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Article

Combined *in silico, ex vivo,* and *in vivo* assessment of L-17, a thiadiazine derivative with putative neuroprotective and antidepressant-like effects

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Abstract: L-17 is a thiadiazine derivative with putative anti-inflammatory, neuroprotective, and antidepressant-like properties. In this study, we applied combined *in silico, ex vivo,* and *in vivo* electrophysiology techniques to reveal the potential mechanism of action of L-17. PASS 10.4 Professional Extended software suggested that L-17 might have pro-cognitive, antidepressant, and antipsychotic effects. Docking energy assessment with AutoDockVina predicted that the binding affinities of L-17 to the serotonin transporter (SERT) and serotonin receptors 3 and 1A (5-HT_3 and 5-HT_{1A}) receptors are compatible to the selective serotonin reuptake inhibitor (SSRI) fluoxetine and selective antagonists of 5-HT_3 and 5-HT_{1A} receptors, granisetron and WAY100135, respectively. Acute pre-treatment with L-17 robustly increased c-Fos immunoreactivity in the amygdala (central nucleus), suggesting increased neuronal excitability in this brain area after L-17 administration. Acute L-17 also dose-dependently inhibited of 5-HT neurons of the dorsal raphe nucleus (DRN). This inhibition was partially reversed by subsequent administration of WAY100135, suggesting the involvement of extracellular 5-HT. Based on *in silico* predictions, c-Fos immunohistochemistry, and *in vivo* electrophysiology, we suggest that L-17 is a potent 5-HT reuptake inhibitor and/or partial 5-HT_{1A} receptor antagonist. Thus, L-17 might be a representative of a new class of antidepressant drugs. Since L-17 also possesses neuro- and cardio-protective properties, it can be useful in post-stroke and post-myocardial infarction (MI) depression. In general, combined *in silico* predictions and *ex vivo* neurochemical and *in vivo* electrophysiological assessment might be a useful strategy for early preclinical assessment of the affectivity and neural mechanism in action of the novel CNS drugs.

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Keywords: thiadizines; serotonin transporter (SERT); serotonin receptors 3 and 1A (5-HT_3 and 5-HT_{1A}) receptors, docking energy, binding affinity, binding mechanisms, c-Fos immunohistochemistry, electrophysiology *in vivo*

1. Introduction

L-17 (2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide; Fig. 1) is a thiadiazine derivative, synthesized by cyclocondensation of α-bromoacetophenone with the original morpholine-4-carbothionic acid hydrazide [1]:

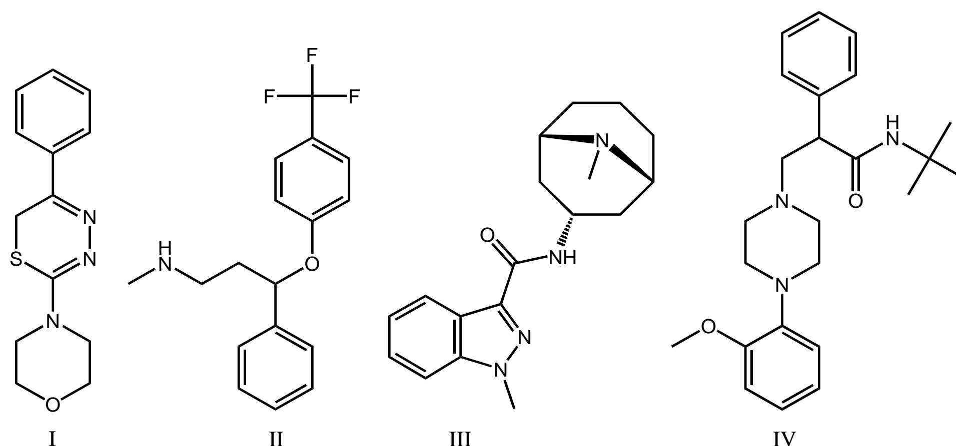

Figure 1. Structural chemical formulas of 2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide (I) (L-17), compared the selective serotonin reuptake inhibitor fluoxetine (II), serotonin-3 (5-HT_3) receptor antagonist granisetron (III) and serotonin-1A (5-HT_{1A}) receptor antagonist WAY100135 (IV).

In our previous studies, L-17 we have shown a putative therapeutic effect of myocardial infarction (MI) [2-4] and pancreatitis [5] in animal models. L-17 has also been reported to attenuate the immune system response to the immobilization stress in rats, suggesting an immunostimulatory effect of this compound during the stress copying [4]. Neuroprotective [1] and antidepressant-like [6] effects of L-17 in rats have been demonstrated, as well. In this study, we aimed to investigate the mechanism of the putative beneficial effect of L-17 on the central nervous system (CNS), using a combination of *in silico*, *ex vivo*, and *in vivo* methods [7].

For *in silico* assessments, we constructed three-dimensional (3D) model of L-17, and examined its interaction with different targets, such as serotonin (5-HT) transporter (SERT) and 5-HT receptors 3 and 1A (5-HT_3/5-HT_{1A}), used the calculation of the minimum binding energy. SERT and 5-HT_3 were chosen because they play a primary role in the pathophysiology and treatment of CNS disorders.

Immunohistochemical assessment of proto-oncogene Fos across the brain is an *ex vivo* experimental technique used for the investigation of mechanism of action of novel CNS drugs. The activation of the proto-oncogene Fos, resulting in an increased expression of its protein product c-Fos, is a well-established marker for the neuronal excitability within the specific brain area [8-11]. Increased proto-oncogene Fos expression in the amygdala, indicating increased neural excitability in this brain area, has been observed after administration of various CNS drugs, such as typical [12] and atypical antipsychotics [12-14], tricyclic antidepressants [13], and selective 5-HT reuptake inhibitors

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(SSRIs) [13, 15, 16]. The SSRI citalopram-induced increase in c-Fos immunoreactivity has been shown to be potentiated by the selective antagonist of 5-HT_{1A} receptors WAY100638 [17], suggesting a key role of 5-HT system in the modulation of amygdaloid excitability by CNS drugs. With regards to the prefrontal cortex (PFC), some SSRIs, such as fluvoxamine [16], and some atypical antipsychotics, such as olanzapine [18, 19], increased local c-Fos immunoreactivity. On the other hand, 5-HT depletion led to an increased c-Fos immunoreactivity in the PFC and hippocampus [20], suggesting that extracellular 5-HT suppresses neural excitability and Fos proto-oncogene expression in these brain areas.

Finally, we used single-unit extracellular electrophysiology *in vivo* to examine the effect of L-17 on the excitability of 5-HT neurons in the rat dorsal raphe nucleus (DRN). Multiple antidepressant drugs, such as tricyclic antidepressants, SSRIs, dual 5-HT/norepinephrine, and triple 5-HT/ norepinephrine/dopamine reuptake inhibitors, pyridoindoles (experimental drugs with putative triple reuptake inhibition property), and some atypical antipsychotics, excerpt potent acute inhibitory effect on the excitability of 5-HT neurons of the DRN. This inhibition is usually reversed by the selective 5-HT_{1A} receptors antagonist, suggesting the involvement of the extracellular 5-HT. The assessment of the effect of an experimental CNS drug on the excitability of 5-HT neurons is a key marker for the preclinical assessment of its efficacy.

2. Results

2.1. *In silico* predictions

The following pharmacological activities were predicted for L-17 by the PASS 10.4 Professional Extended [21]: cognition disorders treatment (Pa = 0.489, Pa/Pi = 27.17), phobic disorders treatment (Pa = 0.703, Pa/Pi = 9.37), psychotropic (Pa = 0.258, P /Pi = 1.74), immunostimulant (Pa = 0.191, Pa/Pi = 1.39), and antidepressant (Pa = 0.162, Pa/Pi = 1.14) one. According to joint predictive evaluations in PASS and experimental data [1, 6], the most likely targeted activities corresponding to the serotoninergic effects of compound L-17 were suggested: 5-HT_3 antagonism (Pa = 0.139, Pa/Pi = 1.17), 5-HT release inhibition (Pa = 0.225, Pa/Pi = 1.01), and 5-HT reuptake blockade (Pa = 0.470, Pa/Pi = 6.35).While L-17 compound manifests itself as an atypical mild antipsychotic and antidepressant [1], for the subsequent analysis of its multitarget mechanism of action the serotonin transporter (SERT), the serotonin receptor types 3 (5-HT_3) and 1A (5-HT_{1A}) were chosen as target proteins (Tab 1).

Target	**SERT**			**5-HT_3**			**5-HT_{1A}**		
Molecule	ΔE, kcal/mol	pK	RA	ΔE, kcal/mol	pK	RA	ΔE, kcal/mol	pK	RA
L-17	-8.1	5.90	0.87	-6.6	4.81	0.94	-7.9	5.78	0.85
Fluoxetine	-9.3	6.78	—	—	—	—	—	—	—
Granisetron	—	—	—	-7.0	5.10	—	—	—	—
WAY100135	—	—	—	—	—	—	-9.3	6.78	—

Table 1. Docking energy (ΔE), binding affinity (pK), and relevant affinity (RA) of L-17 to the SERT and 5-HT_3/5-HT_{1A} receptors, compared to the selective SERT, 5-HT_3, 5-HT_{1A} ligands: fluoxetine, granisetron, and WAY100135.

Figure 2 illustrates the binging mechanisms of L-17, fluoxetine, granisetron, and WAY100135 with the SERT and 5-$HT_3$and 5-HT_{1A} receptors, predicted using the LigandScout 4.2.1 software [22].

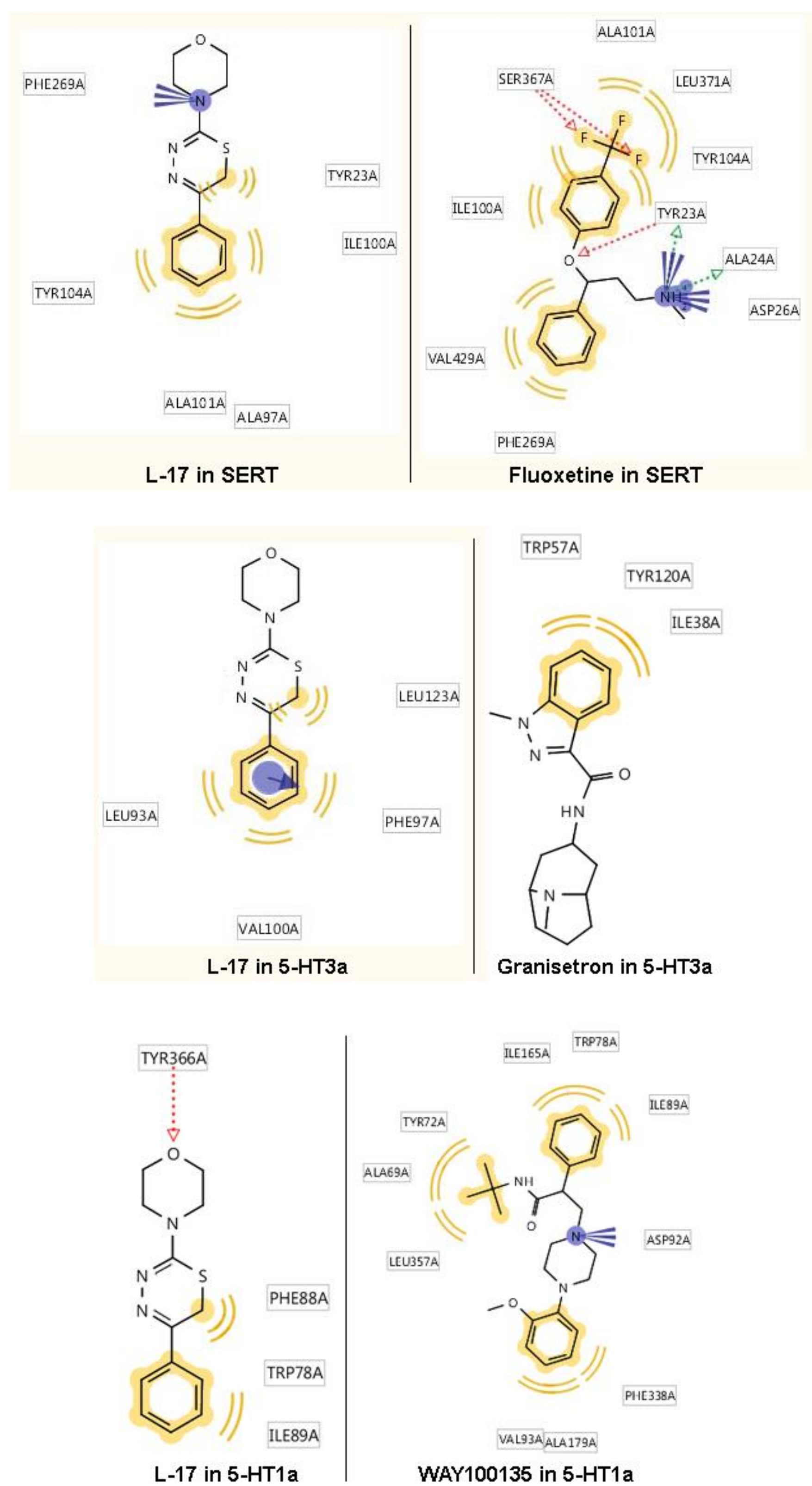

Figure 2. Binging mechanisms of L-17, fluoxetine, granisetron, and WAY100135 with the SERT and 5-HT_3 and 5-HT_{1A} receptors

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Figure 3 illustrates the poses of L-17, fluoxetine, granisetron, and WAY100135 with the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptor:

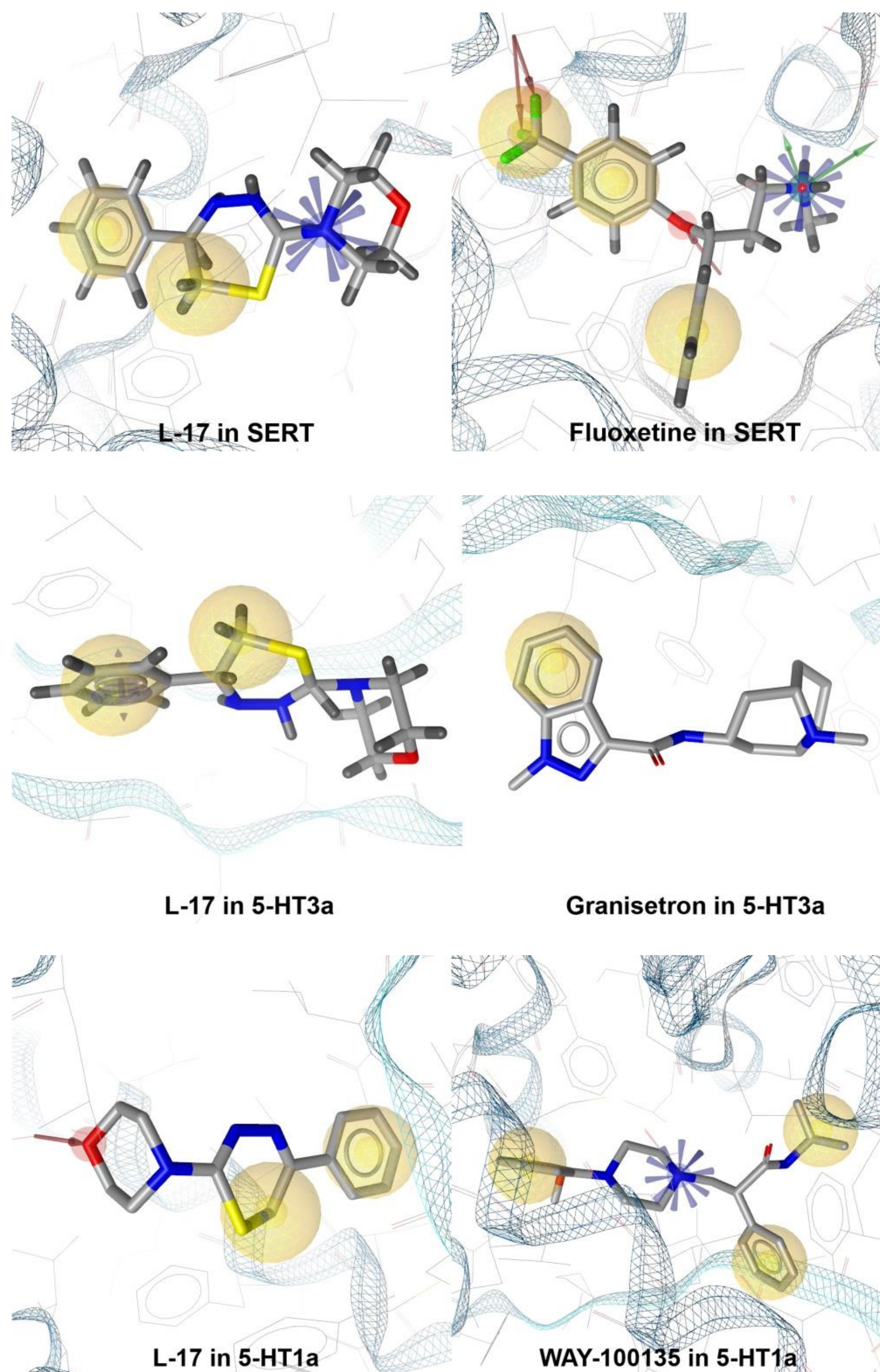

Figure 3. Poses of L-17, fluoxetine, granisetron, and WAY100135 with the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptor

The pose of L-17 within the SERT binding site is remarkably close to this of fluoxetine. There is also a partial similarity between L-17 and WAY100134 poses within the binding site of the 5-HT_{1A} receptor. The poses of L-17 and granisetron within the binding site of the 5-HT_3 receptor are remarkably different.

2.2. Proto-oncogene Fos expression

Figure 4 illustrates the expression of proto-oncogene Fos in three selected brain areas, including prefrontal cortex (PFC, A-C), hippocampus (D-F), and amygdala (G-I) of vehicle- and L-17-preeterated rats:

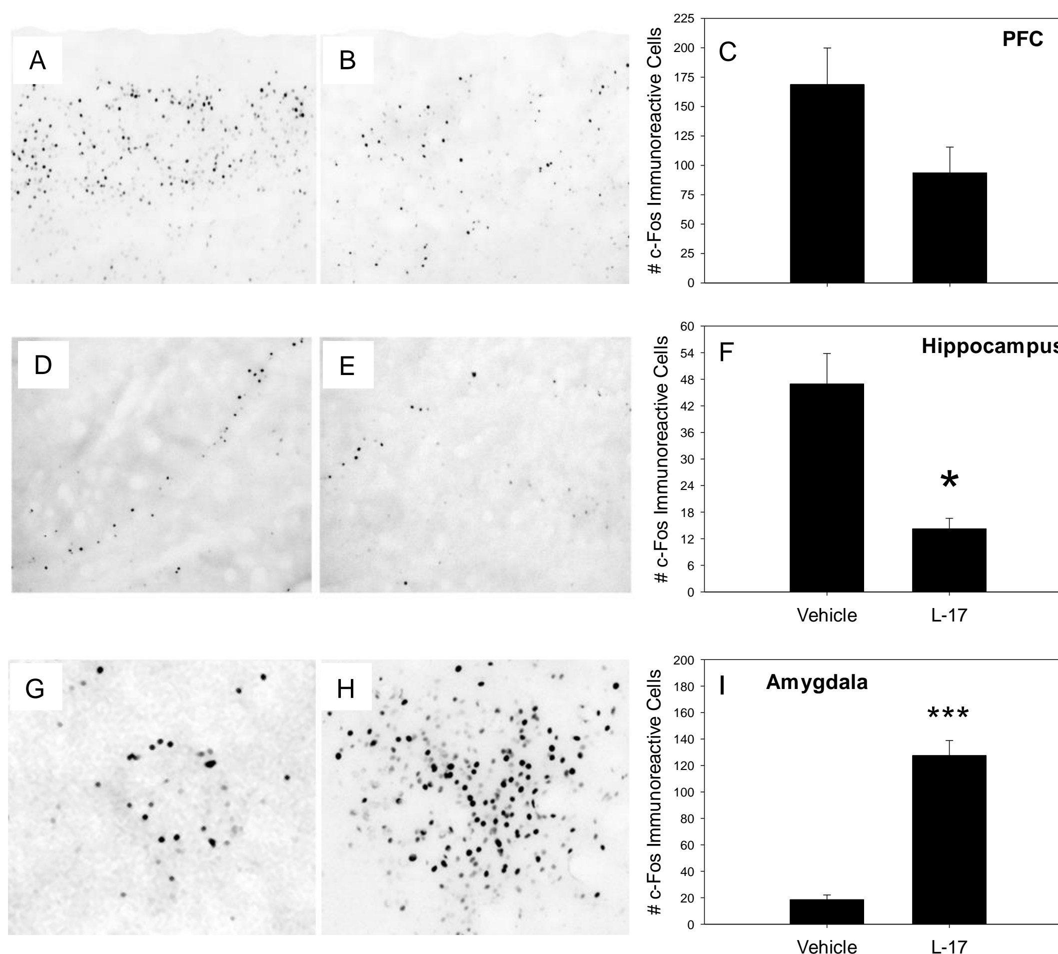

Figure 4. Representative sections illustrating the expression and distribution of c-Fos protein immunolabeled cells in the prefrontal cortex (PFC, A-C), hippocampus (D-F), and the central nucleus of the amygdala (G-I) in vehicle- and L-17-pretreated rats. *p<0.05 and p<0.001, two-tailed Student's t-test.

Pre-treatment with L-17 decreased c-Fos immunoreactivity in the hippocampus and increased it in the amygdala. The expression of c-Fos in the PFC was not statistically different between the groups. Summary quantitative assessments were done from four vehicle- and four L-17-administered rats.

2.3. *In vivo* electrophysiology

The mean basal firing activity was 3.46±0.83 Hz. L-17 (0.1–12 mg/kg, i.v.) significantly and dose-dependently ($F_{8,53}$=4.84, p<0.001, ANOVA for repeated measured) inhibited the firing activity of 5-HT neurons, reaching the maximal 90%-inhibition at 12 mg/kg. WAY100135 partially reversed the L-17-induced inhibition of 5-HT neurons (Fig 4):

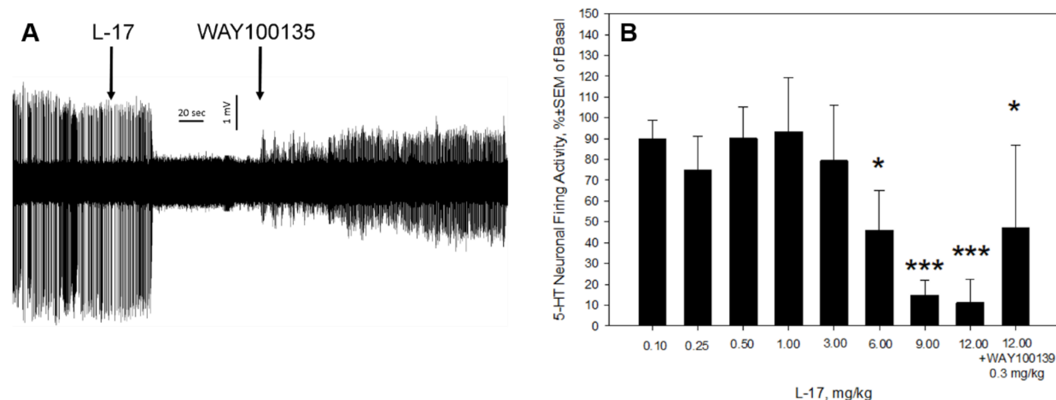

Figure 4: L-17 significantly and dose-dependently inhibited the firing activity of 5-HT neurons. A: representative recording from a 5-HT neuron during L-17 (12 mg/kg) and WAY100135 (0.3 mg/kg) administration. B: Summary effect of L-17 (0.1-12 mg/kg) and WAY100135 (0.3 mg/kg) on the spontaneous firing activity of 5-HT neurons of the DRN (data from 8 neurons from 7 rats).

3. Discussion

In this study, we performed a complex *in silico* assessment of pharmacotherapeutic properties of L-17, as well as in vivo electrophysiological assessment of the effect of this compound on the firing activity of 5-HT neurons in the rat DRN. PASS 10.4 Professional Extended[21] software predicted antidepressant-like properties of L-17. This prediction is consistent with the previously reported antidepressant-like effect of L-17 in rats [6]. PASS 10.4 Professional Extended had also predicted that the antidepressant-like effect of L-17 can be explained, at least in part, *via* its 5-HT reuptake inhibition property.

AutoDockVina 1.1.2 [23] docking energy assessment predicted that L-17 has an affinity to the SERT comparable with fluoxetine,5-HT_3 binding affinity similar to granisetron, and 5-HT_{1A} binding affinity comparable to WAY100135. The prediction of the molecular interactions between L-17 and the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptors with LigandScout 4.2.1 software [22] revealed that the mechanisms of L-17 binding to the SERT and 5-HT_{1A} receptor are similar to fluoxetine and WAY100135, respectively. On the other hand, L-17 binding to 5-HT_3 receptors is completely different from that of granisetron. Consistently, the topographic poses of L-17 within SERT and 5-HT_{1A} receptor binding sites are remarkably similar to those of fluoxetine and WAY100135, respectively. Thus, the morpholine cycle nitrogen atom of L-17 is engaged in a p-π exchange with PHE269 of SERT. If protonated, this nitrogen forms a region of undirected electrostatic interaction. Two more structural elements of L-17 participate in five nonspecific hybrid interactions with TYR23, ALA97, ILE100, ALA101, TYR104 of the SERT. Fluoxetine is being banded to the SERT *via* the electrostatic interaction of protonated NH_2 group. Three additional groups of L-17 form five hydrogen bonds with TYR23, ALA24, ASP26, SER367x. Three more structural elements participate in six nonspecific hybrid interactions with ILE100, ALA101, TYR104, PHE269, LEU371, VAL429. L-17 might bind to the 5-HT_3 receptor by stacking with PHE97. Two structural elements of L-17 participate in four nonspecific hybrid interactions with LEU93, PHE97, VAL100, LEU123. No stacking is observed in granisetron binding to a 5-HT_3 receptor. Fixation is exerted by three nonspecific hydrophobic interactions with ILE38, TRP57, TYR120. The morpholine cycle oxygen atom of L-17 forms a hydrogen bond with TYR366 of the binding site of the 5-HT_3 receptor. Two structural

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elements of L-17 form three nonspecific hydrophobic links with TRP78, PHE88, and ILE89 of the binding site of5-HT_3 receptor. The protonated nitrogen atom of the morpholine cycle of WAY100135 exerts electrostatic interaction with TYR366. Two WAY100135 molecule fragments participate in three nonspecific hydrophobic interactions with ALA69, TYR72, TRP78, ILE89, VAL93, ILE165, ALA179, PHE338, LEU357 of the binding site of the 5-HT_3 receptor.

It has been demonstrated that the pre-treatment with L-17 robustly increased c-Fos immunoreactivity in the amygdala. Amygdala is a fundamental structure in the emotional responses, including fear and anxiety [24]. Thus, it is possible that the modulation of amygdaloid neural excitability is involved in the therapeutic response to antidepressant, anxiolytic, and mood stabilizing medicines. It has indeed been reported that various CNS drugs, such typical [12] and atypical antipsychotics [12-14], tricyclic antidepressants [13], and selective 5-HT reuptake inhibitors (SSRIs) [13, 15, 16], increased c-Fos immunoreactivity in the amygdala, indicating an increased neural excitability in this brain area. In our previous studies, we have found that pyridoindole derivate SMe1EC2M3, a molecule with putative 5-HT reuptake inhibition properties and antidepressant-like behavioral effect [7], stimulated amygdaloid c-Fos immunoreactivity, as well [11]. The fact that SSRI-induced amygdaloid c-Fos immunoreactivity was potentiated by an antagonist of 5-HT_{1A} autoreceptors indicated that 5-HT system is involved in the modulation of amygdaloid neural excitability by antidepressant drugs. Thus, it can be summarized that the induction of amygdaloid c-Fos immunoreactivity is an important marker for the putative mood stabilizing and antidepressant-like effect, and the fact that L-17 stimulated proto-oncogene Fos expression in the amygdala brings an additional support to the hypothesized beneficial effects of this molecule as a future CNS drug.

With regards to proto-oncogene Fos expression in other brain areas, we found that acute administration of L-17 significantly decreased c-Fos immunoreactivity in the hippocampus and tended to decrease it also in the PFC. It has been previously reported that 5-HT depletion led to an increased c-Fos expression in these brain areas [20]. Thus, it is possible that the L-17-induced decrease in forebrain c-Fos immunoreactivity might be explained by the ability of this drug to elevate extracellular 5-HT concentrations. Further studies should be, however, performed to test this hypothesis.

We found that acute intravenous administration of L-17 significantly and dose-dependently inhibited the firing activity of 5-HT neurons of the DRN. Similar inhibitory effects on 5-HT neuronal firing activity has been observed with other SSRIs, such as citalopram [25], escitalopram [25, 26], Wf-516 [27] or paroxetine [28]. Similar to that observed in other SSRIs, the L-17-induced inhibition of 5-HT neuronal firing activity was reversed by WAY100135. It is likely that L-17 is acting as a potent SERT blocker. Inhibition of the SERT by acute L-17 leads to an increase in the extracellular 5-HT levels, activation of 5-HT_{1A} autoreceptors, and reduction of firing activity of 5-HT neurons. The subsequent blockade of 5-HT_{1A} autoreceptors reverses the inhibition of 5-HT neurons.

On the other hand, unlike escitalopram-induced suppression of 5-HT neuronal firing activity, which was completely reversed by 0.1 mg/kg of WAY100135 [25, 26], the L-17-induced inhibition of 5-HT neurons was reversed by WAY100135 only partially, even though WAY100135 was administered at 0.3 mg/kg. Thus, it is possible that L-17 interacts with a molecular target(s) other than SERTs. One of these targets predicted by *in silico* tests is 5-HT receptor. It is possible that L-17 acts, in addition to its function as a SERT blocker, as a partial agonist of 5-HT_{1A} receptors. The competition

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between L-17 and WAY100135 on 5-HT$_{1A}$ receptors putatively prevents complete WAY100135-mediated recovery of firing activity of 5-HT neurons. Another possibility might be, however, a direct interaction of L-17 with α_1-adrenoceptors [29].

Summarizing, the antidepressant-like properties of L-17, reported in previous studies, can be explained, at least in part, by the ability of this compound to modulate the central 5-HT neurotransmission. We cannot, however, exclude the interaction of L-17 with receptors modulation the excitability of 5-HT neurons, such as 5-HT$_{1A}$, 5-HT$_{3,}$ and/or $\alpha_{1/2}$-adrenergic receptors. It is also possible that L-17 directly interacts with other neurotransmitter systems, such as norepinephrinergic, dopaminergic, and/or histaminergic ones. The L-17-induced modulation of 5-HT transmission is likely to be mediated, at least in part, *via* the inhibition of 5-HT reuptake and partial antagonism to 5-HT$_{1A}$ receptors. L-17-induced increase in 5-HT ton? putatively results in increased neuronal excitability in the amygdala and decreased neuronal firing activity in the hippocampus. These L-17-induced changes in neural excitability across the brain might be involved in the neuroprotective and antidepressant-like behavioral effects of this compound, reported in previous studies. This previously reported antidepressant-like properties of L-17 and its modulatory effect on 5-HT neurons of the DRN and on the neural excitability in the amygdala and the hippocampus, reported in this study, suggest that L-17 might be an effective antidepressant drug. Since L-17 also possesses neuro- and cardio-protective properties, it could be useful in affective illness developing due to the general medical condition, such as post-stroke and post-MI depression. It should be also stated that the combined *in silico* (computerized molecular binding mechanisms prediction), *ex vivo* (assessment of the neuronal excitability using c-Fos immunoreactivity measurement) and *in vivo* (direct assessment of excitability of monoamine-secreting neurons) investigation might be a useful strategy for early preclinical assessment of the affectivity and neural mechanism of action in the novel CNS drugs.

4. Materials and Methods

4.1. *In silico* predictions

Using the PASS 10.4 Professional Extended software [21], the presence (Pa) or absence (Pi) probability of 480 systemic types of pharmacotherapeutic activity was calculated. Promising activities were those with Pa ≥0.1 and Pa/Pi ≥1.0.

Further, for a comparative evaluation of the L-17 compound's affinity to the selected biotargets, the docking of the compound to the specific binding sites of these proteins was performed. Five experimental X-ray 3D models of SERT were obtained from Protein Data Bank in Europe[30]. Among these five models, the longest one (PDB code 5I6X), with the maximum resolution including an inhibitor, was chosen to allow the unambiguous determination of the binding site position [31]. The experimental 3D models for 5-HT$_3$ and 5-HT$_{1A}$ receptors were not available in Protein Data Bank in Europe, therefore a search for the best theoretical 3D models from the Database of Comparative Protein Structure Models [32]was conducted. Among the available models, the longest ones, with the highest statistical significance, were selected for 5-HT$_3$ [33] and 5-HT$_{1A}$ [34] receptors.

The 3D models of L-17 and reference molecules were constructed using the molecular mechanics' methods in the MarvinSketch15.6.15software [35], followed by optimization with the semi-empirical quantum chemical method PM7 in the MOPAC2016 software [36]. The docking was performed using AutoDockVina 1.1.2 [23], five times for each compound into each target and then

the spectrum of energies were used to determine the minimum binding energies. To reveal the mechanisms of serotoninergic action of the compound L-17, an analysis of its affinity spectrum in comparison with the affinity spectra of reference compounds was performed. The primary information about reference compounds for target proteins was obtained from the UniProtKB base [37]. For each reference compound found in the UniProtKB, the mechanism of its action was clarified using the DrugBank[38]. In the case of the information in UniProtKB and DrugBank datasets was not sufficient, the search for references was performed in the database of pharmacologically relevant proteins and their ligands IUPHAR [39]. The SSRI fluoxetine and the selective antagonists of 5-HT_3(granisetron) and 5-HT_{1A}(WAY100135) receptors were used as reference molecules. Using the obtained energy spectra of ΔE, the pK values were calculated using the formula:

$$pK = -\lg e^{-\Delta E/RT} ,$$

where R is a universal gas constant (8.314 J×mol^{-1}×K^{-1}) and T is a temperature, set as 300 K. The molecular binging mechanism of L-17, granisetron, and WAT100135 with SERT and with 5-HT_3 and 5-HT_{1A}receptors was furtherly evaluated using the LigandScout 4.2.1 software [22]. The data on binding sites were found in the available literature. For SERT, the key amino acids of the binding site were Gly94, Ala96, Val97, Asn101, Ser336, Asn368, Leu434, Asp437, Ser438 [31]. For 5-HT_3receptor, the key amino acids of the binding site were Tyr229, Phe221, Asn123, Trp85, Trp178, Tyr148, Arg87, Gln146, Tyr138 [33]. For 5HT_{1A}receptor, the key amino acids of the binding site were Tyr56, Gln57, Asp76, Val77, Ser159, Trp318, Phe321, Phe322, Thr339, Gly342, Ala343, Ile345, Asn346 [34].

4.2 Assessment of c-Fos immunoreactivity

For the assessment of the proto-oncogene c-Fos expression, rats were pre-treated with L-17 (60 mg/kg, intraperitoneally, i.p.) twenty-four and one hour before immunoreactivity measurements. C-Fos expression was analyzed, as we have reported previously [11, 40]. The rats were anesthetized by a combined treatment of zoletil (30 mg/kg, Virbac, Carros, France) and xylariem (15 mg/kg, Riemser Germany) in the volumes of 0.1 ml and 0.24 ml/300 g b.w., respectively. Then, they were perfused transcardially with 50 ml of cold isotonic saline containing 150 µl of heparin (5000 IU/ml, Zentiva, Slovakia) followed by 200 ml of fixative containing 4% paraformaldehyde (Sigma-Aldrich, Germany) in 0.1 M phosphate buffer (PB, pH 7.4). Immediately after perfusion, the brains were removed from the skulls, postfixed in a fresh fixative overnight, washed in 0.1 M PB at 4 °C overnight, and infiltrated with 30% sucrose (Slavus, Slovakia) at 4 °C for 48 h. Coronal sections of 35-µm thickness were alternatively cut over the whole brain in a cryocut (Hyrax C-50). The sections were repeatedly washed in 0.1 M PB and pretreated with 0.3% H_2O_2 (Sigma-Aldrich, Germany) in 0.1 M PB for 10 min at room temperature (RT). Thereafter, the sections were rinsed 3 × 10 min in 0.1 M PB and exposed to rabbit anti-Fos polyclonal antibody (1:2000) in 0.1 M PB containing 4% normal goat serum (Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Sigma-Aldrich, Germany), and 0.1% sodium azide (Sigma-Aldrich, Germany) for 48 h at RT. After several rinsing in PB, the sections were incubated with biotinylated goat anti-rabbit IgG (1:500, BA-9200, VectorStain Elite ABC Kit, Vector Lab, Burlingame, CA, USA) in PB for 90 min at RT. Next, the PB rinsing was followed by incubation with the avidin–biotin-peroxidase complex (1:250) for 90 min at RT. After several washings in 0.05 M sodium acetate buffer (SAB, pH 6.0), c-Fos-antigenic sites were visualized by 3,3′-diaminobenzidine tetrahydrochloride (0.0625% DAB) enhanced with 2.5% nickel chloride (Sigma-Aldrich, Germany), in

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SAB containing 0.0006% H_2O_2. Developing time was 6–8 min. The heavy metal intensification of DAB yielded to black staining of c-Fos-labeled nuclei.

The topographic mapping of the c-Fos protein immunolabeled cells was performed in three brain areas (PFC, hippocampus, and central nucleus of the amygdala) identified based on the rat brain atlas [11, 41]. The individual structures investigated were captured unilaterally from 6-10 representative sections using an Axio-Imager A1 light microscope (Carl Zeiss, Jena) coupled to a video camera and monitor. Since the staining of each individual Fos-immunoreactive cell varied from very intense to very light, c-Fos profiles counting was performed on pictures inverted by the Adobe Photoshop 7.0 adjustment INVERT to reach white c-Fos profiles on the black background. The counting of the c-Fos profiles was performed manually in the PC computer by a person blind to the design of the experiment.

4.3 *In vivo* electrophysiology

In vivo electrophysiological assessment of the excitability of 5-HT neurons of the DRN was performed as previously described [7, 42-44]. Adult male Wistar rats, weighing 300-350 g, were ordered from the Animal Breeding Facility of The Institute of Experimental Pharmacology and Toxicology, Center of Experimental Medicine, Slovak Academy of Sciences in DobráVoda, Slovakia. Rats were anesthetized with chloral hydrate (Lambda Life s.r.o., Bratislava, Slovakia; 0.4 g/kg, intraperitoneally: *i.p.*) and maintained in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Rat body temperature was maintained between 36 and 37°C with a heating pad (Gaymor Instruments, Orchard Park, NY, USA). The scalp was opened, and a 3 mm hole was drilled in the skull for insertion of electrodes. Glass-pipettes were pulled with a DMZ-Universal Puller (Zeitz-Instruments GmbH, Martinsried, Germany) to a fine tip approximately 1 μm in diameter and filled with 2M NaCl solution. Electrode impedance ranged from 4 to 6 MΩ. The pipettes were lowered into the DRN (7.8-8.3 mm posterior to bregma and 4.5-7.0 mm ventral to brain surface) [41] by David Kopf Instruments hydraulic micro-positioner. The action potentials generated by 5-HT neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). The 5-HT neurons were identified by bi- or tri-phasic action potentials with a rising phase of long duration and regular firing rate of 0.5–5.0 Hz [45]. All experimental procedures were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (Permit number Ro 3592/15-221) and confirmed to the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes.

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Abbreviations

3D	Three dimensional
5-HT	5-hydroxytryptamine (serotonin)
ANOVA	Analysis of variance
DRN	Dorsal raphe nucleus
L-17	2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide
MI	Myocardial infarction
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor

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