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Activity of the Hypothalamic–Pituitary–Adrenal System in Prenatally Stressed Male Rats on the Experimental Model of Post-Traumatic Stress Disorder

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Using the experimental model of post-traumatic stress disorder (stress–restress paradigm), we studied the dynamics of activity of the hypothalamic–pituitary–adrenal system (HPAS) in adult male rats, whose mothers were daily subjected to restraint stress on days 15-19 of pregnancy. Prenatally stressed males that were subjected to combined stress and subsequent restress exhibited not only increased sensitivity of HPAS to negative feedback signals (manifested under restress conditions), but also enhanced stress system reactivity. These changes persisted to the 30th day after restress. Under basal conditions, the number of cells in the hypothalamic paraventricular nucleus of these animals expressing corticotropin-releasing hormone and vasopressin was shown to decrease progressively on days 1-30. By contrast, combined stress and restress in control animals were followed by an increase in the count of CRH-immunopositive cells in the magnocellular and parvocellular parts of the paraventricular nucleus and number of vasopressin-immunopositive cells in the magnocellular part of the nucleus (to the 10th day after restress). Our results indicate a peculiar level of functional activity of HPAS in prenatally stressed males in the stress–restress paradigm: decreased activity under basal conditions and enhanced reactivity during stress.

Key Words: *post-traumatic stress disorder; corticotropin-releasing hormone; vasopressin; prenatal stress; rat*

Post-traumatic stress disorder (PTSD) belongs to a group of anxiety disorders, whose pathogenesis is closely related to dysfunction of the hypothalamic–pituitary–adrenal system (HPAS) [5,8]. Analysis the neuroendocrine status in PTSD patients revealed elevated content of corticotropin-releasing hormone (CRH) in the cerebrospinal fluid, which does not correspond to activity of the peripheral part of this system. The 24-h urinary excretion of cortisol in these patients decreased, while the concentration of circulating cortisol could be reduced, unchanged, or even elevated [7,12]. Stress activity of HPAS in some patients was shown to be high [8]. Similar results were obtained in experiments with animals on various paradigms of experimental PTSD (*e.g.*, stress–restress and time-dependent sensitization) [10,11]. The cause for this hormonal heterogeneity of PTSD manifestations remains unknown.

Our previous studies showed that prenatal stress causes an increase in the stress activity of HPAS in adult rats, which aggravates the severity and duration of behavioral and hormonal disturbances in the experimental model of PTSD (stress–restress paradigm) [1,3,4]. Only prenatally stressed (PS) rats were characterized by a decrease in the basal activity of this hormonal axis. These changes manifested in a lower level of plasma corticosterone after the stress–restress procedure.

This work was designed to study changes in stress activity of HPAS in PS animals in the stress–restress paradigm and the state of the paraventricular nucleus

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(PVN), the hypothalamic regulatory center of this system.

MATERIALS AND METHODS

Experiments were performed on adult male rats (220-250 g) obtained from primiparous Wistar females. Pregnant females (n=12) were daily immobilized in narrow plastic tubes under excess illumination (60-W lamp at a height of 50 cm) on gestation days 15-19. Control pregnant females (n=12) remained intact. The onset of pregnancy was verified from the presence of spermatozoids in vaginal smears. The offspring was kept with a mother until the 30th day of life. Then the animals were maintained in cages (6-7 specimens per cage) under standard vivarium conditions and had free access to water and food.

The study was performed in accordance with the European Union Council Directives (86/609/EEC) on the Use of Laboratory Animals. The protocol of experiments was approved by the Committee on Animal Welfare (I. P. Pavlov Institute of Physiology).

The stress–restress paradigm was used as the experimental model of PTSD [11]. PS animals and control rats were divided into 2 subgroups. Subgroup 1 males were subjected to severe combined stress (successive procedures of 2-h immobilization, 20-min swimming, and ether stress to loss of consciousness). Restress (30-min immobilization on day 7 after combined stress) served as the trigger for the development of pathology. Subgroup 2 comprised intact males and served as the control for subgroup 1.

Activity of the hypothalamic regulatory center for HPAS (PVN) was evaluated immunohistochemically from the number of cells containing an immunoreactive protein for vasopressin or CRH. The rats of both subgroups were decapitated in 1, 10, and 30 days after restress. The brain was removed, placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 1 h, and then maintained at 4°C for 48 h. Brain tissues were subjected to the standard histological treatment. Tissue samples were treated with alcohols in increasing concentrations (70, 80, 96, and 96%; 1 h for each concentration) and butanol (1 h and 1 night). Then the samples were treated with 4 portions of xylene (15 min) and embedded into paraffin blocks. Series of alternating brain sections (frontal plane, thickness 5-6 μ) were prepared on a microtome. Experiments were performed with primary polyclonal rabbit antibodies to CRH (1:50; Santa Cruz Biotechnology Inc.) and vasopressin (1:500; Millipore Corp.) and universal system of the avidin-biotin complex (ABC; VectorLaboratories Inc.). The diaminobenzidine kit (DAB Substrate kit; VectorLabs) was used to visualize antibody-antigen binding. A quantitative study of CRH- and vasopressin-immunoreactive cells was conducted on a Jenaval light microscope (Carl Zeiss), Baumer CX05c digital camera (Baumer Optronic), and VideoTest-Master (Morfologiya) software (VideoTest). The calculation was performed on 5-6 sections from each animal (area 400×400 μ). Each group consisted of 5 males for each time interval.

Changes in the stress activity of HPAS were evaluated before combined stress, during restress, and 30 days after restress. Some control specimens and PS males (subjected or not subjected to combined stress and restress) were immobilized in narrow plastic tubes for 30 min. The blood was sampled from the caudal vein 30 and 60 min after the start of stress. Plasma corticosterone concentration was measured by radioimmunoassay with antiserum (Sigma) and [1,2,6,7-^{3}H]-corticosterone (specific activity 76.5 Ci/mmol; NEM Life Science Products).

The results were analyzed by nonparametric Mann–Whitney test for immunohistochemical data with Statistica 8.0 software (StatSoft Inc.). Two-way analysis of variance (ANOVA) was used to evaluate the effect of prenatal and combined stress on changes of corticosterone concentration in dependence on the time after the start of immobilization (30 and 60 min). Individual groups were compared by the post-hoc pairwise comparison test (Dunnett's test). The differences were significant at p<0.05.

RESULTS

Table 1 shows changes in stress activity of HPAS in control and PS rats during the development of anxiety in the stress–restress paradigm. Initially, PS males differed from control specimens in a higher level of plasma corticosterone 60 min after immobilization. No differences were found in corticosterone concentration for control and PS animals 30 and 60 min after immobilization. However, the animals of both groups were characterized by a greater rate of HPAS inhibition after stress-induced activation during restress. This conclusion was derived from a sharp decrease in corticosterone concentration 60 min after the start of stress. It should be emphasized that PS rats exhibited a significant increase in the stress reactivity of this system. By the 30th minute of our study the hormonal index in these rats was much higher than that in PS males not subjected to combined stress. The stress reactivity in control animals also increased during restress (statistically insignificant). The stress reactivity of HPAS in PS rats 30 days after restress was similar to that observed during restress. It should be emphasized that the maximum release of corticosterone decreased, but remained high. Activity of HPAS in control males subjected to combined stress and restress was shown

TABLE 1. Blood Corticosterone Concentration (nmol/liter) after 30-min Immobilization

Group		Time after the start of immobilization	
		30 min	60 min
Control	intact (n=10)	791.0±51.4	825.0±91.3
	PS (n=10)	748.0±46.7	1195.0±130.4*
Restress	stress–restress (n=9)	938.0±69.2	326.0±39.1^{+}
	PS stress–restress (n=9)	1457.0±124.2*^{o}	199.0±17.9^{+}
30 days after restress	control stress–restress (n=7)	903.0±78.7	738.0±61.3
	PS stress–restress (n=7)	994.0±88.7*^{o}	437.0±50.1^{+}

Note. p<0.05 in comparison with *corresponding control, $^{+}$30 min, and ^{o}intact subgroup of the control group.

to return to normal after 30 days. The analysis of variance for these studies revealed a significant effect of PS on the stress reactivity of HPAS in intact males ($F_{(1,39)}$=5.98; p=0.018) and animals subjected to combined stress ($F_{(1,35)}$=11.32; p=0.0014 during restress; and $F_{(1,27)}$=6.49; p=0.015 on day 30 after restress).

It should be emphasized that corticosterone level by the 60th minute was lower than that observed 30 min after the start of immobilization. Our results illustrate an increase in the sensitivity of HPAS to negative feedback signals in animals subjected to traumatic stress. The observed changes in activity of this system serve as a diagnostic criterion of PTSD [8]. Clinical studies showed that an increase in the stress reactivity of HPAS is typical of some patients with PTSD [12]. We showed that the post-stress level of corticosterone increases only in PS males. Change in activity of the hormonal axis in these specimens were observed for a long time.

The number of immunopositive cells for CRH and vasopressin in hypothalamic PVN was shown to differ between PS and control males (Table 2). The count of CRH-immunopositive cells in both parts of PVN in control rats increased 1 and 10 days after restress, but returned to normal by the 30th day. The number of vasopressin-immunopositive cells changed only in the magnocellular part of PVN. This index decreased on day 1, but increased by the 10th day after restress. Our results are consistent with published data [10].

The number of CRH-immunopositive cells in hypothalamic PVN of PS rats was higher under basal conditions and increased 1 day after restress (similarly to control animals). This index decreased significantly in the follow-up period and remained unchanged over

TABLE 2. Changes in the Number of CRH- and Vasopressin-Immunopositive Cells in Hypothalamic PVN of Male Rats during the Development of Anxious State in the Model of PTSD (Stress–Restress Paradigm)

Ligand		Group	Time after combined stress and restress			
			Baseline	1 day	10 days	30 days
CRH	parvocellular	control	24.560±3.575	40.90±3.99^{+}	40.20±4.09^{+}	24.5±3.138
		PS	43.000±5.251*	75.400±4.206^{+}	16.40±1.63^{+}	10.00±1.21^{+}
	magnocellular part	control	8.20±1.76	15.0±2.5^{+}	17.20±3.42^{+}	22.10±3.16^{+}
		PS	11.40±1.98	38.00±7.37^{+}	7.80±2.46	6.80±1.54^{+}
Vasopressin						
	parvocellular	control	18.60±2.45	16.60±3.04	18.30±4.48	19.80±2.21
		PS	21.70±5.09	13.90±2.01^{+}	11.80±4.94^{+}	5.0±1.0^{+}
	magnocellular part	control	44.30±7.89	27.20±3.86^{+}	60.10±5.60^{+}	40.30±5.75
		PS	66.50±5.54*	48.30±4.46^{+}	48.10±5.18^{+}	42.60±5.83^{+}

Note. p<0.05 in comparison with *corresponding control, $^{+}$baseline.

30 days. Similar dynamics was revealed for the count of vasopressin-immunopositive cells in PVN of PS rats. Our previous studies showed that combined stress and restress in PS males are followed by a decrease in the basal level of corticosterone [3]. Therefore, it could be hypothesized that the exhaustion of HPAS is manifested at various regulatory levels of this system. However, the hormonal system of PS rats retained the ability to react to stress even 30 days after restress. It seems to be more likely that HPAS in these animals is transformed into another level of functional activity (but not exhausted). These changes are often observed during severe and/or repeated stress exposures [6,9]. It should be emphasized that similar changes in activity of HPAS (long-term decrease in the basal activity and increase in the stress reactivity during the stress–restress paradigm) were previously revealed in PS female rats [2]. However, significant sex differences in the reaction of hypothalamic PVN in PS animals are observed in this paradigm. For example, combined stress and subsequent restress are followed by the decrease of CRH expression in PS males and females. However, this index in females returned to normal by the 30th day. The number of vasopressin-immunopositive cells in PS females subjected to combined stress and restress was shown to increase progressively. These data indicate that an increase in the stress reactivity of HPAS in PS animals in the stress–restress paradigm is not associated with a change in activity of the central regulatory compartment for this system. The observed changes are mediated by other regulatory factors, which require further investigations.

We conclude that in the stress–restress paradigm (experimental model of PTSD), PS males exhibit not only an increase in the sensitivity of HPAS to negative feedback signals (manifested under restress conditions), but also an elevation of the stress system reactivity. These changes persist to the 30th day after restress. The activity of HPAS in these animals is reduced under basal conditions. This feature is manifested in a decrease in the number of CRH- and vasopressin-expressing cells in hypothalamic PVN.

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REFERENCES

1. N. E. Ordyan and S. G. Pivina, *Ross. Fiziol. Zh*., **89**, No. 1, 52-59 (2003).
2. N. E. Ordyan, S. G. Pivina, V. I. Mironova, *et al*., *Ross. Fiziol. Zh*., **100**, No. 12, 1409-1420 (2014).
3. N. E. Ordyan, I. V. Smolenskii, S. G. Pivina, and V. K. Akulova, *Zh. Vyssh. Nervn. Deyat.*, **63**, No. 2, 280 (2013).
4. S. G. Pivina, V. K. Akulova, V. V. Rakitskaya, and N. E. Ordyan, *Bull. Exp. Biol. Med*., **157**, No. 3, 316-319 (2014).
5. C. R. Bailey, E. Cordell, S. M. Sobin, and A. Neumeister, *CNS Drugs*, **27**, No. 3, 221-232 (2013).
6. X. Belda, S. Fuentes, R. Nadal, and A. Armario, *Horm. Behav*., **54**, No. 5, 654-661 (2008).
7. J. D. Bremner, J. Licinio, A. Darnell, *et al*., *Am. J. Psychiatry*, **154**, No. 5, 624-629 (1997).
8. N. P. Daskalakis, A. Lehrner, and R. Yehuda, *Endocrinol. Metab. Clin. North Am*., **42**, No. 3, 503-513 (2013).
9. B. S. McEwen, *Dev. Neurobiol*., **72**, No. 6, 878-890 (2012).
10. V. Mironova, E. Rybnikova, and S. G. Pivina, *Acta Physiol. Hung*., **100**, No. 4, 395-410 (2013).
11. S. Yamamoto, S. Morinobu, S. Takei, *et al*., *Depress. Anxiety*, **26**, No. 12, 1110-1117 (2009).
12. R. Yehuda, *Ann. N.Y. Acad. Sci*., **1179**, 56-59 (2009).