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# Effect of aripiprazole and improved living conditions on behavioral manifestations and neurogenic markers expression in an animal model of PTSD

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**Objective.** Post-traumatic stress disorder (PTSD) is a complex psychiatric disorder that can develop after exposure to a severe traumatic event and is connected with behavioral changes and adult neurogenesis impairment. Atypical antipsychotics including aripiprazole (ARI) or enriched environment can ameliorate disturbances connected to PTSD. This study was aimed to reveal whether ARI treatment supplemented by improved living conditions, i.e. toy rotation (TR), will ameliorate behavioral outcomes and reverse assumed changes in neurogenesis more effectively than sole ARI treatment in a single prolonged stress (SPS) animal model of PTSD.

**Methods.** Adult male Sprague-Dawley rats weighing 176–200 g were randomly assigned to 5 experimental groups: (1) VEH – control non-stressed animals injected with vehicle (VEH, 2% Tween 20 in the saline); (2) SPS – animals exposed to SPS injected with VEH; (3) SPS+ARI – SPS animals injected with ARI (5 mg/kg in VEH); (4) SPS+TR – SPS animals exposed to TR and injected with VEH; (5) SPS+ARI+TR – SPS animals exposed to TR and injected with ARI. Animals in TR groups were housed in the standard cages with two toys per cage, which were replaced every other day. Elevated plus maze (EPM), open field (OF), and novel object recognition test (NOR) were used to assess the anxiety-like behavior and learning/memory. Changes in gene and protein expression of selected neurogenic markers (BDNF, GFAP, Sox2, DCX, NeuN) and transcription factors (ΔFosB, pCREB) in the subventricular zone (SVZ) and the gyrus dentatus (GD) of the hippocampus were determined by semi-quantitative real-time PCR and immunohistochemistry, respectively.

**Results.** SPS animals showed increased anxiety-like behavior that was suppressed by TR and ARI+TR treatment combination. Although SPS did not affect the expression of studied neurogenic markers, ARI treatment increased the expression of doublecortin in the SVZ and TR increased expression of NeuN in the GD of PTSD-like animals. TR enhanced ARI effect on NeuN expression in the SVZ. SPS induced increase of ΔFosB positive cells, which was reduced by ARI+TR complementary treatment.

**Conclusions.** Obtained results indicate that TR, in contrary to ARI, suppressed the anxiety-like behavior in PTSD-like animals. SPS does not affect the neurogenic markers expression in the SVZ or GD, but ARI and TR or their combination seems to increase the survival of the newborn cells.

**Keywords:** post-traumatic stress disorder, single prolonged stress, aripiprazole, improved living conditions, neurogenic markers

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Post-traumatic stress disorder (PTSD) is a complex psychiatric disorder that can develop in a small portion of the human population following exposure to severe traumatic event or series of events (Polimanti and Wendt 2021). The core symptoms of PTSD include the re-experiencing of the traumatic events, heightened anxiety, overgeneralization of threat responses to otherwise safe environments or situations, and reduced extinction of conditioned threat (Fujikawa et al. 2024). Neuroimaging studies have identified the hippocampus (HIP) as one of the key regions that contribute to the behavioral abnormalities in PTSD (Lisieski et al. 2018).

Neurogenesis, a biological process of new neurons formation from neural precursors, is restricted to the *gyrus dentatus* (GD) of the HIP and in mammals also to the subventricular zone (SVZ) of the lateral ventricles. Deficiency in adult neurogenesis is believed to be relevant in many psychiatric disorders including the PTSD (Carli et al. 2021). Impairment of adult neurogenesis as a consequence of stress exposure may disrupt the normal physiology of the HIP and thus underlie the etiology of PTSD (Gomes-Leal 2021). Newly formed neurons added to already existing hippocampal circuits facilitate the formation of new memories as well as remodeling or overwriting the existing ones. This form of neurogenesis-mediated forgetting might induce forgetting of maladaptive memories that underlie hypermnesic conditions, such as PTSD (Fujikawa et al. 2024).

All the above-mentioned facts underline the importance of adult neurogenesis in PTSD pathophysiology. Some of the medications used for PTSD treatment are known to modulate neurogenesis. Atypical antipsychotics can affect neuroplasticity and reverse the behavioral effects of chronic stress by improving adult neurogenesis, cell survival, and neuronal reorganization (Kusumi et al. 2015; Chikama et al. 2017). Atypical antipsychotic aripiprazole (ARI) has been shown to modulate some symptoms associated with PTSD and in monotherapy exhibited promising results in a few clinical studies. However, due to the small number of patients, the high discontinuation rate, and missing placebo group, further studies are required (Gasparyan et al. 2022).

In both *in vitro* and *in vivo* animal experiments, ARI has been shown to increase the number of BrdU-positive newly formed cells as well as enhance the proliferation, survival, and neuronal differentiation of these cells what is evident from elevated numbers of glial fibrillary acidic protein (GFAP, a marker of primary neuronal progenitors), doublecortin (DCX, a marker of immature neurons), and neuronal nuclear antigen (NeuN, a marker of mature neurons) positive cells in the HIP (Yoneyama et al. 2014; Chikama et al. 2017; Chen and Nasrallah 2019). Besides pharmacological also non-pharmacological interventions, like enriched environment (EE), have been shown to support the positive drug effects and also enhance the neurogenesis (Rief et al. 2016). It was proven that rodents living in cages with EE – additional motor and sensory stimuli, exhibited more hippocampal new-born cells than animals living in the standard plain cages (Gomes-Leal 2021). Animals exposed to EE exhibited increased number of BrdU/Sox2/DCX positive cells (Sox2, a marker of neural progenitor cells) and also increase of phosphorylated CREB positive cells (pCREB, initiator of lasting neuroplastic changes in response to various stimuli) (Gronska-Peski et al. 2021). In an animal model of PTSD, EE ameliorated avoidance/numbing-like behavior and elevated hippocampal brain-derived neurotrophic factor (BDNF) expression. However, individual components of EE, i.e. physical exercise or stimulation from learning and new experiences, which is replicated by toy rotation (TR), have been shown to differently affect the PTSD-like behavior (Tanichi et al. 2018).

Based on the previously mentioned outcomes, the aim of this study was to reveal whether ARI treatment supplemented by TR (in standard cages) will more effectively improve behavioral outcomes and reverse alterations in neurogenic markers expression in animal model of PTSD than sole exposure to ARI or TR in SPS rats. We have studied changes in anxiety-like behavior, learning and recognition memory, and gene and protein expression of selected neurogenic markers in the SVZ and GD brain regions.

## Materials and Methods

**Animals.** In the experiment, 70 male Sprague-Dawley rats (176–200 g, Charles River, Germany) were used. Prior to the experiment, animals were acclimatized for 7 days in the animal facility to avoid stress carryover. The rats were housed two per cage in a controlled environment (22±2 °C, 12 h light/dark cycle, lights on at 7 a.m.) with food and water provided *ad libitum*. All experimental procedures were performed in accordance with the Council Directive 2010/63EU of the European Parliament and the of the Council of 22nd September 2010 on the protection of animals used for scientific purposes and were approved by the Committee of the State Veterinary and Food Administration of the Slovak Republic (Approval protocol number 5541/2023-220).

**Experimental design.** After acclimatization period, the animals were randomly assigned to five experimental groups by 14 animals in each. The experimental groups were as follows:

(1) **VEH** – control non-stressed animals intraperitoneally (i.p.) injected with vehicle (VEH);

(2) **SPS** – animals exposed to a single prolonged stress (SPS) and i.p. injected with VEH;

(3) **SPS+ARI –** SPS animals i.p. injected with ARI;

(4) **SPS+TR** – SPS animals exposed to TR and i.p. injected with VEH;

(5) **SPS+ARI+TR** – SPS animals exposed to TR and i.p. injected with ARI.

We used a SPS model for induction of PTSD-like symptoms under following conditions: rats were restrained for 2 h in plastic restrainers (Flat Bottom Rodent Holders, RSTR544, Kent Scientific Co., Torrington, CT, USA). Immediately after restraint, the animals were exposed to 20-min forced swimming in a glass cylinder filled with water (22±2 °C) to two-thirds. Then the animals were dried and allowed to recuperate for 15 min. Afterwards, they were exposed to ether vapor until loss of consciousness. Then they were transferred into clean cages, 2 rats per cage. SPS protocol was followed by 14 days of sensitization period to develop PTSD-like symptoms during which animals were undisturbed and only water and food were replenished. After 14 days, the animals were exposed to the 1st elevated plus maze (EPM) test and afterwards started 28-day long treatment period with daily i.p. injection with VEH (2% Tween 20 in the saline, Sigma-Aldrich, USA), ARI (5 mg/kg dissolved in VEH; Abcam, UK), or exposure to TR. Animals in TR groups were housed in the standard cages (two per cage) and given two toys per cage, which were replaced every other day and some treats. Afterwards, rats underwent the 2nd EPM test and 48 h later, to avoid any stress carryover, the novel object recognition test (NOR). The design of the experiment is graphically illustrated in Figure 1.

**Behavioral tests.**

**Elevated plus maze test.** All animals were in the EPM tested twice, i.e. after 14-day period of sensitization and after 28-day VEH/ARI/TR exposure. The open and closed arms of the maze were 50 cm above the floor, 50 cm long, and 10 cm wide. The animals were tested for 5 min. After each individual trial, the maze was cleaned with 60% ethanol. Animals´ movement was recorded with a digital camera and individual sessions were analyzed with the ANY-maze Video Tracking System 7.1 (Stoelting Co., Wood Dale, IL, USA) computer software. We analyzed percentage of the time spent and the distance travelled in the open arms (OA) of the EPM and the differences between the time spent and the distance travelled in the OA of the EPM during the 1st and the 2nd exposure.

**Open field and novel object recognition tests.** On the first day, each rat was acclimatized to an empty arena for 5 min, i.e. habituation phase, and this trial was evaluated as an open field test (OF), where we measured the distance travelled, mobility, time spent in the central and peripheral zones of the arena. Movements were recorded with a digital camera and individual sessions analyzed with the ANY-maze Video Tracking System 7.1 (Stoelting Co., Wood Dale, IL, USA) computer software. Next day, the rats were returned to the same arena, where two identical objects were placed in opposite corners and rats were allowed to explore them for 3 min (the familiarization phase). On the 3rd day, the rat was reintroduced to the arena, but one of the familiar objects was replaced with a novel one (the trial phase). The rat was allowed to explore the objects for 3 min. The time spent exploring the novel and familiar objects during the familiarization and trial phases was recorded with a digital camera and individual sessions were analyzed with the ANY-maze Video Tracking System 7.1 (Stoelting Co., Wood Dale, IL, USA) computer software. The discrimination index was calculated by subtracting the time spent exploring the familiar object from the time spent exploring the novel object, then dividing the result by the total exploration time.

**Euthanasia of animals and tissue processing.** The rats were euthanized by decapitation (n=50, for gene expression analysis) or transcardial perfusion (n=20, for immunohistochemical analysis) 48 h after the NOR test.

Following decapitation, the brains were carefully removed, frozen on dry ice, and kept at –72 °C until used for biochemical analyses. Afterwards, the brains from decapitated animals were acclimatized to –12 °C in a cryostat (Reichert&Jung, Germany) and cut into 300 µm thick coronal sections. The sections were placed on cooled microscope slides. The SVZ (Bregma =1.2 mm) and HIP (Bregma =–3.8 mm) were microdissected from the frozen sections by a punch technique under stereomicroscope, according to brain atlas (Palkovits and Brownstein 1988). The dissected pieces of the brain tissue were collected in Eppendorf tubes, frozen in liquid nitrogen, and stored at –72 °C until used for further analyses.

Four animals from each experimental group were sacrificed by transcardial perfusion. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Spofa, United Pharmaceutical Works, Czech

Republic) and transcardially perfused with 60 ml of saline containing 450 µl of heparin (5000 IU/l, Zentiva, Slovakia) followed by 250 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The removed brains were postfixed in a fresh fixative overnight, washed twice in 0.1 M PB, infiltrated with 30% sucrose for 2 days at 4 °C, cut into 30 µm thick coronal sections using cryostat (Reichert&Jung, Germany), and collected in a cryoprotectant solution at –20 °C for further immunohistochemical processing.

**RNA isolation and real-time PCR.** Total RNA was isolated using the TRI ReagentÒRT (MRC, Inc., USA) according to the manufacturers' protocol and its concentration was quantified using the NanoDrop 2000 (Thermo Fisher Scientific, USA).

The expression levels of the BDNF, GFAP, Sox2, DCX and NeuN in the SVZ and GD brain regions were determined by semi-quantitative real-time PCR. Reverse transcription of RNA was performed with the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol, using an oligo dT primer. Semi-quantitative real-time PCR was set up in total volume of 25 µl containing 30 ng of template cDNA mixed with 12.5 µl of FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Switzerland), 1 µl of specific primer pair set (Table 1) and water. Each sample was analyzed on QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 1 cycle of 2 min at 50 °C followed by 1 cycle of 10 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were normalized to GAPDH levels and expressed as the relative fold change, calculated using the DDCt method (Livak and Schmittgen 2001). Melting curve analysis was performed to confirm the specificity of the amplified products.

**Immunohistochemistry.** Free floating brain sections were washed 3×5 min in 0.1 M PB (pH=7.4). Afterwards, the sections for immunofluorescent staining were incubated in the blocking solution (0.1 M PB with 3% normal goat serum (NGS, ab 138478, Abcam, UK) and 2% bovine serum albumin (BSA, Sigma-Aldrich, USA) for 1 h and for common immunohistochemical light microscopy staining with 0.3% $H_2O_2$ (Sigma-Aldrich, USA) in 0.1 M PB for 30 min at room temperature (RT). After blocking with $H_2O_2$, the sections were rinsed 3×10 min in 0.1 M PB and incubated with primary antibody for 48 h at 4 °C.

All primary antibodies used, namely anti-ΔFosB (mouse, 1:2000, ab11959, Abcam, UK), anti-phosphorylated cyclic adenosine monophosphate-responsive element-binding protein (pCREB, mouse, 1:400, 35-0900, Invitrogen, USA), anti-sex determining region Y-box 2 (Sox2, rabbit, 1:500, AB5603, Millipore, CA, USA), anti-DCX (rabbit, 1:500, ab18723, Abcam, UK), anti-NeuN (rabbit, 1:1000, ab177487, Abcam, UK) were diluted in PB containing 4% NGS, 1% Triton X-100 (Sigma-Aldrich, USA), and 0.1% sodium azide (Sigma-Aldrich, USA). Then the sections were washed 3×5 min in PB and incubated with secondary antibodies for immunofluorescence Alexa Fluor 555 (1:500, Thermo Fisher Scientific, USA) 90 min at RT in the dark and for the light microscopy with biotinylated goat anti-rabbit/mouse IgG (1:500, Vector Laboratories, Inc., Burlingame, CA, USA) for 90 min at RT, rinsed in 0.1 M PB and with the avidin-biotin peroxidase complex (1:250, Vector Laboratories, Inc., Burlingame, CA, USA) for 90 min at RT. Then the sections were again washed 3×5 min in 0.1 M PB. After washings in the section

**Table 1**
Sequences of BDNF, GFAP, Sox2, DCX, NeuN and GAPDH primers

| Target gene | Primer sequence |
|---|---|
| BDNF | Forward primer 5'-GCGCCCATGAAAGAAGCA AA-3'<br>Reverse primer 5'-TCGTCAGACCTCTCGAACCT-3' |
| GFAP | Forward primer 5'-GAAGAAAACCGCATCACCATTCC-3'<br>Reverse primer 5'-GCATCTCCACCGTCTTTACCA-3' |
| Sox2 | Forward primer 5'-ACAGCATGTCCTACTCGCAG-3'<br>Reverse primer 5'-A GTGGGAGGAAGAGGTAACCA-3' |
| DCX | Forward primer 5'-ACGACCAAGAC GCAAATGGA-3'<br>Reverse primer 5'-ACAGTGGCAGGTACAAGTCC-3' |
| NeuN | Forward primer 5'-CT TACGGAGCGGCACTGG-3'<br>Reverse primer 5'-CAAGAGAGTGGTGGGAACGC-3' |
| GAPDH | Forward primer 5'-TGGACCACCCAGCCCAGCAAG-3'<br>Reverse primer 5'-GGCCCCTCCTGTTGTTATGGGGT-3' |

Abbreviations: BDNF – brain-derived neurotrophic factor; DCX – doublecortin; GFAP – glial fibrillary acidic protein; NeuN – neuronal nuclear protein; Sox-2 – sex determining region Y-box 2.

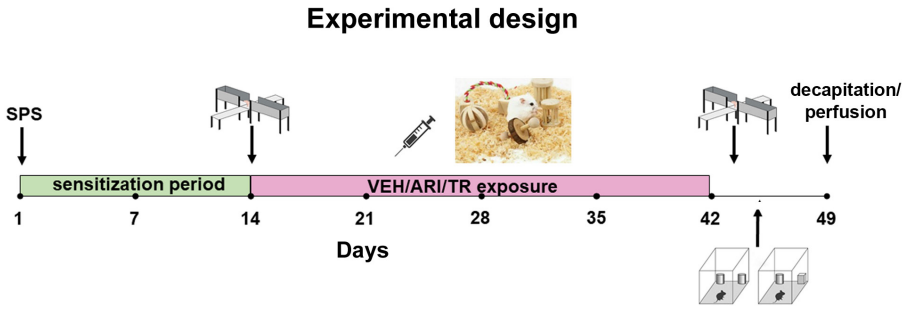


**Figure 1.** A schematic illustration of the experimental design. Animals were randomly assigned to the five experimental groups: (1) **VEH** – non-stressed animals i.p. injected with vehicle (VEH); (2) **SPS –** single prolonged stress (SPS) exposed animals i.p. injected with VEH; (3) **SPS+ARI** – stressed animals i.p. injected with aripiprazole (ARI); (4) **SPS+ toy rotation (TR)** – stressed animals exposed to TR; and (5) **SPS+ARI+TR** – stressed animals exposed to ARI and TR. After SPS, animals were left 14 days undisturbed in their cages to develop PTSD symptoms. After 14-day sensitization period, animals were tested in the elevated plus maze (EPM) and following 28 days exposed to VEH/ARI/TR. Afterwards, rats underwent the 2nd EPM test and 48 h later, the novel object recognition test (NOR), 72 h after the NOR test the animals were sacrificed.

stained by immunofluorescence, the nuclei were visualized by addition of DAPI (1:1000, Thermo Fisher Scientific, USA) for 30 min at RT. Finally, these sections for immunofluorescence were washed 3×5 min in PB, mounted on adhesive slides and coverslipped with Fluoromont (Thermo Fisher Scientific, USA). The light microscopy-stained sections were washed in 0.05 M sodium acetate buffer (SAB, pH6.0) and the ΔFosB antigenic sites were visualized by nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (2.5% nickel chloride, 0.0625% DAB, Sigma-Aldrich, USA), in SAB containing 0.0006% $H_2O_2$ until optimal staining of the ΔFosB labeled nuclei was achieved. The developing process was monitored under the light microscope and the developing time was approximately 5 min. The sections were visualized using Zeiss Axio Imager A1 and AxioCam ERc 5s camera (Carl Zeiss, Germany). Quantification of ΔFosB, pCREB and Sox2 immunopositive cells was performed manually using Fiji/ImageJ software in 3 regions of interest (ROI=10 615 μm$^2$) in the SVZ and GD unilaterally from at least 4 sections/animal. DCX and NeuN immunohistochemistry in the SVZ and GD was evaluated by the Fiji/ImageJ software as percentage of covered area.

**Statistical analysis.** All the data were analyzed with SigmaPlot 11.0 software (Systat Software, Inc.). The data were first tested for normal distribution using the Shapiro-Wilk test. If the data were normally distributed, the two-group means were analyzed using an unpaired two-tailed Student's t-test. Data are reported as mean±SEM. Differences were considered significant at $p<0.05$. The outliers were excluded if the data points ranged more than 1.5 interquartile below the first quartile or above the third quartile.

## Results

**The elevated plus maze test.** In the 1st EPM test which animals underwent after 14-day sensitization period, we did not observe any significant differences in the percentage of distance travelled or time that animals spent in the OA. After the 2nd exposure to EPM, the animals exposed to SPS+ARI+TR spent significantly more time in the OA than SPS ones (p=0.0403) (Figure 2). During the 2nd EPM test, all the experimental groups spent overall less time and some groups travelled also shorter distance in the OA in comparison with the 1st EPM. When the difference between the time spent and the distance travelled in the OA during the 1st and 2nd exposure was calculated, the most pronounced difference was found in SPS animals that spent significantly less percent of time in the OA than the VEH (p=0.0312), SPS+TR (p=0.0123) and SPS+ARI+TR (p=0.0338) ones and travelled shorter distance than the VEH (p=0.0364) and SPS+TR animals (p=0.0117) (Figure 2).

**The open field and novel object recognition tests.** The first day of the NOR test, i.e. habituation phase, was evaluated as an OF test. We found out that the SPS+TR animals in comparison with the SPS ones travelled longer distance (p=0.0173) (Figure 3A) and were active for longer time (p=0.0045) (Figure 3B). SPS+TR animals were more active also compared to the VEH animals (p=0.0058) (Figure 3B). In other parameters studied, no significant differences among experimental groups were observed. Moreover, we did not detect differences in anxiety index among experimental groups examined by the NOR test (Figure 3C).

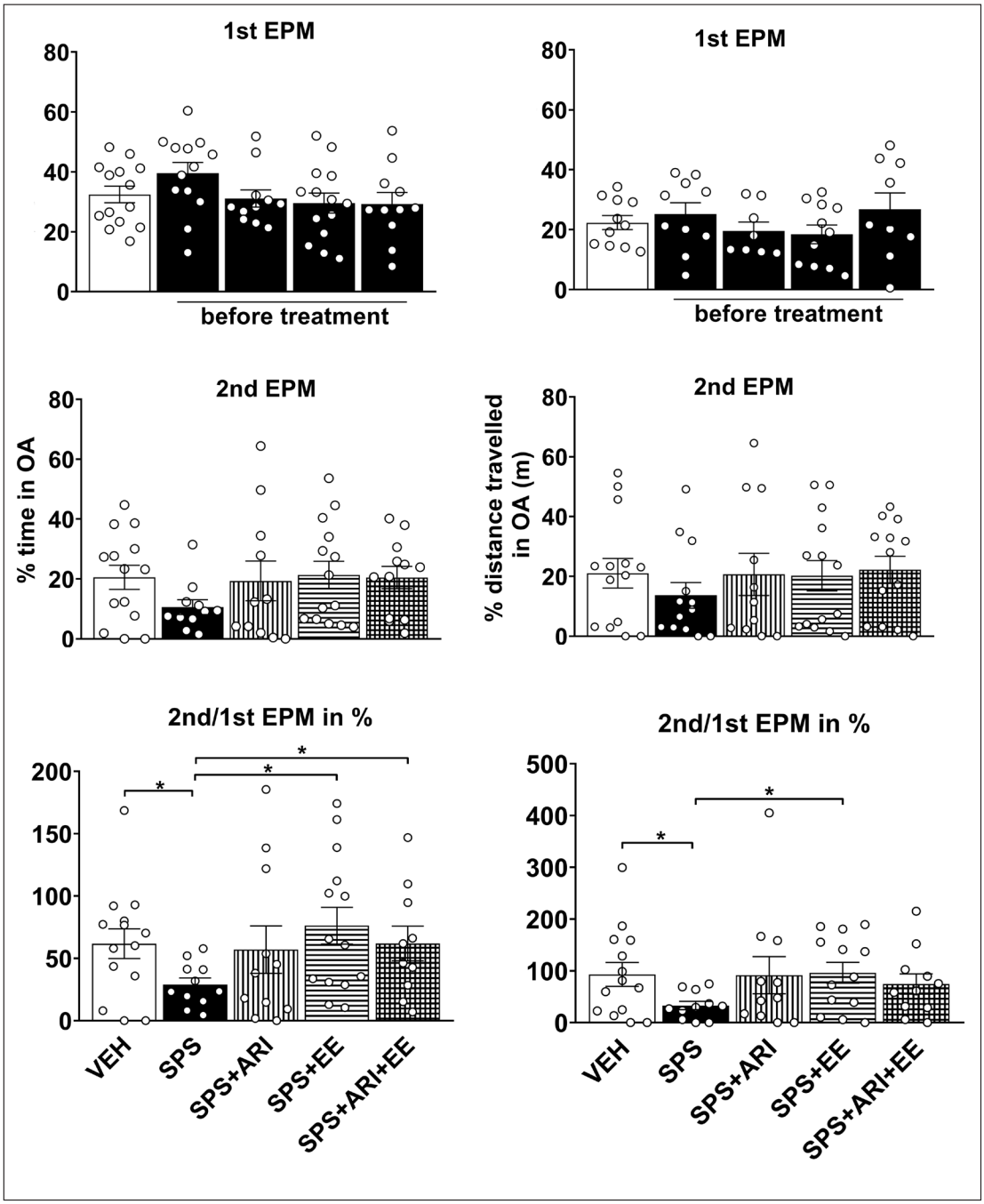


**Figure 2.** Percentage of time spent and distance travelled in the open arms (OA) of the elevated plus maze (EPM) during the 1st and the 2nd exposure and their difference. *p<0.05.

**Genes expression.**

SPS exposure had no significant effect on gene expression of selected neurogenic markers (BDNF, GFAP, Sox2, DCX and NeuN) in both SVZ and GD brain regions compared to controls (VEH group) (Figures 4, 5).

**BDNF and GFAP mRNA.** None of the treatments, ARI, TR, or their combination ARI+TR, significantly affected BDNF or GFAP mRNA levels either in the SVZ or GD of the HIP in SPS animals (Figure 4).

**Sox2 mRNA.** In the SVZ, SPS+TR exposed animals exhibited significantly lower Sox2 mRNA

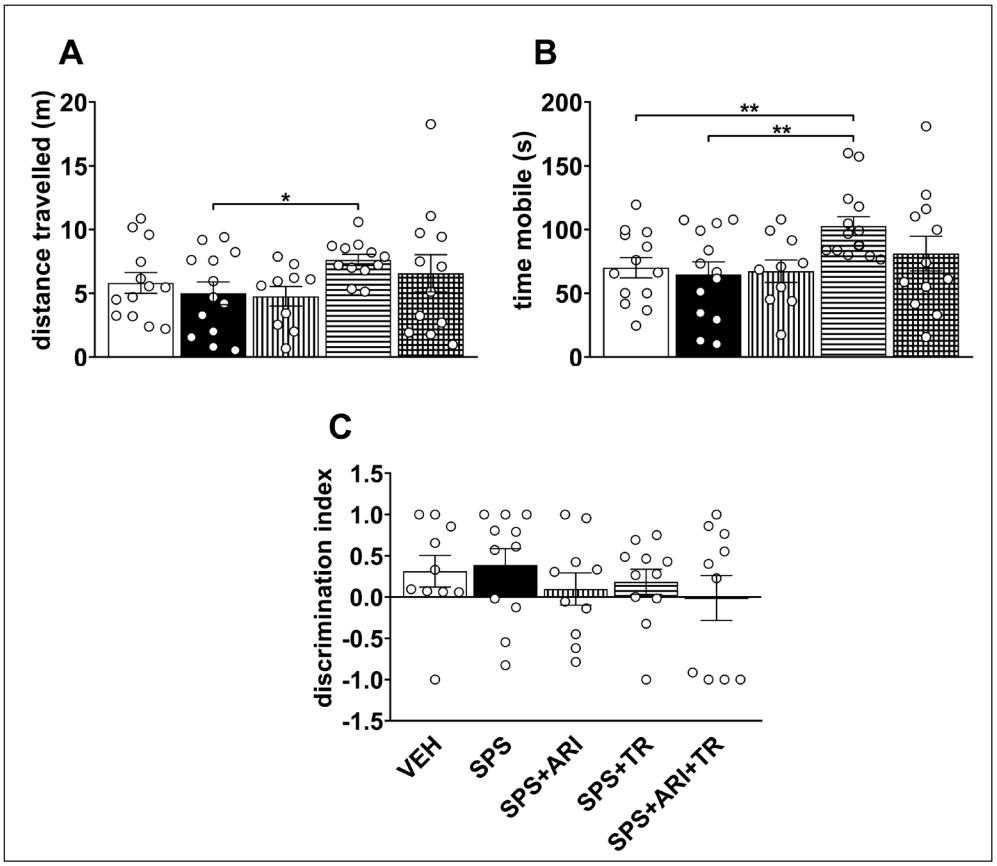


**Figure 3.** Distance travelled (**A**) and mobility (**B**) in the open field test and anxiety index in the novel object recognition test (**C**). *p<0.05, **p<0.01.

levels than the VEH and SPS+ARI+TR ones (p=0.035 and p=0.043, respectively) (Figure 4). In the GD, Sox2 mRNA level was not significantly affected by any treatment (ARI, TR, ARI+TR) in SPS animals (Figure 4).

**DCX mRNA.** In the SVZ, expression of DCX mRNA was significantly elevated in SPS animals after ARI treatment alone (SPS+ARI) and its combination with TR (SPS+ARI+TR) compared to the VEH (p<0.001 for both) and SPS groups (p=0.001 and p<0.001, respectively) (Figure 5). The combined treatment of ARI and TR in SPS rats (SPS+ARI+TR) induced increased DCX mRNA level also in comparison with the SPS+TR group (p=0.0003) (Figure 5). Notably, in the GD of the HIP, we did not find any effect of the ARI or TR on DCX mRNA expression in SPS animals.

**NeuN mRNA.** In the SVZ, exposure of SPS animals to ARI or TR did not markedly affect NeuN mRNA level. Only the combination of ARI and TR treatment in SPS animals (SPS+ARI+TR) significantly increased NeuN gene expression compared to the VEH (p=0.0224) (Figure 5). On the contrary, in the GD of the HIP, SPS animals exposed to TR (SPS+TR) exhibited significantly increased NeuN mRNA levels (p=0.0315) compared to the SPS group. NeuN mRNA level was significantly increased after combined treatment of ARI and TR in SPS animals (SPS+ARI+TR) in comparison with the SPS+ARI ones (p=0.0405) (Figure 5).

**Immunohistochemistry.**

**ΔFosB.** ΔFosB has been shown to initiate and maintain changes in gene expression that continue long after stimulus exposure, remaining in the brain for long periods after acute stress exposure but also after chronic administration of some pharmaceuticals (Nestler et al. 2001, Dietz et al. 2014; Nestler 2015). In the SVZ, we did not detect changes in the number of ΔFosB immunopositive cells among the experimental groups (Table 2, Figure 6). Notably, in the GD, exposure to SPS slightly increased number of ΔFosB cells compared to controls but this increase did not reach significance. On the other hand, SPS+ARI+TR treatment significantly decreased the

number of ΔFosB cells compared to the SPS group (p=0.049, Table 2, Figure 6).

**pCREB.** Either in the GD or SVZ, we did not find significant differences in pCREB protein expression among the experimental groups (Table 2).

**Sox2, DCX, and NeuN.** We did not observe any differences in the number of Sox2 immunopositive cells (Table 2) or the percentage of covered area for DCX and NeuN (data not shown) between the experimental groups.

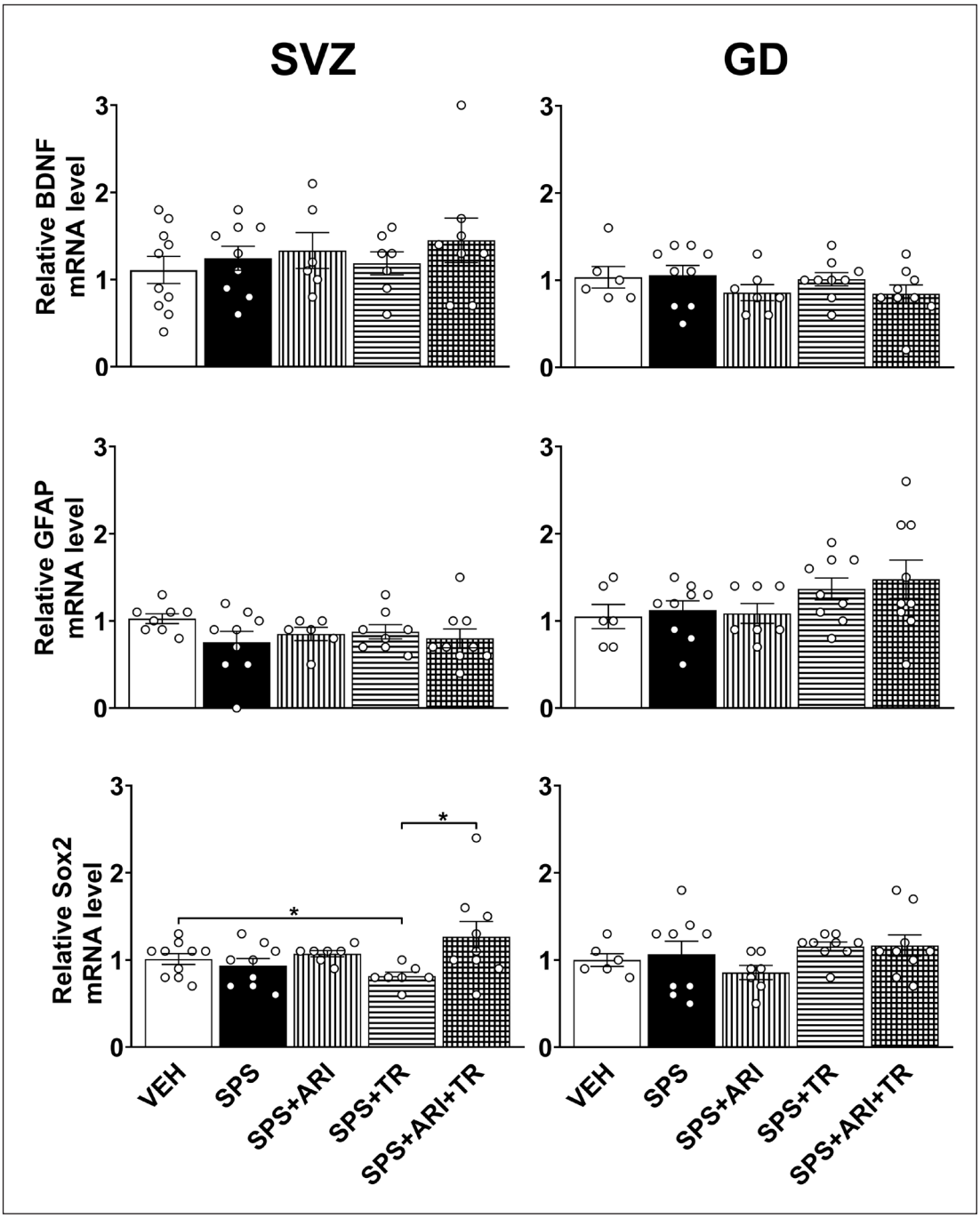


**Figure 4.** Relative brain-derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP), and Sox2 mRNA levels in the subventricular zone (SVZ) and the *gyrus dentatus* (GD). *p<0.05.

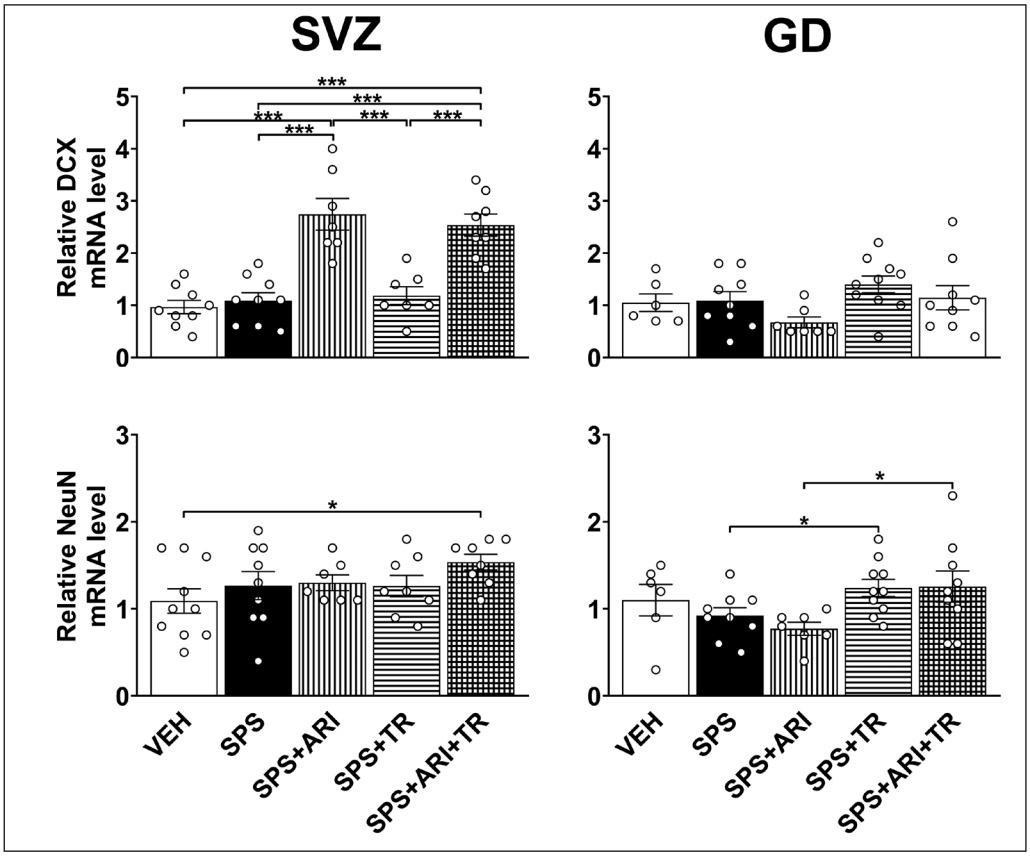


**Figure 5.** Relative doublecortin (DCX) and neuronal nuclear antigen (NeuN) mRNA levels in the subventricular zone (SVZ) and the *gyrus dentatus* (GD). *p<0.05, ***p<0.001.

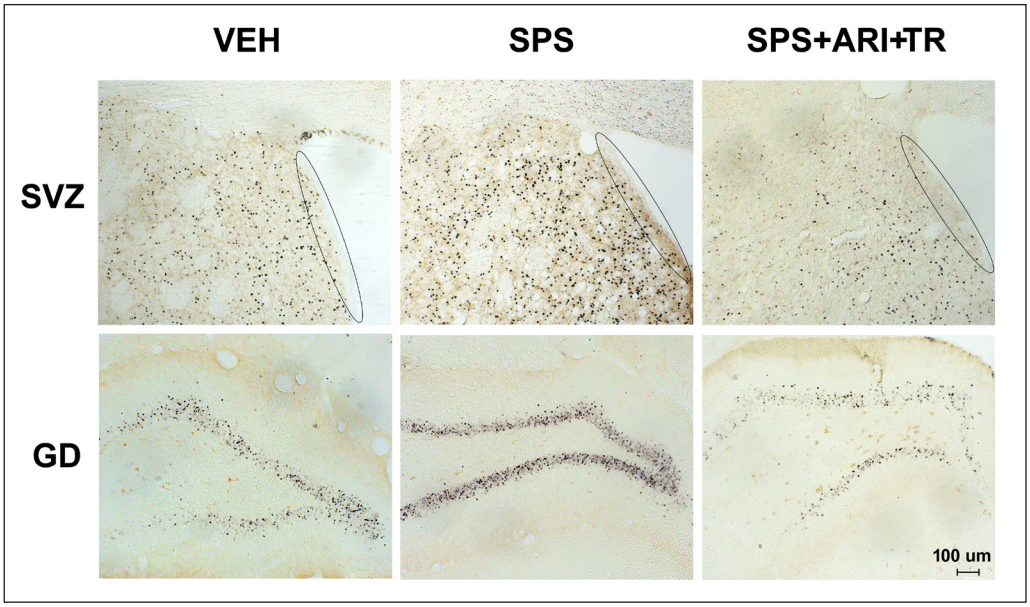


**Figure 6.** Representative images of ΔFosB staining in the subventricular zone (SVZ, circled area) and the *gyrus dentatus* (GD) in non-stressed animals i.p. injected with vehicle (VEH) (**VEH**), animals exposed to single prolonged stress (SPS) i.p. injected with VEH (**SPS**), and stressed animals exposed to aripiprazole (ARI) and toy rotation (TR) (**SPS+ARI+TR**).

**Table 2**
Number of ΔFosB, pCREB and Sox2 immnunopositive cells in the ROI (10 615 μm2) of SVZ and GD brain sections in control and treated rats

| Experimental group | Brain area | Cell number in ROI | | |
|---|---|---|---|---|
| | | ΔFosB | pCREB | Sox2 |
| VEH | SVZ | 43.5±16.9 | 10.6±1.1 | 86.2±3.3 |
| SPS | | 54.4±12.0 | 9.4±5.5 | 91.3±2.8 |
| SPS+ARI | | 47.8±8.9 | 15.4±8.1 | 93.5±9.4 |
| SPS+TR | | 36.3±1.5 | 4.3±1.7 | 88.5±6.3 |
| SPS+ARI+TR | | 41.5±6.2 | 9.3±2.3 | 82.2±3.1 |
| VEH | GD | 28.7±5.0 | 4.6±2.6 | 21.2±1.4 |
| SPS | | 53.8±10.6 | 15.5±9.3 | 20.3±0.8 |
| SPS+ARI | | 36.9±4.5 | 4.9±2.5 | 21.5±1.5 |
| SPS+TR | | 35.9±2.5 | 3.9±3.0 | 18.5±0.9 |
| SPS+ARI+TR | | 26.8±3.1* | 6.6±1.2 | 20.8±0.7 |

Abbreviations: ARI – aripiprazole; pCREB – phosphorylated cyclic adenosine monophosphate-responsive element-binding protein; GD – *gyrus dentatus*; ROI – regions of interest; Sox2 – sex determining region Y-box 2; SPS – single prolonged stress; SVZ – subventricular zone; TR – toy rotation; VEH – vehicle. Data are expressed as mean±SEM. *p<0.05 vs. SPS.

## Discussion

The aim of this study was to assess whether ARI treatment supplemented by TR is more effective in improving behavioral manifestations and affecting neurogenesis in the SVZ and GD in animal model of PTSD than sole tratment with ARI or TR. We have shown that SPS induces anxiety-like behavior in animals, which can be reversed by TR. However, SPS did not influence the expression of studied neurogenic markers. In the PTSD-like animals, ARI treatment increased the expression of DCX in the SVZ and TR elevated expression of NeuN in the GD. TR potentiated the effect of ARI only in the SVZ, where their combination increased the expression of NeuN.

PTSD is characterized by a wide range of behavioral disturbances including hypervigilance, hyperarousal, increased anxiety, generalization of fearful stimuli, and deficits in fear extinction or cognitive deficits (DSM-5, 5th edition, American Psychiatric Association, 2013). Previous studies have shown that PTSD symptoms significantly correlate with the anxiety sensitivity (Chiu et al. 2024) and SPS exposed animals exhibit anxiety-like behavior (Iqbal et al. 2024). Etiology of anxiety is supposed to be connected also with the impaired adult neurogenesis (Gomes-Leal 2021). In the present study, the 1st EPM test was performed 14 days after sensitization period, i.e. before exposure to ARI or TR. This test did not reveal any changes in anxiety-like behavior of PTSD-like animals. However, Serova et al. (2019) have observed decreased OA entries and time spent in OA, and increased anxiety index after the same time period. We believe that this discrepancy might be due to a difference in the SPS protocol, as they used immobilization that is evidently a stronger stressor than restrain stress used in our study. The 2nd EPM was performed 6 weeks after SPS and 4 weeks after the beginning of the application of ARI, TR or their combined application. We detected slight, but not significant changes in the distance travelled and time that animals spent in the OA. As in the 2nd EPM test the animals travelled shorter distance and spent less time in the OA than during the 1st EPM, we calculated the difference between these two measurements. We found that the decline between these two indicators was the most significant in SPS animals. These data indicate that longer time might be necessary to develop the anxiety-like symptoms in our SPS model.

The anxiety-like behavior was effectively reversed by TR. The efficiency of TR could be underlined by previous study, in which EE reversed anxiety-like behavior in an inescapable foot shocks animal model of PTSD (Sun et al. 2016). Notably, we did not observe effect of ARI on anxiety-like behavior, but it is important to point out that the available data concerning ARI anxiolytic effect are unambiguous. The study of Britnell et al. (2017) have even shown that ARI as a monotherapy in a portion of PTSD patients induced anxiety as an adverse effect.

Nevertheless, ARI with respect to its unique receptor profile still represents a reasonable choice for patients who only had a partial response or failed to respond to traditional treatments.

In the OF test, we did not observe any effect of SPS on the anxiety-like behavior what is in the discrepancy with previously published data (Yin et al. 2022; Wislowska-Stanek et al. 2023). This could be explained by different models of PTSD used or different duration of the test itself. We found only increased distance travelled and mobility of SPS+TR animals. Some of the previous studies did not show differences in these parameters in PTSD animals exposed to EE (Hendriksen et al. 2012; Takahashi et al. 2014), but different animal models of PTSD and setups of EE (our improved environment did not include component for physical activity and socialization with more animals) were used.

SPS has been connected with impairments in learning and memory (Eagle et al. 2013). Therefore, we performed NOR test, but we did not observe either negative effect of SPS or positive effect of ARI or TR on learning and recognition memory. Melani and colleagues (Melani et al. 2017) have found that 3-min learning in familiarization phase, commonly used in animal experiments (Antunes and Biala 2012), was not sufficient to develop a long-term memory trace and to induce preferential exploration of the novel object 24 h later in the trial. On the contrary, when the familiarization time was increased to 15 min, animals explored the novel object significantly longer. We assume that short time interval in the familiarization phase was the reason why we did not observe effect of SPS or ARI/TR application.

Animals with PTSD-like phenotype were shown to exhibit increased ΔFosB expression as a marker of repeated neuronal activity in the selected brain areas (Mackenzie et al. 2010; Velasco et al. 2022). We observed a slight increase in the number of ΔFosB cells in the GD what is supported by some previously published data (Schmeltzer et al. 2015). Exposure to ARI or TR decreased activity of the GD, but not significantly, only their combination exerted a significant effect. Previous study has shown that increase in the number of c-Fos cells (that reflects mainly acute activation) in the SVZ was dependent on the type of stressor (Fabianova et al. 2018). According to our data, PTSD does not seem to induce repeated activation of the cells in the SVZ. Previous studies have suggested interconnection between dysregulation of HPA axis and decreased pCREB expression. In PTSD animal models, reduced pCREB and with it connected decreased BDNF expression were detected (Adamec et al. 2011; Chen et al. 2021). We did not find marked differences in pCREB cell number among experimental groups, but animals in the above-mentioned studies were euthanized shorter after traumatic event and another study points to time dependence in the pCREB expression (Adamec et al. 2011).

It is known that adult neurogenesis plays a crucial role in the maintenance of hippocampal capacity for learning and memory formation, which are affected by PTSD. Previous studies have shown decreased number of BrdU and also DCX positive cells in the GD of PTSD-like animals (Peng et al. 2013; Zhou et al. 2019). We determined several markers of different stages of the adult neurogenesis, but we did not observe any changes of them in PTSD-like animals. Schoenfeld with co-workers (2019) have suggested that decline of newborn cells following SPS detected in the above-mentioned studies was a consequence of a decreased cell survival. They monitored cell proliferation and neuronal survival at several time points after SPS and did not observe any effect of SPS on the cell proliferation immediately, 1 or 4 weeks after SPS. However, 30% fewer BrdU/NeuN positive neurons that were born a week after SPS remained also 3 weeks later what probably reflects a lower survival rate for neurons born several days following SPS (Schoenfeld et al. 2019). No changes in expression of neurogenic markers in the SVZ of PTSD-like animals were observed what is in accordance with the outcomes of the study where no changes in Ki67 and DCX cell number in the SVZ of different PTSD animal model have been recorded (Acosta et al. 2013). ARI, like other atypical antipsychotics, is known to stimulate neurogenesis in the HIP (Chikama et al. 2017). In PTSD-like animals, ARI alone or in combination with TR increased only the expression of DCX and NeuN in the SVZ. Interestingly, in the GD of the HIP, we did not find any effect of ARI treatment on neurogenesis, but on the other hand, we found effect of TR. Exposure of SPS animals to TR increased the expression of NeuN in the GD compared to SPS animals and SPS+ARI+TR rats showed elevated NeuN mRNA also compared to SPS+ARI ones. Previous studies have shown positive effect of EE and exercise on the proliferation and survival of neurogenic cells (Benarroch 2013; Tanichi et al. 2018). Tanichi et al. (2018) have emphasized importance of physical exercise components of the EE in the upregulation of neurotrophic factors, which play an important role in the neurogenesis and neuroplasticity. In the experimental group, in which only toys were rotated in the cages without any physical activity components, they did not observe

an increase in neurotrophic factors. This could be the reason why we did not find any pronounced effect of TR on the neurogenic markers in the GD, but it could influence the survival of the newborn neurons, as we detected increased NeuN mRNA level. On the other hand, no increase was seen in the total number of NeuN positive cells in the GD.

## Summary

Our data showed that TR, representing a component of the EE, suppressed the anxiety-like behavior in PTSD-like animals. In contrast, ARI treatment at the administered dose did not induce a significant anxiolytic effect. Moreover, the anticipated potentiating effect of TR in combination with ARI was not observed in our study. Six weeks following SPS exposure, no significant alterations were observed in the expression of neurogenic markers within either the SVZ or the GD, but ARI and TR seems to increase the survival of the newborn cells (increased DCX and NeuN levels after ARI/TR/ARI+TR exposure in the SVZ and GD). We believe that observed changes in the neurogenic markers' expression are likely attributable to the ARI/TR/ARI+TR as SPS itself did not elicit significant changes in these markers.

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***Conflict of interest:*** *The authors declare no conflict of interest.*

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