Cellular Adaptations of Dorsal Raphe Serotonin Neurons Associated with the Development of Active Coping in Response to Social Stress

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Background: Social stress is a risk factor for affective disorders for certain vulnerable individuals. Stress and depression are linked in part through regulation of the dorsal raphe (DR)-serotonin (5-HT) system by the stress-related neuropeptide, corticotropin-releasing factor (CRF). We used a rat social stress model that shows individual differences in coping strategies to determine whether differences in CRF-5-HT interactions underlie individual differences in the vulnerability to social stress.

Methods: Rats were exposed to the resident-intruder model of social stress for 5 days. In vivo single-unit recordings assessed DR-5-HT neuronal responses to CRF and immunoelectron microscopy assessed CRF_1 and CRF_2 cellular localization 24 hours after the last stress.

Results: Rats responded to social stress passively, assuming defeat with short latencies (48%), or actively, with proactive behaviors and longer defeat latencies (LL, 52%). Whereas CRF (30 ng, intra-DR) inhibited 5-HT neuronal activity of control and SL rats, it activated 5-HT neurons of LL rats, an effect that was CRF_2-mediated. Consistent with this, social stress promoted CRF_1 internalization together with CRF_2 recruitment to the plasma membrane of DR neurons selectively in LL rats.

Conclusions: These data suggest that a proactive coping strategy toward social stress is associated with a redistribution of CRF_1 and CRF_2 in DR-5-HT neurons that primes the system to be activated by subsequent stress. The lack of this adaptation in passive coping rats may contribute to their depressive-like phenotype. These studies provide a cellular mechanism for individual differences in stress responses and consequences.

Key Words: Antisauvagine-30, corticotropin-releasing hormone, CRF, receptor internalization, resident-intruder, social defeat

Repeated stress is associated with the development of psychiatric disorders such as depression, anxiety, and drug abuse (1–6). One neuropeptide known to link stress and psychiatric disorders is corticotropin-releasing factor (CRF). In addition to initiating the hypothalamic-pituitary-adrenal axis response to stress, CRF actions in extrahypothalamic regions mediate behavioral, cognitive, and autonomic responses to stress (7–11). Overproduction of CRF as evidenced by increased CRF levels in cerebrospinal fluid, increased CRF expression in paraventricular hypothalamic neurons, and increased CRF-immunoreactivity in the noradrenergic nucleus locus coeruleus (LC), the serotonin (5-HT)-containing dorsal raphe nucleus (DR), and prefrontal cortex have been associated with depressive disorders (12–15). CRF exerts its effects through CRF_1 and CRF_2 receptor subtypes (16,17). Generally, CRF_1 has been associated with anxiogenic and depressive-like responses in animal models (18–21), whereas the consequences of CRF_2 activation are equivocal (22–28). CRF targets monoaminergic systems implicated in affective disorders including the LC (29) and the DR (30). Within the DR, CRF has opposing effects on 5-HT neuronal activity through actions at CRF_1 and CRF_2. At low doses, CRF activates CRF_1, which enhances gamma-aminobutyric acid (GABA)ergic inhibition of DR-5-HT neurons and decreases 5-HT extracellular levels in forebrain and limbic targets (31–34). Higher doses of CRF excite 5-HT neurons through CRF_2 activation and increase extracellular 5-HT in forebrain and limbic targets (31–33,35–39).

Importantly, the dual actions of CRF on the DR-5-HT system are influenced by prior stress exposure. For example, a single exposure to swim stress qualitatively changed the DR-5-HT neuronal responses to CRF from inhibition to excitation (39). This shift was associated with a cellular redistribution of CRF receptors such that CRF_1 became internalized and CRF_2 was recruited to the plasma membrane. This study revealed mechanisms by which prior stress can recruit different receptor subtypes, leading to qualitatively different cellular responses to CRF and ultimately different consequences of subsequent stress.

For humans, a prevalent stressor is social stress, and this has been modeled in rodents through the use of the resident-intruder stress (40,41). We previously identified two phenotypes in the same strain of rats exposed to this stressor (42). One phenotype exhibits a passive coping strategy in response to the stress that is characterized by a short latency to be defeated (SL rats) and develops stress-induced behavioral, neuroendocrine, and cardiovascular end points similar to those associated with depression (18,42). The other phenotype adopts a proactive coping strategy with increased defeat latencies (LL rats) and does not develop the same pathological consequences as that in the SL rats (18,42). To determine the role of CRF-5-HT interactions in these phenotypes, 5-HT neuronal sensitivity to CRF was assessed in rats exposed to repeated social stress through the use of electrophysiological approaches. Additionally, the cellular localizations of CRF_1 and CRF_2 were visualized by means of immunogold labeling and electron microscopy. The results identified a cellular adaptation that occurs selectively in active coping rats that qualitatively

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changes the response of DR-5-HT neurons to CRF and may underlie the resilience of this phenotype.

Methods and Materials

Subjects

Male Sprague Dawley rats (275–300 g) were used as control or social stress "intruder" rats, and male Long-Evans retired breeders (550–850 g) served as residents (Charles River, Wilmington, Massachusetts). Rats were singly housed in a climate-controlled room with a 12-hour light-dark cycle (lights on at 7 AM). Food and water were freely available. Ninety-two rats were used in these studies. Care and use of animals was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee and was in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

Social Stress

The social stressor used in these studies was modified from the resident-intruder model originally developed by Miczek *et al.* (40). Rats were randomly assigned to either a control or social stress exposure for 30 min on 5 consecutive days, identical to that used in Wood *et al.* (18,42). Social stress exposure elicited intruder subordination, termed defeat, and was operationally defined by the intruder exhibiting a supine posture for approximately 3 sec. Once this occurred or 15 min had elapsed, the intruder was placed behind a wire mesh partition in the same cage as the resident, which prevented physical contact between the resident and intruder. The latency to exhibit a supine posture in the presence of the resident aggressor was recorded and averaged over the five exposures. Controls were placed behind the partition in a novel cage for 30 min daily. Rats were returned to their home cage after each session. Twenty-four hours after the fifth exposure to social stress or control manipulation, rats were either subjected to electrophysiological recording or tissue was harvested for electron microscopic or Western blotting studies.

Electrophysiological Recordings in DR

Extracellular single-unit activity from DR-5-HT neurons was recorded in the isoflurane-anesthetized state, as previously described (31,39). Double-barrel glass micropipets were used to record DR neuronal activity and simultaneously microinfuse CRF (30 ng in 30 nL of artificial cerebrospinal fluid) adjacent to the recording electrode, as previously described (31). Spontaneous discharge rate was recorded for 3–5 min immediately preceding the microinfusion and for 5–15 min after CRF infusion. In a separate cohort of rats, the CRF_2 antagonist antisauvagine-30 (ASV; 3 μg in 3 μL) was administered through an intracerebroventricular (i.c.v.) cannula 10 min before CRF. Only one microinjection of CRF was administered to an individual rat. For detailed methods, see Supplement 1.

Histology

At the end of the electrophysiological recording, the recorded neurons were juxtacellularly labeled with neurobiotin (1.5%, Vector Laboratories-SP-1120), as previously described (43,44). Neutral red dye (3 μL, i.c.v.) was injected for later verification of the i.c.v. cannula placement, and the rats were transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Rats remained anesthetized from the beginning of the experiment to termination.

Immunohistochemistry

After perfusion, brains were removed and postfixed for 1 hour at 4°C and stored in 20% sucrose solution containing .1% sodium azide at 4°C. Coronal sections (30 μm) through the DR were cut on a cryostat. Sections were processed to visualize neurobiotin and 5-HT (see Methods in Supplement 1). Only data from juxtacellularly labeled neurons that were 5-HT–immunoreactive were used in the analysis.

Immunoelectron Microscopy

For immunoelectron microscopy, rats were transcardially perfused with 3.8% acrolein and 2% paraformaldehyde in .1 mol/L phosphate buffer 24 hours after the last manipulation. Coronal sections (40 μm) through the DR were cut on the Vibratome and processed for CRF_1 and CRF_2 by means of immunogold detection identical to methods previously described (39,45,46). See details in Supplement 1.

Western Blotting

Brains were flash-frozen in isopentane and stored at –80°C. Coronal sections were cut on the cryostat to the approximate rostral-caudal level of –8.00 mm from the Bregma according to the *Paxinos and Watson Rat Brain Atlas* (47). A 2-mm-deep section of tissue containing the DR was microdissected with the use of a trephine. Samples were homogenized and centrifuged. Protein content was quantified by means of the bicinchoninic acid assay method for each sample. Protein extracts (10 μg) were subjected to SDS-PAGE gel electrophoresis, and proteins were transferred to polyvinylidene fluoride membranes (Immobilon-FL), as previously described (48). CRF_2 has two functional splice variants, α and β (49,50). $CRF_{2\alpha}$ is the major CRF_2 splice variant expressed in rodent brain (51). Therefore, membranes were probed for the full-length CRF_2 receptor ($CRF_{2\alpha}$) with the use of an antibody directed against the transmembrane domain of $CRF_{2\alpha}$ [$CRF_{2\alpha\ (TMD)}$; Santa Cruz, Santa Cruz, California (W-17)]. To examine the possible contribution of a $CRF_{2\alpha}$ splice variant that is not inserted into the plasma membrane ($sCRF_{2\alpha}$), blots were probed with an antibody directed against the unique C-terminus (50) of $sCRF_{2\alpha}$ (Wylie Vale, The Salk Institute, La Jolla, California). Membranes were also probed with an antibody detecting β-arrestin2 (sc-13140). All bands were expressed as a ratio of the corresponding loading controls COX IV (ab14744) or glyceraldehyde 3-phosphate dehydrogenase (G9545; Sigma Chemical, St. Louis, Missouri). Odyssey Infrared Imaging system and software were used to determine the molecular weight and integrated intensity of each band, as previously described (42). Detailed methods are provided in Supplement 1.

Data Analysis

A cluster analysis (JMP 9.0; SAS, Cary, North Carolina) was applied to defeat latencies of all rats exposed to social stress that were used in electrophysiological and protein studies to define phenotypes by defeat latency. Because brains for electron microscopy studies were collected at different times, separate cluster analyses were applied to these. Neuronal discharge rates were determined over 120 sec before the CRF infusion as the mean basal discharge rate. Discharge rate was determined in 30-sec intervals after CRF infusion and expressed as a percentage of the mean basal discharge rate. The mean basal rates were compared between groups by means of a one-way analysis of variance (ANOVA), and the effects of CRF on DR activity over time in different experimental groups were compared by means of a two-way repeated-measures ANOVA with Bonferroni post hoc test.

Quantification of CRF_1 and CRF_2 receptor distribution for the immunoelectron microscopy study was completed in the