Original Paper

Negative modulation of α_5 $GABA_A$ receptors in rats may partially prevent memory impairment induced by MK-801, but not amphetamine- or MK-801-elicited hyperlocomotion

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Abstract

Reportedly, negative modulation of α_5 $GABA_A$ receptors may improve cognition in normal and pharmacologically-impaired animals, and such modulation has been proposed as an avenue for treatment of cognitive symptoms in schizophrenia. This study assessed the actions of PWZ-029, administered at doses (2, 5, and 10 mg/kg) at which it reached micromolar concentrations in brain tissue with estimated free concentrations adequate for selective modulation of α_5 $GABA_A$ receptors, in three cognitive tasks in male Wistar rats acutely treated with the noncompetitive *N*-methyl-D-aspartate receptor antagonist, MK-801 (0.1 mg/kg), as well in tests of locomotor activity potentiated by MK-801 (0.2 mg/kg) or amphetamine (0.5 mg/kg). In a hormetic-like manner, only 5 mg/kg PWZ-029 reversed MK-801-induced deficits in novel object recognition test (visual recognition memory), whereas in the Morris water maze, the 2 mg/kg dose of PWZ-029 exerted partial beneficial effects on spatial learning impairment. PWZ-029 did not affect recognition memory deficits in social novelty discrimination procedure. Motor hyperactivity induced with MK-801 or amphetamine was not preventable by PWZ-029. Our results show that certain MK-801-induced memory deficits can be ameliorated by negative modulation of α_5 $GABA_A$ receptors, and point to the need for further elucidation of their translational relevance to cognitive deterioration in schizophrenia.

Keywords

PWZ-029, Morris water maze, novel object recognition test, social novelty discrimination procedure, dizocilpine, free brain concentration, amphetamine

Introduction

The inhibitory neurotransmission mediated by gamma-aminobutyric acid (GABA) is substantially involved in the regulation of anxiety, memory processes, muscle tone, vigilance, and epileptogenesis in the mammalian central nervous system (Rudolph and Möhler, 2004). In major part, these regulatory roles of GABA are exerted through activation of $GABA_A$ receptors, which most often comprise an α-subunit (α1-6), a β-subunit, and a γ subunit in a 2:2:1 stoichiometry (McKernan and Whiting, 1996). The majority of $GABA_A$ receptors contain the benzodiazepine binding site, found at the interface between the γ_2 subunit and either an α_1, α_2, α_3, or α_5 subunit. Through this binding site, $GABA_A$ receptors are susceptible to both positive and negative modulation (Olsen and Sieghart, 2008). Non-selective negative allosteric modulators (or inverse agonists), such as β-carbolines DMCM, and FG 7142, decrease the inhibitory effects of GABA, and have been known for many years to exert, nearly at the same time, the anxiogenic, proconvulsant, and cognition-enhancing actions in animals (Braestrup et al., 1982; Rossier et al., 1983). Thus, the highly desirable pro-cognitive potential of such modulators could not have been clinically verified and exploited (Dorow et al., 1983).

More recently, genetic studies pointed to the $GABA_A$ receptors containing the α_5 subunit as the major substrate of pro-cognitive effects of reduction of GABAergic neurotransmission (Collinson et al., 2002; Crestani et al., 2002). Based on such data, development of compounds with selectivity for the α_5 subtype of $GABA_A$ receptors, which may enhance cognitive performance without the side-effects associated with non-selective compounds, has been initiated (Maubach, 2003) and resulted, among others, in several patents claiming the effectiveness of α_5-selective negative modulators in cognitive symptoms of schizophrenia, Down syndrome, or mood disorders (Guerrini and Ciciani, 2013; Martínez-Cué et al., 2014). A couple of ligands (α5IA, RO4938581, RY-23, RY-24, PWZ-029) have been reported to demonstrate a considerable degree of affinity and/or efficacy selectivity for α_5 $GABA_A$ receptors *in vitro*, coupled with enhancement of performance of different cognitive tasks, such as the delayed-match-to-position (Ballard et al., 2009;

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Dawson et al., 2006) and the standard spatial version of water maze (Ballard et al., 2009), object retrieval task (Ballard et al., 2009; Soto et al, 2013), or passive avoidance in animals (Savić et al., 2008), as well as prevention of alcohol-induced memory impairment in humans (Nutt et al., 2007). One such ligand is PWZ-029, a partial inverse agonist with both the binding and functional selectivity for α_5-containing $GABA_A$ receptors, at which it reduces control current by 20% when applied at 1 μM. Devoid of effects on anxiety, muscle tone, or convulsion propensity in the tested dose range (2–20 mg/kg), it improved passive avoidance learning task in normal rats (Savić et al., 2008). Moreover, PWZ-029 reversed the scopolamine-induced cognition impairment in novel object recognition, but not water maze task in rats (Milić et al., 2013), and attenuated scopolamine-induced impairment of Pavlovian fear conditioned contextual memory in mice (Harris et al., 2008). The results of the latter kind of experiments, obtained in animals exposed to a compound that induces changes in neurotransmission reminiscent of pathological processes are an improvement on those from tests using intact animals for the pro-cognitive potential of drug candidates (Keeler and Robbins, 2011). However, many of the models employed have limited construct validity and the end point behavioral assessment measures do not always translate well to the clinic for schizophrenia-spectrum disorders (Pratt et al., 2012). In regard to other α_5-selective inverse agonists, RO49838581 has been shown to ameliorate cognitive deficits in rats induced by subchronic and neonatal administration of phencyclidine in novel object recognition and attentional set-shifting performance task, respectively (Redrobe et al., 2012).

Since its discovery, the *N*-methyl-D-aspartate (NMDA) receptor system for the main excitatory neurotransmitter glutamate has been implicated in many essential functions, such as neuronal plasticity, neurotoxicity, learning, and memory (Riedel et al., 2003). Besides phencyclidine, dizocilpine (MK-801) is another experimentally widely used NMDA receptor antagonist, and MK-801-induced cognitive impairments have been validated as a rodent model related to human cognitive deficits associated with dementia (Van der Staay et al., 2011) and schizophrenia (Adell et al., 2012; Andine et al., 1999). Although the MK-801-induced memory deficit model has been assessed as the second most commonly (after scopolamine) used deficit model in preclinical cognition research (Van der Staay et al., 2011), the published results of testing the selective α_5 $GABA_A$ receptor negative modulators in animals treated with MK-801 are limited to only one study, using the incremental repeated acquisition task which allows for the assessment of effects on learning at various levels of task-difficulty (Povroznik et al., 2014). Such data are expected to be of special relevance in the pursuit of novel therapies for cognitive aspects of neuropsychiatric disorders, beginning with the premise that α_5 $GABA_A$ receptors located at the base of dendritic spines of pyramidal cells in the hippocampus and neocortex are strategically located to modulate excitatory glutamatergic input that arrives at the spines of these cells (Rudolph and Möhler, 2014).

The aim of the present study was to investigate the effects of PWZ-029 on the object recognition and social recognition memory, as well as the water maze performance affected by the lowest effective dose of MK-801 in rats (0.1 mg/kg); these three tests were selected in order to explore a wide range of cognitive domains (from visual and social recognition to spatial memory). Besides cognitive impairments, MK-801, administered in somewhat higher doses (0.2 mg/kg), has been validated to induce psychosis, in concordance with the hypothesis of NMDA receptor hypofunction in schizophrenia (Neill et al., 2010; Olney et al., 1999). Hence, we opted to also assess the potential of PWZ-029 to reverse the MK-801- as well as the amphetamine-induced hyperlocomotion. Namely, it was reported that RO49838581 antagonized the stimulating action of the hyperdopaminergic agent amphetamine in normal rats (Redrobe et al., 2012); such a finding with a negative modulator of α_5 $GABA_A$ receptors seems to be at odds with the data that a positive modulator of the same receptors, SH-053-R-CH3-2'F (Savić et al., 2008), was also able to prevent the hyperlocomotor action of amphetamine in methylazoxymethanol acetate (MAM)-treated rats, expressing the schizophrenia-like phenotype (Gill et al., 2011). Finally, we performed the pharmacokinetic study of PWZ-029 dosed at 5 mg/kg and estimated its free plasma and brain concentrations 20 minutes after administering the 2, 5, or 10 mg/kg dose in rats. These studies were aimed to assess if the *in vivo* concentrations attained at the time of behavioral testing enable a selective involvement of α_5 $GABA_A$ receptors (cf. Obradović et al., 2014; Redrobe et al., 2012), when compared with the *in vitro* findings of PWZ-029 (Savić et al., 2008).

Material and methods

Drugs

PWZ-029 (molecular weight = 291.73 g/mol), an α_5 $GABA_A$ receptor selective inverse agonist, was synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee, USA. Binding affinity for PWZ-029 at human recombinant $GABA_A$ receptors containing β_3, γ_2, and α_1, α_2, α_3, or α_5 subunit was determined to amount (Ki, nM): 920, > 300, > 300 and 38.8, respectively (Harris et al., 2008; Savić et al., 2008). (+)-MK-801 hydrogen maleate, a potent NMDA receptor antagonist, and D-amphetamine-sulfate were purchased from Sigma–Aldrich. PWZ-029 was suspended/dissolved with the aid of sonication in the solvent (85% distilled water, 14% propylene glycol, and 1% Tween 80). MK-801 and amphetamine were dissolved in saline. All compounds were administered intraperitoneally (i.p.) in a volume of 2 mL/kg, 20 min before the behavioral testing, with the exception of Experiment V, in which the animal's behavior was recorded immediately after respective treatments.

For plasma protein and brain tissue binding studies, stock solutions of PWZ-029 were prepared in dimethyl sulfoxide (DMSO).

Pharmacokinetic study of PWZ-029

Rats were divided in five groups which corresponded to predetermined time intervals (5, 10, 20, 40, and 60 min), each containing three animals. PWZ-029, dosed at 5 mg/kg, was administered by i.p. injection in a volume of 2 mL/kg. Additional experiments were performed in order to determine brain and plasma concentration, as well as to calculate free brain and plasma levels of PWZ-029 dosed at 2, 5, and 10 mg/kg, 20 min after i.p. injection. The blood samples were collected in heparinized syringes via cardiac puncture of rats anesthetized with ketamine solution (10% Ketamidor, Richter Pharma AG, Wels, Austria), dosed at

100 mg/kg (i.p.), and centrifuged at 2500 rpm for 10 min to obtain plasma. Thereafter, rats were decapitated and brains were weighed, homogenized in 5 mL of methanol and centrifuged at 6000 rpm for 20 min. To determine the concentration of PWZ-029 in plasma and supernatants of brain tissue homogenates, PWZ-029 was extracted from these samples by solid phase extraction, using Oasis HLB cartridges (Waters Corporation, Milford, MA). The procedure of sample preparation and determination of PWZ-029 by ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) with Thermo Scientific Accela 600 UPLC system connected to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA), equipped with electrospray ionization (ESI) source, was described in detail by Obradović et al. (2014).

Plasma protein and brain tissue binding studies

The rapid equilibrium dialysis assay used to determine free fraction of PWZ-029 in rat plasma and brain tissue was the same as in Obradović et al. (2014).

Behavioral experiments

Experiments were carried out on nine weeks old (adults, 320 in total, weighing 200–250 g) or 21 days old (juvenile, 16 in total) outbred Wistar albino male rats supplied by Military Farm, Belgrade, Serbia. Adult rats were housed in Makrolon type III cages in groups of four and juvenile in groups of eight and had free access to food and water. The temperature of the animal room was 22 ± 1 °C, relative humidity 40–70%, illumination 120 lx, with a 12 h light/dark cycle (lights on at 06:00 h). All experiments took place during the light phase of the diurnal cycle (09:00–15:00 h). For social discrimination novelty procedure adults were isolated in individual cages two days before testing. The behavior was recorded by a ceiling-mounted camera and analyzed by ANY–maze Video Tracking System software (Stoelting Co., USA). Experiments were carried out in accordance with the EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade. Throughout the study, experimentally naïve animals were used.

Novel object recognition test

The protocol and statistical analysis used for the novel object recognition test (NORT) were in accordance with those given by Bevins and Besheer (2006), and have been described in detail by Milić et al. (2013). The apparatus consisted of a rectangular chamber (65 cm × 45 cm × 45 cm) which was illuminated with indirect white lighting (20 lx). Animals were habituated to an empty chamber for 10 min, 24 h before the beginning of experiment. The NORT was divided in two stages: familiarization (T1) and testing (T2). In the familiarization phase two identical objects were placed 10 cm from the sides of the chamber and 25 cm apart. Twenty minutes after receiving appropriate treatment, each rat was placed in the arena between the two objects facing the opposite wall and allowed to explore them for 5 min. Animal was then removed from the apparatus and returned to the cage for 1 h. To test for object recognition, one of the familiar sample objects was replaced with a novel object and animal was returned to the apparatus and allowed to explore for 3 min. Object exploration was recorded when the animal was in close proximity (2 cm or less) and oriented to the object, and if the animal sniffed or pawed the object. Furthermore, the animals that did not explore both objects for a minimum of 2 s each were excluded from the data analyses. Object set consisted of a semi–transparent pyramid (base 8 cm × 8 cm, height 7 cm) and a nontransparent dome–shaped paperweight (base diameter 9 cm, height 10 cm), which could not be displaced by rats. Each object was available in triplicate and all combinations and locations of objects were used in a balanced manner to reduce potential biases. After each trial, the apparatus and objects were cleaned with 70% ethanol to reduce olfactory cues.

In Experiment I, the influence of PWZ-029 (2, 5 or 10 mg/kg) on MK-801-induced memory impairment in NORT was examined. The dose of MK-801 (0.1 mg/kg) was selected based on our preliminary study (unpublished data), which is in accordance with previous findings (De Lima et al., 2005; Van der Staay et al., 2011). There were five groups of rats which received one of the following treatments before the familiarization phase: saline + solvent (N = 23), MK-801 (0.1 mg/kg) + solvent (N = 20) and MK-801 (0.1 mg/kg) in combination with PWZ-029 (2, 5, and 10 mg/kg, N = 16, 17, and 19, respectively). The amount of time exploring two identical objects during T1 (Ta and Tb) and familiar (Tf) and novel (Tn) objects in T2 was automatically scored, and discrimination index ((Tn –Tf)/(Tf + Tn)) were calculated. The effect of treatment on exploratory behavior was assessed by investigating total exploration times in T1 (Ta + Tb) and T2 (Tf + Tn).

Social novelty discrimination procedure

Social novelty discrimination (SND) experiments compared the social investigation times of an adult rat with a familiar and a novel juvenile rat. The procedure is replicated from previous studies (Engelmann et al., 1995; Terranova et al., 2005). Testing consisted of two consecutive juvenile presentations periods to an adult subject: period 1 (P1) and period 2 (P2). At the beginning of P1, one juvenile was placed into the adult home cage and the time spent by the adult investigating the juvenile (anogenital sniffing, licking, close pursuing, and pawing) was recorded manually for 5 min. During P2, the same juvenile and a second, novel juvenile were placed in the cage together with the adult, and the times spent by the adult investigating each juvenile were measured independently for 5 min. A different pair of juvenile rats was presented to each adult tested. Manual scoring was conducted in a blinded manner. We used a pharmacological deficit protocol, in which SND was impaired by pharmacological intervention, P1 was 30 min, and there was no delay between P1 and P2. In such conditions, SND was expected to be high in control rats, while disrupted by MK-801 (0.1 mg/kg, i.p.), administered 20 min before P1.

In Experiment II, the influence of PWZ-029 (2, 5 or 10 mg/kg) on MK-801-induced impairment in SND was examined. The dose of MK-801 (0.1 mg/kg) was selected based on our preliminary study (unpublished data). There were five groups of rats (N = 6 per each treatment group) which received one of the following treatments 20 min before P1: saline + solvent, MK-801

(0.1 mg/kg) + solvent, and MK-801 (0.1 mg/kg) in combination with PWZ-029 (2, 5, and 10 mg/kg). The amount of time investigating familiar (Tf) and novel (Tn) juvenile during P2 was manually scored, and discrimination ratio (Tn/Tf) was calculated. Total exploration time during P1 and P2 was also manually recorded.

Morris water maze (MWM)

Experiments were performed in a 2 m diameter circular pool filled to a height of 30 cm with water at 22 ± 1 °C. The escape platform (15 cm × 10 cm) of the same color as the pool was submerged 2 cm below the water surface. An indirect illumination (20 lx) in the experimental room was provided by white neon tubes fixed on the walls and many distal cues were present. On each of the five consecutive days rats were given one swimming block, consisting of four trials. For each trial the rat was placed in the water facing the pool at one of four pseudo–randomly determined starting positions. Since the platform was hidden in the middle of the NE quadrant during training sessions, the four distal start locations were: S, W, NW, and SE. Once the rat has found and mounted the escape platform it was permitted to remain on the platform for 15 s. The rat was guided to the platform by the experimenter if it failed to locate it within 120 s. During the acquisition phase, treatments were applied once daily before the swimming block. On the sixth day, rats were given a treatment-free probe test (60 s) without the platform. In order to ensure that any spatial bias is a consequence of the spatial memory of escape location, rather than of a specific swim strategy, the probe test was started from the novel, most distant SW location (Vorhees and Williams, 2014).

In Experiment III we tested the effects of PWZ-029 on MK-801-induced memory impairment; the dose of MK-801 (0.1 mg/kg) was selected as the lowest dose that disrupted acquisition in our water maze setting (unpublished data). The rats were divided into five groups which received one of the following treatments 20 minutes before swimming at each of five learning days: saline + solvent (N = 8), MK-801 (0.1 mg/kg) + solvent (N = 8), and combination of MK-801 (0.1 mg/kg) and PWZ-029 (2, 5, and 10 mg/kg; N = 7, 8, and 8, respectively). The pool was virtually divided into four quadrants, three concentric annuli and a target region consisting of the intersection of the platform quadrant and the platform (middle) annulus, as represented in Savić et al. (2009). Dependent variables chosen for tracking during the acquisition trials were: escape latency (s), total distance traveled (m), mean speed (m/s) and path efficiency (the ratio of the shortest possible path length to actual path length). The selected parameters in the probe test were the time in the target zone (s) and the time spent in the peripheral ring (s).

Locomotor activity (LA)

Activity of single rats in a clear Plexiglas chamber (40 × 25 × 35 cm^3) under dim red light (20 lx) was recorded for a total of 60 min, without any habituation period but with 20 min of acclimatization to testing room, using ANY-maze software for MK-801-induced hyperlocomotion. Different doses of PWZ-029 (2, 5, and 10 mg/kg) and MK-801 (0.2 mg/kg) were applied 20 min before testing. The same apparatus was used for amphetamine-induced locomotor activity but different protocol was used. In this behavioral test rats received appropriate treatment (solvent, saline, 2, 5, and 10 mg/kg PWZ-029) and were immediately placed in LA arena. Behavior was recorded for 20 min and then rats were injected with saline or 0.5 mg/kg amphetamine and locomotor activity was recorded for an additional 60 min.

The influence of 2, 5, and 10 mg/kg PWZ-029 on hyperlocomotion induced by 0.2 mg/kg MK-801 was assessed in Experiment IV (treatment groups: saline + solvent (N = 9), MK-801 (0.2 mg/kg) + solvent (N = 8), and MK-801 (0.2 mg/kg) in combination with PWZ-029 (2, 5, and 10 mg/kg, N = 9, 9, and 8, respectively)). Finally, the influence of PWZ-029 (2, 5, and 10 mg/kg) on amphetamine-induced hyperactivity was assessed in Experiment V (treatment groups: saline + solvent (N = 8), amphetamine (0.5 mg/kg) + solvent (N = 8) and amphetamine (0.5 mg/kg) in combination with PWZ–029 (2, 5, and 10 mg/kg, N = 10, 7 and 7, respectively)).

Statistics

All numerical data presented in the figures are given as the mean ± SEM, except for discrimination ratio where data are given as median with interquartile range. Pharmacokinetic parameters were calculated using PK Functions for Microsoft Excel software (by Joel Usansky, Atul Desai, and Diane Tang-Liuwere). In NORT, the differences between exploration times spent with familiar and novel object for each group were evaluated using Student's paired t-test and two-way analysis of variance (ANOVA). Discrimination index was assessed by one-way ANOVA and one-sample t-test per treatment group. In SND procedure the discrimination ratios were analyzed by Kruskal–Wallis test, and significant results were further analyzed by a Student–Newman–Keuls (SNK) multiple comparison test. Total exploration times from NOR test and SND procedure were assessed by one-way ANOVA. The data from the acquisition days in the Morris water maze were averaged for each rat (total data/total number of trials per day) and analyzed using two-way ANOVA with repeated measures (factors: treatment and days) with days as the repeated measure. In the case of significant interaction, separate one-way ANOVAs were conducted to assess the influence of treatment within individual levels of factor Days. The data from the probe test were assessed using one-way ANOVA. In LA test one-way ANOVA (for overall effect) or two-way ANOVA with repeated measures (factors: treatment and time) with time as the repeated measure (for effect during 5-min bins) were applied. *Post hoc* comparisons, where applicable, were performed using SNK test. Statistical analysis was performed using SigmaPlot 11 (Systat Software Inc., Richmond, USA) software. Differences were considered significant when $p < 0.05$, while $0.1 > p > 0.05$ was considered as a trend toward significance.

Results

Pharmacokinetic and free fraction study of PWZ-029

The concentration–time profile of PWZ-029 in rat plasma and brain after intraperitoneal administration of 5 mg/kg dose, with the calculated pharmacokinetic parameters, is given in Figure 1.

The concentrations of PWZ-029 in rat plasma (nmol/L) and brain tissue (nmol/kg), both total and free (estimated), obtained 20 min after i.p. injection of PWZ-029 (dosed at 2, 5, and 10 mg/kg) are shown in Table 1. Free concentrations of PWZ-029 were calculated by multiplying the measured total plasma and brain concentrations with the appropriate free fractions (23.95% for plasma and 7.91% for brain tissue) determined by rapid equilibrium dialysis.

The estimated free PWZ-029 brain concentrations, purposefully presented as volume concentrations (Rempp et al., 1994), were incorporated in concentration–effects curves given in Savić et al. (2008), depicting the modulation of GABA-elicited currents by PWZ-029 on Xenopus oocytes expressing rat recombinant α_1-, α_2-, α_3-, and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors (Figure 2).

Novel object recognition test

For Experiment I, the effects of MK-801 + PWZ-029 combination on the rats' behavior in the NORT are presented in Figure 3. Paired t-test revealed no significant differences between the time that rats spent exploring two identical objects (object A and B) in T1 (data not shown). The same statistical analysis applied on the time spent in exploring the familiar and the novel object during T2 showed that only the control group and the group treated with combination 0.1 mg/kg MK-801 and 5 mg/kg PWZ-029 spent significantly more time in exploring the new object (t = 2.383, p = 0.026; t = 3.489, p = 0.003, respectively, Figure 3(a)). Similarly, discrimination indices were significantly different from zero (i.e. chance level) for the control group (t = 2.446; p = 0.023) and the group treated with the same combination (t = 4.220; p < 0.001; Figure 3(b)), notwithstanding the fact that one-way ANOVA revealed no significant difference in this parameter between treatment groups (F(4,90) = 1.85, p = 0.126). One-way ANOVA was significant for total exploration time in T1 (F(4,90) = 6.05, p < 0.001), and SNK *post hoc* revealed differences between control and all other treatment groups (p < 0.01 in all four cases). One-way ANOVA for total exploration time in T2 was not significant (F(4,90) = 0.96, p < 0.435). Two-way ANOVA for exploration times (new vs. familiar) revealed a significant effect for factor object (F(1,180) = 8.44, p < 0.004), but not for treatment as a factor (F(4,180) = 1.04, p = 0.389), or interaction (F(4,180) = 1.26, p = 0.286).

To assess the replicability of our findings (cf. Loscalzo, 2012), especially having in mind that only the middle dose of PWZ-029 was effective in NORT, we opted to perform an additional experiment, as presented in Supplementary material.

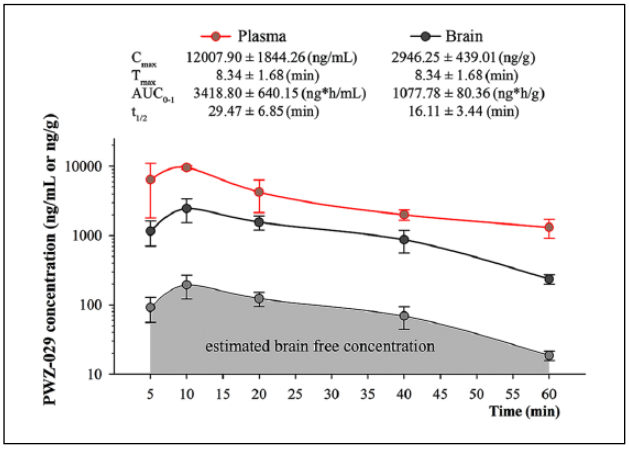

Figure 1. Plasma and brain concentration–time profiles of PWZ-029 (5 mg/kg) after intraperitoneal administration (n = 3 per time point).

Social novelty discrimination

In the SND test, there were no significant differences in total investigation times during the first 5 min of both P1 (one-way ANOVA: F(4,25) = 1.02, p = 0.414) and P2 (one-way ANOVA: F(4,25) = 0.45, p = 0.774, Figure 4). Following an extended P1 period of 30 min and no delay between P1 and P2, subjects administered with vehicle spent more time investigating the novel juvenile than the familiar one, giving a high novel/familiar discrimination ratio, which represents a relatively good ability to socially discriminate (Figure 4). Kruskal–Wallis test revealed significant differences between treatment groups in regard to discrimination ratios (H(4) = 12.378, p = 0.015). The administration of MK-801, irrespective of absence (saline) or presence of PWZ-029 (2, 5 and 10 mg/kg) significantly decreased the discrimination ratio when compared to control rats (p < 0.05 for all treatment groups), as shown by SNK *post hoc* test.

Morris water maze

The results of two-way repeated measures ANOVA and SNK *post hoc* tests for Experiment III are given in Table 2; significant effects of factor treatment and factor days were observed during acquisition for all parameters measured. After the *post hoc* analysis was performed, it appeared that all groups treated with MK-801, irrespective of absence (saline) or presence of PWZ-029 (2, 5, and 10 mg/kg), had longer escape latencies when compared to the control group (respective p values: p < 0.001, p = 0.035, p = 0.002, and p < 0.001; Figure 5(a)), and similarly had traveled longer distances (respective p values: p < 0.001, p = 0.005, p = 0.004, and p = 0.006; Figure 5(b)). Interestingly, *post hoc* analysis of the latency to reach the platform also revealed a significant difference between groups

Table 1. Total and estimated free concentrations of PWZ-029 (dosed at 2, 5, and 10 mg/kg) in plasma and brain samples after 20 min.

Dose (mg/kg)		2	5	10
Plasma (nmol/L)	Total	4647.93 ± 880.16	11943.70 ± 2878.27	20087.07 ± 3997.23
	Free	1113.18 ± 210.80	2860.51 ± 689.35	4810.85 ± 957.34
Brain (nmol/kg)	Total	2323.12 ± 129.98	4074.59 ± 923.30	7002.81 ± 1505.01
	Free	183.76 ± 10.28	322.30 ± 73.03	553.92 ± 119.05

treated with the combination of 0.1 mg/kg MK-801 and 2 mg/kg PWZ-029 and those treated with 0.1 mg/kg MK-801 in combination with solvent ($p = 0.022$) or 10 mg/kg PWZ-029 ($p = 0.042$), suggesting that the lowest dose of PWZ-029 exhibited a partial reversal of MK-801-induced impairment. Moreover, two-way ANOVA for mean speed showed significant effect for treatment, but *post hoc* revealed that only combination of 10 mg/kg PWZ-029 and 0.1 mg/kg MK-801 swam slower than control rats ($p = 0.028$; Figure 5(c)). Rats treated with MK-801 had lower path efficiencies compared to the control group ($p < 0.001$); the addition of PWZ-029 (2, 5, and 10 mg/kg) did not significantly influence this parameter (respective p values: 0.002, <0.001, and <0.001; Figure 5(c)).

One-way ANOVA applied on results from probe test revealed a significant influence of treatment on both parameters measured: the time spent in the target region ($F(4,34) = 8.67$, $p < 0.001$; Figure 6(a)), and the time spent in the peripheral ring ($F(4,34) = 5.99$, $p = 0.001$; Figure 6(b)). Rats treated either with MK-801 or with combination of MK-801 and PWZ-029 (2, 5, and 10 mg/kg) spent less time in the target region ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively) and more time searching in the peripheral ring ($p = 0.002$, $p = 0.002$, $p = 0.002$, and p = 0.003, respectively), when compared to control rats.

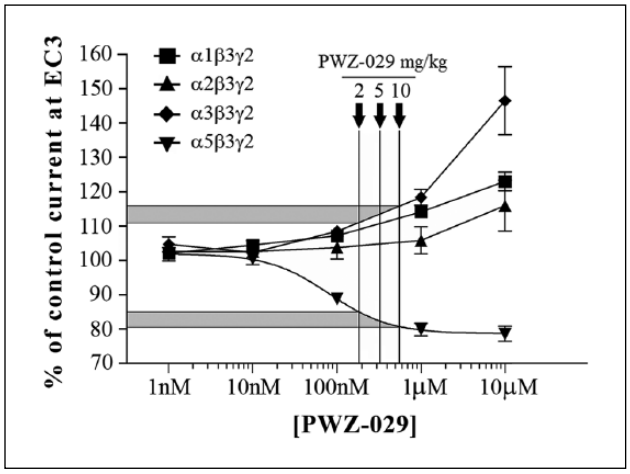

Figure 2. The approximated electrophysiological responses of estimated free PWZ-029 brain concentrations (given in Table 1), presented on the concentration-response curves of PWZ-029 at rat recombinant α1-, α2-, α3,- and α5β3γ2 $GABA_A$ receptors given in Savić et al. (2008).

Locomotor activity

One-way ANOVA applied on data from Experiment IV has shown a statistically significant effect of treatment ($F(4,38) = 42.34$, $p < 0.001$), and *post hoc* comparisons revealed highly significant ($p < 0.001$) differences between all treatment groups (0.2 mg/kg MK-801 alone and in combination with 2, 5, or 10 mg/kg PWZ-029) and control rats. Additionally, there was a significant difference between the MK-801 group and the MK-801 + PWZ-029 (10 mg/kg) group ($p = 0.012$, Figure 7(a)). Analysis of distance traveled in 5-min bins revealed a significant effect of factor treatment and time (treatment: $F(4,38) = 42.34$, $p < 0.001$; time: $F(11,418) = 15.514$, $p < 0.001$; interaction $F(44,418) = 0.960$, $p = 0.547$; Figure 7(b)).

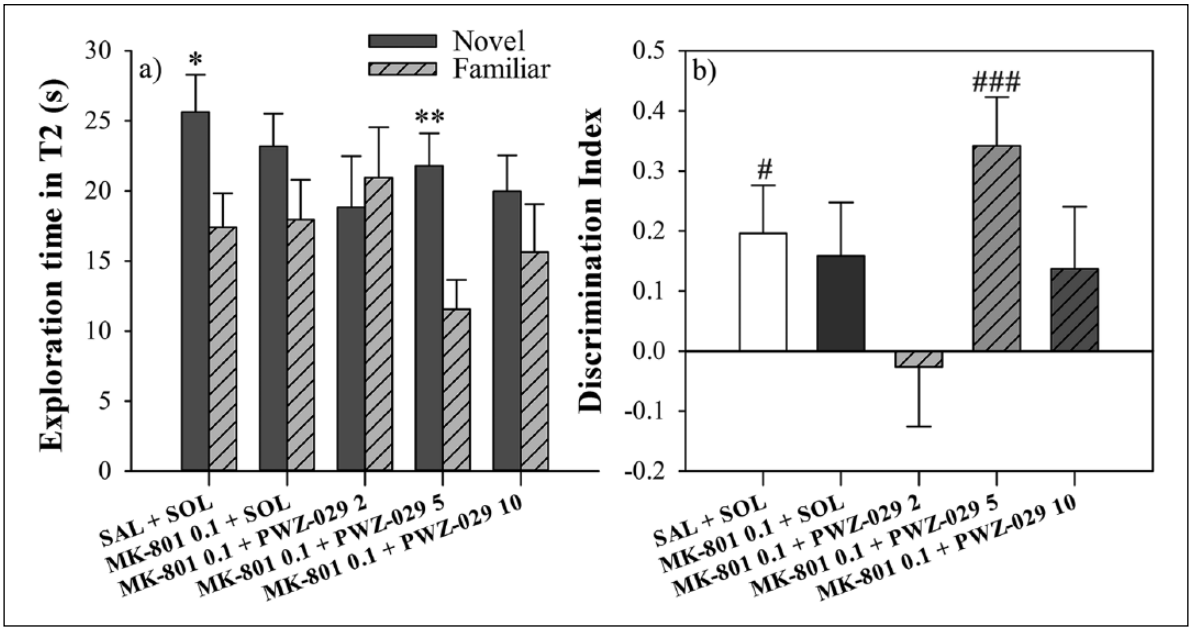

Figure 3. The effects of 0.1 mg/kg MK-801 (MK-801 0.1 + SOL) and combination of 0.1 mg/kg MK-801 and PWZ-029 (2, 5, and 10 mg/kg) on the time exploring familiar and novel object in T2 (a) and on discrimination index (b) in novel object recognition test. Data are represented as mean + SEM. *$p < 0.05$ and **$p < 0.01$ for the familiar vs. novel exploration times (paired-samples t-test). A significant difference from zero for discrimination index is indicated with # (one sample t-test, #$p < 0.05$, ###$p < 0.001$). Number of animals per treatment, for SAL + SOL through MK-801 0.1 + PWZ-029 10, respectively: 23, 20, 16, 17, and 19. SAL = saline, SOL = solvent.

In Experiment V, the tendency of decreasing the locomotor activity by the investigated doses of PWZ-029 on their own did not reach significance (one-way ANOVA for total distance traveled in first 20 min: $F(3,28) = 2.73$, $p = 0.063$). One-way ANOVA applied on the overall data from 60 min recordings after amphetamine was injected revealed a significant effect on total distance traveled ($F(4,35) = 8.01$, $p < 0.001$); *post hoc* results are given in Figure 8(a). Two-way repeated measures ANOVA, applied to 5-min bins, confirmed that amphetamine-treated rats pretreated with any of three doses of PWZ-029 traveled nearly the same distance as those pretreated with vehicle (treatment: $F(4,35) = 8.01$, $p < 0.001$, time: $F(11,385) = 5.06$, $p < 0.001$, interaction: $F(44,385) = 1.17$, $p = 0.216$; Figure 8(b)).

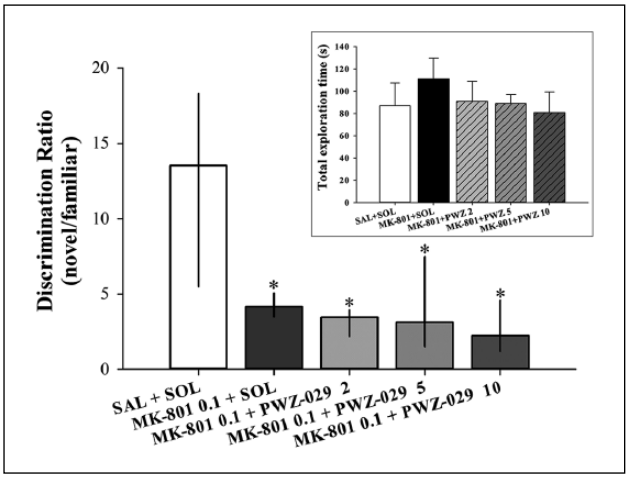

Figure 4. The effects of 0.1 mg/kg MK-801 (MK-801 0.1 + SOL) and combination of 0.1 mg/kg MK-801 and PWZ-029 (2, 5, and 10 mg/kg) on discrimination ratio and total exploration time in P2 in SND procedure. A significant difference is indicated with *(SNK multiple comparison after Kruskal–Wallis test, $p < 0.05$). Data are represented as median ± (25–75%). Number of animals per each treatment group was 6. SAL = saline, SOL = solvent.

Discussion

In the present study, the NMDA receptor hypofunction induced by MK-801, usually seen as a model of memory impairment and psychotic-like behaviors, exerted reliable deficits in three varied cognitive tasks in rats. Our results reveal that negative modulation of α_5 $GABA_A$ receptors was able to prevent only certain aspects of such cognitive disruption. While beneficial effects of PWZ-029 on deficit in hippocampus-dependent spatial learning in the Morris water maze appeared to be partial, and confined to the lowest dose used (2 mg/kg) and the acquisition phase, in NOR test, which predominantly measures visual recognition memory as a type of declarative memory (Young et al., 2009), 5 mg/kg PWZ-029 completely reversed MK-801-induced cognitive deficits. The latter finding was replicated in an additional experiment.

Van der Staay et al. (2011) demonstrated that 0.1 mg/kg MK-801 can reduce the total time the rats spent exploring the objects during T1 in the NOR test. Decrease of total exploration time during familiarization phase observed in our study further suggests that MK-801-induced deficits with 0.1 mg/kg cannot be seen as unambiguously selective for cognition, and emphasizes the necessity for discussing possible motivational and sensorimotor changes. Taking into account the evidence that lack of α_5 $GABA_A$ receptors could itself produce sensorimotor disturbances (Hauser et al., 2005) it is not surprising to see decreased exploration times in combination (MK-801 and PWZ-029) groups even during T2, as presented for the additional

Table 2. The effects of MK-801 and PWZ-029 administration on the rat's behavior in the MWM. Two-way repeated measures ANOVA and overall *post hoc* results for latency (s), total distance (m), mean speed (m/s), and path efficiency.

Two–way repeated measures ANOVA				
Factor	Latency	Total distance	Mean speed	Path efficiency
Treatment: F(4,34)	9.497	5.847	3.341	10.431
p	<0.001	0.001	0.021	<0.001
Days: F(4,136)	33.248	26.229	1.592	19.729
p	<0.001	<0.001	0.180	<0.001
Interaction: F(12,136)	1.410	0.662	1.157	2.100
p	0.410	0.826	0.311	0.481
SNK *post hoc* for treatment				
MK + SOL vs. SAL + SOL	<0.001	<0.001	ns	<0.001
MK + PWZ 2 vs. SAL + SOL	0.035	0.005	ns	0.002
MK + PWZ 5 vs. SAL + SOL	0.002	0.004	ns	<0.001
MK + PWZ 10 vs. SAL + SOL	<0.001	0.006	0.028	<0.001
MK + PWZ 2 vs. MK + SOL	0.022	ns	ns	ns
MK + PWZ 5 vs. MK + SOL	ns	ns	ns	ns
MK + PWZ 10 vs. MK + SOL	ns	ns	ns	ns
MK + PWZ 2 vs. MK + PWZ 5	ns	ns	ns	ns
MK + PWZ 2 vs. MK + PWZ 10	0.042	ns	ns	ns

SAL: saline; SOL: solvent; MK: 0.1 mg/kg MK-801; PWZ 2: 2 mg/kg PWZ-029; PWZ 5: 5 mg/kg PWZ-029; PWZ 10: 10 mg/kg PWZ-029; ns: not significant.

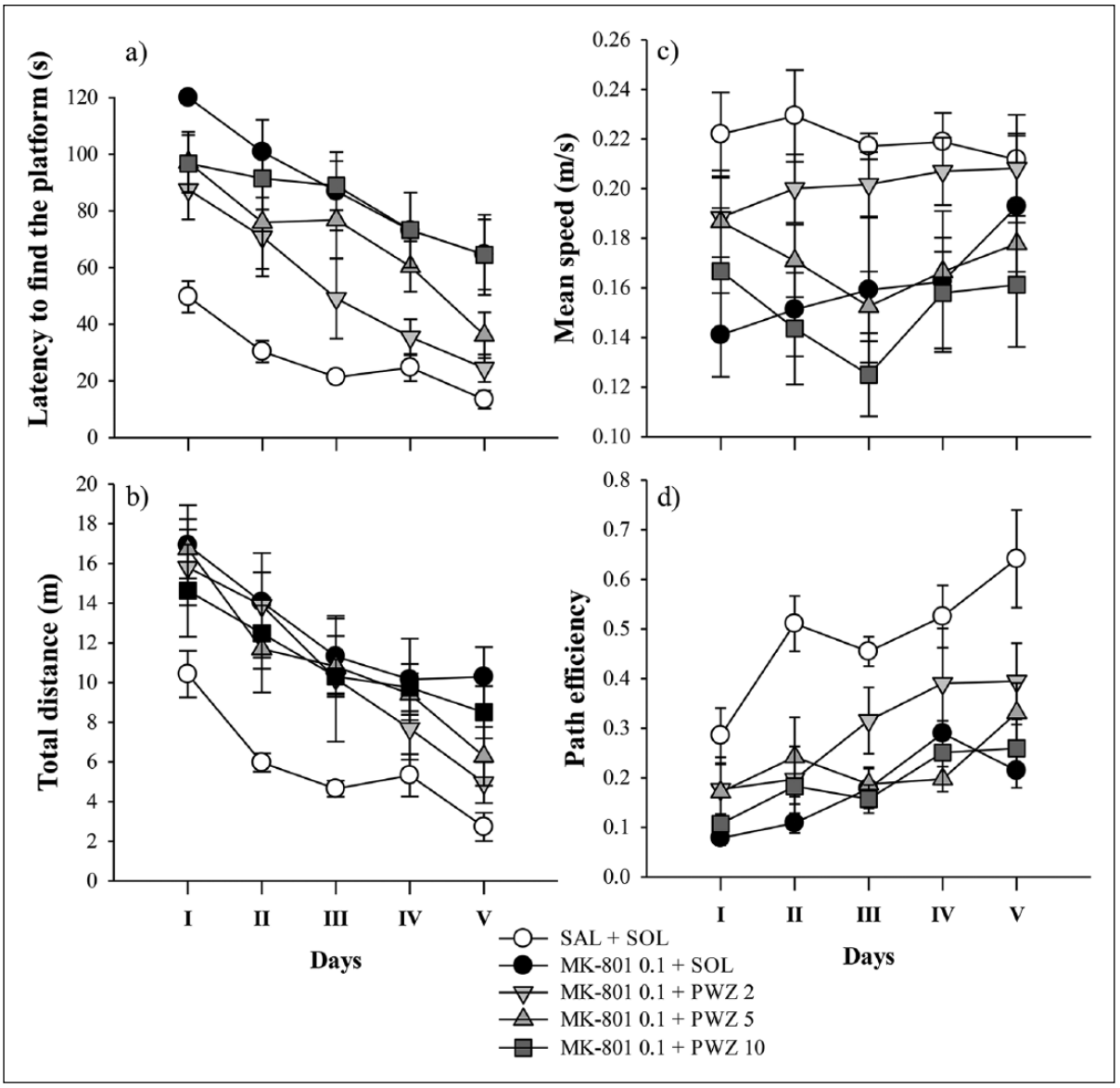

Figure 5. The effects of addition of PWZ-029 (2, 5, and 10 mg/kg) to 0.1 mg/kg MK-801 on the latency to reach the platform (a), the total distance (b), mean speed (c), and the path efficiency (d) during the learning phase of Experiment III. Each data point represents the mean of average values calculated for each rat for the respective day. Number of animals per treatment, for SAL + SOL through MK-801 0.1 + PWZ 10, respectively: 8, 8, 7, 8, and 8. SAL = saline, SOL = solvent, PWZ = PWZ-029.

experiment (see Supplementary material online). Nevertheless, a significant correlation between the time spent investigating two identical objects in T1 and the degree of preference for a novel object does not exist (Gaskin et al., 2010), and any reduction in exploratory activity in treatment groups cannot decisively affect the cognitive ability of the rat. Similarly, non-cognitive influences may also have contributed to the observed differences in two experiments when the exploration times of the novel vs. old object during T2 were assessed by two-way ANOVA of absolute values, but not by the statistical analysis of the discrimination index, as a relative measure of discrimination that corrects for activity and thus is less biased toward any factors affecting exploration, including those related to the sensorimotor performance (Akkerman et al., 2012).

It is notable that PWZ-029 dosed at 5 mg/kg also enhanced formation of declarative memory in normal rats, assessed through the passive avoidance test (Savić et al., 2008). On the other hand, recognition memory deficits in the SND procedure, which engages different brain regions (Watson et al., 2012), were not affected by negative modulation of α_5 $GABA_A$ receptors. Motor hyperactivity induced with 0.2 mg/kg MK-801, reflecting positive symptoms of schizophrenia (Andine et al., 1999), was susceptible to only slight attenuation by PWZ-029, dosed at 10 mg/kg but not 2 and 5 mg/kg. Hyperlocomotion after acute administration of amphetamine was unaffected by previous application of 2, 5, or 10 mg/kg PWZ-029, which is notably different than in the study with RO4938581, another α_5-selective negative modulator (Redrobe et al., 2012). Nonetheless, in the latter study,

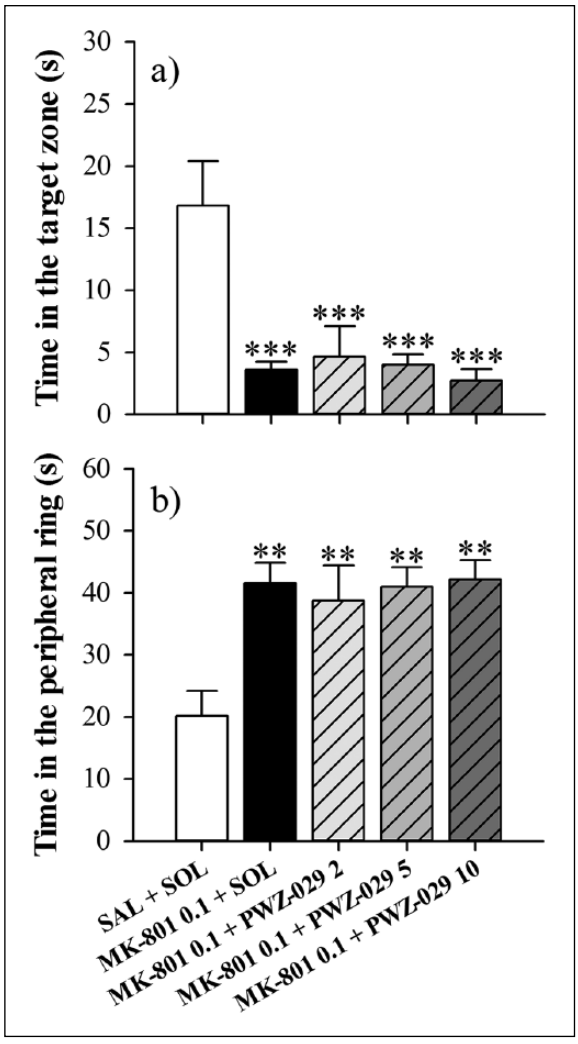

Figure 6. The effects of addition of PWZ-029 (2, 5, and 10 mg/kg) to 0.1 mg/kg MK-801 on the time spent in the target zone (a) and the time spent in the peripheral ring (b) during the probe test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control (SAL + SOL) group. All data are presented as the mean + SEM. Number of animals per treatment, for SAL + SOL through MK-801 0.1 + PWZ-029 10, respectively: 8, 8, 7, 8, and 8. SAL = saline, SOL = solvent.

complete prevention of this behavioral effect of amphetamine was far from accomplished by negative $GABA_A$ receptor modulation (Redrobe et al., 2012), suggesting that possible trials aimed to ameliorate positive symptoms by such an approach would give results which are inferior to those attainable by standard antipsychotic treatment (e.g. Sun et al., 2009).

The calculated pharmacokinetic parameters indicated that PWZ-029 reaches high concentrations in plasma and brain tissue, with relatively fast elimination rate. The approximated electrophysiological responses (Figure 2) of estimated free PWZ-029 brain concentrations (Table 1) suggested that PWZ-029 at 1 μM, which is the concentration roughly twice higher than free brain concentration after the 10 mg/kg dose, could elicit maximally 20% and 15% potentiation at $\alpha_3\beta_3\gamma_2$ receptors and $\alpha_1\beta_3\gamma_2$ receptors, respectively. For comparison, the standard non-selective neutral modulator flumazenil exhibits at 1 μM as much as 56% and 30% of positive modulation at $\alpha_3\beta_3\gamma_2$ and $\alpha_2\beta_3\gamma_2$ receptors, respectively, without significant effects at $\alpha_1\beta_3\gamma_2$ receptors (Ramerstorfer et al., 2010). Having also in mind that modulation of control GABA currents by 1 μM PWZ-029 at $\alpha_3\beta_3\gamma_2$ and $\alpha_1\beta_3\gamma_2$ receptors constitutes no more than 3% and 7%, respectively, of maximum modulation by diazepam, the prototypic positive modulator (personal communication, W. Sieghart), it seems unlikely that partial positive modulation by PWZ-029 on α_3 or α_1 $GABA_A$ receptors would induce noticeable behavioral consequences. However, it cannot be ruled out that even slight modulation by PWZ-029 at receptors other than those containing the α_5 subunit may have some influence on the behavioral changes elicited by MK-801. In regards to modulation by 1 μM PWZ-029 at $\alpha_5\beta_3\gamma_2$ receptors, it amounts to approximately 40% and more than 100% of the maximum negative modulation by the prototypic full negative modulator DMCM and the partial negative modulator FG7142, respectively (personal communication, W. Sieghart), and is thus expected to determine the behavioral profile of PWZ-029 (Savić et al.,2008).

Apparently, while PWZ-029, in the dose range between 2 and 10 mg/kg, did not exert marked differences in selectivity at benzodiazepine-sensitive $GABA_A$ receptors, differences in its behavioral outputs in cognitive tasks were clearly manifested. This may mean that modest changes in negative modulation of α_5 $GABA_A$ receptors, in the settings of NMDA receptor dysfunction elicited by the dose of 0.1 mg/kg MK-801, can give rise to much more complex variability in behavioral outcomes. In freely moving rats, acute i.p. injection of MK-801 (0.1–0.2 mg/kg) increased the neuron firing rates and gamma-band amplitude, while decreased neuronal synchronization in the medial prefrontal cortex, one of the cortical regions most consistently implicated in the etiology of schizophrenia (Molina et al., 2014). In a similar electrophysiological analysis of neuronal activity in medial prefrontal cortex of rats after a single 0.1 mg/kg dose of MK-801, it was found that blockade of NMDA receptors results in excitation of pyramidal neuron activity, which was most probably indirectly caused by inhibition of GABA interneurons through blockade of highly sensitive NMDA receptors which they express (Homayoun and Moghaddam, 2007).

In settings of NMDA blockade-induced increased excitation, therefore, it may be expected that PWZ-029-mediated negative modulation of α_5 $GABA_A$ receptors, with reduction of the amplitudes of the inhibitory postsynaptic potentials (cf. data for another α_5-selective negative modulator, IAα5, given in Ali and Thomson, 2008), leads to further neuronal desynchronization and deterioration of cognitive and other behavioral functions in rats. However, in behavioral paradigms used in our study, PWZ-029 either exhibited no effect (SND) or improved MK-801-induced deficit, slightly to moderately (MWM and LA), or completely (NORT). A similar finding in NORT was also observed with RO4938581, another α_5-selective negative modulator, administered to rats exposed to phencyclidine (Redrobe et al., 2012). Furthermore, we reported that PWZ-029 may induce dose-dependent hypolocomotion in rats, significant at the dose of 10 mg/kg, and susceptible to antagonism by flumazenil as a non-selective, but not βCCt as an α_1-selective neutral modulator (Savić et al., 2008); such an effect of PWZ-029 cannot be easily related to diminished inhibition, i.e. excitation.

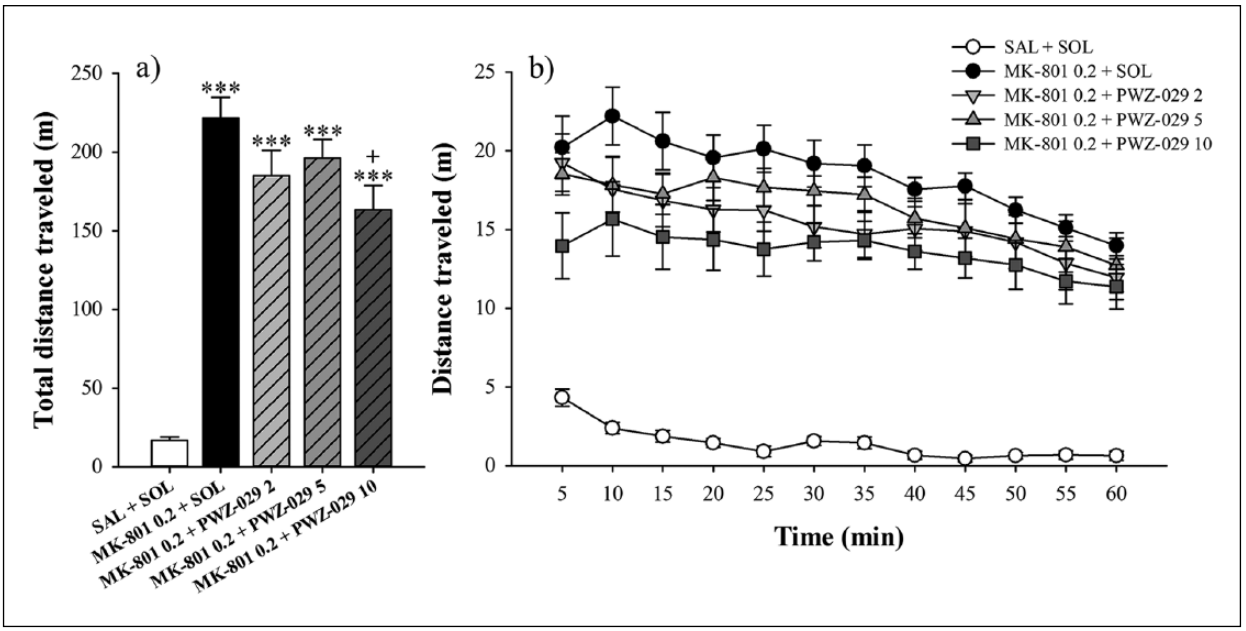

Figure 7. The effects of addition of 2, 5, and 10 mg/kg PWZ-029 to 0.2 mg/kg MK-801 on the total distance traveled (a) and on distance traveled in 5-min bins (b) during 60 min in LA. ***$p < 0.001$ compared to control (SAL + SOL) group; +$p < 0.05$ compared to 0.2 mg/kg MK-801 (MK-801 0.2) group. All data are presented as the mean +/± SEM. Number of animals per treatment, for SAL + SOL through MK-801 0.1 + PWZ-029 10, respectively: 9, 8, 9, 9, and 8. SAL = saline, SOL = solvent.

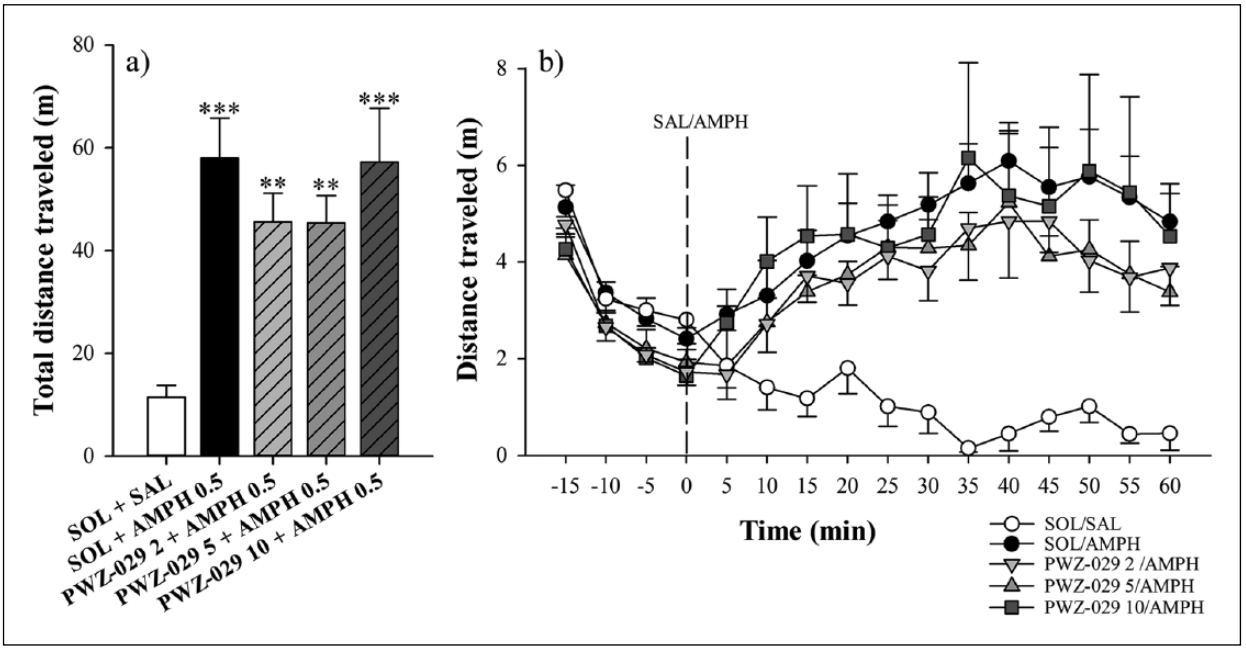

Figure 8. The influence of addition of 2, 5, and 10 mg/kg PWZ-029 to 0.5 mg/kg amphetamine on the total distance traveled during 60 min after amphetamine was injected (a) and on distance traveled in 5-min bins during all testing (b) in LA test. **$p < 0.01$ and ***$p < 0.001$ compared to control (SAL + SAL or SOL + SAL) group. All data are presented as the mean ± SEM. Number of animals per treatment, for SOL + SAL through PWZ-029 10 + AMPH 0.5, respectively: 8, 8, 10, 7, and 7. SAL = saline, SOL = solvent, AMPH = amphetamine.

Receptors containing the α_5 subunit are substantially expressed in brain regions such as the hippocampus, the olfactory bulb and deeper layers of the neocortex (Pirker et al., 2000; Sieghart and Sperk, 2002), and are located at extrasynaptic, but also synaptic sites (Serwanski et al., 2006). With respect to the synaptic population of α_5 $GABA_A$ receptors, they are predominantly found at distal dendritic locations at pyramidal neurons (Ali and Thomson, 2008), but also at inhibitory interneurons, at

least in the hippocampus of an adult rat (Salesse et al., 2011). It was shown that the negative α_5-selective allosteric modulator L655,708, unexpectedly, can decrease the discharge rates of neocortical neurons during episodes of ongoing neuronal activity, in the manner similar to diazepam (Drexler et al., 2013). As proof of receptor selectivity, the excitatory-like action of the α_5-selective positive modulator SH-053-2'F-R-CH3 was absent in brain slices from α_5(H105R) mice, devoid of the benzodiazepine binding site at α_5 $GABA_A$ receptors (Drexler et al., 2013). All of these data together suggest that modulation of α_5 $GABA_A$ receptors, present in pyramidal neurons but also interneurons, elicits the consequences at the electrophysiological and behavioral levels which are not a mere fraction of the sum of effects otherwise seen when all four populations of benzodiazepine-sensitive $GABA_A$ receptors are modulated (cf. Zarnowska et al., 2009). It seems possible that one sub-population of α_5 $GABA_A$ receptors is expressed in a position privileged to control the activity of inhibitory interneurons projecting to pyramidal neurons, which could provide explanation why a negative modulator active at α_5 $GABA_A$ receptors might, at a distinct level of modulation of interneurons vs. pyramidal cells, produce an inhibitory effect.

The actual initiatives aimed to improve treatment of cognitive disruption, especially in schizophrenia, emphasize translational limitations of the current rodent cognitive models, including those used in the present study, and encourage more elaborate analysis of the obtainable results in a wider context of brain functioning (Keeler and Robbins, 2011; Young et al., 2009). Although rather negative (e.g. the probe test in MWM did not confirm the acquisition phase results), our results correspond with the conclusion of Young et al. (2012) that the animal models of cognitive disruption relevant to schizophrenia which demonstrate low to moderate ameliorating effects of pharmacological treatments may represent a "real life" scenario. In this vein, future pharmacological interaction studies with ligands which selectively modulate GABAergic neurotransmission and evidently do not affect the behavior of naïve animals, in parallel with standard antipsychotic drugs, may add to the translational value of preclinical cognitive models.

In conclusion, the present results demonstrated a variable capability of negative modulation of α_5 $GABA_A$ receptors to ameliorate the MK-801-induced deficits of learning and memory in rats, dependent on the cognitive task (declarative-like component of memory was much more susceptible to prevention than spatial, while deficits in social recognition domain were resistant), but also on the subtle changes in degree of receptor modulation (dose dependence of beneficial effects of PWZ-029 was in an apparent inverted-U or even inverted J shape). While hyperlocomotion induced by MK-801 was amenable to only modest, if any, amelioration by PWZ-029, this ligand did not affect amphetamine-induced hyperactivity, suggesting low susceptibility of positive symptoms of schizophrenia to such a treatment. The further research of potential clinical benefit of negative modulation of α_5 $GABA_A$ receptors in schizophrenia should be focused on 'add-on' prevention of deterioration of distinct cognitive domains which are in substantial number of patients resistant to the existing therapeutic options (Citrome, 2014).

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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