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RESEARCH**

## Research Report

**PWZ-029, a compound with moderate inverse agonist functional selectivity at GABA<sub>A</sub> receptors containing  $\alpha$ 5 subunits, improves passive, but not active, avoidance learning in rats****Miroslav M. Savić<sup>a,\*</sup>, Terry Clayton<sup>b</sup>, Roman Furtmüller<sup>c</sup>, Ivana Gavrilović<sup>a</sup>, Janko Samardžić<sup>d</sup>, Snežana Savić<sup>e</sup>, Sigismund Huck<sup>c</sup>, Werner Sieghart<sup>c</sup>, James M. Cook<sup>b</sup>**<sup>a</sup>Department of Pharmacology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia<sup>b</sup>Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201, USA<sup>c</sup>Center for Brain Research, Medical University Vienna, A-1090 Vienna, Austria<sup>d</sup>Department of Pharmacology, Medical Faculty, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia<sup>e</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia

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## ABSTRACT

Benzodiazepine (BZ) site ligands affect vigilance, anxiety, memory processes, muscle tone and epileptogenic propensity through modulation of neurotransmission at GABA<sub>A</sub> receptors containing  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5 subunits, and may have numerous experimental and clinical applications. The ability of non-selective BZ site inverse agonists to enhance cognition, documented in animal models and human studies, is clinically not feasible due to potentially unacceptable psychomotor effects. Most investigations to date have proposed the  $\alpha$ 1 and/or  $\alpha$ 5 subunit-containing GABA<sub>A</sub> receptors as comprising the memory-modulating population of these receptors. The novel ligand PWZ-029, which we synthesized and characterized electrophysiologically, possesses in vitro binding selectivity and moderate inverse agonist functional selectivity at  $\alpha$ 5-containing GABA<sub>A</sub> receptors. This ligand has also been examined in rats in the passive and active avoidance, spontaneous locomotor activity, elevated plus maze and grip strength tests, primarily predictive of the effects on the memory acquisition, basal locomotor activity, anxiety level and muscle tone, respectively. The improvement of task learning was detected at the dose of 5 mg/kg in the passive, but not active avoidance test. The inverse agonist PWZ-029 had no effect on anxiety or muscle tone, whereas at higher doses (10 and 20 mg/kg) it decreased locomotor activity. This effect was antagonized by flumazenil and also by the lower (but not the higher) dose of an agonist (SH-053-R-CH3-2'F) selective for GABA<sub>A</sub> receptors containing the  $\alpha$ 5 subunit. The hypolocomotor effect of PWZ-029 was not antagonized by the antagonist  $\beta$ -CCt exhibiting a preferential affinity for  $\alpha$ 1-subunit-containing receptors. These data suggest that moderate negative modulation at GABA<sub>A</sub> receptors containing the  $\alpha$ 5 subunit is a sufficient condition for eliciting enhanced

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encoding/consolidation of declarative memory, while the influence of higher doses of modulators at these receptors on motor activity shows an intricate pattern whose relevance and mechanism await to be defined.

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## 1. Introduction

The majority of fast inhibitory neurotransmission in the mammalian central nervous system is mediated by GABA<sub>A</sub> receptors. They are, as a whole, profoundly involved in the regulation of vigilance, anxiety, memory processes, muscle tone and epileptogenic propensity (Rudolph and Möhler, 2004). Beside the multiplicity of subunits comprising the GABA<sub>A</sub> receptor pentamer (19 subunits have been identified to the present; Simon et al., 2004), the variety of possibilities for fine tuning of neurotransmission stems from a number of allosteric modulatory sites. It is well established that ligands of the benzodiazepine (BZ) binding site may exert their effects through modulation of four distinct populations of GABA<sub>A</sub> receptors, containing the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5 subunit in addition to the  $\gamma$ 2 and a  $\beta$  subunit (Sieghart and Sperk, 2002; Rudolph and Möhler, 2004). The recent genetic studies with mice carrying a point mutation of histidine to arginine in  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5 subunits, rendering the respective GABA<sub>A</sub> receptors selectively insensitive to effects of BZ site ligands, suggested a specific contribution of individual receptor subtypes to the spectrum of behavioral actions of these compounds (Rudolph and Möhler, 2004). These genetic advances have encouraged synthesis of new, selective BZ site ligands, that possess affinity and/or efficacy profiles which enable separation of wanted from unwanted effects (Sieghart and Ernst, 2005).

One desirable property in this regard is the pro-mnesic activity of BZ site inverse agonists, repeatedly reported in animal models (e.g. Venault et al., 1986; Jensen et al., 1987), as well as in human volunteers (e.g. Dorow et al., 1983; Duka et al., 1996). However, this desirable effect is confounded by different concomitant psychomotor effects (increased vigilance, anxiogenic and/or proconvulsant state), some of which have been described in memory studies with non-selective inverse agonists in humans, urging their early termination (Dorow et al., 1983). Point mutated mice could not be used to identify the receptor subtypes mediating the pro-mnesic activity of inverse agonists because an unexplained switch to the agonist mode of action occurs when an inverse agonist at wild type diazepam-sensitive recombinant GABA<sub>A</sub> receptors is tested at the respective point-mutated receptors (Benson et al., 1998; Crestani et al., 2002a). Thus, inverse agonists exerted agonistic-like sedative and anticonvulsant effects in mice with the point-mutated  $\alpha$ 1 subunits (Crestani et al., 2002a), while corresponding experiments in models of learning and memory were not performed. Nevertheless, behavioral examination of genetically modified animals conducted to date has indicated the  $\alpha$ 1 and  $\alpha$ 5 subunit-containing GABA<sub>A</sub> receptors comprise the 'memory-modulating' population of these receptors (Rudolph et al., 1999; Collinson et al., 2002; Crestani et al., 2002b). It is notable that GABA<sub>A</sub> receptors containing the  $\alpha$ 5 subunit are abundantly expressed in the hippocampus (Pirker et al., 2000; Sieghart and Sperk, 2002), the structure substantially involved

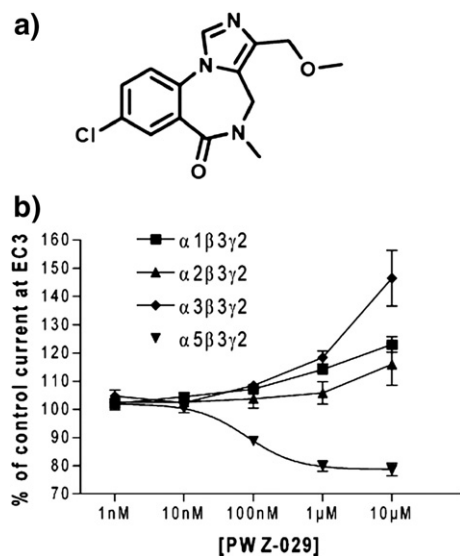
in memory formation (Izquierdo and Medina, 1997). Recent evidence from animal studies with affinity-selective (Atack et al., 2006a) or efficacy-selective ligands (Chambers et al., 2003; Dawson et al., 2006; Collinson et al., 2006) has confirmed that the  $\alpha$ 5 subunit was significantly involved in cognition enhancement mediated by the negative modulation of GABA<sub>A</sub> receptor functions. Moreover, it was shown in humans that pre-treatment with an  $\alpha$ 5 efficacy-selective inverse agonist significantly reduces the amnesic effect of alcohol on learning a word list (Nutt et al., 2007). However, the affinity- or efficacy-selectivity of the ligands, as well as the diversity of the behavioral tasks used in their characterization to date, was of limited extent, which necessitates screening of newer BZ site negative ligands, to determine the putative therapeutic role of such compounds in various disorders with diminished cognitive capabilities in humans (Maubach, 2003).

In this regard, the BZ site ligand PWZ-029 was synthesized (Fig. 1a). Its efficacy profile was examined by two-electrode voltage clamp experiments in *Xenopus* oocytes expressing recombinant GABA<sub>A</sub> receptor subtypes. The behavioral effects on adult male Wistar rats were evaluated in the passive and active avoidance, spontaneous locomotor activity, elevated plus maze and grip strength tests, primarily predictive of detecting the changes in the memory acquisition, basal locomotor activity, anxiety level and muscle tone, respectively.

## 2. Results

### 2.1. Electrophysiological experiments

In vitro data for PWZ-029 (Fig. 1b) demonstrated that at concentrations up to 1  $\mu$ M this ligand engendered a significant partial inverse agonist efficacy at the  $\alpha$ 5-containing GABA<sub>A</sub> receptors (reduction of control current by 20%), whereas its activity at the other three types of receptors tended to be weakly and for  $\alpha$ 1- and  $\alpha$ 3-containing receptors significantly agonistic. Similarly, at 10  $\mu$ M concentrations PWZ-029 exhibited a weak but significant partial agonistic effect at  $\alpha$ 1- and  $\alpha$ 3-containing receptors. The lower potencies of PWZ-029 for  $\alpha$ 1-,  $\alpha$ 2- and  $\alpha$ 3-, relative to the  $\alpha$ 5-containing GABA<sub>A</sub> receptors, deduced from the respective efficacy curves, conform with the explicit binding affinity data (Table 1). Hence, PWZ-029 shows a distinct affinity-, potency-, as well as efficacy-selectivity for the  $\alpha$ 5-containing GABA<sub>A</sub> receptors. PWZ-029 exerted negligible activity in 42 other receptor and enzyme assays (B. Roth et al., NIMH Psychoactive Drug Screening Program, UNC, unpublished results, available at <https://kidbdev.med.unc.edu/pdsp>). The in vitro concentration-effects curves for SH-053-R-CH3-2F, the ligand used for interaction study in the locomotor activity test, showed that it is a high-efficacy agonist at the  $\alpha$ 5-containing GABA<sub>A</sub> receptors, with very low efficacies at  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3-containing subtypes (Rowlett, Furtmüller, Cook, unpublished



**Fig. 1** – Concentration–effects curve for modulation of GABA elicited currents by PWZ-029 (a) on *Xenopus* oocytes expressing GABA<sub>A</sub> receptor subtypes  $\alpha_1\beta_3\gamma_2$ ,  $\alpha_2\beta_3\gamma_2$ ,  $\alpha_3\beta_3\gamma_2$ , and  $\alpha_5\beta_3\gamma_2$  (b). Concentrations of GABA that elicit 3% of the maximum GABA-triggered current of the respective cells were applied alone and with various concentrations of PWZ-029. Control currents represent responses in the absence of PWZ-029. Data points represent means  $\pm$  SEM from 4 oocytes from  $\geq 2$  batches. 1  $\mu$ M PWZ-029 resulted in  $114 \pm 4\%$ ,  $105 \pm 8\%$ ,  $118 \pm 5\%$  and  $80 \pm 4\%$  of control current (at GABA EC<sub>3</sub>) in  $\alpha_1\beta_3\gamma_2$ ,  $\alpha_2\beta_3\gamma_2$ ,  $\alpha_3\beta_3\gamma_2$ , and  $\alpha_5\beta_3\gamma_2$  receptors, respectively. All these values except the one for  $\alpha_2\beta_3\gamma_2$  receptors were significantly different from that of the respective control currents ( $p < 0.01$ , Student's *t*-test).

data); the profile was comparable with that published for its close congener SH-053-R-CH3 (Savić et al., 2008), except for greater potencies and  $\alpha_5$ -efficacy of SH-053-R-CH3-2'F.

## 2.2. Passive avoidance

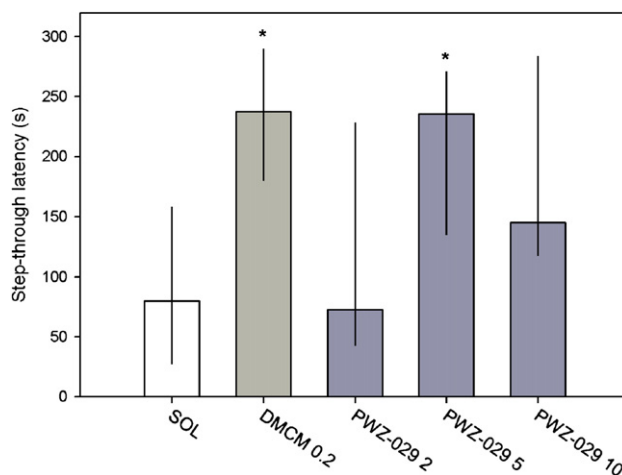
The acquisition session latencies did not differ significantly irrespective of the treatment administered 15 min before the

**Table 1** – Binding affinity (K<sub>i</sub>, nM) at human recombinant GABA<sub>A</sub> receptors containing  $\beta_3$ ,  $\gamma_2$  and named  $\alpha$  subunit, stably expressed in mouse fibroblast L(tk<sup>-</sup>) cells<sup>a</sup>

$\alpha_1$	$\alpha_2$	$\alpha_3$	$\alpha_4$	$\alpha_5$	$\alpha_6$
>300	>300	>300	ND	38.8	>300
920 <sup>b</sup>				30.0 <sup>b</sup>	

<sup>a</sup> Affinity was measured using [<sup>3</sup>H]flumazenil (for  $\alpha_1$ -,  $\alpha_2$ -,  $\alpha_3$ -, and  $\alpha_5$ -containing recombinant receptors), and [<sup>3</sup>H]Ro 15-4513 (for  $\alpha_6$ -containing recombinant receptors) as radioligands, according to the method described in Huang et al., 1996. ND — not determined.

<sup>b</sup> Recent binding affinity, based on the radioligand binding assay described in Lameh et al., 2001, obtained using the transiently transfected Sf9 insect cell line; data by DeLorey et al., unpublished results.



**Fig. 2** – The effects of DMCM (0.2 mg/kg) and PWZ-029 (2, 5 and 10 mg/kg) on retention performance in a passive avoidance task (\* $p < 0.05$  compared to solvent (SOL) group). Number of animals per treatment: 10.

session (data not shown). The influence of the pre-acquisition treatment on the retention trial latency was significant [ $F(4) = 11.45$ ,  $p = 0.022$ ] (Fig. 2). Subsequent Dunn's test indicated that DMCM (0.2 mg/kg) and PWZ-029 at 5 mg/kg, administered before the acquisition session, significantly increased retention session latency relative to the control group.

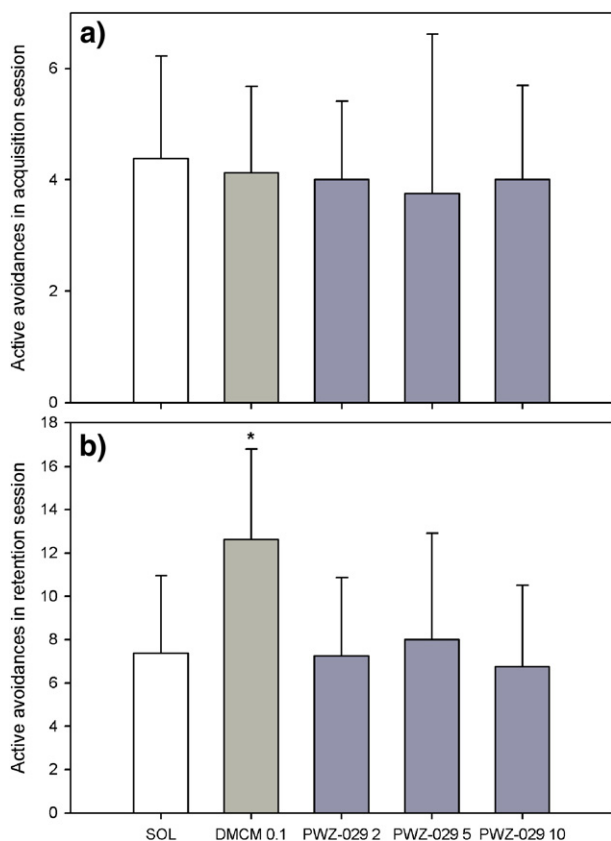
## 2.3. Active avoidance

Fig. 3a shows that DMCM (0.1 mg/kg) and PWZ-029 (2–10 mg/kg) did not affect acquisition of avoidance responses on the training day [ $F(4, 35) = 0.19$ ,  $p = 0.98$ ]. Retention AA performance on the second day was significantly altered by the treatment before the acquisition session [ $F(4, 35) = 2.82$ ,  $p = 0.039$ ]; however, the DMCM (0.1 mg/kg), but not the PWZ-029, was effective (Dunnnett's test) (Fig. 3b). Neither of the motor parameters measured (habituation crossings, intertrial crossings) differed significantly on the training or testing day (data not shown).

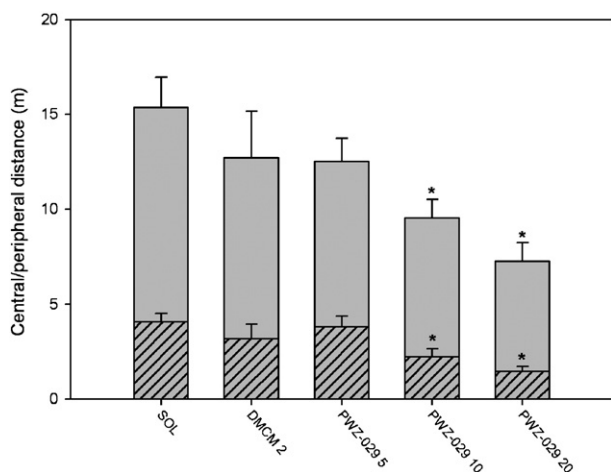
## 2.4. Locomotor activity assay

### 2.4.1. Whole chamber-activity

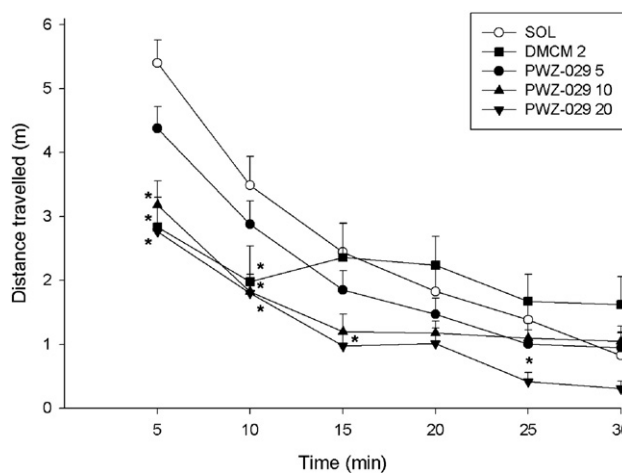
An ANOVA showed a significant effect of treatment on total distance travelled during 30 min of monitoring ( $F(4,43) = 4.06$ ,  $p = 0.007$ ) (Fig. 4, whole bars). According to Dunnnett's test, the two higher doses of PWZ-029 (10 and 20 mg/kg) exerted the activity-decreasing effect related to solvent. When the ANOVA with post hoc analysis was applied on the 5-min intervals of travelled distance (Fig. 5), it turned out that locomotor activity was significantly depressed in the time periods 0–10 min (DMCM 2 mg/kg and PWZ-029 10 and 20 mg/kg), 10–15 min and 20–25 (PWZ-029 20 mg/kg). It is notable that in the periods 15–20 min, 20–25 min and 25–30 min the rats treated with DMCM (2 mg/kg) travelled on average for 23%, 21% and 96% greater distance, respectively, than control animals; the difference did not reach statistical significance at either of the periods (Student's *t*-test).



**Fig. 3 – The effects of DMCM (0.2 mg/kg) and PWZ-029 (2, 5 and 10 mg/kg) on acquisition (a) and retention (b) performance in an active avoidance task (\* $p < 0.05$  compared to solvent (SOL) group). Number of animals per treatment: 8.**



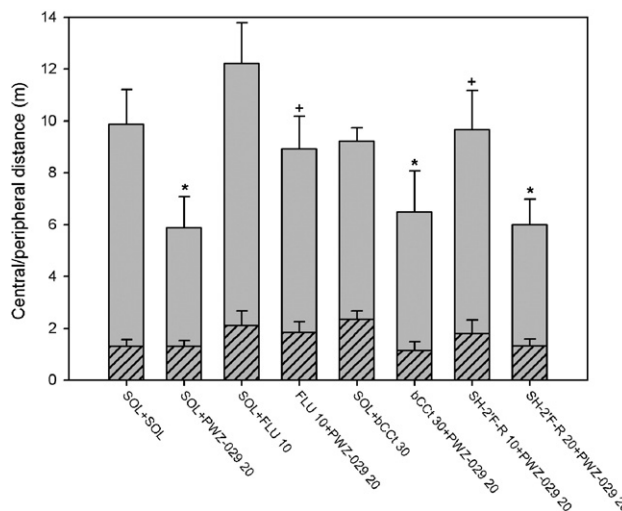
**Fig. 4 – The effects of DMCM (2 mg/kg) and PWZ-029 (5, 10 and 20 mg/kg) on distance travelled in the central (hatched bars) and peripheral (open bars) zone of the activity chamber during 30 min of recording (total activity corresponds to the height of the whole bar). \* $p < 0.05$  compared to solvent (SOL) group. Number of animals per treatment was 10, except for PWZ-029 (8 rats).**



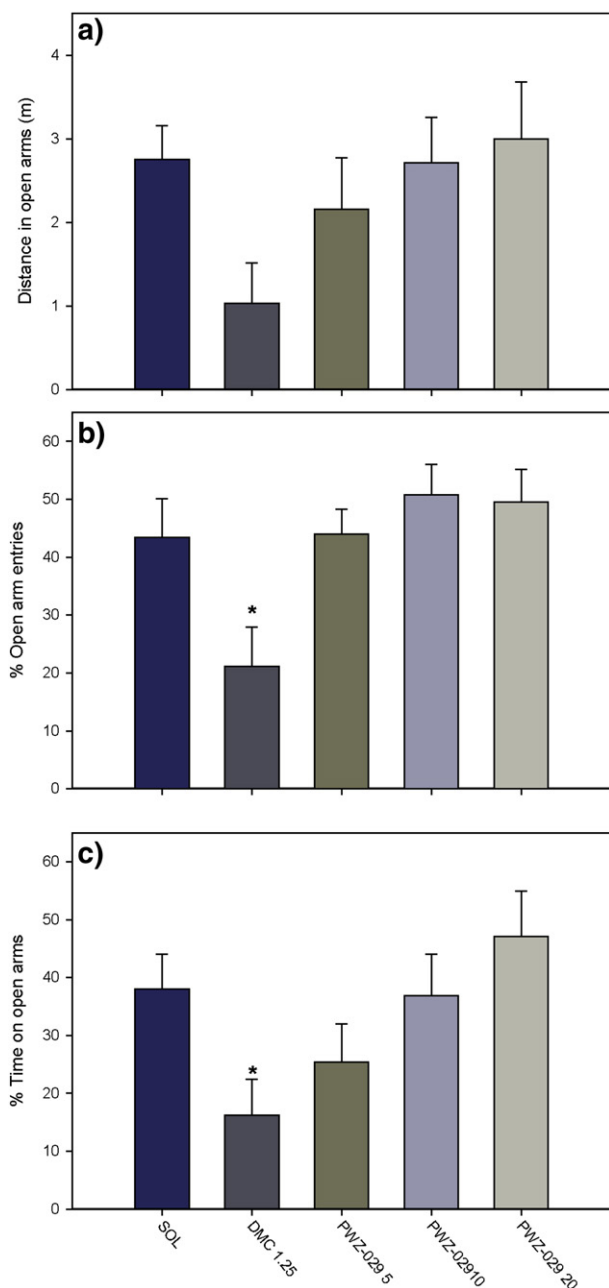
**Fig. 5 – The effects of DMCM (2 mg/kg) and PWZ-029 (5, 10 and 20 mg/kg) on the distance travelled in 5-min intervals in the activity assay. \* $p < 0.05$  compared to solvent (SOL) group. Number of animals per treatment was 10, except for PWZ-029 (8 rats).**

2.4.2. Central/peripheral distance

An ANOVA showed a significant effect of treatment on distance travelled in the arbitrarily set central parts of the chamber during 30 min of monitoring ( $F(4,43)=4.35, p=0.005$ ) (Fig. 4, hatched bars); the effects of PWZ-029 10 and 20 mg/kg were



**Fig. 6 – The influence of addition of antagonists (flumazenil — FLU and  $\beta$ -CCt — hCCt) and the agonist SH-053-R-CH<sub>3</sub>-2'F (SH-2'F-R) on the effect of PWZ-029, administered with solvent (SOL+PWZ-029), on distance travelled in the central (hatched bars) and peripheral (open bars) zone of the activity chamber during 30 min of recording (total activity corresponds to the height of the whole bar). \* $p < 0.05$  compared to solvent (SOL+SOL) group. \* $p < 0.05$  compared to SOL+PWZ-029 group, Student's t-test. Number of animals per treatment, for SOL+SOL through SH-053-R-CH<sub>3</sub>-2'F, respectively, was: 7, 7, 7, 6, 7, 8, 8, and 8.**



**Fig. 7** – The effects of DMCM (1.25 mg/kg) and PWZ-029 (5, 10 and 20 mg/kg) on the a) distance travelled on open arms, b) percentage of entries in open arms and c) percentage of time spent on open arms of the EPM. Number of animals per treatment group was 8. \* $p < 0.05$  compared to solvent (SOL) group.

statistically significant. According to ANOVA, the influence of treatment on activity in peripheral parts of the chamber (Fig. 4, open bars) was similar:  $F(4,43) = 2.91$ ,  $p = 0.033$ ; PWZ-029 20 mg/kg significantly decreased activity (Dunnett's test).

#### 2.4.3. Antagonism of the effect of PWZ-029

Both  $\beta$ -CCt (30 mg/kg) and flumazenil (10 mg/kg), the  $\alpha$ 1-affinity-selective and non-selective antagonist of BZ site, respectively, failed to affect on their own the total distance travelled in the

whole chamber, whereas only flumazenil reversed the suppression of locomotion induced by PWZ-029. The  $\alpha$ 5-selective agonist SH-053-R-CH3-2'F shows a dose-dependent hypolocomotor influence on rats in the spontaneous locomotor activity assay, with the effective dose of 30 mg/kg (Savić, Furtmüller, Cook, unpublished data). When combined with PWZ-029 20 mg/kg, the dose of 10 mg/kg, but not 20 mg/kg, of SH-053-R-CH3-2'F antagonized the effect of the inverse agonist. There were no significant differences among groups in distances travelled in the central zone of the activity chamber (Fig. 6).

## 2.5. Elevated plus maze

### 2.5.1. Activity-related parameters

The influence of treatment on closed arm entries ( $F(4,35) = 0.23$ ,  $p = 0.922$ ), total arm entries ( $F(4,35) = 1.54$ ,  $p = 0.213$ ) and total distance travelled ( $F(4,35) = 1.44$ ,  $p = 0.241$ ) did not reach statistical significance.

### 2.5.2. Anxiety-related parameters

The overall influence of treatment on distance travelled in open arms (Fig. 7a) was not significant ( $F(4,35) = 2.04$ ,  $p = 0.11$ ). There were significant effects of treatment on the percentage of open arm entries ( $F(4,35) = 4.21$ ,  $p = 0.007$ ) (Fig. 7b) and percentage of time spent on open arms ( $F(4,35) = 3.13$ ,  $p = 0.026$ ) (Fig. 7c). In both cases, the effective treatment was DMCM 1.25 mg/kg.

## 2.6. Grip strength test

According to ANOVA, the overall influence of treatment on grip strength was not significant ( $F(4,35) = 2.31$ ,  $p = 0.077$ ). The grip strength (mean  $\pm$  SEM) of rats treated with solvent, DMCM 1.25 mg/kg, PWZ-029 5 mg/kg, PWZ-029 10 mg/kg and PWZ-029 20 mg/kg was  $505.0 \pm 11.4$  g,  $561.1 \pm 29.3$  g,  $546.5 \pm 42.7$  g,  $468.0 \pm 23.3$  g, and  $467.2 \pm 27.1$  g, respectively.

## 3. Discussion

The present results demonstrated that PWZ-029, a novel BZ site ligand, possesses in vitro binding selectivity as well as moderate inverse agonist functional selectivity for the  $\alpha$ 5-containing GABA<sub>A</sub> receptors, which has translated into the pro-mnesic effect detected in the PA, but not AA, learning task in rats. It has been shown that the non-selective inverse agonist DMCM enhances retention performance when administered at a small dose before the acquisition session of the active (Savić et al., 2005a), or passive (File and Pellow, 1988; Savić et al., 2005b) avoidance test, and these findings were replicated as a positive control in the present study. The interactions between DMCM and the antagonists flumazenil and  $\beta$ -CCt have suggested that the  $\alpha$ 1-subunit is substantially involved in processing of both of these memory tasks, whereas the interactions between agonists (midazolam and zolpidem) and antagonists pointed to the importance of some other  $\alpha$ -subunit(s), in addition to the  $\alpha$ 1-subunit, in the passive, but not active avoidance task (Savić et al., 2005a,b). The latter agrees well with the memory-enhancing effect of the  $\alpha$ 5-selective inverse agonist PWZ-029, a ligand supposedly largely inactive at  $\alpha$ 1-containing GABA<sub>A</sub> receptors, in the PA task only.

Although seemingly similar, these two tests differ in some important characteristics, including the type of memory assessed and the brain regions involved in memory processing. Namely, the one-trial step-through PA test assesses declarative memory (to the point that terms such as 'declarative' or 'explicit' can be applied to rodent experiments) (Izquierdo et al., 1999), and for the retention of the task requires the preserved hippocampus (Izquierdo and Medina, 1997). On the other hand, the two-way AA test is an implicit, i.e. procedural (Squire, 1992), hippocampal-independent (Gray and McNaughton, 1983; Collinson et al., 2002) memory task. However, as aversively motivated tests, both 'measure' memory of emotionally arousing experiences, and critically involve activity of the amygdala, especially the basolateral amygdala (McGaugh, 2004) in which the  $\alpha 1$  subunit is the most abundant of the six  $\alpha$  subunits (Pirker et al., 2000; Fujimura et al., 2005). It is felt that this fact at least in part explains the uniform involvement of the  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors in modulation of memory acquisition, found for the present (Rudolph et al., 1999; Savić et al., 2005a,b) as well as similar, aversively motivated, memory tasks, such as the water-lick suppression test (Rudolph et al., 1999).

Receptors containing the  $\alpha 5$  subunit constitute a modest fraction of BZ-sensitive GABA<sub>A</sub> receptors (approximately 5%), but are substantially expressed in several brain regions, most notably the hippocampus, the olfactory bulb and layers V and VI of the neocortex (Pirker et al., 2000; Sieghart and Sperk, 2002). Studies with untreated mice with the point mutation at the  $\alpha 5$  subunit, which unexpectedly displayed a partial loss, restricted to the hippocampus, of the expression of this subunit, have indicated the significance of the  $\alpha 5$  subunit for learning of certain aversively- as well as appetitively-motivated memory tasks which require temporal integration (Crestani et al., 2002b; Yee et al., 2004). On the other hand, in line with the present findings, mice with the deleted  $\alpha 5$  subunit did not differ in comparison to the wild type controls in acquisition of the two-way AA task (Collinson et al., 2002). The results with PWZ-029 in the present memory tests suggest that moderate negative modulation of GABA-ergic transmission mediated by GABA<sub>A</sub> receptors containing the  $\alpha 5$  subunit is a sufficient, but possibly not prerequisite condition for eliciting enhanced encoding/consolidation of declarative memory assessed by the PA test.

PWZ-029 had no effect on anxiety-related parameters in the EPM, in agreement with results with the other  $\alpha 5$ -selective inverse agonist tested in this model (Dawson et al., 2006); the  $\alpha 5$  affinity-selective inverse agonist L-655,708 exerted an unusual U-shaped anxiogenic effect in the murine EPM, but with the use of a non-standard statistical analysis (Navarro et al., 2002). The effect of PWZ-029 on muscle tension measured by the grip strength test also was absent. Hence, its hypolocomotor effect seen in the locomotor activity assay could not be ascribed to any muscle relaxation. Further, there was no hint of convulsive activity in any of the rats treated with PWZ-029 throughout the behavioral studies. Decreases of locomotor activity may also reflect an increase in emotional reactivity, i.e. anxiety (cf. Atack et al., 2005). However, such an activity was not seen with PWZ-029 in the EPM test, nor was its hypolocomotor effect disproportionately more pronounced in the central, more exposed, and hence 'emotional', zone of the chamber, but was notably different from standard patterns of activity of a non-

selective inverse agonist (i.e. DMCM). Namely, as a rule, when rodents explore a novel environment under the influence of a negative modulator of GABA<sub>A</sub> receptors, locomotor activity is depressed, but usually in a relatively short period of habituation (e.g. Jaskiw et al., 2003); afterwards, a type of rebound hyperlocomotion may ensue (Savić et al., 2006). However, as we have concluded from the experiment with DMCM and the  $\alpha 1$ -subunit-selective antagonist  $\beta$ -CCt, hyperlocomotion in habituated animals may depend primarily on the negative modulation at  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors (Savić et al., 2006), and this was not observed with PWZ-029. Instead, the temporal pattern of the extended motor depression with PWZ-029 (20 mg/kg) was more similar to activity of an agonist, rather than an inverse agonist, and the most parsimonious explanation would be that, at the higher doses, PWZ-029 binds to enough  $\alpha 1$ -containing GABA<sub>A</sub> receptors to manifest its slight partial agonistic activity. Nevertheless, the effect of PWZ-029 was mediated by BZ site(s) (antagonism of the effect by flumazenil) other than those at the  $\alpha 1$ -containing GABA<sub>A</sub> receptors (lack of antagonism by  $\beta$ -CCt). Comparative analysis of in vitro affinity and efficacy data of flumazenil and  $\beta$ -CCt (Cox et al., 1995; June et al., 2003; Furtmüller and Sieghart, unpublished data) suggests that the principal difference between them lies in activity at  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors. Namely, flumazenil and  $\beta$ -CCt show qualitatively similar mixed antagonist/agonist efficacy profiles, with the main distinction being at  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors at which  $\beta$ -CCt is a weak inverse agonist, and flumazenil an antagonist/weak agonist. Moreover, a 150-fold lower affinity of  $\beta$ -CCt for the  $\alpha 5$  over the  $\alpha 1$  subtype of GABA<sub>A</sub> receptors (Cox et al., 1995) should plausibly preclude its in vivo binding at  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors and influencing any effect mediated by them. Hence, it is hardly conceivable that  $\beta$ -CCt may antagonize the effects of PWZ-029 at these receptors. Similarly, the slight positive modulation at  $\alpha 2$  or  $\alpha 3$  subunit-containing GABA<sub>A</sub> receptors, also observed at high doses of PWZ-029, could hardly have contributed to its hypolocomotor activity (cf. Low et al., 2000; Atack et al., 2006b).

Standard tests of locomotor activity were not usually employed in reported studies with  $\alpha 5$  subunit-selective ligands (Atack et al., 2006a; Chambers et al., 2003; Collinson et al., 2006; Dawson et al., 2006). Based on the results with three novel BZ site agonists functionally silent at the  $\alpha 1$  subunit, we recently set out the hypothesis that positive modulation at the GABA<sub>A</sub> receptors containing  $\alpha 5$  subunits may contribute to sedative properties of BZ site agonists (Savić et al., 2008). The finding that SH-053-R-CH3-2'F at the lower (10 mg/kg), but not higher dose (20 mg/kg), antagonized the hypolocomotor effect of PWZ-029 adds to the notion that there may exist certain discontinuous 'effective windows' of the modulation of locomotion exerted by neurons expressing the  $\alpha 5$ -subunit-containing GABA<sub>A</sub> receptors. Namely, the maximal level of receptor inhibition obtained with PWZ (80% of the basal GABA effects sustained by the  $\alpha 5$ -subunit-containing receptors) is clearly different from that present in mice lacking this subunit at all (0% of the basal GABA effects at the affected receptors; Collinson et al., 2002) or in mice harboring a point mutation at the  $\alpha 5$  subunits (approximately 70% of the basal GABA effects in the hippocampus related to the  $\alpha 5$ -subunit-containing receptors; Crestani et al., 2002b; Hauser et al., 2005).

Nevertheless, the respective locomotor outputs (a decrease with higher doses of PWZ-029, no changes in knockout mice, stimulation/no changes in mice with a point mutation at the  $\alpha 5$  subunits) were apparently random in relation to the level of receptor modulation, but certainly dependent on the stress level and experimental settings (cf. an increase of spontaneous locomotor activity in the mice with point-mutated  $\alpha 5$  GABA<sub>A</sub> receptor subunits in Hauser et al., 2005, vs. lack of changes in Crestani et al., 2002a,b). The present hypothesis of the protean influences of the  $\alpha 5$ -subunit-containing receptors on locomotion, possibly related to the intricacy of the different processes which are concomitantly modulated by these receptors (such as sensorimotor gating; Hauser et al., 2005, and firing of the layer V pyramidal neurons of the neocortex; Yamada et al., 2007), should be checked through various locomotor tests employing a range of doses of different  $\alpha 5$  subunit-selective ligands, both as single treatments and in combination.

In conclusion, examination of the present results suggests that the negative modulation engendered by the novel BZ site ligand PWZ-029, which demonstrates binding and functional selectivity for the  $\alpha 5$ -containing GABA<sub>A</sub> receptors, may be sufficient to effect enhanced formation of declarative memory. The sedative-like effects, seen at doses higher than those affecting memory formation, reduce the probability of generation of anxiogenic or proconvulsant states. The molecular and neuronal substrates in regard to the role of  $\alpha 5$ -subunit-containing GABA<sub>A</sub> receptors in control of vigilance, including the proposed phenomenon of the 'on/off switch', may be numerous and await to be further elucidated.

## 4. Experimental procedures

### 4.1. Drugs

PWZ-029 (methyl(8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5- $\alpha$ ][1,4]benzodiazepin-3-yl)methyl ether), SH-053-R-CH3-2'F (the (R) stereoisomer of 8-ethynyl-6-(2-fluorophenyl)-4-methyl-4H-2,5,10b-triaza-benzo[e]azulene-3-carboxylic acid ethyl ester) and the preferential  $\alpha 1$ -subunit-selective antagonist  $\beta$ -carboline-3-carboxylate-t-butyl ester ( $\beta$ -CCT) were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin — Milwaukee. The non-selective antagonist flumazenil was donated from F. Hoffman-La Roche (Basel, Switzerland) and the non-selective inverse agonist DMCM was purchased from Research Biochemicals Incorporated (Natick, MA, USA).

### 4.2. Electrophysiological experiments

Cloning of GABA<sub>A</sub> receptor subunits  $\alpha 1$ ,  $\beta 3$  and  $\gamma 2$  into pCDM8 expression vectors (Invitrogen, CA) has been described elsewhere (Fuchs et al., 1995). cDNAs for subunits  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 5$  were gifts from P. Malherbe and were subcloned into pCI-vector. After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMessage mMachine T7 transcription kit (Ambion, TX). The capped transcripts were polyadenylated using yeast poly(A) polymerase (USB, OH) and were diluted and stored in diethylpyrocarbonate-treated water at  $-70$  °C.

The methods used for isolating, culturing, injecting and defolliculating of the oocytes were identical as described previously (Sigel, 1987; Sigel et al., 1990). Briefly, mature female *Xenopus laevis* (Nasco, WI) were anaesthetized in a bath of ice-cold 0.17% Tricain (Ethyl-*m*-aminobenzoate, Sigma, MO) before decapitation and removal of the frog's ovary. Stages 5 to 6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at 18 °C in modified Barths' Medium (MB, containing 88 mM NaCl, 10 mM HEPES–NaOH (pH 7.4), 2.4 mM NaHCO<sub>3</sub>, 1 mM KCl, 0.82 mM MgSO<sub>4</sub>, 0.41 mM CaCl<sub>2</sub>, 0.34 mM Ca(NO<sub>3</sub>)<sub>2</sub>) that was supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Oocytes with follicle cell layers still around them were injected with a total of 2.25 ng of cRNA. cRNA ratio used was 1:1:5 for the  $\alpha$  subunits,  $\beta 3$  and  $\gamma 2$ , respectively. After injection of cRNA, oocytes were incubated for at least 36 hours before the enveloping follicle cell layers were removed. To this end, oocytes were incubated for 20 min at 37 °C in MB that contained 1 mg/ml collagenase type IA and 0.1 mg/ml trypsin inhibitor I-S (both Sigma). This was followed by osmotic shrinkage of the oocytes in doubly concentrated MB medium supplied with 4 mM Na-EGTA and manually removing of the follicle cell layer. After peeling off the follicle cell layer, the cells were allowed to recover overnight before being used in electrophysiological experiments.

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer solution (XR, containing 90 mM NaCl, 5 mM HEPES–NaOH (pH 7.4), 1 mM MgCl<sub>2</sub>, 1 mM KCl and 1 mM CaCl<sub>2</sub>). The oocytes were constantly washed by a flow of 6 ml/min XR which could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO-solutions resulting in a final concentration of 0.1% DMSO perfusing the oocytes. Drugs were preapplied for 30 s before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. For current measurements the oocytes were impaled with two microelectrodes (2–3 m $\Omega$ ) which were filled with 2 mM KCl. All recordings were performed at room temperature at a holding potential of  $-60$  mV using a Warner OC-725C two-electrode voltage clamp (Warner Instruments, Hamden, CT). Data were digitised, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA). Results of concentration response experiments were graphed using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA). Data were graphed as mean  $\pm$  SEM of at least four oocytes from at least two batches.

### 4.3. Behavioral experiments

Experiments were carried out on male Wistar rats (Military Farm, Belgrade, Serbia), weighing 220–250 g. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Medical Faculty in Belgrade. The rats were housed in transparent plastic cages, six animals per cage, and had free access to pelleted food and tap water. The temperature of the animal room was  $22 \pm 1$  °C, the relative humidity 40–70%, the illumination 120 lux, and the 12/12 h light/dark period (light on at 6:00 h). All handling and testing took place during the light phase of the diurnal cycle. Throughout

the study the animals were used only once, with exception of the grip strength measurement, which was done immediately after the tracking of behavior on the elevated plus maze. Spontaneous locomotor activity and elevated plus maze behavior were analyzed by the ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA). All drugs were dissolved/suspended with the aid of sonication in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween 80, and were administered intraperitoneally in a volume of 1 ml/kg, 15 min before behavioral testing (for active and passive avoidance test, before the acquisition session). Time of administration, as well as the doses of DMCM in various tests were chosen based on our previous studies (Savić et al., 2005a,b; 2006). For interaction studies in the locomotor activity assay, the first treatment indicated in combination was administered into the lower left quadrant of the peritoneum 20 min before testing, and the second treatment 5 min later into the lower right quadrant of the peritoneum.

#### 4.4. Passive avoidance (PA) paradigm

The experiments were performed in an adapted Automatic reflex conditioner (Ugo Basile, Milan, Italy, Model 7051), as described earlier (Savić et al., 2005b). In short, the apparatus consisted of a shuttle-box, equipped with a grid floor and divided with a sliding door into a lit and a dark compartment, and a programming unit. The animals were submitted to two, 24-h-separated sessions. The acquisition session started by placing individual subjects in the illuminated compartment. After 30 s, the entrance to the dark compartment was opened, and as soon as the rat had entered it with all four paws, the footshock (2 s, 0.3 mA) was delivered. Immediately afterwards, the rat was returned to its home cage. The same procedure was repeated 24 h later (retention session), without footshock. A cut-off time of 180 s was used on the training day, whereas, on the retention trial, a ceiling of 300 s was imposed.

#### 4.5. Two-way active avoidance (AA) paradigm

The AA test was performed in automated two-way shuttle-boxes (Campden Instruments, Sileby, UK), as described earlier (Savić et al., 2005a). In short, the animals were submitted to two, 24-h-separated sessions. Training and test sessions were procedurally identical. Animals were placed singly into the shuttle-box and left to freely explore for 15 min, and habituation crossings were automatically counted. Afterward, they received 30 tone foot shock trials. During the first 5 s of each trial, a sound signal was presented (broadband noise of 69 dB), allowing the animal to avoid shocks by moving to the other compartment (avoidance response). If the animal did not respond within this period, a foot shock of 0.5 mA (7-s duration) was applied. Crossing to the adjacent compartment during the shock discontinued its delivery. The animal could move freely in the apparatus between trials (18-s intertrial intervals), and the intertrial crossings were automatically counted.

#### 4.6. Measurement of locomotor activity

Activity of single rats in a clear Plexiglas chamber (40×25×35 cm) under dim red light (20 lux) was recorded for a total of 30 min, without any habituation period, using ANY-

maze software (as described above). For purposes of improving data analysis, the central 20% of the chamber (200 cm<sup>2</sup>) was virtually set as a central zone. The minimum percentage of animal that must have been in the zone for an entry to occur was set at 70%, and 50% of the animal must have remained in the zone for an exit not to occur.

#### 4.7. Behavior on the elevated plus maze (EPM)

The apparatus consisted of two open (50×10 cm, with a ledge of 0.3 cm) and two enclosed arms (50×10×40 cm), connected by a junction area (10×10 cm). The illumination in the experimental room was 10 lux on the surface of the arms. At the beginning of the experiment, single rats were placed in the center of the maze, facing one of the enclosed arms, and their behavior was recorded for 5 min. An entry into an open or closed arm was scored when 90% of the animal crossed the virtual line separating the central square of the maze from the arm, whereas an exit occurred when more than 90% of the animal left the respective arm. After each trial, the maze was cleaned with dry and wet towels.

#### 4.8. Grip strength test

Muscle strength was assessed by the grip strength meter (Ugo Basile, Milan, Italy, model 47105). When pulled by the tail, the rat grasps the trapeze connected to a force transducer, and the apparatus measures the peak force of experimenter's pull (in g) necessary to overcome the strength of the animal's forelimbs grip. Each animal was given three consecutive trials, and the median value was used for further statistics.

#### 4.9. Statistical analysis

All numerical data presented in the figures were given as the mean±SEM, except for results from PA test (median latency with 25th, 75th interquartile range; data were non-parametric because the procedure involved a cut-off). For electrophysiological data Student's t-test was used for statistical analysis. Data from PA test were assessed by a Kruskal–Wallis non-parametric ANOVA, with post hoc comparison relative to solvent control by a Dunn's test ( $\alpha=0.05$ ). Data from the AA, EPM, grip strength and activity assay were assessed by a one-way ANOVA. If the ANOVA was significant, each treatment condition was compared with control by a Dunnett's test ( $\alpha=0.05$ ). Where appropriate, the assessment of the antagonist influence on the inverse agonist effect was conducted by a Student's t-test. Statistical analyses were performed with ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA) and SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA).

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