Sex Differences in Corticotropin-Releasing Factor Receptor-1 Action Within the Dorsal Raphe Nucleus in Stress Responsivity

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Background: Women are twice as likely as men to suffer from stress-related affective disorders. Corticotropin-releasing factor (CRF) is an important link between stress and mood, in part through its signaling in the serotonergic dorsal raphe (DR). Development of CRF receptor-1 (CRFr1) antagonists has been a focus of numerous clinical trials but has not yet been proven efficacious. We hypothesized that sex differences in CRFr1 modulation of DR circuits might be key determinants in predicting therapeutic responses and affective disorder vulnerability.

Methods: Male and female mice received DR infusions of the CRFr1 antagonist, NBI 35965, or CRF and were evaluated for stress responsivity. Sex differences in indices of neural activation (cFos) and colocalization of CRFr1 throughout the DR were examined. Whole-cell patch-clamp electrophysiology assessed sex differences in serotonin neuron membrane characteristics and responsivity to CRF.

Results: Males showed robust behavioral and hypothalamic-pituitary-adrenal axis responses to DR infusion of NBI 35965 and CRF, whereas females were minimally responsive. Sex differences were also found for both CRF-induced DR cFos and CRFr1 co-localization throughout the DR. Electrophysiologically, female serotonergic neurons showed blunted membrane excitability and divergent inhibitory postsynaptic current responses to CRF application.

Conclusions: These studies demonstrate convincing sex differences in CRFr1 activity in the DR, where blunted female responses to NBI 35965 and CRF suggest unique stress modulation of the DR. These sex differences might underlie affective disorder vulnerability and differential sensitivity to pharmacologic treatments developed to target the CRF system, thereby contributing to a current lack of CRFr1 antagonist efficacy in clinical trials.

Key Words: Corticotropin releasing factor, CRF receptor-1, dorsal raphe nucleus, GABA, parvalbumin, serotonin, sex, stress

Stress-mediated affective disorders such as depression and anxiety show a marked sex disparity, affecting women at nearly twice the rate of men (1,2). Corticotropin-releasing factor (CRF) represents an important link between stress and mood regulation (3–6). Studies have suggested that stress-induced elevations in CRF contribute to neuropsychiatric disease development through excessive activation of its type 1 receptor, corticotropin-releasing factor receptor-1 (CRFr1) (7–15). Consequently, CRFr1 has received considerable attention as a novel pharmaceutical target for the treatment of stress-related affective disorders; GlaxoSmithKline, Pfizer, Neurocrine Biosciences, DuPont/Bristol-Myers Squibb, and others have developed CRFr1 small molecule antagonists toward this end [recently reviewed in Paez-Pereda *et al.* (16)]. However, despite compelling results for antidepressant-like and anxiolytic-like effects of these drugs in pre-clinical studies in rodents and nonhuman primates (17–25), none of the CRFr1 antagonists brought to clinical trial over the past decade have successfully completed a Phase III trial [reviewed in Koob and Zorrilla (26)].

Considerable evidence supports the involvement of CRFr1 in stress modulation of the serotonergic (5-HTergic) dorsal raphe nucleus (DR) in regulation of mood and affect (27–30). Robust sex differences exist across the stress-serotonin system, where females exhibit greater corticosterone and behavioral (anxiogenic) responses to acute selective 5-HT reuptake inhibitor (SSRI) treatment (31–34). A disruption in the ability of CRF to regulate 5-HT circuits during chronic stress is implicated in affective disorder pathophysiology (35–38). Thus, we hypothesized that sex differences in CRFr1 activation within the DR might contribute, in part, to an increased female predisposition to stress-induced affective disorders and might underlie disparities between predicted outcomes from preclinical studies and those in clinical trials for CRFr1 antagonists.

Methods and Materials

Subjects

A total of 268 adult male and female littermate mice were used for all experiments. Mice were maintained under a 12-hour light/dark cycle with ad libitum access to food and water. For behavioral experiments and electrophysiological studies, C57Bl/6:129S/J F1 hybrid mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) or bred in house. For CRFr1 colocalization studies, mice with fluorescent-labeled CRFr1 containing neurons were generated as previously described (39). Mice received implantation between ages 7 and 8 weeks, were allowed to recover for at least 1 week, and were behaviorally

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tested in age-matched cohorts at 8–20 weeks of age. Mice were singly housed after cannulation to prevent disturbance of the cannulae. For electrophysiological experiments, slices were obtained from mice at 9–13 weeks of age. Mice were individually housed for 7–12 days before recording, to mimic the housing conditions of behavioral studies. All studies were conducted in accordance with experimental protocols approved by the University of Pennsylvania Institutional Animal Use and Care Committee and, where applicable, by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Stereotaxic Surgery and Placement Verification

Mice were anesthetized with isofluorane and implanted with a 26-gauge guide cannula (Plastics One, Roanoke, Virginia) with a stereotaxic instrument (Kopf, Tujunga, California) positioned 1 mm from the DR with the following coordinates (from brain surface): AP −4.36 mm, ML + 1.5 mm, DV −2.0 mm, angled 26 degrees (40). At the end of each study, mice were transcardially perfused, and cannula placement was verified on the basis of the termination point of the injector as estimated from the location of scar tissue in 50-μm sections through the DR. Mice with incorrect cannulae placement were dropped from the statistical analysis. Group sizes reported represent the final group size after subjects with incorrect placements were omitted.

Drugs and Microinfusion

All drugs were reconstituted in distilled water, aliquotted, and frozen until the day of use. Fresh aliquots were dissolved in artificial cerebrospinal fluid (ACSF) (Tocris, Bristol, United Kingdom) immediately before behavioral testing. NBI 35965 (Tocris), a highly selective CRFr1 antagonist, was used at .44 ng, 1000 times the Ki (41). Ovine CRF (Sigma, St. Louis, Missouri) was used because of its higher affinity for CRFr1 (42). Doses (1 ng and 50 ng) were selected on the basis of previous studies of DR infusion of this peptide to preferentially target CRFr1 (43,44). Drug in .25 μL ACSF was infused over 1 min through a microinjector attached to polyethylene tubing connected to a 10 μL Hamilton syringe on an infusion pump (KD Scientific, Holliston, Massachusetts). Drug or ACSF (.50 μL) was perfused through the microinjector to ensure patency between injections.

Hypothalamic-Pituitary-Adrenal Axis Assessment

Testing was performed during a 4-hour period beginning 1 hour after lights-on. Tail blood (10 μL) was collected immediately before DR infusion and at 30, 45, 60, and 120 min post injection. Between the 30- and 45-min collections, mice in the NBI 35965 study were restrained in a 50-mL conical tube with a 5-mm air hole. Corticosterone was measured as described previously (45).

Behavioral Testing

The tail suspension test (TST) and light-dark box (LD) were performed on separate cohorts of mice 30 min after drug or ACSF infusion. Methods were similar to those described previously (36,46) (details in Supplement 1).

cFos Immunohistochemistry

To assess CRF-induced neuronal activation in the DR, double labeling immunohistochemistry for cFos and tryptophan hydroxylase (TPH) was performed on DR sections from mice sacrificed 90 min after CRF or ACSF infusion. Methods were similar to those described previously (47,48) (details in Supplement 1).

Gene Expression Analysis

Brains were collected from experimentally naive adult male and female mice. Female brains were collected in diestrus. Gene expression of CRFr1, CRFr2, CRF binding protein, TPH2, and γ-aminobutyric acid (GABA) receptor subunits alpha-1, alpha-2, delta, and gamma-2 were determined by quantitative Taqman real-time polymerase chain reaction as previously described (49,50) (details in Supplement 1).

Immunofluorescence and CRFr1 Localization

Dual immunofluorescence was performed to detect enhanced green fluorescent protein (GFP) and TPH or parvalbumin in DR sections from paraformaldehyde-fixed male and female CRFr1-GFP mice (51) (details in Supplement 1).

Electrophysiology

A modified procedure based on the method of Challis *et al.* (52) was used (details in Supplement 1).

Data Analysis and Statistics

Total corticosterone was analyzed by multivariate analysis of variance (ANOVA) (drug × time). Behavioral measures were analyzed by two-way ANOVA (sex × drug). Subsequent one-way analyses were performed on data within sex, with Dunnett's test used to identify significant post hoc comparisons. Student *t* test was used to compare gene expression between males and females. To determine CRFr1 counts, a generalized linear mixed model was employed to analyze GFP count × sex × subregion with a Poisson distribution. Assuming a binomial distribution, further analyses were made to predict the likelihood that a given GFP-immunoreactive (-ir) cell co-expressed parvalbumin or TPH. Data are reported as estimated effect size ± 95% confidence intervals. Significance was determined as $p < .05$, with 95% confidence intervals not bounding zero. Statistics were performed in R software. For electrophysiological studies, results of 5-HTergic neuron response to CRF were compared between males and females via two-way repeated measures ANOVA and post hoc Tukey tests employing sex and drug (6,7-dinitroquinoxaline-2, 3-dione [DNQX] vs. DNQX + CRF) as the independent variables. Statistics were performed with JMP8 (SAS, Cary, North Carolina) software; data are reported as mean ± SEM.

Results

DR Infusions of NBI 35965 or CRF Preferentially Alter Male Corticosterone Production

The 5-HT output from the DR has modulatory activity on the hypothalamic-pituitary-adrenal (HPA) axis (34,35). The CRF regulation of DR neurons could therefore influence HPA responsiveness. Thus, we assessed the effect of CRFr1 antagonism within the DR on the corticosterone response to restraint stress (Figure 1). The NBI significantly blunted corticosterone levels in males ($F_{1,9} = 7.085$, $p = .026$). The effect of NBI was manifested as a reduction in the rise time from 0 to 30 min before restraint ($t_9 = 3.191$, $p = .011$) and a reduction in total corticosterone produced throughout the course of the experiment (area under the curve) ($t_9 = 2.794$, $p = .021$). In females, NBI did not significantly impact corticosterone production ($F_{1,10} = .1180$, $p = .7383$). We next tested the effect of CRF infusion on the HPA axis. In males, CRF significantly increased corticosterone ($F_{1,17} = 5.926$, $p = .026$). In females, CRF did not significantly affect corticosterone ($F_{1,18} = 1.28$, $p = .27$).