or risperidone on haloperidol-induced catalepsy measured 45 min (A) and 90 min (B) after haloperidol administration. The compounds were administered 30 min before haloperidol injection. Doses in mg/kg are indicated in parentheses. Data are presented as the means ± SEM. N = 6–7/group.

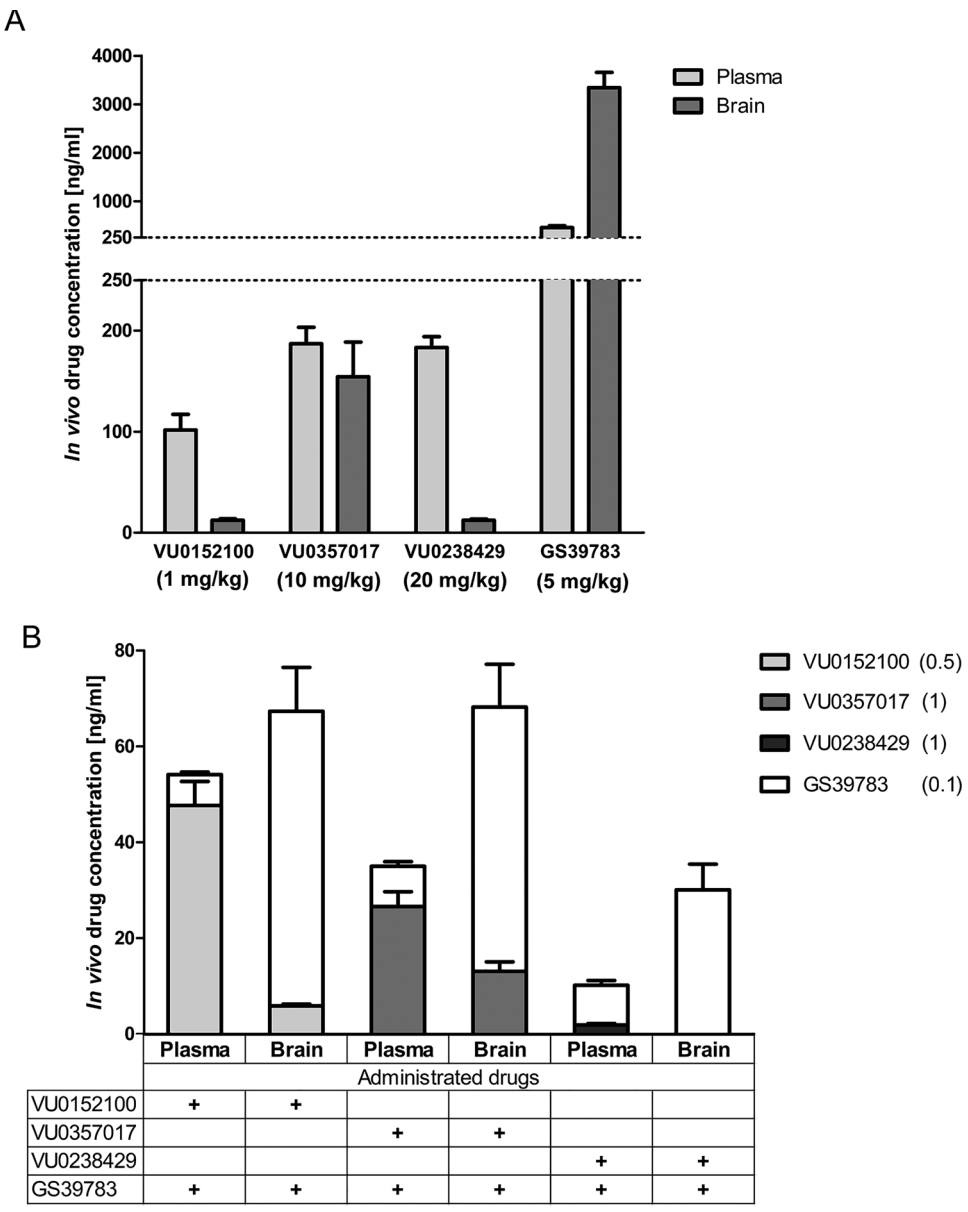

Fig. 12. Compounds concentration in plasma and brain when administered at the top doses (A) and when co-administered together at the subthreshold doses (doses of the compounds are indicated in parenthesis). Plasma and tissue samples were collected 30 min after administration. (A) Bars represent concentration level of administered drugs in plasma (light grey) or brain samples (grey). (B) Bars represent concentration level of co-administered drugs in plasma or brain samples. All compounds were co-administered with GS39783 (white color). In the table below the graph, drug co-administered in the sample were marked (+). Additionally each compound has a separate color on the graph: VU0152100 (light grey), VU0357017 (grey) and VU0238429 (dark grey).

interactions, which may be both hydrophobic and hydrogen. It should be noted that although VU0238429 displays a low micromolar in vitro potency (EC50 = 1.16 μM) at the M_5 mAChR subtype with limited brain exposure [33], it is still active in DOI-induced head twitch, novel object recognition and motor coordination tests, and its activity is blocked by the administration of specific antagonist. It may be possible that the compound breaks down into active metabolites. However, it remains to be established.

Ligands of these particular receptors were chosen for investigation because of their previously defined roles. Cholinergic and $GABA_B$ receptors have been shown to be expressed in brain circuits that are important in antipsychotic treatment, decreasing dopamine release via presynaptically expressed receptors in the striatum [43] and nucleus accumbens [44–49] and controlling the activity of entorhinal and perirhinal cortex projections, thus contributing to an improvement in cognitive functions [50–55]. This would be in line with our results showing, the efficacy of the compounds (and their combinations) in NOR rather than in social interaction test.

Therefore, the simultaneous stimulation of those receptors may result in an additive effect on the striatal dopaminergic system and cognitive processes.

Declarations of interest

The authors declare no conflict of interest.

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