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

Alexis R. Howerton, Alison V. Roland, Jessica M. Fluharty, Anikó Marshall, Alon Chen, Derek Daniels, Sheryl G. Beck, and Tracy L. Bale

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 serotoninergic 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 serotoninergic 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 pharmacological 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 serotoninergic (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/6j: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...
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.

Stereoaxic Surgery and Placement Verification
Mice were anesthetized with isoflurane 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 CRF1 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 CRF1 (42). Doses (1 ng and 50 ng) were selected on the basis of previous studies of DR infusion of this peptide to preferentially target CRF1 (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 CRF1 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’s 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 modular 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 CRF1 antagonism within the DR on the corticosterone response to restraint stress (Figure 1). The NBI significantly blunted corticosterone levels in males (F1,9 = 7.085, p = .026). The effect of NBI was manifested as a reduction in total corticosterone produced throughout the course of the experiment (area under the curve) (t9 = 2.794, p = .021). In females, NBI did not significantly impact corticosterone production (F1,10 = .1180, p = .7383). We next tested the effect of CRF infusion on the HPA axis. In males, CRF significantly increased corticosterone (F1,17 = 5.926, p = .026). In females, CRF did not significantly affect corticosterone (F1,18 = 1.28, p = .27).
Dorsal raphe infusion of the corticotropin-releasing factor receptor-1 (CRFr1) small molecule antagonist NBI 35965 (A–D) or 50 ng CRF (E–H) had male-specific effects on hypothalamic-pituitary-adrenal responsiveness. (A,C) Time course of corticosterone response to a 15-min restraint (indicated by shaded region) in males (A) and females (C). (B,D) Area under the curve analysis demonstrated a significant reduction of corticosterone output by NBI 35965 in males (B) but not in females (D). (E–H) Corticosterone response to infusion of 50 ng CRF in males (E,F) and females (G,H). Area under the curve analysis demonstrated that CRF enhancement of corticosterone release was specific to males (F). Data are presented as mean values ± SEM ($n = 8$). *$p < .05$ in comparison with artificial cerebrospinal fluid (ACSF).

**Male-Specific Effects of DR Infusion of NBI 35965 and CRF on Stress Coping and Anxiety-Like Behaviors**

To assess the role of DR CRFr1 in modulating stress coping behavior of male and female mice, the TST was performed 30 min after infusion of NBI 35965 or vehicle (ACSF) (Figure 2). We detected a significant interaction of sex and NBI on latency to become immobile ($F_{1,37} = 6.654, p = .014$), where NBI increased latency in males ($p = .015$) but not in females ($p = .37$). There was a trend toward an interaction effect of NBI on immobility in the TST, where NBI reduced immobility in males and increased immobility in females, although this effect failed to reach statistical significance ($F_{1,40} = 2.651, p = .11$).

The observed sex difference in response to the CRFr1 antagonist suggested potential sex differences in the response to CRF. To address this possibility, we next assessed the effect of two doses (1 ng, 50 ng) of CRF infusion on behavior in the TST. The 1-ng dose of CRF was ineffective to change immobility or latency to become immobile on either test, suggesting ineffective local concentrations were achieved. The 50-ng dose of CRF decreased immobility ($F_{2,26} = 3.467, p = .046$) and increased latency to become immobile ($F_{2,26} = 8.684, p = .001$) in males and was without effect in females.

To assess anxiety-like behavior, mice were tested in the LD. The NBI had no effect in either sex on any parameter. However, as with behavioral outcomes in the TST, where 1-ng dose CRF was without effect, 50-ng dose CRF had sex-specific effects on behavior, increasing latency to exit the light compartment ($F_{2,26} = 5.313, p = .011$), and total time spent in the light compartment ($F_{2,26} = 5.024, p = .014$) in males but not females. In males, 50 ng CRF also reduced the number of transitions between compartments ($F_{2,26} = 4.915, p = .016$) but did not affect distance traveled in the light (normalized to time spent in the light), indicating that the animals did not freeze while in the light compartment.

**DR Infusion of CRF Increases cFos in Males But Decreases cFos in Females**

To determine whether sex differences in behavioral responsiveness to CRF in the DR were associated with differential patterns of neuronal activation, we next assessed cFos immunoreactivity after infusion of CRF or ACSF (Figure 3, Table 1). In males, CRF increased the number of cFos-positive cells across the DR ($F_{1,6} = 14.79, p < .001$); whereas in females, CRF reduced the number of cFos-positive cells ($F_{1,56} = 7.563, p = .008$). Subregion analysis indicated that this interaction was present in dorsomedial ($F_{1,17} = 6.216, p = .023$), ventromedial ($F_{1,19} = 6.319, p = .029$), and lateral wing subregions of the DR (rostal, $F_{1,24} = 7.300, p = .013$; caudal, $F_{1,21} = 7.359, p = .013$). The CRF had a similar effect of increasing cFos-positive cells in both male and female rostral DR ($F_{1,21} = 12.38, p = .002$), and no significant main effects were found in the caudal DR.

**CRF1 Is Expressed Differentially Throughout Subdivisions of the DR in Males and Females**

We next assessed whether the observed sex differences in responsiveness to the CRF1 antagonist or CRF were due to differences in transcript levels of genes relevant to CRF and 5-HT signaling. We quantified the relative expression of CRF1, CRF2, and CRF-binding protein messenger RNA (mRNA) in DR micro-punches from experimentally naive male and female mice (Figure 4A). We observed no difference in the mRNA levels of any of these relevant transcripts. Because we predict that some of our observed behavioral and physiological differences might be GABA-mediated, we also quantified the relative expression of GABA receptor subunits alpha-1, alpha-2, delta, and gamma-2, which play important roles in receptor kinetics. We observed no sex difference in mRNA in any of these receptor subunits. As has been previously described, we detected differences in TPH2, with females expressing 1.62-fold higher levels relative to males ($t_{1,8} = 2.652, p = .029$) (53–55).
Figure 2. Males and females show divergent behavioral responses to corticotropin-releasing factor receptor-1 (CRFr1) antagonism (A–D) or CRF (E–H) infusion into the dorsal raphe. (A,B) Effects of CRFr1 antagonist NBI 35965 on total immobile time (A) and latency to first bout of immobility (B) in the tail suspension test (TST). Males showed a greater latency to immobility. (C,D) CRFr1 antagonism had no effect in the light-dark box (LD) on measures of total time spent in the light compartment (C) or latency to first exit from the light compartment (D). (E–H) Behavioral effects of 1-ng and 50-ng corticotropin-releasing factor (CRF) infusion demonstrated no effect of the 1-ng dose but male-specific effects of the 50-ng dose. The 50-ng CRF infusion reduced immobile time (E) and increased latency to immobility (F) in males in the TST and increased both total light time (G) and latency to exit the light (H) in males in the LD. Data are presented as mean values ± SEM (n = 8), *p < .05 in comparison with artificial cerebrospinal fluid (ACSF).

After finding no differences in CRFr1 gene expression, we hypothesized that sex differences in responsivity to CRFr1 antagonist or CRF might be due to differences in the neurotransmitter cell type expressing CRFr1. Because current available antibodies are unable to distinguish between CRFr1 and CRFr2, we used a CRFr1-GFP transgenic mouse in which GFP is transcribed under the control of the CRFr1 promoter to identify CRFr1 positive neurons in the DR (Figure 4b) (39). Sections throughout the DR from these mice were dual labeled for either GFP and parvalbumin to identify putative GABAergic neurons expressing CRFr1 or GFP and TPH to identify serotonergic neurons expressing CRFr1. In accordance with our CRFr1 mRNA data, there were no sex differences in overall number of GFP-ir cells (−.542 ± −1.59, +.053; p = .0796). However, we observed a sex difference in co-localization of GFP-ir cells throughout regions of the DR. In females, the probability that a given GFP-ir cell co-expressed parvalbumin was lower than in males (−2.535 ± −4.819, −.259; p = .0291). The probability that a given GFP-ir cell co-expressed TPH was <.25% in all subdivisions, regardless of sex, except within the rDR, where males displayed significantly more co-localization with TPH than females (−2.16 ± −3.736, −.583; p = .007).

5-HT Neurons in Females Demonstrate Reduced Excitability and a Blunted Response to CRF

To investigate physiological differences in DR 5-HT neurons of males and females, whole-cell electrophysiological recordings were conducted with current-clamp and voltage-clamp techniques (Figure 5, Table 2). Data from a total of 21 neurons, with current-clamp recordings, were analyzed (9 from 6 male mice, 12 from 7 female mice). Cellular characteristics that were measured, included resting membrane potential, resistance, time constant (tau), after hyperpolarization, and action potential amplitude,
regulation of DR circuits are a key determinant in affective disorder vulnerability and might be important in predicting therapeutic outcomes. In our studies, male and female mice received an infusion of the CRFr1 small molecule antagonist, NBI 35965, or one of two doses of CRF directly into the DR and were evaluated for changes in physiological and behavioral stress responsiveness. The NBI 35965 in the DR significantly blunted corticosterone levels in response to a restraint stress only in males. Similarly, CRF infused into the DR in the absence of restraint significantly elevated corticosterone production above the levels induced by intracranial injection only in males (34,59). The 5-HT system is a known activator of the HPA axis, where selective SSRIs and 5-HT agonists increase corticosterone production during and independent of stress (60). Although direct innervation of the paraventricular nucleus (PVN) has been reported (61–63), 5-HTergic fibers from the DR also heavily innervate the GABAergic neurons of the PVN-surround (64). Therefore, CRF-mediated changes in 5-HT output could modulate this axis through a disinhibition of medial parvocellular neurons. We have previously demonstrated sex differences in this pathway, with females showing a greater corticosterone response to peripheral SSRI administration compared with males (34,47). Thus, a male-specific response to administration of NBI 35965 and CRF directly into the DR suggests a unique sex-specific mechanism upstream of the PVN, including potential differences in CRFr1 co-localization or signaling within the DR.

In our assessment of behavioral stress coping strategies, including the TST, only males again showed a significant effect of NBI 35965 to increase the latency to immobility and of CRF infusion to reduce the immobile time. Although NBI 35965 infusion produced no significant changes in male or female mice in the LD, CRF at the higher dose again increased time spent in the light and escape latency only in males. These findings are consistent with behavioral effects reported in previous studies for systemically administered CRFr1 antagonists and CRFr1 gene deletion, implicating the DR as a key brain region mediating these outcomes (9,10). Interestingly, in our current studies, both CRF and NBI 35965 infusions produced similar effects in the TST.

### Table 1. Dorsal Raphe Nucleus cFos Response

<table>
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<tr>
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<th>ACSF</th>
<th>CRF (50 ng)</th>
<th>Delta (CRF–ACSF)</th>
<th>Sex Effect</th>
<th>CRF Effect</th>
<th>Interaction</th>
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<tr>
<td>Male (6,7)</td>
<td>41.333 ± 7.923</td>
<td>83.857 ± 14.825</td>
<td>42.524</td>
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<td>.6555</td>
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<td>Female (6,6)</td>
<td>29.883 ± 6.161</td>
<td>62.667 ± 9.535</td>
<td>32.784</td>
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<tr>
<td>Male (6,6)</td>
<td>86.833 ± 22.237</td>
<td>151.167 ± 26.734</td>
<td>64.334</td>
<td>.4301</td>
<td>.713</td>
<td>.0233</td>
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<td>Female (4,5)</td>
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<td>73.200 ± 17.971</td>
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<tr>
<td>Male (6,6)</td>
<td>42.667 ± 10.899</td>
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<td>.2026</td>
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<td>52.333 ± 9.175</td>
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<tr>
<td>Male (5,7)</td>
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<tr>
<td>Male (7,7)</td>
<td>212.429 ± 24.094</td>
<td>250.143 ± 36.476</td>
<td>37.714</td>
<td>.1836</td>
<td>.1472</td>
<td>.0125</td>
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<tr>
<td>Female (6,8)</td>
<td>254.167 ± 38.640</td>
<td>122.625 ± 20.671</td>
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<tr>
<td>Male (7,7)</td>
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<td>216.714 ± 36.973</td>
<td>92</td>
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<td>Female (4,7)</td>
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<td>119.143 ± 25.842</td>
<td>−85.607</td>
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Sample sizes vary because some sections were excluded from data analysis due to poor tissue quality. ACSF, artificial cerebrospinal fluid; cLW, caudal lateral wing; CRF, corticotropin-releasing factor; rLW, rostral lateral wing.

**Discussion**

Stress-mediated affective disorders display significant sex differences in incidence and treatment efficacy (1,56). The CRFr1 is a key mediator of neuroendocrine and behavioral stress responses, in part through signaling in the 5-HTergic DR (27–30). Because there are known sex differences in the DR, dysregulation of 5-HTergic signaling might contribute to increased disease risk in females (57,58). Development of CRFr1 small molecule antagonists has been a major focus in clinical trials for more than a decade, but these compounds have been largely unsuccessful. Although preclinical studies have predominantly been conducted in males, the majority of clinical trials have either focused on female patient populations or been underpowered to evaluate gender differences in study outcomes (Table 3). Therefore, we hypothesized that sex differences in CRFr1

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It might be that interactions with CRFr2, which is also found in the DR, and/or alternate DR projections might account for differences in this behavior (36,65,66). Because the DR has a heterogeneous cell population involved in the complex regulation of 5-HT neurotransmission throughout the brain, the localization of CRF receptors on different cell types and their recruitment likely determines specificity in 5-HT release (27,28).

Similar paradoxical findings have been reported for other CNS receptor systems involved in complex behaviors (67,68). In support of our hypothesis, these data demonstrate striking sex differences in stress responses to DR infusion of a CRFr1 antagonist or CRF.

To test whether sex differences in neuronal activation by CRF might underlie sex differences we detected in physiological and behavioral stress responses, we analyzed numbers of cFos positive cells after infusion of the behaviorally effective 50-ng dose of CRF into the DR. No significant sex differences in basal cFos staining after vehicle infusion were detected, suggesting similar basal cellular activation in the DR. However, CRF infusion into the DR resulted in a dramatic increase in the number of cFos positive cells in males and a paradoxical reduction in females. This lack of an appreciable effect of CRF infusion in females supports the specificity of CRF administration to the DR with minimal diffusion into the cerebral aqueduct, because the latter would be
responsive GABAergic neurons in females could account for the dendrites (27,72,73). Thus, differences in the number of CRF

Baseline

regions, where CRF evidence supports a dense CRF innervation of these DR sub-

trollable stress and anxiety(28,70,71). Immunohistochemical

These sex differences were most apparent in the dorsomedial and

 DR might be due to differences in CRFr1 localization on

within the DR might be due to differences in CRFr1 localization on functionally distinct neuronal populations, we used a CRFr1-
driven GFP transgenic reporter mouse line to quantify sex

differences in co-expression of CRFr1 (39). Because the available

antibodies for the CRF receptors are known to be of poor quality

expected to augment corticosterone irrespective of sex (34,69).

These sex differences were most apparent in the dorsomedial and lateral wings of the DR, regions previously implicated in uncontrol-
trollable stress and anxiety (28,70,71). Immunohistochemical
evidence supports a dense CRF innervation of these DR sub-

regions, where CRF fibers primarily contact GABA-containing
dendrites (27,72,73). Thus, differences in the number of CRF-
responsible GABAergic neurons in females could account for the observed reduction in activation detected in these mice.

Table 2. Cell Characteristics

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<th>Active Cell Characteristics</th>
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<td>DNQX</td>
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<td>CRF</td>
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<td></td>
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<tr>
<td>Delta (CRF–DNQX)</td>
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AHP, after hyperpolarization; AP, action potential; AUC, area under the curve; CRF, corticotropin-releasing factor; DNQX, 6,7-dinitroquinoxaline-2,3-
dione.

Significant sex differences on the basis of t test.

Figure 5. Males and females exhibit differences in baseline characteristics as well as divergent responses to bath applied corticotropin-releasing factor (CRF). (A) Representative inhibitory postsynaptic current trace. (B,C) Scaled inhibitory postsynaptic currents averaged from a single male (B) and female (C) illustrating the effect of DNQX and CRF on half-width. Insets (B’,C’) illustrate sex difference in effect of CRF on rise time. (D–F) CRF increases rise (D), half-width (E), and decay time (F) in males and decreases these measures in females. (G) Males exhibit increased excitability compared with females to a series of current injections. Values represent the difference in response of CRF–DNQX (n = 7–9). *p < .05.
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<tr>
<td><strong>NBI-34041</strong></td>
<td>High-affinity CRF1 receptor antagonist NBI-34041: preclinical and clinical data suggest safety and efficacy in attenuating elevated stress response</td>
<td>Elevated stress response</td>
<td>Male</td>
<td>24 (24/0)</td>
<td>Phase 2a</td>
<td>Completed</td>
<td>Success</td>
<td>Ising et al. (2007) (83)</td>
</tr>
<tr>
<td><strong>BMS-562086 (Pexacerfont)</strong></td>
<td>Multi-site, double-blind, placebo-controlled study in major depression</td>
<td>Depression</td>
<td>Female</td>
<td>271 (0/271)</td>
<td>Phase 1/2</td>
<td>Completed</td>
<td>Undisclosed</td>
<td>NCT00135421</td>
</tr>
<tr>
<td><strong>BMS-562086 (Pexacerfont)</strong></td>
<td>Multi-site, double-blind, placebo-controlled study in generalized anxiety disorder</td>
<td>Anxiety</td>
<td>Female</td>
<td>260 (0/260)</td>
<td>Phase 2/3</td>
<td>Completed</td>
<td>Fail</td>
<td>NCT00481325</td>
</tr>
<tr>
<td><strong>GSK561679 (Verucerfont)</strong></td>
<td>Multi-site, double-blind, placebo-controlled study in major depression</td>
<td>Depression</td>
<td>Female</td>
<td>150 (0/150)</td>
<td>Phase 2</td>
<td>Completed</td>
<td>Undisclosed</td>
<td>NCT00733980</td>
</tr>
<tr>
<td><strong>GSK561679 (Verucerfont)</strong></td>
<td>Effects of CRH1 antagonism on stress-induced craving in alcoholic women with high anxiety</td>
<td>Anxiety</td>
<td>Female</td>
<td>37 (0/37)</td>
<td>Phase 2</td>
<td>Suspended</td>
<td>Undisclosed</td>
<td>NCT01187511</td>
</tr>
<tr>
<td><strong>NBI-30775 (R121919)</strong></td>
<td>Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated</td>
<td>Depression</td>
<td>Both</td>
<td>24 (13/11)</td>
<td>Phase 2a</td>
<td>Terminated</td>
<td>Elevated liver enzymes</td>
<td>Zobel et al. (2000) (84)</td>
</tr>
<tr>
<td><strong>CP-316,311</strong></td>
<td>Multi-site, double-blind, placebo controlled study in major depression</td>
<td>Depression</td>
<td>Both</td>
<td>28 (17/11)</td>
<td>Phase 2</td>
<td>Terminated</td>
<td>Fail</td>
<td>NCT00143091</td>
</tr>
<tr>
<td><strong>GW876008 (Emicerfont)</strong></td>
<td>A 12-week flexible dose study of GW876008, placebo, and active control (paroxetine) in the treatment of SocAD</td>
<td>Anxiety</td>
<td>Both</td>
<td>280 (undisclosed)</td>
<td>Phase 2</td>
<td>Completed</td>
<td>Undisclosed</td>
<td>NCT00397722</td>
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<tr>
<td><strong>ONO-2333Ms</strong></td>
<td>Placebo-controlled study of ONO-2333Ms in patients with recurrent MDD</td>
<td>Depression</td>
<td>Both</td>
<td>278 (undisclosed)</td>
<td>Phase 2</td>
<td>Completed</td>
<td>Undisclosed</td>
<td>NCT00514865</td>
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<tr>
<td><strong>SSR125543A</strong></td>
<td>A trial evaluating the efficacy and tolerability of SSR125543 in outpatients with MDD</td>
<td>Depression</td>
<td>Both</td>
<td>580 (undisclosed)</td>
<td>Phase 2</td>
<td>Completed</td>
<td>Undisclosed</td>
<td>NCT01034995</td>
</tr>
</tbody>
</table>

CRH1, corticotropin-releasing hormone receptor 1; F, female; M, male; MDD, major depressive disorder; SocAD, social anxiety disorder.

Sponsor: Neurocrine/GlaxoSmithKline.

Sponsor: Bristol-Myers-Squibb.

Sponsor: GlaxoSmithKline.

Sponsor: Neurocrine/Janssen.

Sponsor: Pfizer.

Sponsor: Ono Pharma.

Sponsor: Sanofi-Aventis.
and lack sufficient specificity, this reporter mouse provided an excellent tool to identify CRFr1-positive neurons in the DR. We quantified sex differences in co-expression of GFP with TPH, a marker of 5-HTergic neurons, or with parvalbumin, a marker of a subset of GABAergic neurons. Parvalbumin was used to mark GABAergic neurons, because 87% of GAD67–ir neurons in the DR co-express parvalbumin (75). These neurons might display some functional differences compared with the broader population of GABAergic neurons but were selected on the basis of reliable somal immunoreactivity for co-expression analysis with CRFr1 (76). Overall numbers of CRFr1 neurons did not differ between males and females, as indicated by similar numbers of GFP-positive neurons in the DR. This was also confirmed by similar expression levels of CRFr1 mRNA in the DR of males and females. As expected, few GFP-positive neurons were co-expressed with TPH, consistent with previous reports that demonstrate CRF primarily acts on GABAergic neurons and that CRFr1 shows little expression overlap with 5-HT neurons (77). Surprisingly, females had reduced GFP co-labeling with parvalbumin compared with males. This outcome supports a revised model; rather than females demonstrating greater CRFr1-mediated GABA tone, sex differences in CRF-induced neuronal activation might be due to differences in CRFr1 intracellular signaling, trafficking, or receptor kinetics (78). Such sex differences in CRF signaling have recently been demonstrated in the locus coeruleus (44,79). Alternatively, CRF might be activating a population of parvalbumin-negative GABA neurons in the female DR.

On the basis of our observed sex differences in CRFr1 co-expression, we hypothesized that DR 5-HT neurons in males and females might receive differential GABA input in response to CRF. To examine this, we used whole-cell electrophysiological recordings to measure GABAergic IPSCs in 5-HT neurons before and after application of CRF. We observed a striking sex difference in CRF responsivity, where CRF increased IPSC decay time in males but decreased decay time in females. The IPSC decay time can correlate with the number of activated axonal inputs during the IPSC, suggesting sex differences in presynaptic GABAergic input onto 5-HT neurons (80). This is consistent with the differential co-expression of CRFr1 on parvalbumin neurons between males and females. Furthermore, the reduced IPSC half-width in response to CRF exhibited by females also supports this sex difference in the number of GABAergic release sites onto 5-HT neurons. These differences in IPSC kinetics constitute a significant functional difference between males and females that could alter 5-HT neuron excitability and neurotransmission and thereby influence stress physiological and behavioral measures.

In gathering basal characteristics before assessing sex differences in 5-HTergic neuron responses to CRF, we uncovered an unexpected sex difference in 5-HT neuronal excitability. With whole-cell patch clamp recordings from 5-HT neurons from male and female dorsomedial DR slices, we found reduced neuronal excitability in females in response to a series of current injections. In addition to sex differences in CRFr1 localization and GABAergic responses to CRF, this suggests that 5-HT neurons in females require a greater depolarizing stimulus to generate neuronal firing and subsequent 5-HT release, even at baseline. Compared with males, this might translate to 5-HTergic hypofunction in females, an underlying risk factor for the development of affective disorders during stress exposure (81,82).

Overall, these studies revealed intriguing sex differences in behavioral and physiological effects of CRFr1 antagonist and CRF in the DR, which were mechanistically associated with sex differences in receptor co-expression and divergent electrophysiological responses to CRF in 5-HTergic neurons. The blunted response of females points to a potential explanation for the lack of efficacy in CRFr1 antagonists in clinical trials, which have focused primarily on female participants due to their increased disease prevalence (Table 3). These findings support the importance of identifying sex differences in central stress pathways to understand the heightened predisposition of females toward these disorders and in identifying sex-appropriate and potentially sex-specific pharmacological treatments.

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