Reorganization of Synaptic Inputs to the Hypothalamic Paraventricular Nucleus During Chronic Psychogenic Stress in Rats

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Background: Chronic stress in humans precipitates hyper-reactivity of the hypothalamic-pituitary-adrenocortical (HPA) axis and triggers symptoms associated with certain forms of depression. Reorganization of neuronal networks has been implicated in development of depression, however it remained unknown how chronic exposure to psychogenic challenges affects excitatory and inhibitory inputs to corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus that govern neuroendocrine stress response.

Methods: Rats ($n = 32$) were exposed for 21 days to chronic variable stress and their behavioral (sucrose preference) and hormonal (corticosterone) responses were followed together with electron microscopic stereologic analysis of excitatory and gamma-aminobutyric acid (GABA)-containing, inhibitory synapses on the CRH synthesizing neurons.

Results: Chronic stress in rats resulted in weight loss, anhedonia, and hyperactivity of hypothalamic-pituitary-adrenocortical axis. Following 3 weeks' exposure to variable psychologic stressors the number of synapses has been doubled in the paraventricular nucleus. Asymmetrical excitatory as well as GABAergic inhibitory synaptic contacts were increased on CRH neurons; however, the excitatory/inhibitory input ratio remained constant. In response to chronic stress, we found rearrangement of inhibitory GABA-containing inputs with the increase of contacts on dendrites and decrease at the soma region of CRH neurons.

Conclusions: Significant remodeling of synaptic contacts was found on CRH neurons in response to chronic stress. This morphologic plasticity might be related to the hyperactivity of the HPA axis and to development of stress-related psychopathologies such as depression.

Key Words: Corticotropin-releasing hormone, electronmicroscopic disector, GABA, hypothalamus, rat, synaptic plasticity

Stress-induced activity of the hypothalamic-pituitary-adrenocortical (HPA) axis is governed by corticotropin-releasing hormone (CRH), synthesized by neurosecretory neurons in the parvocellular subdivision of the hypothalamic paraventricular nucleus (PVH) (1,2). Chronic stress is one of the most important environmental factors that precipitate affective disorders in humans. Disregulated HPA axis activity has been implicated in many stress-related mood disorders including depression (3). Patients with major depression have increased drive to CRH neurons, overproduction of corticotropin and cortisol, and enlarged pituitary and adrenal glands (4). They also have impaired negative glucocorticoid feedback as revealed by dexamethasone suppression test (5).

The chronic variable stress (CVS) paradigm is an animal model with high face- and predictive validity for the pathophysiology of depressive disorders. This paradigm in rats produces symptoms such as decreased locomotor activity, social withdrawal, and anhedonia (6,7). Animals after CVS display basal hypersecretion of corticosterone, ACTH and prolactin, adrenal hypertrophy, upregulation of CRH mRNA levels in the stress-related neurosecretory neurons of the PVH and downregulation of glucocorticoid and mineralocorticoid receptors in the hippocampal formation (8).

There are two major mechanisms constraining the HPA axis for which impaired ability to inhibit basal and stress-induced activity might contribute to HPA abnormalities seen in chronic stress and depression: 1) the corticosteroid negative feedback and (9) 2) the neuronal inhibition posed by gamma-aminobutyric acid (GABA)ergic inputs on CRH cells (10–12). Morphology and physiology of GABAergic inputs to the hypothalamic stress-related neurons display plasticity (13). Following adrenalectomy, the number of GABA-containing terminals on CRH neurons is significantly increased and miniature inhibitory postsynaptic currents (mIPSC) frequencies significantly enhanced in parvocellular PVH neurons (14,15). In contrast, in rats subjected to chronic unpredictable stress, decreased spontaneous release of GABA-containing vesicles was detected by recording mIPSCs frequencies in parvocellular neurons in vitro (16).

According to the "network" hypothesis for depression, the disease is associated with changes in neuronal connectivity rather than changes solely in chemical–neurotransmitter balance (17). Severe stress during critical developmental periods has lasting effects on brain morphology. To support this view, reduced hippocampus volume is commonly detected in unipolar depression (18,19) and in adult depressed patients who suffered childhood trauma (20). Morphologic alterations have been revealed in various other brain areas such as the prefrontal cortex, anterior cingulate cortex, amygdala, and bed nucleus of stria terminalis, which are affected by chronic stress and depression (21–25). However, changes in the neuronal network and synaptic connectivity in the stress-related medial parvocellular subdivision of the PVH has not been examined.

In this study, we addressed the hypothesis that reorganization of synaptic inputs to hypothalamic CRH neurons contributes to hyperactivity of HPA axis in rats exposed to chronic variable psychogenic stress.

Methods and Materials

Animals

Adult male Wistar rats (Crl[WI]BR SPF; 300 ± 12g; Toxi-Coop, Budapest, Hungary) were kept under controlled conditions (three per cage, temperature: 21° ± 1°C, humidity 65%, lights on 7 AM–7 PM). Standard rat food and tap water were available ad libitum. All

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animal procedures were carried out in accordance with the European Communities Council Directive (86/609 EEC) and Hungarian Government directive 243/98. Experiments were approved by the Animal Care and Use Committee at the Institute of Experimental Medicine.

Chronic Variable Stress Paradigm

Rats were stressed according to the chronic variable-stressor (CVS) paradigm adopted from Herman *et al.* (8). Stressed animals were housed in the same room as control rats but on different shelves and were transported into a separate room for stress. Animals were exposed to different psychogenic stressors twice daily according to the schedule in Table S1 in Supplement 1. The schedule was repeated three times, so that rats received chronic unpredictable stress for 21 days. Control rats were transported into a separate room and handled twice daily.

Sucrose Preference Test

Sucrose preference test was performed on the last day of CVS. Animals had free choice for 24 hours to drink .8% sucrose or tap water. To prevent possible side preference, the place of the bottles was switched after 12 hours. Animals were not deprived of water before the test. The consumption of fluids was measured by weighing the bottles, and the sucrose preference was calculated with the following formula: sucrose/(sucrose + water) × 100.

Procedures

One day following the last stress session, rats were anesthetized with intraperitoneal injection of a cocktail of 50 mg/kg ketamine (Vetoquinol, Poland), 10 mg/kg Xylazine (Spofa, Czech Republic), and 5 mg/kg Pipolphen (Egis, Hungary), and a blood sample was collected from a tail nick. Plasma corticosterone was measured by radioimmunoassay as described previously (26). Rats were perfused through the ascending aorta with saline at room temperature and then with 4% paraformaldehyde, .5% glutaraldehyde, .2% picric acid in ice cold .1 M phosphate buffer (PB, pH ≈ 7.2). Thymus and adrenal weights were recorded. Brains were postfixed in fixative without glutaraldehyde for 4 hour at 4°C, and then the hypothalamic blocks were sectioned (50 μm) by Vibratome (Technical Product International Inc., St. Louis, Missouri).

To visualize CRH immunoreactive (-ir) cells, half of the animals were anesthetized and received stereotaxic injection of colchicine (75 μg in 15 μL saline; Sigma-Aldrich Ltd, Budapest, Hungary) into the right lateral cerebral ventricle on the day of the last stress session (1 day before perfusion).

Pre- and postembedding procedures were performed as described (27), with minor modifications (14).

Pre-embedding CRH Immunocytochemistry

Sections were cryoprotected and freeze-thawed in an aluminum-foil boat over liquid nitrogen three times to enhance the penetration of antisera. Sections were treated with 1% borohydride and with .3% H_2O_2, incubated in 2% normal goat serum (Vector, Burlingame, California), then placed in rabbit anti-rat CRH antibody (1:4000 in .1 M PB Peninsula Laboratories, Belmont, California) for 48 hours at 4°C. This antibody fails to label after preadsorption with the CRH peptide (28). The antigen was visualized by conventional avidin-biotin-immunoperoxidase protocol (Vector Elite Kit, Vector) using 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) as chromogen. After incubating in 1% OsO_4 (TAAB, Aldermaston, United Kingdom) and dehydration, the sections were contrasted with 2% uranyl acetate (Merck, Hamburg, Germany) and flat-embedded in Durcupan ACM (Fluka, Buchs, Switzerland) between slides and coverslips. Using these sections, the area of CRH-ir cells were identified on bright field images, captured using SPOT-RT camera (Diagnostic Instruments, Sterling Heights, Michigan), and measured by Image-J 1.32 program (National Institutes of Health, Bethesda, Maryland). The volume of the medial dorsal parvocellular area was estimated using Cavalieri's principle (http://www.stereology.info/cavalieri-estimator) (29,30).

Postembedding GABA Immunogold Staining

Serial, ultrathin sections (50 nm) at the dorsal-medial parvocellular subdivision of the PVH were cut on Leica Ultramicrotome (Leica Microsystems, Wetzlar, Germany). The sections were collected on 1% Formvar-coated one-hole nickel grids, then consecutively incubated in 2% periodic acid, 2% sodium periodate, 1% ovalbumin, and anti-GABA antibody No. 9 (courtesy of Dr. Peter Somogyi, Medical Research Council, Anatomical Neuropharmacology, Oxford, United Kingdom) at 1:2000 dilution. The specificity of the antibody was reported previously (27). Control grids were omitted from incubating with the primary antibody. GABA-positive elements were revealed using gold-labeled anti-rabbit serum raised in goat (Auroprobe, GAR 15 nm; Amersham, Buckinghamshire, England). The sections were contrasted with saturated uranyl acetate and lead citrate solution (Ultrastain 2; Leica, Vienna, Austria).

Table 1. Mean Numerical Density Values (× 10^6) of Synapses per mm^3 in the Medial Dorsomedial Parvocellular Paraventricular Nucleus in Control and Stressed Animals

	Total Synapses	Total GABA Synapses	Total Non-GABA Synapses	GABA Synapses on CRH Neurons	Non-GABA Synapses on CRH Neurons	GABA Synapses on Non-CRH Neurons	Non-GABA Synapses on Non-CRH Neurons
Contol Rats (No Colchicine; $n = 4$)	29.4 ± 1.1	16.2 ± 1.4	13.2 ± .5	—	—	—	—
Chronic Stress for 3 Weeks (No Colchicine; $n = 4$)	62.4 ± 3.7[a]	38.1 ± 2.8[a]	24.3 ± 1.2[a]	—	—	—	—
Control Rats with Colchicine ($n = 4$)	33.7 ± 1.9	21.1 ± 1.4	12.7 ± .9	9.1 ± 1.4	3.9 ± 1.1	11.9 ± 1.3	8.7 ± .6
Chronic Stress for 2 Weeks with Colchicine ($n = 3$)	52.3 ± 9.3	34.3 ± 3.3[a]	17.9 ± 6.4	10.9 ± 3.8	6.2 ± 3.8	23.4 ± 2.0[a]	11.7 ± 4.6
Chronic Stress for 3 Weeks with Colchicine ($n = 4$)	66.1 ± 3.5[a]	40.6 ± 3.8[a]	25.6 ± .8[a]	14.9 ± .5[a]	7.1 ± .9	25.7 ± 3.5[a]	18.5 ± 1.2[a]

Data are represented as means ± SEM.
CRH, corticotropin-releasing hormone; GABA, gamma-aminobutyric acid.
[a]Significantly greater than corresponding values in control or control + colchicine treated rats at $p < .05$, Mann-Whitney test.