Article

Increased excitability of serotoninergic neurons and reduced concentrations of circulating corticosteroids two weeks after chronic stress in rats: mechanisms involved.

Eliyahu Dremencov[1,2,*], Daniil Grinchii[1], Katarina Buzgoova[2], Maxim Lapshin[3], Maria Komelkova[3,4,5], Jan Graban[2], Agnesa Puhova[2], Olga Tseilikman[3,4], Vadim Tseilikman[3], and Daniela Jezova[2*]

[1] Institute of Molecular Physiology and Genetics, Center of Biosciences, Slovak Academy of Sciences, Bratislava, Slovak Republic
[2] Institute of Experimental Endocrinology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovak Republic
[3] School of Medical Biology, South Ural State University, Chelyabinsk, Russian Federation
[4] Faculty of Fundamental Medicine, Chelyabinsk State University, Chelyabinsk, Russian Federation
[5] Institute of Immunology and Physiology, Ural Division of the Russian Academy of Sciences, Ekaterinburg, Russian Federation
* Correspondence: Eliyahu.Dremencov@savba.sk; Daniela.Jezova@savba.sk

Abstract: Exposure to predator scent (PS) has been used as a model of stress associated with danger to life and body integrity. We tested the hypothesis that repeated PS exposure alters the excitability of serotonin (5-HT) neurons of the dorsal raphe nucleus. To study the mechanisms involved, we approached serum and adrenal corticosterone and aldosterone concentrations, as well as cortical brain-derived neurotrophic factor (BDNF) expression. Adult male Sprague-Dawley rats were exposed to PS for ten minutes daily for ten consecutive days. Two weeks after the last exposure, electrophysiological and biochemical assessments were performed. Measurements by *in vivo* electrophysiology showed increased spontaneous firing activity of 5-HT neurons in rats exposed to PS. PS exposure resulted in reduced serum corticosterone and aldosterone concentrations. Concentrations of both corticosteroids in the adrenal glands, as well as the relative weight of the adrenals, were unaffected. The gene expression of hippocampal BDNF of rats exposed to PS remained unaltered. In conclusion, repeated exposure of rats to PS leads to enhanced firing activity of 5-HT neurons accompanied by reduced serum, but not adrenal aldosterone and corticosterone concentrations. Reduced corticosteroid concentrations in the blood appear to be the result of increased metabolism and/or tissue uptake rather than altered steroidogenesis. The decrease in circulating corticosterone in rats experienced repeated PS may represent part of the mechanisms leading to increased excitability of 5-HT neurons. The increase in 5-HT neuronal firing activity might be an important compensatory mechanism designated to diminish the harmful effects of the repeated PS exposure on the brain.

Keywords: predator scent, aldosterone, corticosterone, adrenal gland, dorsal raphe nucleus (DRN), serotonin (5-HT), electrophysiology

1. Introduction

Monoaminergic systems of the brain are the primary targets for most known antidepressant drugs, as well as for numerous mood stabilizers and atypical antipsychotics [1]. The majority of antidepressants stimulate serotonin (5-HT) neurotransmission, either directly or indirectly. Different

antidepressant drugs can however have stimulatory, as well as inhibitory effects on other monoamines neurotransmission [2, 3]. It may be suggested that even though central catecholamines (noradrenaline and dopamine) are important in the pathophysiology and treatment of depression as well as related stress and anxiety disorders, the 5-HT system plays a dominant role in mentioned conditions. Stress is therefore likely to alter the excitability of brain 5-HT neurons.

The effects of different stressors on 5-HT excitability were indeed documented. Social defeat [4] and forced swimming [5] had both enhanced the γ-aminobutyric acid (GABA)-mediated inhibition of excitability of 5-HT neurons of the dorsal raphe nucleus (DRN). Stress of maternal immune activation decreased the firing activity of 5-HT neurons in the offspring [6]. Chronic unpredictable stress also led to a decreased firing activity of 5-HT neurons, accompanied with desensitization of DRN serotonin-1A (5-HT$_{1A}$) autoreceptors [7]. A similar decrease in the sensitivity of 5-HT$_{1A}$ receptors of the DRN was observed during stress of novel uncontrolled environmental [8]. An increased sensitivity of 5-HT$_{1A}$autoreceptors of the DRN was induced by early parental separation [9].

Stress- and antidepressant drug-induced changes in 5-HT neurotransmission are interconnected with neurotrophins and other factors involved in brain plasticity. The brain-derived neurotrophic factor (BDNF) is a primary regulator of neuroplasticity, and pro-neuroplastic effects of antidepressant drugs are associated with their ability to boost BDNF expression in the brain [10]. On the other hand, stress is generally associated with impaired neuroplasticity and reduced BDNF expression. Reduced BDNF expression was detected following the exposure to different types of stressors, such as maternal immune activation [11], maternal restraint [12], single prolonged stress [13], and chronic unpredictable mild stress [14].

The excitability of central 5-HT neurons is under the control by multiple central and peripheral factors. Among them are the main components of the stress response, catecholamines and corticosteroids. The regulation of 5-HT excitability by noradrenaline and dopamine has been extensively studied [15-17]. The corticosteroid-mediated modulation of 5-HT excitability had received less attention. An *ex vivo* perfusion of rat DRN slices with corticosterone reduced the 5-HT$_{1A}$ receptor agonist-induced inhibition of 5-HT neurons. This effect was prevented by an antagonist and mimicked by an agonist of glucocorticoid receptors (GR). Administration of the mineralocorticoid hormone aldosterone did not alter 5-HT$_{1A}$ receptor agonist-induced inhibition of 5-HT neuronal firing activity [18].

An exposure to predator scent (PS) has been used as a model for severe stress associated with a danger to the life or body integrity [19]. Electrophysiological data on 5-HT neurotransmission in this model is not yet available. Two recent studies have, however, evaluated the concentrations of 5-HT in different brain regions of rats repeatedly exposed to PS. The results showed that repeated PS exposure led to a long-lasting increase in thalamic 5-HT concentrations [20], decrease in 5-HT concentrations in the midbrain, hippocampus, and neocortex of rats characterized by a high-anxiety phenotype, an and no changes in hypothalamic and forebrain 5-HT levels [21]. It is notable that repeated PS exposure resulted in a decrease of 5-HT concentrations mainly in brain areas innervated by the DRN, rather than in those innervated by the median and rostral raphe nuclei [22]. It can be therefore hypothesized that repeated PS exposure alters the excitability of 5-HT neurons of the DRN. Since exposure to PS alters circulating concentrations of corticosteroids [19, 21, 23], PS-induced

alterations in DRN 5-HT excitability may be linked to changes in circulating corticosteroids. Finally, the PS-induced changes in 5-HT neurotransmission may lead to altered BDNF expression. This study aimed to test these hypotheses.

2. Results

2.1. Repeated PS exposure increased the firing activity of 5-HT neurons of the DRN

The excitability of 5-HT neurons of the DRN was affected by used stress stimulus (Figure 1). The mean spontaneous firing activity of 5-HT neurons in the DRN of rats experienced repeated PS was significantly higher than that in the controls ($p < 0.01$, two-tailed Student's post-hoc test):
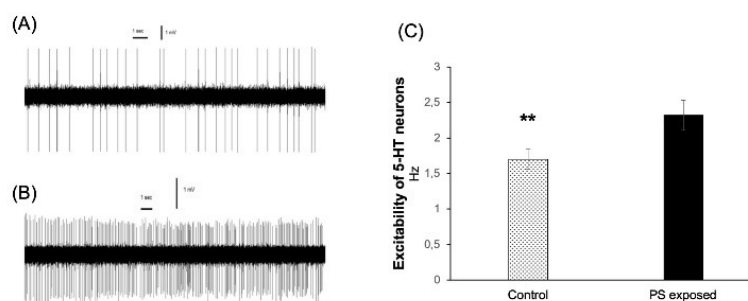

Figure 1. Effect of repeated PS on the excitability of 5-HT neurons of the DRN. A and B: representative recordings from a control and PS rat; C: summary effect from 74 neurons from 10 control and 56 neurons from 10 PS rats; **$p < 0.01$, two-tailed Student's t-test.

2.2. Repeated PS exposure reduced serum, but not adrenal hormone concentrations nor adrenal weights

Serum corticosterone concentrations (Figure 2A) were significantly lower in PS exposed group when compared to the control animals ($p < 0.05$). Similarly, PS exposure led to a significant decrease in serum aldosterone concentrations ($p < 0.01$) in comparison with those in controls (Figure 2B). Interestingly, concentrations of corticosterone in the right adrenal did not differ between the groups (Figure 2C). The concentration of aldosterone in the adrenal gland was unaffected by the repeated PS exposure (Figure 2D). The repeated PS exposure failed to modify the relative weight of the adrenal glands. The relative weight of both left and right adrenals was comparable in PS exposed and control rats (Figure 2E).

2.3. Repeated PS exposure failed to modify BDNF gene expression in the prefrontal cortex.

The gene expression of BDNF in the prefrontal cortex was unaffected by PS exposure. In animals exposed to repeated PS, the concentrations of mRNA coding for BDNF (0.82±0.05 a.u.) were comparable to those in the control animals (1.06±0.22 a.u.).
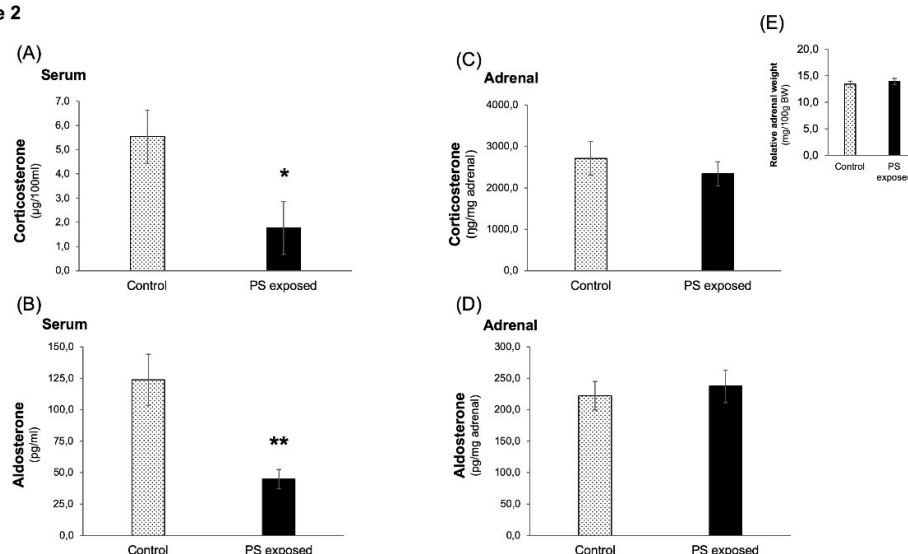

Figure 2. Effect of repeated PS on the serum corticosterone (A) and aldosterone (B) concentrations, on the adrenal gland corticosterone (C) and aldosterone (D) contents, and on the relative weight of the adrenal glands (E); *$p<0.05$ and **$p<0.01$, two-tailed Student's t-test.

2.4. Repeated PS exposure did not induce changes in adrenal steroidogenic factors.

To better understand the findings presented on Figure 2, we have analyzed selected factors involved in the adrenal steroidogenesis. The gene expression of steroidogenic factor-1 (SF-1) in the left adrenal gland was unaffected by PS exposure (Figure 3A). The mRNA levels of steroidogenic acute regulatory protein (StAR) in the adrenal gland showed a declining trend in the group of animals exposed to PS (Figure 3B), but the difference did not reach the statistical significance ($p=0.059$). Independently of the stress stimulus, the gene expression of steroid 11β-hydroxylase (CYP11B1) in the adrenal gland was comparable in both groups of animals (Figure 3C).
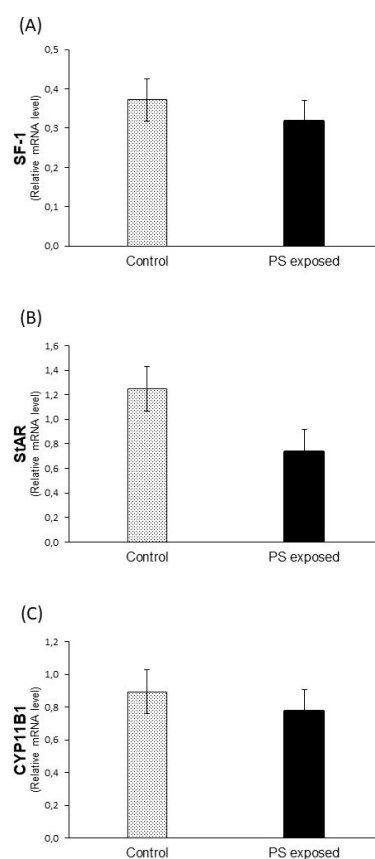
Figure 3

(A)

(B)

(C)

Figure 3. Effect of repeated PS on the gene expression of SF-1 (A), StAR (B) and 11CYPB1 (C) in the left adrenal gland.

3. Discussion

The results of the present study demonstrate that rats exposed to the repeated PS exert increased spontaneous firing activity of 5-HT neurons of the DRN compared to that in unexposed controls. Repeated PS exposure resulted in decreased serum concentrations of corticosterone and aldosterone. The concentrations of both steroid hormones within the adrenal glands, as well as the relative weight of the adrenals, were unaffected by the PS. The expression of BDNF in the hippocampus of rats exposed to the repeated PS was not statistically different from the controls.

In this study we detected, for the first time, the enhancing effect of repeated PS exposure on the excitability of 5-HT neurons of the DRN. This observation is startling, since other stressors, such as maternal immune activation [6]and chronic unpredictable stress [7], led to decreased 5-HT neuronal firing activity. It is possible that increased excitability of 5-HT neurons is lined with decreased brain serotonin concentrations in the midbrain and neocortex, observed in our previous study, though using a rats from a different source [21]. A decrease in midbrain 5-HT can diminish the tonic activation of 5-HT$_{1A}$autoreceptors of the DRN [24], resulting in an increased firing activity of 5-HT

neurons. Cortical 5-HT can attenuate the excitability of 5-HT neurons of the DRN as well, *via* a mechanism involving 5-HT$_{1A}$ receptor-mediated inhibition of cortical neurons projecting to the DRN and positively regulating 5-HT neuronal firing activity [25]. Alternatively, increased firing activity of 5-HT neurons after repeated PS exposure might be related to decreased circulating glucocorticoid levels [18].

The repeated exposure to PS in the present experiments failed to modify gene expression of BDNF, an important marker of brain plasticity, in the hippocampus. In general, chronic and intensive stressors are inducing negative effects on brain plasticity. A decreased expression of hippocampal BDNF was observed also in studies investigating the consequences of exposure to PS, particularly those using protocols including situational trauma reminder to simulate post-traumatic stress symptoms [26, 27]. In a recent study, stress of post-weaning isolation did not alter hippocampal BDNF, but it led to a reduced gene expression of VGF (non-acronymic) and tyrosine receptor kinase B (TrkB) receptor [28]. Thus, the modulation of VGF/BDNF/TrkB signaling by repeated PS exposure cannot be excluded.

Although increased release of glucocorticoids in response to repeated stressors is a well-known phenomenon [29], repeated PS exposure resulted in lower serum corticosterone concentrations.. It is consistent with the results of previous studies with repeated exposure to PS [21, 30, 31]. As mentioned above, changes in circulating glucocorticoid availability may be related to central serotonin neurotransmission. Since corticosterone is known to enhance 5-HT$_{1A}$ autoreceptors-induced inhibition of 5-HT neurons [18], low serum corticosterone concentrations and/or decreased glucocorticoid signaling can be associated with reduced excitability of central 5-HT neurons. It may be suggested that decreased corticosterone concentrations in rats after repeated PS exposure contribute, at least in part, to the increased firing activity of 5-HT neurons. A previously reported enhanced 5-HT neuronal firing activity induced by the inhibition of glucocorticoid metabolism supports this hypothesis [32].

The present study shows that repeated exposure to PS leads to a decrease in serum concentrations of the mineralocorticoid hormone aldosterone. This finding is surprising, since other stressors, such as insulin-induced hypoglycaemia, and immune challenge were shown to increase aldosterone concentrations in the rat blood [33, 34]. Aldosterone is a somewhat neglected stress hormone and its involvement in stress-related mental disorders is being explored only recently [29]. To our best knowledge, concentrations of aldosterone have not been investigated in animal models using PS exposure.

Interestingly, exposure to PS led to decreased corticosterone and aldosterone concentrations blood serum, but not their content in the adrenal gland. The relative weight of the adrenal glands remained unchanged as well. Corticosteroids are not being stored in the adrenal cortex and changes in their circulating levels in response to stress stimuli usually occur with parallel changes in their concentration in the adrenals [35]. The present results thus provide indirect evidence that reduced serum concentrations of these steroids in rats experienced repeated PS resulted from their excessive metabolism and/or tissue uptake, rather than reduced release or synthesis within the adrenal cortex.

It is in agreement with our previous suggestion that increased metabolism of glucocorticoids contributes to decreased glucocorticoid signaling in stress-related psychopathologies [23, 36].

To further analyze the findings mentioned above, we decided to measure gene expression of steroidogenic factors. We have chosen two factors involved in the early stages of steroidogenesis, namely SF-1 and StAR. Moreover, we have focused on the CYP11B1, the enzyme involved in the biosynthesis of adrenal corticosteroids. The gene expression of none of these factors was modified by the exposure to the stressor. These findings are in support of increased metabolism and/or tissue uptake of adrenal steroids.

In conclusion, repeated exposure of rats to PS leads to enhanced firing activity of 5-HT neurons in the DRN accompanied by reduced serum, but not adrenal concentrations of aldosterone and corticosterone. Reduced corticosteroid concentrations in the blood appear to be the result of increased metabolism and/or tissue uptake rather than altered steroidogenesis. The decrease in circulating corticosterone in rats experienced repeated PS may represent part of the mechanisms leading to increased excitability of 5-HT neurons of the DRN. Since serotonin is a potent pro-neuroplastic agent, this increase in 5-HT neuronal firing activity might be an important compensatory mechanism designated to diminish the harmful effects of the repeated PS exposure on the brain.

4. Materials and Methods

4.1. Animals

Adult male Sprague-Dawley rats, weighting 200-250 g, were ordered from Velaz, s.r.o. (Prague, Czech Republic). They were kept under standard housing conditions with a constant 12:12h light/dark cycle (lights on at 06.00 h), temperature (22±2 °C) and humidity (55±10%). Animals were housed two per cage in standard cages with free access to rat chow and water. 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.

4.2. Repeated exposure to PS

After an acclimatization period of one week, the rats were randomly divided to control (n=20) and PS exposed (n=20) groups. Sand containing fresh cat urine was collected daily from the litterbox used by two domestic cats belonging to one of the authors (ED). This sand was stored at the room temperature in a closed plastic container for 3-5 h before the exposure. This sand was laid in plastic dish and placed in a 20×30×50 cm exposition cage. Rats were transferred to the exposition cage for 10 min daily, between 13:00 and 14:00, for 10 consecutive days. Control unstressed rats were exposed to the sand containing clean water, using the same schedule. Control and PS exposed rats were kept and exposed to the stressor in different rooms with separate air conditioning systems. After the last exposure, the rats were kept under non-stress conditions for 2 weeks.

4.3. *In vivo* electrophysiology

Thefirst half of the animals (10 control and 10 PS exposed rats) was used for electrophysiological measurements. Fourteen days after the last exposure to the sand containing clean water or cat urine,

those rats were anesthetized by chloral hydrate (400 mg/kg, i.p.) and mounted in the 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 inserted into the DRN, 7.8-8.3 mm posterior to bregma and 4.5-7.0 mm ventral to brain surface [37] by hydraulic micro-positioner (David Kopf Instruments, Tujunga, CA). The action potentials generated by monoamine-secreting neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). The 5-HT neurons were identified by their regular firing rate of 0.5–5.0 Hz and positive action potential of long duration of 0.8-1.2 msec. [38-40]. After completion of electrophysiological recordings, the animals were euthanized by overdose of chloral hydrate.

4.4. Blood and organ collection

The other half of the animals (10 control and 10 PS exposed rats) was decapitated fourteen days after the last exposure and their blood and tissues were collected. The 2 rats from the same cage were killed within 30 s. Trunk blood was collected into polyethylene tubes without anticoagulant. The clotted blood was spun at 3000 rpm for 15 min at 4 °C and the serum was separated. The separated adrenal glands were quickly weighted, and frozen in liquid nitrogen. The brain was quickly removed from the skull, the prefrontal cortex was dissected and frozen in liquid nitrogen. Serum aliquots and collected tissues were stored at -80 °C until analyzed.

4.5. Hormone measurements

Serum corticosterone was measured by double-antibody radioimmunoassay (Corticosterone Double Antipoints RIA Kit, MP Biomedicals, USA). Both intra- and inter-assay coefficients of variation (CVs) were < 5%. Serum aldosterone was analyzed by a coated-tube radioimmunoassay (RIAZENco Aldosterone kit, ZenTech, Belgium), according to the manufacturer's instructions [41]. The intra- and inter-assay CVs were 3.8% and 6.2%, respectively and the detection limit was 1.4 pg/ml. For the measurement of adrenal corticosterone and aldosterone concentrations, the right adrenal gland was homogenized (D1000 Handheld Homogenizer, Benchmark Scientific, USA) in necessary amount of saline and processed as described previously [35]. The concentrations of corticosterone and aldosterone were analyzed using the same commercially available kits mentioned above.

4.6. Analysis of gene expression

The total mRNA was isolated by TRIzol® Reagent (Life technologies, California, USA) from prefrontal cortex and left adrenal homogenates according to manufacturer protocol as described previously [42]. Concentration and purity of mRNA preparations was measured by absorption spectroscopy (Nanodrop 2000). The isolated mRNA (1 μg) from prefrontal cortex and left adrenal gland was reverse-transcribed to cDNA using oligo (dT) nucleotides by M-MuLV reverse transcription system (ProtoScript First Strand cDNA Synthesis Kit, New England BioLabs, Ipswich,

Massachusetts, United States). Primer BLAST NCBI software [43] was used to design primers specific for studied genes (Table 1):

Gene	Forward primer 5´- 3´	Revers primer 5´- 3´
BDNF	AGCAGAGGAGGCTCCAAGG	ACCATAAGGACGCGGACTTG
SF-1	GTCCAGTGTGTGGTGACAAGGT	GACTCTCGGTGCACGTGTAATG
StAR	GGAGCTCTCTACTTGGTTCTCAACTG	AGGCCTTTTGCATAGCTTCCTCT
CYP11B1	CCCAGAAGGCCATGTCAGA	GGGTAGAGCCTCAAGGTCTCTTT
PPIA	AAGCATACAGGTCCTGGCATCT	CATTCAGTCTTGGCAGTGCAG
RPS29	GCTGAACATGTGCCGACAGT	GGTCGCTTAGTCCAACTTAATGAA

Table 1. Nucleotide sequence of primers used for gene expression measurements (BDNF: brain derived neurotrophic factor; SF-1: steroidogenic factor-1; StAR: steroidogenic acute regulatory protein; CYP11B1: steroid 11β-hydroxylase; PPIA: peptidyl prolyl isomerase A; RPS29: ribosomal protein S29)

Quantitative PCR was used for evaluation of mRNA concentrations. Analysis was performed in a reaction volume of 10 µl by Luna Universal qPCR Master Mix, New England BioLabs Inc.) as described previously [42, 44]. Primers (Table 1) were used at a concentration of 0.25 pmol/µl. 5 ng of BDNF, SF-1, StAR and CYP11B1 gene cDNA was added to the final reaction volume of 10 µl. [45]. Quantitative PCR was performed by QuantStudio 5 Fast Real-Time PCR System (ThermoFisher, USA). All data obtained by quantitative PCR analysis were evaluated as ng of mRNA (cDNA) according to a standard curve and was normalized to gene expression of peptidyl prolyl isomerase A (PPIA) and Ribosomal Protein S29 (RPS29) as reference genes both in prefrontal cortex and adrenal samples. Gene expressions were evaluated by ΔΔCt calculation and normalized to PPIA and RPS29 housekeeping genes as arbitrary units.

4.7. Statistical analysis

Data was expressed as mean ± SEM. Two-tailed Student's t-test was used to determine the effects of PS exposure on excitability of 5-HT neurons in the DRN and on stress related parameters. The probability of $p \leq 0.05$ was considered significant.

Author Contributions: Conceptualization, ED, OT, VT, and DJ; planning of experiments, ED, OT, VT, and DJ; writing—original draft preparation, conductance of the experiments, ED, DG, KB, ML, MK, JG, and AP; data analysis: ED, DG, KB, JG, and DJ; writing—review and editing, E.D. and DJ; funding acquisition, E.D, OT, VT, and DJ. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
BDNF	brain-derived neurotrophic factor
CYP11B1	steroid 11β-hydroxylase
DRN	dorsal raphe nucleus
GABA	γ-aminobutyric acid
GR	glucocorticoid receptor
PPIA	peptidyl prolyl isomerase A
PS	predator scent
RPS29	ribosomal protein S29
SF-1	steroidogenic factor-1
StAR	steroidogenic acute regulatory protein
TrkB	tyrosine receptor kinase B

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