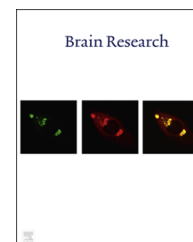


Available online at www.sciencedirect.com**ScienceDirect**www.elsevier.com/locate/brainres**Research Report****Sh-I-048A, an *in vitro* non-selective super-agonist at the benzodiazepine site of GABA_A receptors: The approximated activation of receptor subtypes may explain behavioral effects**

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ABSTRACT

Enormous progress in understanding the role of four populations of benzodiazepine-sensitive GABA_A receptors was paralleled by the puzzling findings suggesting that substantial separation of behavioral effects may be accomplished by apparently non-selective modulators. We report on SH-I-048A, a newly synthesized chiral positive modulator of GABA_A receptors characterized by exceptional subnanomolar affinity, high efficacy and non-selectivity. Its influence on behavior was assessed in Wistar rats and contrasted to that obtained with 2 mg/kg diazepam. SH-I-048A reached micromolar concentrations in brain tissue, while the unbound fraction in brain homogenate was around 1.5%. The approximated electrophysiological responses, which estimated free concentrations of SH-I-048A or diazepam are able to elicit, suggested a similarity between the 10 mg/kg dose of the novel ligand and 2 mg/kg diazepam; however, SH-I-048A was relatively more active at α_1 - and α_5 -containing GABA_A receptors. Behaviorally, SH-I-048A induced sedative, muscle relaxant and ataxic effects, reversed mechanical hyperalgesia 24 h after injury, while it was devoid of clear anxiolytic actions and did not affect water-maze performance. While lack of clear anxiolytic actions may be connected with an

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enhanced potentiation at α_1 -containing GABA_A receptors, the observed behavior in the rotarod, water maze and peripheral nerve injury tests was possibly affected by its prominent action at receptors containing the α_5 subunit. The current results encourage further innovative approaches aimed at linking *in vitro* and *in vivo* data in order to help define fine-tuning mechanisms at four sensitive receptor populations that underlie subtle differences in behavioral profiles of benzodiazepine site ligands.

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1. Introduction

Ionotropic receptors for the inhibitory neurotransmitter γ -aminobutyric acid (GABA) are the most widely distributed receptors in the central nervous system (Watanabe et al., 2002) and at the same time successfully exploited drug targets (Sieghart, 2006). Among drugs acting through GABA_A receptors, benzodiazepines (BZs) have been established as especially versatile GABA-modulatory drugs. However, a plethora of regulatory roles of GABA itself is reflected in a wide range of pharmacological actions of BZs and an increased burden of side effects. The recognition that the molecular substrate of BZ action comprises four populations of GABA_A receptors which contain the α_1 , α_2 , α_3 or α_5 subunit, in addition to the γ_2 subunit, has given an impetus for synthesis and development of new ligands. It was hypothesized that four populations of BZ-sensitive GABA_A receptors mediate distinct sets of behavioral responses, enabling separation of desirable effects mediated by one, from undesirable effects supposedly mediated by the other subtype of receptors (Olsen and Sieghart, 2008; Rudolph and Möhler, 2006).

A number of subtype selective ligands acting at the BZ site have been reported in recent years, with the final aim of obtaining the optimal balance between efficacy and safety and possibly broadening the current spectrum of indications (Rudolph and Knoflach, 2011). This concept pursued the results of genetic studies, which have attributed the sedative and ataxic properties of BZs to the predominantly expressed α_1 subunit-containing GABA_A receptors, the anxiolytic, muscle relaxant and antihyperalgesic actions to the α_2/α_3 subtype-containing receptors, and anticonvulsant activity to all, the $\alpha_1/\alpha_2/\alpha_3$ GABA_A receptors (reviewed in Rudolph and Möhler, 2006). Despite promising preclinical data, no clear clinical breakthroughs have been made to date (Atack et al., 2011) and, indeed, the whole concept has been questioned to a certain degree (Skolnick, 2012). Especially puzzling were the results suggesting that substantial improvement in the behavioral profile may be accomplished by non-selective ligands active at all four populations of BZ-sensitive GABA_A receptors (Auta et al., 2010; Rabe et al., 2007), or even predominantly active at GABA_A receptors containing the α_1 subunit (Lippa et al., 2005; Popik et al., 2006). Such findings were clearly at odds with results from genetic studies showing that sedation and motor impairments exerted by BZs depend to a high degree on positive modulation of α_1 GABA_A receptors (Atack, 2010). The predominantly, though not exclusively hippocampus-based α_5 GABA_A receptors are thought to contribute to amnesic effects of BZs (Rudolph and Möhler, 2006), but their biological role is also the subject of debate, because a number of results suggest that

activation of these receptors may facilitate memory processing (Koh et al., 2013; McEown and Treit, 2013), as well as reduce psychotic symptoms (Gill et al., 2011).

To tackle the problem in a stepwise way, we hereby report on a newly synthesized chiral positive modulator of GABA_A receptors characterized by high affinity, high efficacy and non-selectivity, which was examined for its brain exposure and pharmacodynamic profile in rats. In order to estimate pharmacologically active drug concentrations, we calculated the unbound concentrations in brain (Hammarlund-Udenaes, 2010; Read and Braggio, 2010). *In vitro* and *in vivo* properties, as well as brain exposure of SH-I-048A were directly compared with data generated, under identical experimental conditions, for the standard non-selective BZ site positive modulator, diazepam. The chosen battery of behavioral tests included those assessing motor status of the animal (spontaneous locomotor activity, rotarod, and grip strength), its anxiety level (open field and elevated plus maze), learning and memory capability (Morris water maze) and pain susceptibility (model of peripheral nerve injury). We hypothesized that a ligand with an *in vitro/ex vivo* profile demonstrating superiority or, at least, non-inferiority to diazepam, in terms of availability at the receptor site and its subsequent modulation, should exert a set of *in vivo* actions comparable to those repeatedly reported for diazepam (Rudolph and Knoflach, 2011). Potential failures to support the hypothesis would prove that assigning various behavioral effects to any of specific GABA_A receptor populations demands further refining and obtaining more comprehensive experimental data than usually presented (cf. Skolnick, 2012).

2. Results

2.1. *In vitro* affinity

The *in vitro* binding data for SH-I-048A, in parallel with those for diazepam, are presented in Table 1. The novel ligand showed

Table 1 – Binding affinity (K_i, nM) of SH-I-048A and diazepam at human recombinant GABA_A receptors containing β_3 , γ_2 and named α subunit, stably expressed in mouse fibroblast L(tk⁻) cells; ND – not detected. [³H] flunitrazepam was used as the radiolabelled ligand in the competition binding assay. Data for diazepam were published in Savi et al. (2010).

Ligand	α_1	α_2	α_3	α_4	α_5	α_6
SH-I-048A	0.77	0.17	0.38	ND	0.11	ND
Diazepam	14.0	7.8	13.9	ND	13.4	ND

very high (subnanomolar) affinity for BZ-sensitive recombinant human GABA_A receptors, considerably higher than that of diazepam. Furthermore, no major selectivity in binding at one over the other subtype was noticed (quite similar to diazepam).

2.2. Electrophysiological experiments

Electrophysiological behavior of SH-I-048A and the reference benzodiazepine diazepam in a wide range of concentrations (1 nM–10 μM) is presented in Fig. 1. The novel ligand exerted higher positive modulation than diazepam at all four rat recombinant GABA_A receptor subtypes. In addition, it seemed to interact with a second binding site at concentrations > 1 μM, as indicated by the mathematical fit of the data points by a biphasic dose-response simulation using GraphPad Prism. EC₅₀ values used by this program for fitting the SH-I-048A data were

14 nM and 2560 nM; 14 nM and 1410 nM; 20 nM and 2830 nM; or 12 nM and 2670 nM, for α₁β₃γ₂, α₂β₃γ₂, α₃β₃γ₂, or α₅β₃γ₂ containing receptors, respectively. Although an interesting finding, we did not further investigate this low potency interaction of SH-I-048A because the high free concentrations of the ligand required to generate relevant effects via this second site (probably well above 100 nM) were not reached under the conditions used (see Section 2.3.1) and thus could not have contributed to the results of the present study.

2.3. Estimation of free plasma and brain concentrations

2.3.1. Quantification in rat plasma and brain tissue

The concentrations of SH-I-048A in rat plasma (nmol/L) and brain tissue (nmol/kg), both total and free (estimated), are shown in Table 2. Free concentrations of SH-I-048A were

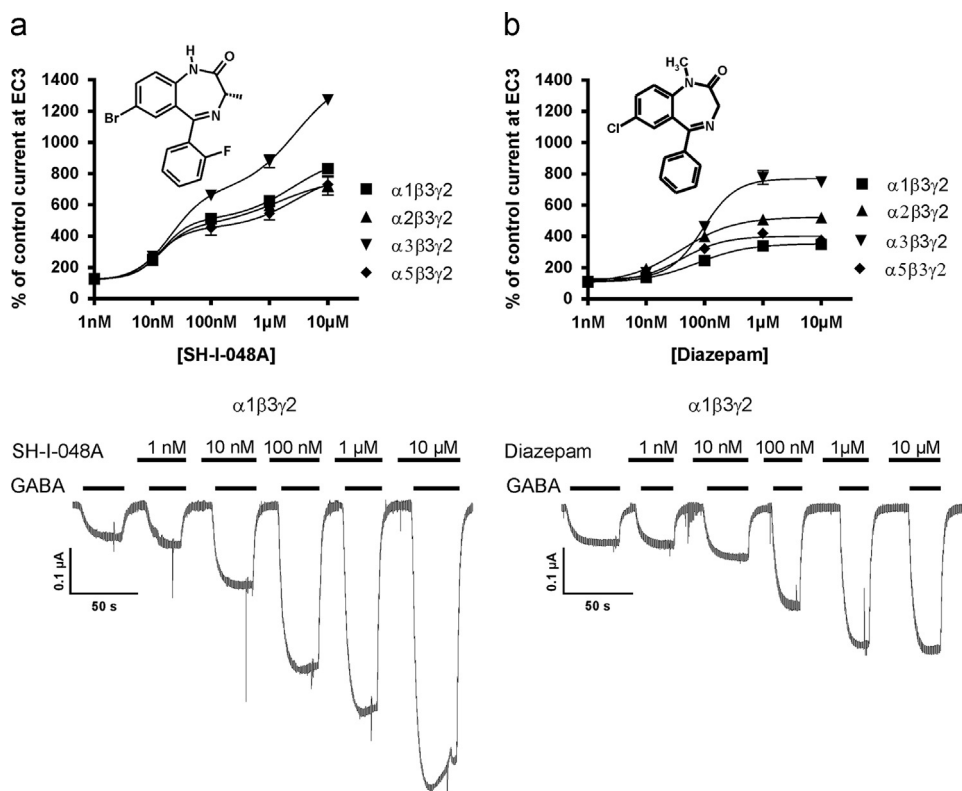


Fig. 1 – Effects of SH-I-048A and diazepam at different GABA_A receptor subtypes. (a) Concentration–response curves of SH-I-048A at α₁β₃γ₂, α₂β₃γ₂, α₃β₃γ₂ and α₅β₃γ₂ receptors (*n* = 3–5). Below: Individual current traces of GABA EC₃ in the absence or presence of increasing SH-I-048A concentrations at α₁β₃γ₂ receptors. (b) Concentration–response curves of diazepam at α₁β₃γ₂, α₂β₃γ₂, α₃β₃γ₂ and α₅β₃γ₂ receptors (*n* = 5–9). Below: Individual current traces of GABA EC₃ in the absence or presence of increasing diazepam concentrations at α₁β₃γ₂ receptors. Data are mean values ± SEM.

Table 2 – Total and estimated free concentrations of SH-I-048A (dosed at 0.5, 2 and 10 mg/kg) in plasma and brain samples after 20 and 60 min.

Dose (mg/kg)		0.5		2		10	
Time (min)		20		60		60	
Plasma (nmol/L)	Total	187.59 ± 26.66	818.82 ± 57.06	617.87 ± 149.35	1591.41 ± 5.15	1396.19 ± 492.70	
	Free	8.43	36.79	27.76	71.50	62.73	
Brain (nmol/kg)	Total	231.12 ± 52.27	750.59 ± 307.14	739.37 ± 182.94	2096.33 ± 858.32	1035.23 ± 421.19	
	Free	3.26	10.60	10.45	29.62	14.63	

calculated by multiplying the measured total plasma and brain concentrations with the appropriate estimated free fractions.

2.3.2. Plasma protein and brain tissue binding studies

Free fractions of SH-I-048A in rat plasma and brain tissue determined by rapid equilibrium dialysis were 4.49% and 1.41%, respectively.

2.3.3. Assessment of in vivo efficacy

The exact in vitro efficacy values for SH-I-048A and diazepam at each of four recombinant GABA_A receptor subtypes, used for the mathematical fit presented in Fig. 2, were applied to calculate linear equations between two concentrations adjacent to the estimated in vivo free brain concentrations, which are presented in Table 2 for SH-I-048A, while the estimated free concentration of 2 mg/kg diazepam equals 58.46 nmol/kg (Savić et al., unpublished data). For example, in vitro efficacies of SH-I-048A at $\alpha_1\beta_3\gamma_2$ receptors are 124% and 256%, for concentrations of 1 nM and 10 nM, respectively, giving rise to potentiation of 157% when in vivo free concentration of 3.26 nM was applied to the corresponding linear equation; the values calculated by this approach are presented in Fig. 7.

2.4. Behavioral experiments

2.4.1. Spontaneous locomotor activity

The influence of SH-I-048A (dosed at 2, 5 and 10 mg/kg) on the total distance traveled during 30 min of monitoring did not reach statistical significance, as assessed by a one-way ANOVA ($F(3,28)=2.76, p=0.061$) (the inset of Fig. 2a). When

the analysis of distance traveled was developed into 5 min bins (Fig. 2a), it turned out that rats treated with SH-I-048A, dosed at 5 and 10 mg/kg, traveled shorter distances than the solvent-treated animals during the second time interval (5–10 min) ($p=0.017$ and $p=0.008$, respectively).

2.4.2. Elevated plus maze

A one-way ANOVA showed no significant effect of treatment on the number of closed arm entries ($F(3,24)=2.64, p=0.072$; Fig. 2b). Regarding the anxiety-related parameters, the same statistical analysis revealed that the effect of treatment on the percentage of the time spent on open arms was not significant, but again a statistical trend was noted ($F(3,24)=2.37, p=0.095$; Fig. 3a). However, ANCOVA revealed that the trend of SH-I-048A to increase this parameter, related to anxiolytic-like activity, disappeared when the concomitant influence on closed arm entries was taken into account ($F(3,23)=2.22, p=0.113$). The doses of 0.5 and 2 mg/kg tested in a preliminary study were devoid of any influences on the EPM parameters (data not shown).

2.4.3. Open field test

A one-way ANOVA revealed a significant effect of treatment on the total distance traveled in the open field arena ($F(4,30)=11.48, p<0.001$; Fig. 2c). Dunnett's post hoc test showed that only SH-I-048A (10 mg/kg) significantly decreased the total distance traveled, when compared to the solvent group ($p<0.001$). Anxiety-related parameter (the percent of time spent in the central zone) was not significantly affected, as assessed by a one-way ANOVA ($F(4,3)=0.60, p=0.669$; Fig. 3b).

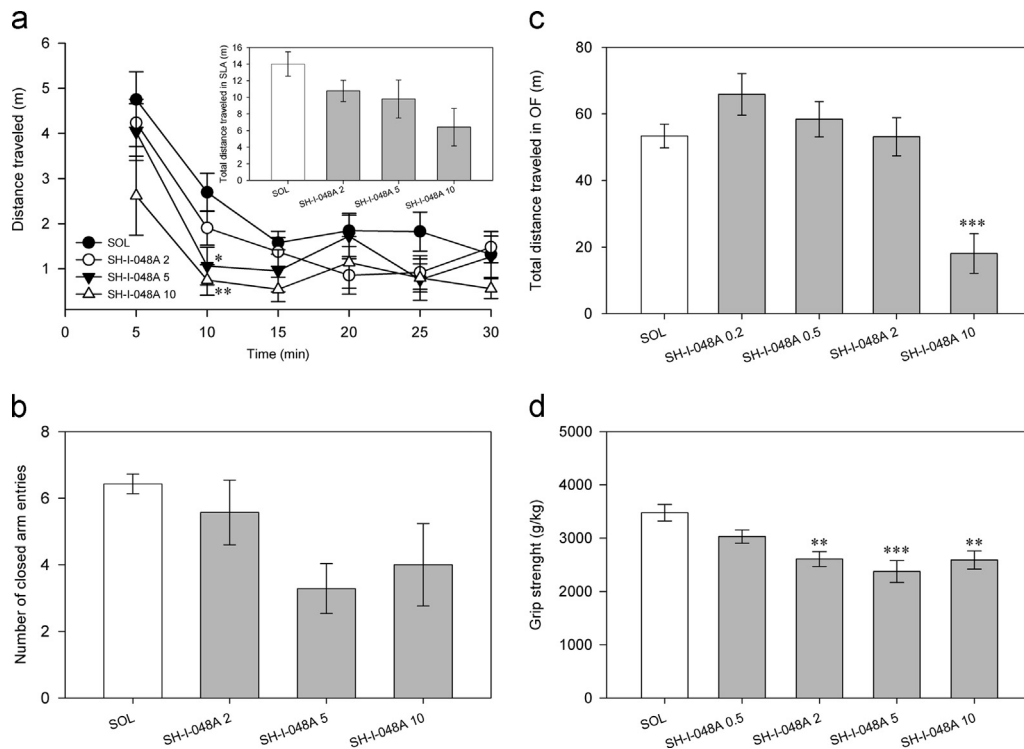


Fig. 2 – The effects of SH-I-048A on the (a) total distance traveled in spontaneous locomotor activity test, (b) number of closed arm entries of elevated plus maze, (c) total distance traveled in open field test, and (d) grip strength. ** $p<0.01$ and *** $p<0.001$, compared to the solvent (SOL) group. Data represent mean \pm SEM. Number of animals per each treatment group in (a) and (d) was 8, while in (b) and (c) was 7.

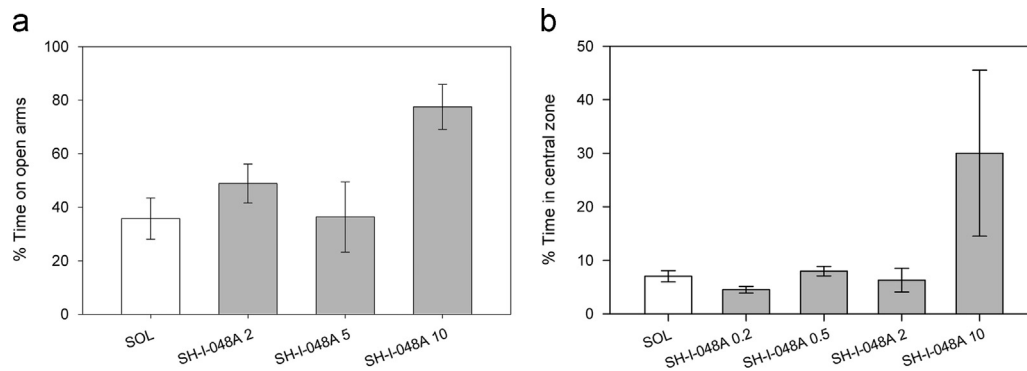


Fig. 3 – The effects of SH-I-048A (2, 5 and 10 mg/kg) on the (a) percentage of time spent on open arms and (b) percentage of spent in the central zone of the open field arena. Data represent mean \pm SEM.

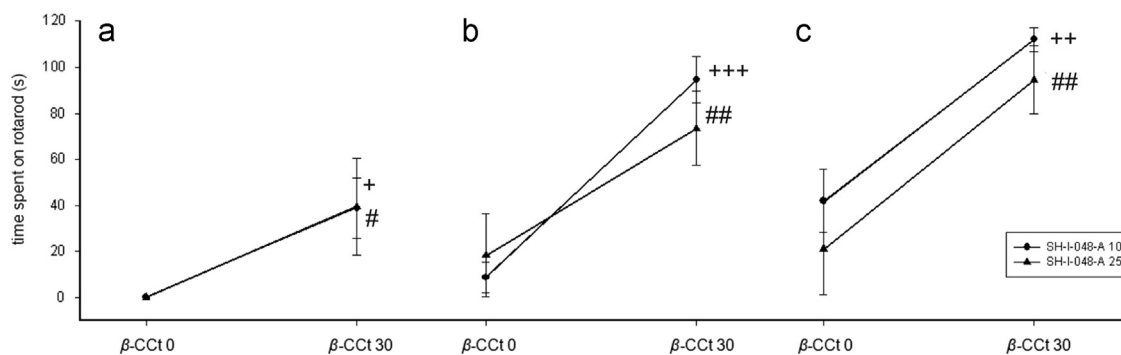


Fig. 4 – The effects of SH-I-048A (10 and 25 mg/kg) in the absence (β -Cct 0) and the presence of 30 mg/kg β -Cct (β -Cct 30) on ataxia in the rotarod test: (a) 30 min, (b) 60 min, and (c) 90 min after treatment. $^+p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to SH-I-048A 10 + β -Cct 0 group; $^#p < 0.05$ and $^{##}p < 0.01$ compared to SH-I-048A 25 + β -Cct 0 group. Data represent mean \pm SEM. Number of animals per treatment varied from 6 to 8.

2.4.4. Grip strength test

A one-way ANOVA revealed a significant effect of treatment on grip strength ($F(4,35)=7.48$, $p < 0.001$; Fig. 2d). Post hoc Dunnett's test showed that rats treated with SH-I-048A (2, 5 and 10 mg/kg) experienced significant muscle relaxation compared to the control (solvent) group ($p=0.002$, $p < 0.001$ and $p=0.002$, respectively).

2.4.5. Rotarod test

When three separate two-way ANOVAs (SH-I-048A and β -Cct, as factors) were applied at each time point, the only significant factor was β -Cct (after 0.5 h: $F(1,24)=9.91$, $p=0.004$; after 1 h: $F(1,24)=30.02$, $p < 0.001$, and after 1.5 h: $F(1,24)=26.38$, $p < 0.001$). Post hoc Tukey test showed that the addition of β -Cct to both 10 and 25 mg/kg SH-I-048A increased latency to fall from the revolving rod at 0.5 h ($p=0.031$ and $p=0.040$, respectively; Fig. 4a), after 1 h ($p < 0.001$ and $p=0.008$, respectively; Fig. 4b), and finally after 1.5 h ($p=0.001$ and $p=0.002$, respectively; Fig. 4c).

2.4.6. Morris water maze

Regarding the dose response study of SH-I-048A in the water maze, a two-way ANOVA with repeated measures revealed that only factor Days were significant, for both the latency to find the platform (treatment: $F(3,24)=0.88$, $p=0.467$; day: $F(4,96)=46.77$, $p < 0.001$; and treatment \times day: $F(12,96)=0.59$, $p=0.843$; Fig. 5a) and the path efficiency (treatment: $F(3,24)=0.89$,

$p=0.463$; day: $F(4,96)=28.40$, $p < 0.001$; and treatment \times day: $F(12,96)=0.61$, $p=0.825$; Fig. 5b). A one-way ANOVA applied on the data from the probe test revealed no significant effect of factor treatment on both parameters measured: the latency to the first entry to the target zone ($F(3,24)=0.28$, $p=0.841$) and the path efficiency to the first entry to the target zone ($F(3,24)=0.73$, $p=0.542$).

2.4.7. Peripheral nerve injury model

A two-way ANOVA with repeated measures showed that both factors were significant (treatment: $F(3,20)=7.83$, $p=0.001$; time: $F(2,40)=124.50$, $p < 0.001$; Fig. 6). Since a significant interaction between these two factors was also determined ($F(6,40)=3.07$, $p=0.015$), we decided to use one-way ANOVAs (for each of three levels of factor Time) to analyze simple main effects. A one-way ANOVA followed by Tukey's post hoc analysis showed no significant differences between all groups at the baseline time (0 h). After 24 h, the same statistical procedure showed that rats treated with three doses of SH-I-048A (10 mg/kg) exhibited significantly higher paw withdrawal threshold compared to the solvent group ($p=0.015$), those treated with only one dose ($p < 0.001$), as well as to rats treated with two doses of SH-I-048A ($p=0.019$). A one-way ANOVA applied on the data measured 48 h after surgery revealed no significant effect of factor Treatment on paw withdrawal threshold ($F(3,2)=1.93$, $p=0.157$). Regarding the

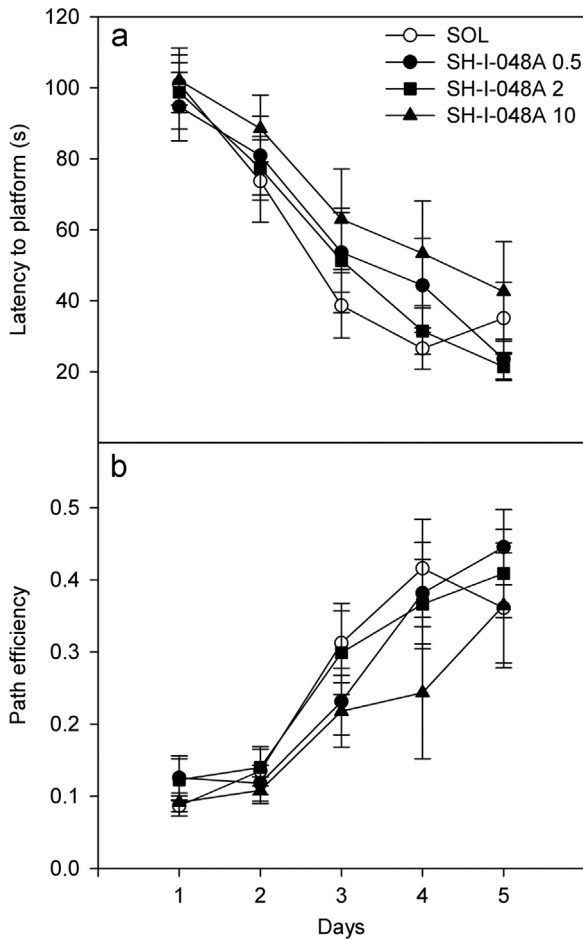


Fig. 5 – The effects of SH-I-048A (0.5, 2 and 10 mg/kg) on the water maze parameters: (a) the latency to reach the platform and (b) the path efficiency. Data represent mean \pm SEM. Number of animals per each treatment was 7.

analysis of data for left (uninjured) hind paws, a two-way ANOVA with repeated measures showed that both factors, as well as their interaction, were without significant effect (treatment: $F(3,20)=1.99$, $p=0.148$; time: $F(2,40)=1.073$, $p=0.352$; and treatment \times time $F(6,40)=1.98$, $p=0.091$).

2.4.8. Studies with diazepam as positive control

Table 3 demonstrates the results of behavioral studies with 2 mg/kg diazepam in comparison to the appropriate control group and, in parallel, the above-presented data for 10 mg/kg SH-I-048A and its control. Although the effects of ligands were generally similar, the observed behavioral inactivity of SH-I-048A in MWM was distinctly different from the incapacitating effect of diazepam during five days of acquisition. This finding was not accompanied by significant differences in the overall latencies during five acquisition days for solvent-treated animals from experiments with diazepam and SH-I-048A, respectively ($F(1,69)=3.169$, $p=0.100$). The only significant difference between controls in two experiments was for the percent time spent in the central zone in OF (data not shown); this discrepancy was probably related to subtle differences in illumination used and also to the well-known variability of results obtained in this test (Prut and Belzung, 2003).

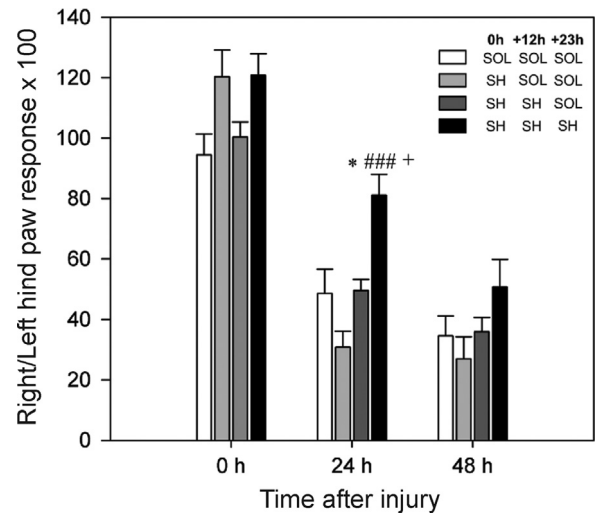


Fig. 6 – The effects of three treatment combinations comprising solvent (SOL) and/or 10 mg/kg SH-I-048A (SH), administered immediately after surgery (0 h), 12 h later (+ 12 h) and after 23 h (+23 h), on the ratios between the right (injured) hind paw and left hind paw responses, obtained by dividing the two values and then multiplying the result by 100, in the peripheral nerve injury model, measured at the baseline (0 h), 24 and 48 h after injury. * $p < 0.05$ compared to 3 \times SOL group, ### $p < 0.001$ compared to 1 \times SH-I-048A+2 \times SOL group, and + $p < 0.05$ compared to 2 \times SH-I-048A+1 \times SOL group. Data represent mean \pm SEM. Number of animals per each treatment group was 6.

3. Discussion

In recent years, several research groups, including ours, have reported on a number of ligands for the BZ site of GABA_A receptors which possessed *in vitro* selectivity for one or two populations of BZ-sensitive receptors, and as a rule low activity at GABA_A receptors containing the α_1 subunit (e.g. Atack et al., 2011; Griebel et al., 2001; Mirza et al., 2008; Savić et al., 2010). Their *in vivo* profiles were mainly in line with the results of genetic studies, which, as a finding of special clinical relevance, associated motor impairing effects with activation of receptors containing the α_1 subunit (reviewed in Rudolph and Möhler, 2006). At the same time, there have been some promising developments also of ligands reportedly non-selective in affinity and efficacy at GABA_A receptors (Auta et al., 2010; Rabe et al., 2007), or even predominantly active at receptors containing the α_1 subunit (Lippa et al., 2005; Popik et al., 2006), but still showing a low potential for motor deficits in animals. In order to shed some additional light on these apparently contradictory results, we discovered and pharmacologically characterized SH-I-048A, a non-selective ligand that exhibited an exceptional, subnanomolar capacity to bind to all four BZ-sensitive GABA_A receptors and also strongly potentiated the inhibitory effect of GABA, mediated through these receptors. Its overall ability to change the rat's behavior was similar, but far from identical, to that characterizing diazepam at the most commonly used 2 mg/kg dose.

Table 3 – Numeric values for diazepam (DZP, 2 mg/kg), SH-I-048A (10 mg/kg) and their appropriate solvent controls (SOL) in behavioral experiments. Each value is the mean \pm SEM of 6–8 rats. Data for SH-I-048A and its solvent control are also graphically presented in Figs. 2–5.

Test/parameter	SOL	DZP (2 mg/kg)	p values (SOL vs. DZP)	SOL	SH-I-048A (10 mg/kg)	p values (SOL vs. SH-I-048A)
SLA total distance (m)	16.6 \pm 1.51	7.17 \pm 1.13***	$p < 0.001$	14.01 \pm 1.47	6.42 \pm 2.26*	$p = 0.014$
EPM number of closed arm entries % time on open arms	5.36 \pm 0.89	4.09 \pm 0.77	$p = 0.306$	6.43 \pm 0.30	4.00 \pm 1.23	$p = 0.080$
OF total distance (m) % time in central zone	40.00 \pm 9.17	76.67 \pm 6.67**	$p = 0.009$	35.71 \pm 7.68	67.43 \pm 12.34*	$p = 0.050$
GS grip strength (g/kg)	53.91 \pm 4.68	24.50 \pm 5.34***	$p = 0.001$	53.36 \pm 3.56	15.39 \pm 5.35***	$p < 0.001$
RR time on rotarod (s)	2.11 \pm 0.42	3.09 \pm 1.15	$p = 0.437$	7.04 \pm 1.06	30.01 \pm 15.49	$p = 0.165$
MWM latency (s)	2698.33 \pm 211.36	1722.50 \pm 197.65**	$p = 0.007$	3477.30 \pm 154.85	2588.83 \pm 169.52**	$p = 0.002$
Day 1	120.00 \pm 0.00	41.70 \pm 9.79***	$p < 0.001$	120.00 \pm 0.00	0.25 \pm 0.16***	$p < 0.001$
Day 2	73.92 \pm 9.14	103.45 \pm 10.64	$p = 0.057$	101.10 \pm 5.97	102.11 \pm 9.11	$p = 0.927$
Day 3	64.84 \pm 13.09	92.82 \pm 6.40	$p = 0.079$	73.75 \pm 11.58	88.52 \pm 9.40	$p = 0.342$
Day 4	31.57 \pm 6.37	77.83 \pm 15.52*	$p = 0.017$	38.68 \pm 9.21	62.98 \pm 14.13	$p = 0.175$
Day 5	37.66 \pm 8.79	59.02 \pm 9.78	$p = 0.130$	26.56 \pm 5.82	53.39 \pm 14.74	$p = 0.116$
	17.61 \pm 3.10	56.40 \pm 10.20**	$p = 0.003$	35.11 \pm 10.09	42.65 \pm 13.99	$p = 0.670$

* $p < 0.05$.
 ** $p < 0.01$.
 *** $p < 0.001$ compared to the respective SOL.

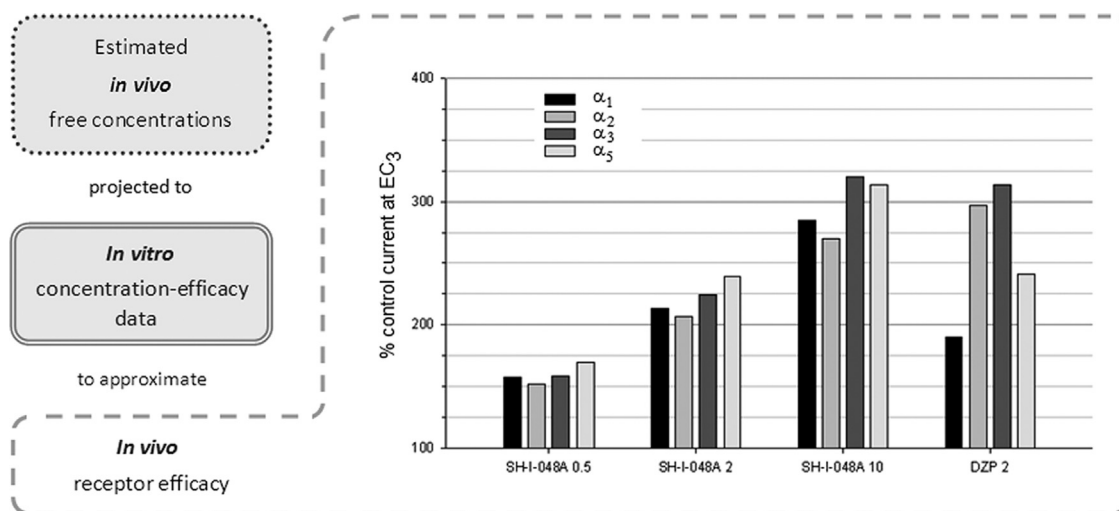


Fig. 7 – Approximated electrophysiological behavior of the estimated free brain concentrations, based on the total brain concentrations measured 20 min after i.p. administration of SH-I-048A (0.5, 2 and 10 mg/kg) and diazepam (2 mg/kg; DZP 2). The estimated free concentrations of SH-I-048A are given in Table 2, while the estimated free concentration of 2 mg/kg diazepam equals 58.46 nmol/kg (Savić et al., unpublished data). The bars indicate the stimulation of the individual $\alpha_x\beta_3\gamma_2$ GABA_A receptors at these concentrations, as extrapolated from data presented in Fig. 1.

The ligand SH-I-048A is structurally closely related to typical benzodiazepines such as diazepam and flurazepam, except that it contains a chiral methyl function at C-3 with the S-configuration. Previously, in another series of ligands, it was demonstrated that the binding, efficacy and *in vivo* activity of ligands which contain a chiral S-CH₃ group at C-4 (similar to the C-3 S-CH₃ function in SH-I-048A) is different than the R-CH₃ isomer (Fischer et al., 2010; Savić et al., 2010). Importantly, the R-enantiomer of SH-I-048A was almost completely inactive in the same oocyte assay (not shown), consequently stimulating interest in the study of chiral S-enantiomer described in this manuscript. Hence this chiral function would be expected

to alter the profile of SH-I-048A vs. diazepam. The question was to what extent and how.

It is generally accepted that the receptor effects, beside affinity and efficacy, depend on the available unbound ligand (Hammarlund-Udenaes, 2010; Read and Braggio, 2010). Here, we present an innovative approach aimed to compare brain exposure and electrophysiological data obtained for SH-I-048A to the results for diazepam, observed in the same experimental settings. The approximated electrophysiological responses, which the estimated free concentrations of two ligands are able to elicit (Fig. 7), suggest that the 2 mg/kg dose of diazepam corresponds to a five times higher dose of the novel ligand, at

least after intraperitoneal administration in Wistar rats. Such a relation could not have been predicted solely from knowledge of the *in vitro* properties. Moreover, any discrepancies in *in vivo* profiles could be governed by relative differences in the approximated receptor activity of diazepam and SH-I-048A. Most remarkably, a substantial difference related to α_1 - and α_5 -containing GABA_A receptors, at which SH-I-048A is relatively more active than diazepam, could be noted in Fig. 7.

SH-I-048A dose-dependently affected parameters of general motor activity and dexterity, in a manner similar to diazepam (Milić et al., 2012; Savić et al., 2009; Siemiakowski et al., 2000). Among parameters of locomotor activity, the statistical significance of the sedative effect was reached for distance traveled during the recording period 5–10 min in SLA, at doses of 5 and 10 mg/kg, and the total distance traveled in OF, at the dose of 10 mg/kg. Grip strength, measuring muscle relaxant effect, was moderately, but significantly reduced at doses of 2–10 mg/kg. Having in mind non-selectivity of SH-I-048A, these two sets of results conform with the postulate that sedation is mediated by α_1 GABA_A receptors, and muscle relaxation by α_2 and α_3 GABA_A receptors (Rudolph and Knoflach, 2011).

While our recent results showed that the administration of β -CCt fully antagonized the impairment of motor coordination in rats induced by diazepam (Milić et al., 2012), the antagonist effect of this α_1 -selective ligand on the prolonged rotarod incapacitation induced by SH-I-048A was significant, but still not sufficient enough to enable the rats to regain the control performance, especially at the 30 min time point, when they spent less than 40 s on the revolving rod, far different from the basal value of 120 s. Since our analysis (Fig. 7) suggested a similarity of the effects of SH-I-048A and diazepam elicited at α_2 and α_3 GABA_A receptors, the difference in the behavioral effect has probably been contributed to the population of α_5 -containing GABA_A receptors, which are more activated by SH-I-048A than by diazepam.

Diazepam in a range of moderate doses commonly exerted anxiolytic-like actions in the EPM (Savić et al., 2010) and OF (Prut and Belzung, 2003), whereas SH-I-048A, applied in a wide range of doses (0.5–10 mg/kg in EPM and 0.2–10 mg/kg in OF), did not significantly affect the parameters of anxiety-related behavior in either of these two anxiety tests. Moreover, the observed tendency of SH-I-048A to induce anxiolytic-like changes at the dose of 10 mg/kg appeared to be confounded by locomotor influences, as indicated by the results of statistical analysis which involved the number of closed arm entries as a covariate. We hypothesize that the motor effects mediated by an enhanced potentiation at α_1 and/or α_5 GABA_A receptors, relative to diazepam, probably hampered the anxiolytic actions of SH-I-048A expected to be effected by α_2 and/or α_3 GABA_A receptors (Rudolph and Knoflach, 2011). The results from OF and tests of motor activity suggest that even diazepam at the dose of 2 mg/kg was prone to elicit suppressing motor effects which may compromise the assessment of anxiety-like behaviors.

The results obtained in MWM demonstrated a lack of BZ-characteristic impairment of learning and memory parameters during acquisition sessions and probe trial. This finding is clearly different from the well-established incapacitating actions of diazepam (Arolfo and Brioni, 1991) and

generally unexpected, especially bearing in mind the noticeable activity of SH-I-048A at α_1 and α_5 GABA_A receptors, which were shown to contribute to the water-maze incapacitation exerted by diazepam (Savić et al., 2009). Noteworthy, the rats dosed with 10 mg/kg SH-I-048A, apparently heavily sedated immediately before being placed into the water-maze, appeared vigilant when confronted with a challenging task. While seemingly unexplainable in the scope of the results of genetic studies (Rudolph and Knoflach, 2011), the observed 'behavioral silence' of SH-I-048A in MWM may be connected with the recent finding that potentiation of α_5 GABA_A receptors increases firing rates of cortical neurons during episodes of ongoing neuronal activity (up states) (Drexler et al., 2013). In this vein, the observed higher activity at α_5 GABA_A receptors of SH-I-048A compared to diazepam may have contributed to a kind of compensatory effect on MWM performance, noted in the current investigation.

The decrease of GABA neurotransmission in the spinal cord contributes to the enhanced pain sensitivity observed in pain states, especially those of neuropathic nature (Munro et al., 2009). At the behavioral level, it was shown that GABA_A receptor positive allosteric modulators, such as diazepam, attenuate nociceptive transmission in animal models of neuropathic pain (Knabl et al., 2009; Malan et al., 2002). The present study has focused on sciatic crush injury (Bester et al., 2000), which mimics a common and refractory disability. SH-I-048A, administered in three 10 mg/kg doses, has reversed mechanical hyperalgesia 24 h after injury, as measured 1 h after administration of the last dose. Motor incapacitation elicited by the novel ligand, demonstrated in tests of motor function, cannot be seen as substantially confounding these results, having in mind normal responses of uninjured hind paws. Although the antihyperalgesic influence of treatment was statistically not significant 48 h after injury, there was still a certain level of the residual effect in the group injected thrice with SH-I-048A. In the more common chronic constriction injury model, which could be regarded as similar to the crush injury model used in the present study, it was shown that diazepam was able to attenuate hind paw hypersensitivity at the highest dose tested (10 mg/kg) (Munro et al., 2008). On the contrary, zolpidem, a positive modulator highly effective at α_1 -, moderately effective at $\alpha_{2/3}$ - and ineffective at α_5 GABA_A receptors (Savić et al., 2010), was completely inactive in the same model (Munro et al., 2008). Genetic and pharmacological studies have focused on the role of benzodiazepines in central pain transmission on the population of $\alpha_{2/3}$ GABA_A receptors (Di Lio et al., 2011; Knabl et al., 2008). However, a number of studies have pointed to α_5 -containing receptors as possibly contributing to GABA-mediated antihyperalgesia (Nickolls et al., 2011). A substantial potential of antihyperalgesic action of SH-I-048A dosed thrice at 10 mg/kg may be seen as further adding to the possible role of α_5 GABA_A receptors, otherwise moderately present in the spinal cord (Paul et al., 2012), in modulation of pain transmission.

In conclusion, this paper presented SH-I-048A, a novel, chiral, highly potent and efficacious positive allosteric modulator at the BZ site of recombinant GABA_A receptors. Apparently small differences in the simulated *in vivo* environment, approximated on the basis of electrophysiological, equilibrium

dialysis and rat brain exposure studies, have been reflected in a number of differences in behavioral profiles of SH-I-048A and diazepam, which may be connected with relatively higher activity of SH-I-048A at α_1 and/or α_5 GABA_A receptors. It appears crucial to establish firmer links between *in vitro* and *in vivo* data in order to help define the criteria for declaring selectivity or non-selectivity of BZ site ligands, and the presented approximation of activation of GABA_A receptor subtypes may be a valuable approach. The current results suggest that diazepam, commonly seen as a prototype of non-selective BZ site ligands, does possess a kind of 'semi-selectivity' towards α_2 and α_3 GABA_A receptors, far different from SH-I-048A, which thus proved to be a useful experimental tool.

4. Experimental procedures

4.1. Drugs

The novel chiral compound SH-I-048A ((S)-7-bromo-5-(2-fluorophenyl)-3-methyl-1H-benzo[e][1,4]diazepin-2(3H)-one) and β -Cct (t-butyl- β -carboline-3-carboxylate), the preferential α_1 -containing GABA_A receptor selective antagonist, were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin – Milwaukee, while diazepam was obtained from Galenika (Belgrade, Serbia).

4.2. Competition binding assays

Competition binding assays were performed in a total volume of 0.5 mL at 4 °C for 1 h using [³H]flunitrazepam as the radiolabelled ligand. A total of 6 μ g of cloned human GABA_A receptor DNA containing the desired α subtype along with β_2 and γ_2 subunits were used for transfecting HEK 293T cell lines using Fugene 6 (Roche Diagnostic) transfecting reagent. Cells were harvested 48 h after transfection, washed with Tris-HCl buffer (pH 7.0) and Tris-acetate buffer (pH 7.4) and the resulting pellets were stored at –80 °C until assayed. On the day of the assay, pellets containing 20–50 μ g of GABA_A receptor protein were resuspended in 50 mM Tris-acetate pH 7.4 at 4 °C and incubated with the radiolabel as previously described (Choudhary et al., 1992). Nonspecific binding was defined as radioactivity bound in the presence of 100 μ M diazepam and represented less than 20% of total binding.

Membranes were harvested with a Brandel cell harvester followed by three ice-cold washes onto polyethyleneimine-pretreated (0.3%) Whatman GF/C filters. Filters were dried overnight and then soaked in Ecoscint A liquid scintillation cocktail (National Diagnostics; Atlanta, GA). Bound radioactivity was quantified by liquid scintillation counting. Membrane protein concentrations were determined using an assay kit from Bio-Rad (Hercules, CA) with bovine serum albumin as the standard.

4.3. Electrophysiological experiments

cDNAs of rat GABA_A receptor subunits were used for generating the respective mRNA's that were then injected into *Xenopus laevis* oocytes (Nasco, WI) as described previously (Ramerstorfer et al., 2010). 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 MgCl₂, 1 mM KCl and 1 mM CaCl₂). 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. To test for modulation of GABA-induced currents by drugs, a concentration of GABA was used that was titrated to trigger 3% of the respective maximum GABA-elicited current of the individual oocyte (EC₃). At this low GABA concentration, within-day and between-day currents are reproducible and effects of drugs (% of modulation) are much higher than that at higher GABA concentrations, again contributing to the accuracy of the results. In addition, such low GABA concentrations correspond to those acting at extrasynaptic receptors that represent the majority of GABA_A receptors in the brain (Kasugai et al., 2010). In contrast, GABA concentrations acting at synaptic GABA_A receptors in many cases fully activate these receptors. At such GABA concentrations benzodiazepines cannot enhance GABA currents anymore, but they prolong current decay. This effect, however, is antagonized by a simultaneously occurring rapid receptor desensitization making measurements unreliable. The GABA EC₃ concentration was applied to the cell together with various concentrations of drugs. For current measurements the oocytes were impaled with two microelectrodes (2–3 m Ω) 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 digitized, 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).

Maximum GABA-evoked currents (1 mM GABA) were measured in each oocyte and were in the 4.5–7 μ A range for all receptor subtypes investigated, indicating an adequate expression of the different receptor subtypes. From these data, GABA EC₃ values were calculated and the oocytes were then stimulated by the respective GABA concentration, confirming the calculated value. The GABA EC₃ current was then modulated by 1 μ M diazepam, which should nearly maximally modulate the receptors. Further measurements with the oocyte were only performed if the extent of modulation by diazepam was in a range corresponding to that shown in Fig. 1b for the respective receptor subtype.

4.4. Estimation of free plasma and brain concentrations

4.4.1. Quantification in rat plasma and brain tissue

Eighteen Wistar male rats (180–220 g, Military Farm, Belgrade, Serbia) were divided into six treatment groups (3 animals per group) that intraperitoneally (i.p.) received 0.5 mg/kg SH-I-048A 20 min before decapitation, 2 or 10 mg/kg SH-I-048A 20 or 60 min before decapitation, or solvent. Blood samples were collected by cardiac puncture into heparinized tubes then

centrifuged at 2500g for 10 min to obtain plasma. Thereafter, brains were rapidly removed, measured and homogenized at 16,000 rpm for 2 min by a rotor–stator blender (T 25 digital Ultra-Turrax, IKA, Germany) in 2 mL of methanol. The final volume was adjusted to 5 mL with methanol and after centrifugation (6000g for 20 min) supernatants were collected.

Concentrations of SH-I-048 A in plasma and brain tissue were determined by ultra performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS). The sample pre-treatment procedure was carried out using solid-phase extraction (SPE) on Oasis HLB cartridges (Waters, Milford, MA, USA). The procedure was adapted from the method reported by Mercolini et al. (2009). Briefly, cartridges preconditioned with methanol and water were loaded with samples (plasma or supernatant of brain tissue homogenate) and the internal standard solution. Endogenous impurities were removed by washing the cartridges with water and methanol. Finally, analyte elution was carried out with 1 mL of methanol for 1 min.

4.4.2. Plasma protein and brain tissue binding studies

A 48-well rapid equilibrium dialysis (RED) device (Thermo Scientific) was used to determine SH-I-048 A free fraction in fresh rat plasma and brain tissue. Brain samples were diluted with 4 volumes of PBS and homogenized. The sample chambers of the RED device insert were loaded with 190 μ L of plasma or brain tissue homogenate and 10 μ L of SH-I-048A working solution in DMSO (100 μ M) in order to obtain a final SH-I-048A concentration of 5 μ M. A 350 μ L of PBS were transferred into the buffer chamber of the insert. After incubation at 37 °C on an orbital shaker for 4 h, 50 μ L of buffer, plasma and brain homogenate aliquots were transferred to microcentrifuge tubes containing equal volume of DMSO. A 50 μ L volume of control buffer was added to the plasma and brain homogenate samples and 50 μ L of either control brain homogenate or control plasma was added to the buffer sample to yield an identical matrix between buffer and nonbuffer samples. Sample extraction was performed by the addition of 300 μ L of chilled acetonitrile containing midazolam as an internal standard. After vortexing and centrifuging, 120 μ L of supernatant were transferred to vials for UPLC/MS/MS analysis. The same protocol was applied for the matrix preparation for standard curves except that 50 μ L of drug-free dialyzed plasma, brain homogenate and buffer were added to microcentrifuge tubes containing 50 μ L of SH-I-048A standard solutions in DMSO over the concentration range from 50 μ M to 0.5 μ M.

The unbound fraction in plasma was determined as the ratio of concentrations determined in buffer to that determined in plasma. The unbound fraction in undiluted brain was calculated by using the following equation: undiluted $f_u = \{1/D * [(1/ f_{u(\text{apparent})}) - 1] + 1/D\}$ (Kalvass and Maurer, 2002), where D and $f_{u(\text{apparent})}$ represent dilution factor and free fraction determined as the ratio of concentrations in buffer vs. diluted brain tissue, respectively.

4.5. Behavioral experiments

In vivo studies were carried out on male Wistar rats (Military Farm, Belgrade, Serbia), weighing 180–220 g at the beginning of the experiments. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethical

Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade, or, for the peripheral nerve injury model, Medical Faculty in Belgrade. The rats were group-housed four per cage (in the peripheral nerve injury model two per cage) in transparent plastic cages with tap water and food pellets available *ad libitum* and kept under a 12 h light/dark cycle (light on at 6:00 h). The temperature of the animal room was 22 ± 1 °C, the relative humidity is 40–70% and the illumination is 120 lx. With the exception of the second treatment in the peripheral nerve injury model, all handling and testing took place during the light period of the cycle. Ligands were dissolved/suspended with the aid of sonication in solvent containing 85% distilled water, 14% propyleneglycol, and 1% Tween 80, and were administered i.p. in a volume of 1 mL/kg.

Separate groups of naïve animals were used for seven behavioral paradigms. For all behavioral studies, with the exception of the peripheral nerve injury model, the positive control experiments (2 mg/kg diazepam, with the accompanying solvent control) have been performed separately during the period when experiments with SH-I-048 A were carried out. The behavior in spontaneous locomotor activity (SLA), open field (OF), Morris water maze (MWM) and elevated plus maze (EPM) was recorded by a ceiling-mounted camera and analyzed by the ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA). The rats' behavior in rotarod (RR) and grip strength (GS) tests, as well as in peripheral nerve injury (PNI) model, was recorded automatically by the apparatus. Where applicable, olfactory traces left in the apparatus were removed after each testing using diluted ethanol.

4.5.1. Spontaneous locomotor activity test

The rats' spontaneous locomotor activity was monitored in a clear Plexiglas open chamber (40 × 25 × 35 cm) without any prior habituation to this apparatus. Twenty minutes after receiving the appropriate treatment, a single rat was placed in the chamber, under dimmed red light (20 lx), and activity was recorded for a total of 30 min. The total distance traveled (m) was the activity parameter evaluated in four treatment groups (solvent and SH-I-048 A, dosed at 2, 5 and 10 mg/kg). Besides the total distance traveled, behavior was analyzed by breaking the locomotor activity data into 5-min bins.

4.5.2. Elevated plus maze test

The apparatus was constructed of sheet metal, with a black rubber floor. It consisted of a maze elevated to a height of 50 cm with two open (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm), connected by a junction area measuring 10 × 10 cm. A ledge of sheet metal (0.3 cm high) surrounded the open arms. The red illumination on the surface of the closed arms was 10 lx. 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 80% of the animal crossed the virtual line separating the central square of the maze from the arm, whereas an exit occurred when more than 80% of the animal left the respective arm. In this experiment we used four treatment groups: solvent and SH-I-048A dosed at 2, 5 and 10 mg/kg. In a preliminary study, we tested also the 0.5 mg/kg dose of SH-I-048A.

4.5.3. Open field test

The OF arena consisted of a square wooden box (100 × 100 × 50 cm³), with a black rubber floor. Twenty minutes after receiving the appropriate treatment, and without any prior habituation, a single animal was placed in the center of the box. The central 16% of the floor surface (40 × 40 cm²) was virtually set as a central zone. Animal activity under dimmed red light (20 lx) was recorded for 15 min. The total distance traveled (*m*) was evaluated as an activity parameter, while the percent of time spent in the central zone, as an anxiety-related parameter, was estimated in the first 5-min period. In this experiment we used five treatment groups: solvent and SH-I-048A dosed at 0.2, 0.5, 2 and 10 mg/kg.

4.5.4. Grip strength test

Myorelaxant properties of SH-I-048A, dosed at 0.5, 2, 5 and 10 mg/kg, were examined in this study. Twenty minutes after administration of the appropriate treatment rats were allowed to grasp with their front paws a metal trapezoid wire grid attached to a grip-strength meter (Ugo Basile, Italy), while being lifted by the tail with increasing firmness until they loosened their grip. Every testing was performed by the same experimenter. The apparatus measured the pull force (expressed in grams) necessary to overcome the animal's forelimbs grip-strength. Each animal was given three consecutive trials and the median value was taken in the statistics. Dependent variable chosen was calculated by dividing the pull force with the rat's body weight.

4.5.5. Rotarod test

Motor performance was assessed using an automated rotarod (Ugo Basile, Italy). Before testing, rats were trained for three days until they could remain on a revolving rod for 120 s with acceleration from 15 rpm to 25 rpm. During the training days, all animals were given three training sessions of 2 min each, with a 30 min inter-session interval. On the fourth day, selection was made and rats that fit the given criteria were included in the experiment. Four groups received one of the following treatments: SH-I-048A (10 and 25 mg/kg) alone or in combination with β -CCt (30 mg/kg). Latency to fall from the rotarod was recorded automatically for each animal after 30, 60 and 90 min.

4.5.6. Morris water maze test

The water maze consisted of a black cylindrical pool (diameter, 200 cm; height, 60 cm) filled to a height of 30 cm with 23 °C (± 1 °C) water. The escape platform made of black plastic (15 × 10 cm) was submerged 2 cm below the water surface. An indirect illumination in the experimental room was provided by white neon tubes. The rats received the appropriate treatment 20 min before a swimming block, each day for 5 consecutive days of spatial acquisition. Each block consisted of 4 trials, lasting a maximum time of 120 s, the intertrial interval being 60 s. For each trial the rat was placed in the water facing the pool at one of four pseudorandomly determined starting positions. Since the platform was hidden in the middle of the NE quadrant during spatial learning, the four distal start locations were S, W, NW and SE. Once the rat found and mounted the escape platform, it was permitted to

remain on it for 15 s. The rat was guided to the platform by the experimenter if it did not locate the escape within 120 s. For each training day and parameter measured in the water maze the mean value was calculated for each rat (total data/total number of trials). To assess the reference memory at the end of the learning period, a probe trial was conducted 24 h after the last acquisition day. The probe trial, starting from the novel, most distant SW location, with the platform omitted, lasted 60 s and was performed without any pre-treatment. The tracking software virtually divided the pool into four quadrants, three concentric annuli and a target region consisting of the intersection of the platform quadrant and the platform (middle) annulus, as graphically represented in [Savić et al. \(2009\)](#).

Dependent variables chosen for tracking during the acquisition trials were the latency to reach the platform (time from the start to the goal) and the path efficiency (the ratio of the shortest possible path length to actual path length). In addition, the latency to the first entry to the target zone and the path efficiency to the first entry to the target zone were assessed during the probe trial. In the water maze study we used four treatment groups: solvent and SH-I-048A dosed at 0.5, 2 and 10 mg/kg.

4.5.7. Peripheral nerve injury model

The animals were habituated in individual Plexiglas chambers for 30 min before testing. Electronic von Frey apparatus (Model 2390, IITC Life Science – USA) was used to determine paw withdrawal threshold expressed in grams. An increasing pressure of maximum 80 g was intermittently applied to the right and left rat hind paw for three times each, until the animal showed a typical paw withdrawal reaction. Baseline withdrawal scores were obtained before injury (0 h). To induce PNI model, we performed a crush injury to the right sciatic nerve. Rats were anesthetized with ketamine solution (10% Ketamidol, Richter Pharma Ag, Wels, Austria). After the right external thigh and buttock area had been shaved, the skin was properly prepared with antiseptic solution and alcohol, and small incision was made. The *m. vastus lateralis* and *m. biceps femoralis* were bluntly separated at the mid-thigh level. The nerve was kept *in situ*. At approximately 7 mm from the point of its emergence, the common sciatic nerve was mobilized and traumatized by a single 30-s crush with a serrated hemostat ([Bridge et al., 1994](#)). After surgery animals were separated in four groups. Treatments were administered three times: immediately after surgery, after 12 h and finally after 23 h. The treatment groups were solvent three times, one dose of SH-I-048A (10 mg/kg); solvent two times, two doses of SH-I-048A (10 mg/kg); and solvent once and three doses of SH-I-048A (10 mg/kg). After 24 and 48 h, mechanical hyperalgesia was determined in both hind paws. The graph shows the ratios between the right hind paw and left hind paw responses, obtained by dividing the two values and then multiplying the result by 100 for a better graph representation.

4.6. Statistical analysis

For electrophysiological data, as well as for behavioral data presented in [Table 3](#), Student's *t*-test was used. Other

behavioral data sets were checked for homogeneity of variance and normality prior to analysis by a one-way ANOVA (SLA, OF, EPM and GS tests), or a two-way ANOVA (RR test). In order to fully investigate the rats' behavior in the EPM, an analysis of covariance (ANCOVA) was performed for the percentage of time spent on open arms, as an anxiety-related parameter, using the number of enclosed arm entries as covariate. Data from MWM and PNI model were assessed with a two-way ANOVA with repeated measures (treatment as between-subject factor and day or time, respectively, as within-subject factor). In the case of significant interaction, separate one-way ANOVAs were conducted to assess the influence of one factor within individual levels of other factor. For *post hoc* analysis, Dunnett's or Tukey's test were used. Statistical analyses were performed with ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA) and Sigma Plot 11 (Systat Software Inc., Richmond, CA, USA). Differences were considered to be statistically significant when p was less than 0.05, while $0.1 > p > 0.05$ was considered as a trend toward significance.

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