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# Corticotropin-Releasing Factor Inhibits Maternal Aggression in Mice

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Lactating females that fiercely protect offspring exhibit decreased fear and anxiety. The authors tested whether decreased corticotropin-releasing factor (CRF), an activator of fear and anxiety, plays a functional role in maternal aggression. Intracerebroventricular (icv) injections of CRF (1.0 and 0.2 $\mu$g, but not 0.02 $\mu$g) significantly inhibited maternal aggression but not other maternal behaviors. The CRF antagonist D-Phe-$CRF_{12-41}$ had no effect. Maternal aggression and icv CRF (0.2 $\mu$g) induced Fos in 11 of the same regions, including the lateral and medial septum, the bed nucleus of the stria terminalis, the medial and central amygdala, the periaqueductal gray, the dorsal raphe, and the locus coeruleus. These findings suggest that decreased CRF is necessary for maternal aggression and may act by altering brain activity in response to an intruder.

Maternal aggression toward intruders is a highly adaptive behavior that plays a critical role in the protection of offspring. Female rodents rapidly respond to a threat to offspring by attacking an intruder. The attacking females appear to be fearless toward the intruders. At the time of high aggression, lactating females exhibit decreased indices of fear and anxiety, relative to virgin females, in several experimental paradigms, including acoustic startle, open field, elevated plus-maze, defensive burying, punished drinking, and light–dark choice test (for review, see Lonstein & Gammie, 2002). Further, an inverse relationship between fear and anxiety and maternal aggression is found among individuals and strains of mice (Maestripieri & D'Amato, 1991; Parmigiani, Palanza, Rogers, & Ferrari, 1999). Therefore, it is possible that decreased fear and anxiety facilitates maternal aggression by allowing dams to be less hesitant to attack a potentially threatening and normally fear-evoking stimulus.

Corticotropin-releasing factor (CRF) is a neuropeptide that can act either centrally [released within the central nervous system (CNS)] or peripherally (described below). CRF released within the CNS mediates stress-induced behaviors, including elevating indices of fear and anxiety in the same test paradigms described above (for reviews, see Koob & Heinrichs, 1999; Smagin, Heinrichs, & Dunn, 2001). CRF messenger RNA (mRNA) is decreased in the central amygdala (CeAMY) and paraventricular nucleus (PVN) in late (Postpartum Days 7–15) lactating relative to virgin female rats (Deschamps, Woodside, & Walker, 2003; Walker, Tilders, & Burlet, 2001; Windle, Brady, et al., 1997). Further, adrenergic input to the PVN is decreased during lactation (Toufexis et al., 1998), and the CNS of lactating rats is less responsive to intracerebroventricular (icv) CRF relative to the CNS of virgin females (da Costa, Kampa, Windle, Ingram, & Lightman, 1997). Thus, decreased synthesis of CRF (later in lactation), decreased activation of CRF release, or decreased responsiveness of the CNS to CRF could contribute to the decreased fear and anxiety that occurs during lactation.

In terms of peripheral action, CRF plays a critical role in the hypothalamic–pituitary–adrenal axis. CRF from the PVN acts on the anterior pituitary to trigger the peripheral release of adrenocorticotropin-releasing hormone (ACTH) that in turn causes the release of corticosterone from the adrenal glands (Vale, Spiess, Rivier, & Rivier, 1981). In response to many stressors, both ACTH and corticosterone rise in lactating females, but to lower levels as compared with those of virgin females (da Costa, Wood, Ingram, & Lightman, 1996; Neumann, 2001; Neumann, Torner, & Wigger, 2000; Stern, Erskine, & Levine, 1973; Walker, Trottier, Rochford, & Lavallee, 1995; Windle, Wood, et al., 1997; but see Deschamps et al., 2003). Thus, decreases in CRF synthesis or release (from the PVN) may contribute to the stress hyporesponse in lactating females.

Application of stressors (which elevates central CRF release) decreases maternal aggression in mice (Maestripieri & D'Amato, 1991; Pardon, Gerardin, Joubert, Perez-Diaz, & Cohen-Salmon, 2000), suggesting that CRF needs to be low for maternal aggression to be expressed. Previously, an inhibitory role for CRF in the control of some maternal behaviors was identified in hormone-treated virgin female rats (Pedersen, Caldwell, McGuire, & Evans, 1991), but maternal aggression was not examined. Recently, a quantitative trait loci (QTL) mapping study in mice identified 12 QTL that affect maternal behavior, including maternal aggression (Peripato et al., 2002). One of these QTL was located within a chromosomal region that contains the gene for CRF, and another QTL was located in the vicinity of the gene for the primary receptor of CRF. Although these results are intriguing, further work is needed to narrow the size of the implicated chromosomal regions to obtain more conclusive evidence. Ultimately, it will be necessary to identify whether polymorphisms in these two CRF genes or regulatory regions that affect expression of these genes contribute to variability in maternal behavior. Despite a wide range

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of studies suggesting that CRF signaling plays a critical role in the expression of maternal aggression, no work to date has directly examined how CRF regulates this important social behavior.

The aim of this study was to test the hypothesis that reduced CRF during lactation is necessary to express maternal aggression. If maternal aggression depends on reduced CRF neurotransmission, then icv injection of CRF should inhibit this behavior. If, during normal maternal aggression, there is a slight inhibition of peak aggression that occurs because of a small central release of CRF in response to an intruder male, then icv injection of a CRF receptor antagonist might further elevate levels of aggression. Alternatively, if other neuromodulators control the intensity (peak levels) of aggression and lowered CRF neurotransmission acts to open a gate that allows the expression of maternal aggression, then icv injection of a CRF receptor antagonist would not be expected to alter levels of aggression. In this study, we asked three questions: (a) Does icv CRF inhibit maternal aggression? (b) Do icv injections of a CRF antagonist affect maternal aggression? (c) If icv CRF inhibits maternal aggression, then where in the brain might CRF be suppressing the behavior? We used lactating mice to determine which brain regions show increased activity (using Fos immunoreactivity) with either maternal aggression testing (maternal aggression circuitry) or CRF injections (CRF responsive circuitry). An analysis of overlaps or connections between the respective circuitries then allowed for speculation on how CRF may affect maternal aggression.

## Method

### *Mice*

Outbred Harlan–Sprague–Dawley: Institute of Cancer Research mice (Harlan Teklad, Madison, WI) were used. Females were housed with a single breeder male, and following impregnation (~2 weeks), each female mouse was housed individually for the remainder of the study. Just prior to parturition, female mice were given precut nesting material. Polypropylene cages were changed once weekly, but cages were not changed after parturition for the duration of the experiments. Intruder male mice were sexually naive and group housed (4 mice/cage). Intruder males (~2 months old) were given ad-lib access to regular chow (LabDiet, Brentwood, MO). Intruder males were never used more than once per day and were used for ~3 tests each. All mice were housed on a 14:10-hr light–dark cycle, with lights on at 0600 central standard time. Female mice were given ad-lib access to breeder chow (Harlan Teklad Breeder Diet 7004) and tap water.

### *Cannula Surgeries*

Under isoflurane anesthesia, through the use of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), a midline incision across the top of the skull was made and the periosteum removed. Prior to the cut, the fur above the skull was shaved, and the skin was cleaned and treated with alcohol and Betadine (Purdue Frederick, Stamford, CT). A 1-mm hole was drilled −0.6 mm posterior to and 1.6 mm lateral to bregma. A 26-gauge stainless-steel indwelling cannula (Plastics One, Roanoke, VA) was implanted in the hole to −2.5 mm below the skull surface into the lateral ventricle. Two small screws were drilled into the skull adjacent to the guide cannula. Each cannula was secured to the skull with dental cement (Plastics One). A dummy cannula was inserted to maintain patency. Injections were made with a 33-gauge stainless-steel injector attached to PE-50 tubing (Becton Dickenson, Sparks, MD) fitted to a 10-$\mu$l syringe (Hamilton, Reno, NV). Female mice were fitted with cannulas to the lateral ventricle on approximately Days 3–4 postpartum.

### *Intracerebroventricular Injections*

Beginning 3 days following surgery, single injections were delivered each day for up to 4 consecutive days (depending on experiment, described below) to mice under light isoflurane anesthesia. The day for first injection was approximately Days 6–7 postpartum. All injections were made with a 1-$\mu$l volume. Infusions were verified by following movement of an air bubble in the tubing. The cannulas remained in place for 60 s following each injection. Thirty minutes after injection, each female was tested for maternal aggression for 10 min as described below. At the completion of the testing series and just prior to perfusion (within 10 min), 1 $\mu$l of 0.01% Chicago blue (Sigma Chemical, St. Louis, MO) in saline was injected to verify cannula placement. Only results from cannulas correctly directed to the lateral ventricle were used. The following doses were tested—human–rat CRF (Sigma Chemical, St. Louis, MO): 0.02, 0.2, and 1.0 $\mu$g in 1.0 $\mu$l of saline, with saline (1.0 $\mu$l) as a control; D-Phe-$CRF_{12-41}$ (Bachem, San Carlos, CA): 1.0 and 5 $\mu$g dissolved in 1.0 $\mu$l saline, with saline (1.0 $\mu$l) as a control. For testing 1.0 $\mu$g CRF versus saline, 8 mice were used, and the order of injection was counterbalanced so that an equal number of mice received CRF (1.0 $\mu$g) or saline for the first test. For testing 0.02 and 0.2 $\mu$g CRF and saline, 15 different mice were used. Again, the sequence of injections was counterbalanced such that an equal number of mice received each of the three doses on the 3 different test days. Two mice tested for 0.2 $\mu$g CRF and saline were not tested for 0.02 $\mu$g CRF. For injections of the CRF antagonist, 9 additional mice were used, and the order of injection was also counterbalanced. Doses were chosen on the basis of previous icv studies of the behavioral effects of CRF and antagonist in rodents (e.g., Menzaghi et al., 1994; Smagin et al., 2001).

### *Maternal Aggression Behavioral Testing*

Immediately after injections, females were returned to their home cages with the pups still in the cage. Thirty minutes later, each female was exposed to an intruder male for 10 min in her home cage between 1000 and 1500. The pups were removed from the cage 2 min prior to the behavioral test. Removal of the pups from a dam just before an aggressive test does not diminish the expression of maternal aggression in mice (Svare, Betteridge, Katz, & Samuels, 1981). The range for timing of the injections (Postpartum Days 6–10) occurred within the window of peak maternal aggression that occurs from Postpartum Day 4 to 10 in mice (Svare, 1990). An intruder male mouse was placed in the dam's home cage, and each test session was recorded on videotape and subsequently analyzed offline to quantify maternal aggression. Maternal aggression scoring was conducted by individuals blind to experimental conditions and treatments. For quantification of maternal aggression, the following features were measured: latency to first attack, number of attacks, and total duration of attacks (Gammie, Huang, & Nelson, 2000; Gammie & Nelson, 1999). In addition, the amount of time attacking different regions of the male (including head and/or neck, flank and/or back, or a combination of these two general regions) and the amount of time lunging or clawing (without physical contact) were recorded. From our preliminary results, we found that a subset of mice (10%–20%) exhibited no aggression under any circumstances. To eliminate unnecessary and unusable experiments, we screened each female for maternal aggression for 2 min on approximately Postpartum Days 3–4. Only females exhibiting aggression were fitted with cannulas.

### *Immunohistochemistry for Fos*

Nineteen mice were randomly assigned to four groups: saline-injected, no intruder ($n = 4$); saline-injected, plus intruder ($n = 5$); 0.2 $\mu$g CRF-injected, no intruder ($n = 5$); 0.2 $\mu$g CRF-injected, plus intruder (n = 5). The dose of 0.2 $\mu$g CRF icv was chosen because it was the lowest dose of CRF to effectively reduce maternal aggression. For the plus intruder groups, maternal aggression testing was conducted for 10 min as described

above (30 min following injection), and for the no intruder groups, no testing occurred. To control for any effect that pup removal itself might have on Fos (rather than effects related to the intruder per se), we removed pups from the home cage of nontested mice for 10 min, 30 min after injections. Of the 19 mice included in this study, 15 were previously used in the experiment looking at the effect of saline and 0.2 and 0.02 $\mu$g CRF on maternal aggression (described above). They were recruited into the study 1 day after the final behavioral test (approximately Postpartum Day 9). Three additional mice were implanted with cannulas just for the Fos study (one of which was excluded, see below), and 2 were taken from the group examining the effect of 1.0 $\mu$g CRF compared with saline. The prior history of CRF treatment was used as a cofactor in the analysis of Fos levels and was not significant.

Mice were perfused 2 hr ($\pm$ 10 min) following the icv injection described above. Mice were given an intracardial perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were postfixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose in PBS for 2 days. Brains were frozen on a platform, cut into 40-micron-thick sections with a sliding microtome (Leica, Microsystems, Heidelberg, Germany), and stored in a cryoprotectant solution at $-20$ °C until processing for immunohistochemistry. One set of alternate sections was washed in PBS in the presence of 0.2% Triton X-100 (PBS-X), blocked in 5% normal goat serum for 1 hr, and incubated for 2 days at 4 °C with rabbit anti-c-Fos antibodies (1:20,000; Oncogene Research Products, Cambridge, MA). After washes in PBS-X, the sections were incubated for 90 min at room temperature in biotinylated goat anti-rabbit secondary antibodies (1:500; Vector Laboratories, Burlingame, CA), washed in PBS-X, exposed to an avidin–biotin complex (Vector Laboratories) for 1 hr, washed again in PBS-X, and visualized through the use of diaminobenzidine (Sigma Chemical) as a chromagen, enhanced with 0.008% nickel chloride. The sections were mounted, dehydrated, and coverslipped.

### *Analysis of c-Fos Immunoreactivity*

For counting of Fos cells, the sections were projected in bright field from an Axiocam Zeiss microscope (Zeiss, Gottingen, Germany), through an Axiocam Zeiss high-resolution digital camera attached to the microscope, and interfaced with a computer. KS300 software (Zeiss) was used for thresholding and cell counting through the use of a similar paradigm previously used (Gammie & Nelson, 2001). Cells were automatically counted within a box, and the dimensions and locations are shown in Figure 1. The following steps were taken to ensure Fos immunoreactivity was measured consistently between samples: (a) all sections were exposed to diaminobenzidine for exactly 10 min, (b) the background was normalized by adjusting light levels, (c) a threshold level of staining was used to automatically distinguish Fos-positive cells, (d) all slides were coded and the counting was performed by one individual, blind to the experimental

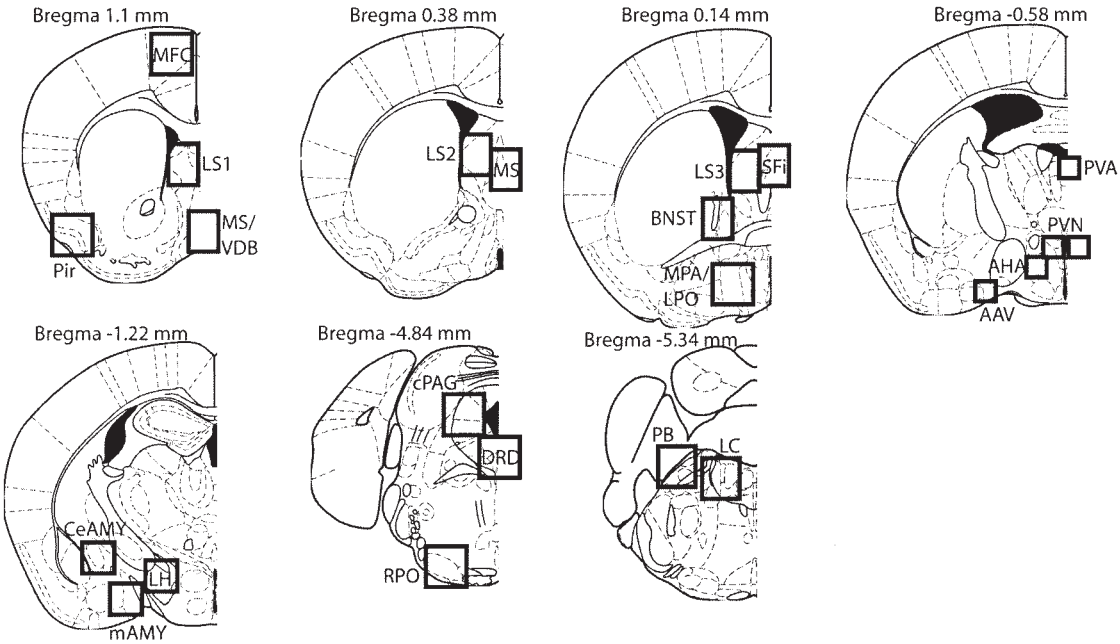


*Figure 1.* Schematic representation of the brain regions analyzed. For bregma 1.1 mm, 0.38 mm, and 0.14 mm, the largest boxes represent an 870 $\times$ 870 $\mu$m region, and the smaller regions are 638 $\times$ 870 $\mu$m. For bregma $-0.58$ mm, all regions are 435 $\times$ 435 $\mu$m. The PVN was counted with two boxes placed bilaterally. For bregma $-1.22$ mm, all regions are 690 $\times$ 675 $\mu$m. For bregma $-5.34$, both boxes are 807 $\times$ 870 $\mu$m. Reprinted from *The Mouse Brain in Stereotaxic Coordinates,* 2nd ed., G. Paxinos and K. B. J. Franklin, Figures 22, 28, 30, 36, 41, 71, and 75, Copyright 2001, with permission from Elsevier. MFC = medial frontal cortex; LS = lateral septal nucleus; Pir = piriform cortex; MS/VDB = medial septal nucleus/vertical limb of the diagonal band; MS = medial septal nucleus; SFi = septofimbrial nucleus; BNST = bed nucleus of the stria terminalis; MPA/LPO = medial preoptic area/lateral preoptic area; PVA = paraventricular thalamic nucleus; PVN = paraventricular nucleus of the hypothalamus; AHA = anterior hypothalamic area; AAV = anterior amygdaloid area; CeAMY = central amygdala; LH = lateral hypothalamus; mAMY = medial amygdala; cPAG = caudal periaqueductal gray; DRD = dorsal raphe nucleus; RPO = rostral periolivary region; PB = parabrachial nucleus; LC = locus coeruleus.

conditions, and (e) only Fos-positive nuclei within a specified size range were counted. One mouse (from the saline, no test group) was removed from the data because it showed unusually high background staining throughout the brain.

### *Data Analysis*

Maternal aggression variables were analyzed with either paired tests or repeated measures to account for the fact that same individuals were measured multiple times at the different doses. For analysis of CRF action, paired tests were conducted instead of a repeated measures analysis of variance (ANOVA) because not all doses of CRF were tested in the same mouse. In the cases in which the distribution of the differences was not normally distributed, a nonparametric pairwise Wilcoxon rank sum test was used. For analysis of CRF antagonist effect on aggression, a one-way repeated measures ANOVA was used. In the case of time to first attack, if a mouse was not aggressive, a time of 600 s was assigned (the maximum possible for the test). Lunges and clawing, which represent a minor form of aggression, were quantified to examine the relative proportions of all forms of agonistic behavior. The numbers of pups per dam (12.3 ± 0.5) were included as covariates in analyses of maternal aggression but were not found to be significant and therefore were removed from the final analyses.

Separate *t* tests for Fos analysis were used to test (a) the effect of maternal aggression testing within saline-injected mice; (b) the effect of CRF versus saline injections in nontested mice; and (c) the effect of maternal aggression on CRF-injected mice. Brain regions previously implicated in either maternal aggression or CRF signaling were examined. Because multiple tests (66 total *t* tests) were conducted comparing Fos counts between 4 different groups in 22 brain regions, we determined the global, experiment-wide, false discovery rate that would occur for our data if we were to apply the standard *p*-value cutoff of .05 to determine positive results (Storey, 2002). This was done with open-source software called QVALUE (Dabney & Storey, 2002). As it turns out, for our data, which yielded many small *p* values (34 out of 66 total tests yielded a *p* value of less than .05), applying the standard *p*-value cutoff of .05 would yield a global false discovery rate of 3% (i.e., 3 out of 100 positive results will be false positives). Thus, even though multiple tests were conducted, in this case and for these data, the application of the standard *p* value cutoff of .05 is appropriate.

## Results

### *Effects of icv CRF and CRF Antagonist on Maternal Aggression*

Injection of CRF to the lateral ventricle significantly inhibited expression of maternal aggression in a dose-dependent manner (see Figure 2). The lowest dose of CRF (0.02 μg) had no effect on any of the measures of aggression relative to saline injections (see

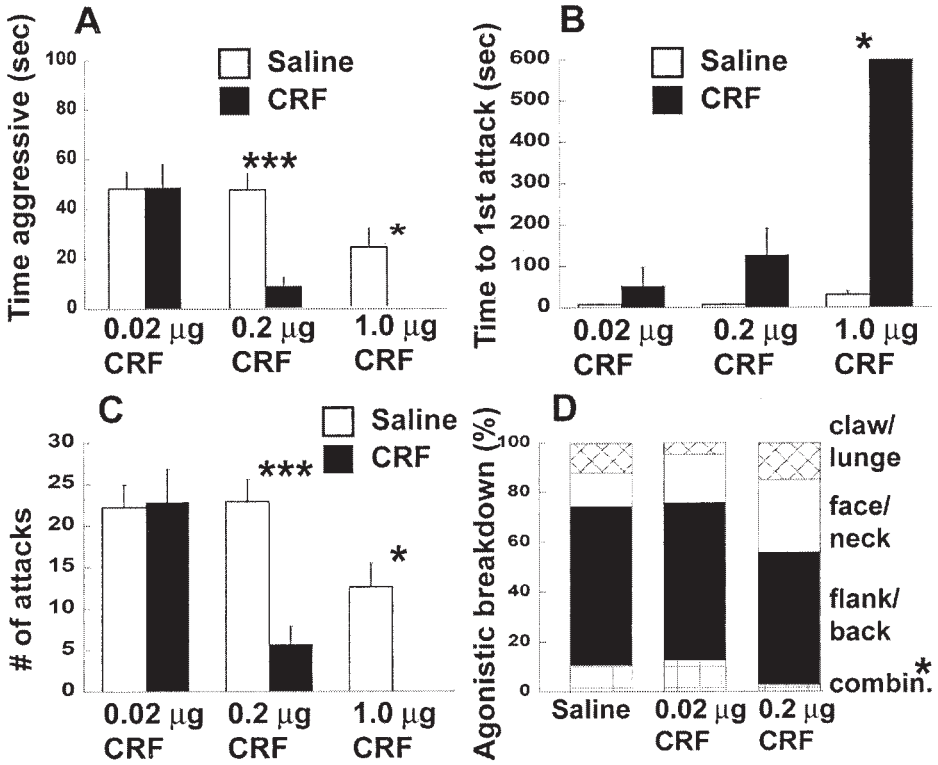


*Figure 2.* Effects of intracerebroventricular corticotropin-releasing factor (CRF) on measures of maternal aggression in outbred mice. A: Mean (± *SE*) time aggressive is significantly decreased by 0.2 μg CRF and 1.0 μg CRF, but not 0.02 μg CRF, relative to saline. B: Mean time (± *SE*) to first attack is significantly delayed by 1.0 μg CRF relative to saline. C: Mean number (± *SE*) of attacks is significantly reduced by 0.2 and 1.0 μg CRF relative to saline. D: Breakdown by percentage of total agonistic behaviors for saline and 0.02 and 0.2 μg CRF. Mice receiving 0.2 μg CRF exhibited a significantly smaller percentage of combination (combin.) attacks to both the face and/or neck and back and/or flank region. *$p < .05$. ***$p < .01$. (See Results for specific statistical tests performed.) For all tests, order of injections was counterbalanced.

Figures 2A–2C). The dose of 0.2 μg CRF caused a significant decrease in mean time aggressive: paired *t* test, $t(14) = 5.91$, $p < .01$; and number of attacks: paired *t* test, $t(14) = 5.18$, $p < .01$; but not time to first attack, relative to saline injections (see Figures 2A–2C). The dose of 0.2 μg CRF also caused a significant decrease in mean time aggressive: paired *t* test, $t(12) = 4.78$, $p < .01$; and number of attacks: paired *t* test, $t(12) = 4.19$, $p < .01$; relative to 0.02 μg CRF injections. The highest dose of CRF (1.0 μg) reduced aggression to zero in all mice, and the effect was significantly different from saline in terms of mean time aggressive, time to first attack, and number of attacks: Wilcoxon matched pairs tests for each, $z(1, 7) = 2.52$, $p < .05$; (see Figures 2C–2E). The lower mean time aggressive for saline-injected mice that were also examined with 1.0 μg CRF (compared with saline-injected mice examined with the lower doses of CRF) reflects that different batches of mice were used for the different comparisons (see the Methods section).

Mice receiving 1.0 μg CRF moved normally in their home cage after injections but tended to remain stationary once the intruder male was placed into the cage. Further, they would often sniff in the direction of the male as opposed to actively follow and sniff and/or attack the intruder, as occurred for saline or 0.02 μg CRF injections. Mice receiving 0.2 μg CRF injections did not exhibit overt behavioral differences before the male was introduced, but they became less active once the male was present.

Lunges or clawing represent mild forms of aggression. For saline and 0.02 and 0.2 μg CRF injections, lunges or clawing represented only ~10% of total agonistic encounters, and there were no differences between groups in this measure (see Figure 2D). Bites to the face or neck region constituted ~20% of total agonistic encounters, and bites to the flank or back constituted ~60% of total agonist encounters for the three groups; there were no differences between the three groups in either of these measures. However, mice receiving 0.2 μg CRF exhibited significantly lower percentage (2.6%) of attack events in which the lactating female bit both the head and/or neck and flank and/or back regions in one encounter (termed a *combination attack*), as compared with mice receiving either saline (10.6%) or 0.02 μg CRF (12.6%): Kruskal–Wallis one-way ANOVA on ranks, $H(2, 12) = 9.0$, $p < .05$. A combination attack usually occurs during the expression of high levels of aggression, and the decrease in this type of attack likely reflects the overall decrease in aggression in mice receiving 0.2 μg CRF injections.

Immediately after behavioral testing, pups were returned to the home cage and interaction of dams with offspring was observed. For all injections, dams were observed nursing offspring when examined 5 min after return of pups to the home cage. Crude observations of maternal behaviors did not reveal any effect of injections, but quantitative measures were not taken. No mortality of pups occurred following any injections.

Neither 1.0 nor 5.0 μg D-Phe-$CRF_{12-41}$, a nonspecific CRF receptor antagonist, had any effect on maternal aggression, relative to saline injections, in terms of mean time aggressive, $F(2, 7) = 1.3$, $p = .29$; mean number of attacks, $F(2, 7) = 1.9$, $p = .17$; or mean time to first attack, $F(2, 7) = 0.7$, $p = .50$ (one-way repeated measures ANOVA). For the saline and 1.0 and 5.0 μg D-Phe-$CRF_{12-41}$ groups the mean times aggressive were 66.8 ± 7.1, 71.4 ± 5.4, and 59.7 ± 8.5 s, respectively; the mean numbers of attacks were 27.4 ± 2.2, 32.6 ± 2.2, and 27.6 ±1.8, respectively; and the mean times to first attack were 4.5 ± 1.4, 2.8 ±0.6, and 4.6 ± 1.1 s, respectively. Further, neither dose of D-Phe-$CRF_{12-41}$ had an effect on the profile of agonistic behavior (e.g., percentage of attacks to head and/or neck; one-way repeated measures ANOVA, for each category, $p > .1$; data not shown).

### *Effect of Maternal Aggression Testing and icv Injections of CRF on Fos Immunoreactivity*

The saline-treated mice that were measured for maternal aggression and subsequently examined for Fos ($n = 5$) exhibited an average of 42.6 ± 6.6 s of aggression, an average of 23.4 ± 4.3 attacks, and a mean latency to first attack of 3.8 ± 0.7 s. In contrast, mice that were injected with 0.2 μg CRF, then tested for maternal aggression and subsequently analyzed for Fos ($n = 5$), exhibited only 5.2 ± 1.9 s of aggression, 4.0 ± 1.5 attacks, and a mean latency to first attack of 125.0 ± 118.7 s.

Maternal aggression testing in saline-injected mice was associated with significant elevation of Fos levels in 15 out of 22 brain regions examined, relative to the Fos levels of nontested control mice (see Figures 3 and 4 and Table 1). In nontested mice, 0.2 μg CRF was a potent activator of Fos and elicited a significant increase in Fos in 18 of 22 regions, relative to saline injections (see Figure 4 and Table 1). Maternal aggression testing in 0.2 μg CRF-injected mice (that resulted in only low maternal aggression) was never associated with increases in Fos relative to Fos levels in nontested, CRF-injected controls (see Table 1). However, whether a lack of effect of aggression testing in CRF-injected mice was due to a ceiling effect of CRF action alone could not be determined. Eleven of the 15 brain regions that showed increased Fos levels with maternal aggression testing in saline-injected mice also showed elevated Fos with CRF-injected, nontested mice (see Figure 4), suggesting that CRF was acting on regions implicated in the production of maternal aggression.

## Discussion

When exhibiting maternal aggression, lactating females rodents also exhibit decreased fear and anxiety, decreased CRF mRNA (during Postpartum Days 7–15 in rats), and decreased responsiveness of the CNS to CRF (da Costa et al., 1997; Toufexis et al., 1998; Walker, Toufexis, & Burlet, 2001). Because CRF is a potent activator of indices of fear and anxiety (Koob & Heinrichs, 1999; Smagin et al., 2001), decreased CRF signaling can account for the decreased fear and anxiety during lactation. Our finding that CRF inhibits maternal aggression, but not other maternal behaviors, is the first work to date to provide a mechanistic basis for understanding the inverse relationship between fear and anxiety and maternal aggression that has been reported in a number of studies. The finding that the CRF receptor antagonist does not alter aggression suggests that decreases in CRF may be necessary, but not sufficient, for the normal expression of maternal aggression. Further, our identification of common regions between maternal aggression circuitry (increased Fos with maternal aggression) and the CRF responsive regions of the CNS (increased Fos with CRF) suggests the possibility that decreased CRF activity at specific sites in the CNS removes inhibition and opens the gate for maternal aggression.

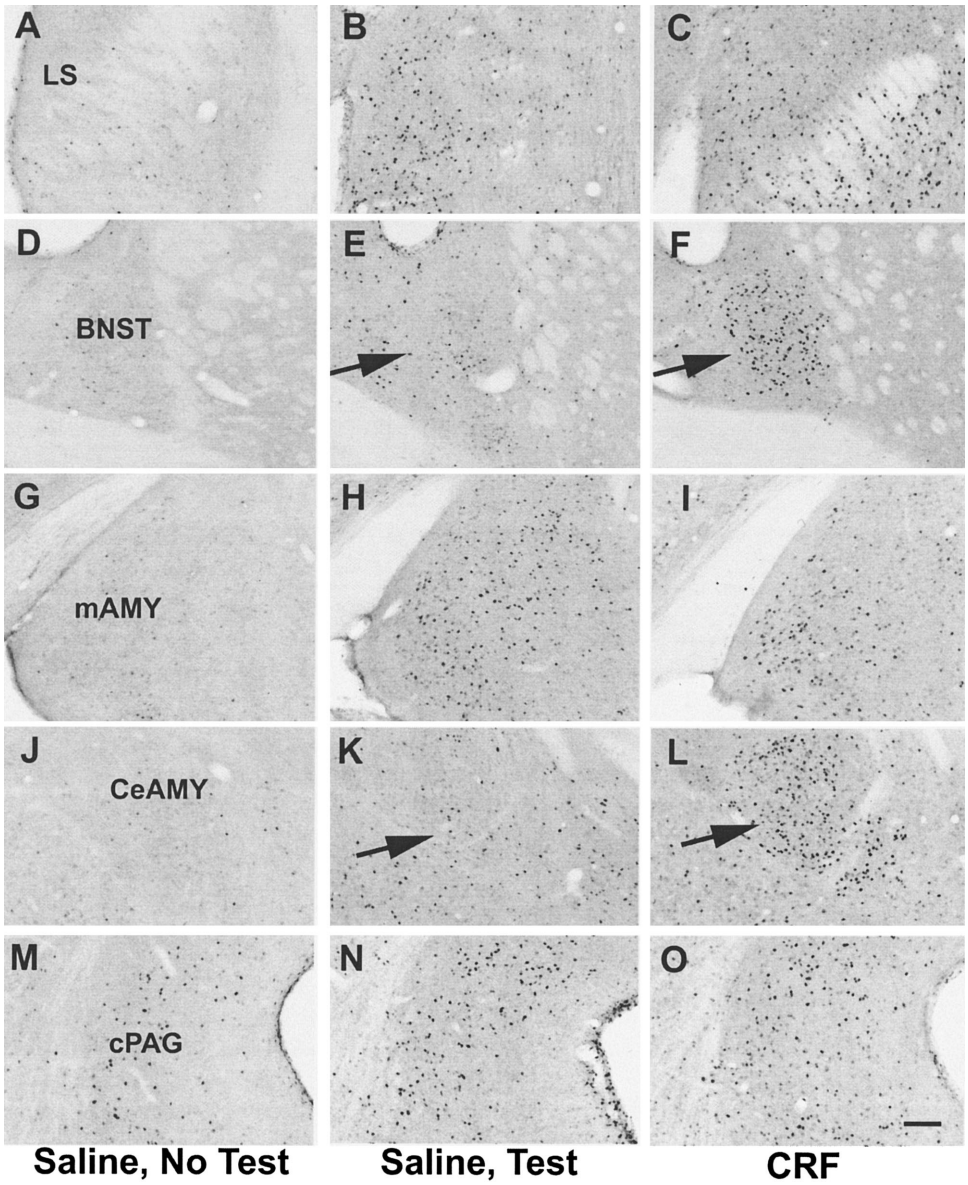


*Figure 3.* Examples of Fos immunoreactivity 2 hr following intracerebroventricular infusion of either saline or corticotropin-releasing factor (CRF; 0.2 $\mu$g). At 30 min after injection, mice were either tested for maternal aggression or given a sham test for 10 min. Common sites for significant increases in Fos with testing (in saline-injected mice) and with CRF relative to saline injections occurred in the lateral septal nucleus (LS; A, B, C), the bed nucleus of the stria terminalis (BNST; D, E, F), the medial amygdala (mAMY; G, H, I), the central amygdala (CeAMY; J, K, L), and the caudal periaqueductal gray (cPAG; M, N, O). Levels of Fos were equivalent with CRF injections regardless of testing (see Table 1), and examples of CRF-injected, tested mice are shown.

### *Inhibitory Effects of CRF on Maternal Aggression*

The aim of this study was not to demonstrate that CRF can elevate fear and anxiety (because that has been documented numerous times) but rather to link alterations of CRF levels specifically to maternal aggression expression. The finding that centrally administered CRF inhibits maternal aggression suggests that decreased CRF neurotransmission is necessary for maternal aggression to be properly expressed. Although we cannot exclude the possibility that inhibition of maternal aggression by CRF was caused indirectly by elevated corticosterone (Song, Earley, & Leonard, 1995), this is not likely because injection of the synthetic glucocorticoid, dexamethasone, does not affect maternal aggression in mice (Al-Maliki, 1980).

Although decreased CRF mRNA synthesis occurs later in lactation in rats (Postpartum Days 7–15, described above), levels of maternal aggression are actually higher earlier during lactation

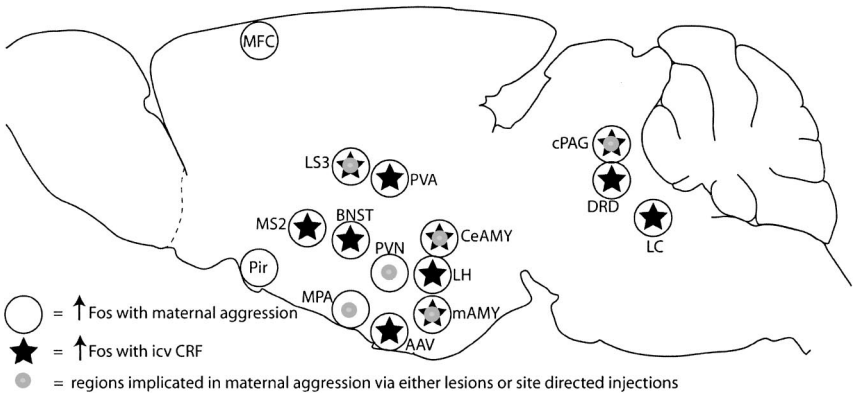


*Figure 4.* Schematic (sagittal) diagram of brain regions exhibiting significant increases in Fos due to testing for maternal aggression relative to no testing (in saline-injected mice) and due to corticotropin-releasing factor (CRF) injections relative to saline injections within those regions (in nontested mice). Areas previously implicated in maternal aggression via either lesion or site-directed injections of neurotransmitters or antagonists are shown. See the Discussion section for references. Additional regions showing increased Fos due to CRF that occurs in regions not implicated in maternal aggression are not shown. MFC = medial frontal cortex; cPAG = caudal periaqueductal gray; LS = lateral septal nucleus; PVA = paraventricular thalamic nucleus; DRD = dorsal raphe nucleus; MS = medial septal nucleus; BNST = bed nucleus of the stria terminalis; CeAMY = central amygdala; LC = locus coeruleus; Pir = piriform cortex; PVN = paraventricular nucleus of the hypothalamus; LH = lateral hypothalamus; MPA = medial preoptic area; mAMY = medial amygdala; AAV = anterior amygdaloid area. Reprinted from *The Mouse Brain in Stereotaxic Coordinates*, 2nd ed., G. Paxinos and K. B. J. Franklin, Figure 108, Copyright 2001, with permission from Elsevier.

when RNA levels are unchanged, or even elevated, relative to those of virgin females (da Costa, Ma, Ingram, Lightman, & Aguilera, 2001; Deschamps et al., 2003). Thus, if decreased CRF neurotransmission supports maternal aggression in early lactation in rats, then it may occur through decreased release or response to CRF, but not decreased synthesis of CRF.

### *Possible Interpretations for Lack of Effect of the CRF Antagonist, D-Phe-CRF$_{12–41}$, on Maternal Aggression*

One possible explanation for a lack of effect of D-Phe-CRF$_{12–41}$ is that reduced CRF is necessary but not sufficient for maternal aggression. CRF neurotransmission is already low in lactating females (described above), and it is possible that reducing CRF activity further by application of the antagonist has no added benefit (i.e., a floor effect). Reduced CRF, then, may be a necessary gate that, when lowered, allows the expression of maternal aggression to be produced by other neuromodulators. In this scenario, the antagonist would not be predicted to elevate maternal aggression because the gate has already been lowered (in association with lactation) and the positive regulators of maternal aggression do not involve CRF.

Other approaches, though, might reveal an effect of the antagonist on aggression. Because chronic stress decreases maternal aggression in mice, long-term icv injections of the antagonist in stressed lactating mice might abate the inhibitory effect of endogenously released CRF on aggression. Also, levels of maternal aggression wane later during lactation, and if this decrease is due in part to elevated CRF neurotransmission, then injections of the antagonist at times of lower aggression during lactation might restore high levels of aggression.

### *Possible Link Between Maternal Aggression Neuronal Circuitry and CRF Responsive Brain Regions*

Using indirect markers of neuronal activity (Fos, phosphorylated cyclic adenosine monophosphate response element binding protein [pCREB], and citrulline), researchers have identified 11 brain regions as being part of the maternal aggression circuitry (Gammie & Nelson, 1999, 2000, 2001). Five of these 11 brain regions have also been implicated in maternal aggression circuitry via either lesion or direct neurotransmitter and/or antagonist injection studies, including the lateral septal nucleus (LS; Flannelly, Kemble, Blanchard, & Blanchard, 1986), the medial preoptic area (lesions eliminate maternal behaviors, but maternal aggression per se was not tested in females; Numan, 1994; Rosenblatt, Hazelwood, & Poole, 1996), the PVN (Consiglio & Lucion, 1996; Giovenardi, Padoin, Cadore, & Lucion, 1997, 1998), the medial amygdala (mAMY; De Almeida & Lucion, 1997; Hansen & Ferreira, 1986), and the caudal periaqueductal gray (Lonstein & Stern, 1997). The findings of elevated brain activity in association with maternal aggression testing in this study (see Table 1 and Figures 3 and 4) are in accordance with the earlier work establishing maternal aggression circuitry. Further, our identification of a possible role for CeAMY in maternal aggression circuitry concurs with recent work implicating this region in maternal aggression via pharmacological injections (Lubin, Elliott, Black, & Johns, 2003).

A dose of 0.2 $\mu$g CRF triggered significant increases in Fos, relative to saline injections (in nontested mice), in 18 of 22 brain regions examined (see Table 1 and Figure 4). These results are consistent with earlier work showing icv injection of CRF in male rats increased Fos in similar regions, including the LS and the mAMY (Bittencourt & Sawchenko, 2000). Although our study is

Table 1
*Mean (± SE) Number of Fos-Positive Nuclei Within Tested and Nontested Lactating Mice*

| | Group | | | |
|---|---|---|---|---|
| | Saline injected | | CRF injected | |
| Site | Not tested | Tested | Not tested | Tested |
| LS1[a] | 84 ± 14 | 150 ± 23 | 194 ± 36 | 229 ± 24 |
| MS1[a] | 26 ± 16 | 60 ± 10 | 117 ± 29 | 111 ± 25 |
| MFC | 20 ± 5 | 153 ± 32** | 182 ± 63 | 191 ± 57 |
| Pir | 107 ± 34 | 257 ± 41* | 241 ± 51 | 297 ± 41 |
| LS2[a] | 33 ± 13 | 61 ± 9 | 284 ± 57 | 221 ± 46 |
| MS2[a] | 12 ± 3 | 22 ± 2* | 147 ± 33 | 129 ± 15 |
| LS3[a] | 20 ± 5 | 60 ± 7** | 209 ± 36 | 198 ± 41 |
| SFi[a] | 6 ± 2 | 14 ± 3 | 81 ± 15 | 91 ± 10 |
| BNST[a] | 22 ± 8 | 51 ± 7* | 98 ± 18 | 116 ± 19 |
| MPA | 97 ± 19 | 246 ± 23** | 258 ± 45 | 233 ± 37 |
| PVN | 52 ± 8 | 229 ± 49* | 188 ± 48 | 210 ± 59 |
| PVA[a] | 53 ± 14 | 112 ± 14* | 129 ± 9 | 99 ± 16 |
| AAV[a] | 14 ± 5 | 65 ± 17* | 110 ± 19 | 101 ± 12 |
| AHA[a] | 30 ± 11 | 61 ± 8 | 89 ± 14 | 81 ± 14 |
| mAMY[a] | 18 ± 8 | 103 ± 16** | 140 ± 24 | 124 ± 14 |
| LH[a] | 26 ± 11 | 89 ± 4** | 93 ± 16 | 97 ± 5 |
| CeAMY[a] | 20 ± 7 | 47 ± 6* | 73 ± 14 | 134 ± 35 |
| cPAG[a] | 101 ± 13 | 181 ± 20* | 198 ± 22 | 198 ± 19 |
| DRD[a] | 32 ± 9 | 95 ± 13** | 209 ± 40 | 148 ± 10 |
| RPO[a] | 113 ± 30 | 283 ± 75 | 430 ± 81 | 315 ± 96 |
| LC[a] | 49 ± 8 | 98 ± 17* | 136 ± 25 | 142 ± 9 |
| PB[a] | 70 ± 26 | 114 ± 34 | 165 ± 21 | 178 ± 36 |

*Note.* LS = lateral septal nucleus; MS = medial septal nucleus; MFC = media frontal cortex; Pir = piriform cortex; SFi = septofimbrial nucleus; BNST = bed nucleus of the stria terminalis; MPA = medial preoptic area; PVN = paraventricular nucleus of the hypothalamus; PVA = paraventricular thalamic nucleus; AAV = anterior amygdaloid area; AHA = anterior hypothalamic area; mAMY = medial amygdala; LH = lateral hypothalamus; CeAMY = central amygdala; cPAG = caudal periaqueductal gray; DRD = dorsal raphe nucleus; RPO = rostral periolivary region; LC = locus coeruleus; PB = parabrachial nucleus.
[a] Regions showing significantly higher levels of Fos in CRF-injected compared with saline-injected mice in the nontested condition. No differences occurred between tested and nontested CRF-injected mice. Unpaired Student's tests used for all comparisons.
* $p < .05$. ** $p < .01$.

the first to examine increases in Fos protein in response to icv CRF in lactating mice, a similar study was conducted in lactating rats using a higher dose of icv CRF (5 $\mu$g) and examining Fos mRNA (da Costa et al., 1997). In the lactating rat study, Fos mRNA increased in CRF-injected rats in the CeAMY, the locus coreuleus (LC), the dentate gyrus of hippocampus, and the nucleus tractus solitarius. Other regions, such as the LS and the PVA, almost doubled levels of Fos mRNA with CRF but did not reach significance (da Costa et al., 1997). Our finding of a higher number of CRF-responsive regions in lactating mice (with 0.2 $\mu$g CRF) compared with those found in the rat study could be due to several different methodological approaches including differences in species, histological techniques (in situ hybridization for mRNA vs. immunohistochemistry for protein product), time course following icv infusion (0.5 hr vs. 2.0 hr), brain regions chosen for examination (only 10 similar regions were examined), and the method of counting (mean optical density vs. cell counting with an automated program). No behavioral testing was conducted in the rat study, but the lactating female CNS was found to be less responsive to CRF than the virgin female CNS (da Costa et al., 1997), supporting the idea that CNS responsiveness to CRF is decreased during lactation.

As shown in Figure 4, 11 common regions were found in this study in which Fos was elevated in association with both maternal aggression testing (maternal aggression circuitry) and icv CRF (CRF responsive circuitry). Among these regions were: bed nucleus of the stria terminalis, the LS, the mAMY, the CeAMY, and the caudal periaqueductal gray, regions strongly implicated in both maternal aggression and CRF responses. An important question is how to interpret the finding that certain brain regions show Fos increases with aggression and also with CRF even though CRF injections inhibit the behavior. Increased Fos does not indicate whether a neuron received excitatory or inhibitory inputs, only that a change in second messenger activity occurred. One interpretation is that maternal aggression and CRF-induced fear involve similar regions, but whether similar or different neurotransmitters or neurons within the same regions are involved with each respective behavior is not known. Hence, CRF could be inhibiting maternal aggression by acting on or near maternal aggression circuitry neurons.

The stress hyporesponse during lactation (that involves peripheral actions of CRF from the PVN as part of the hypothalamic–pituitary–adrenal axis) does not occur in all paradigms, as evidenced by the finding that predator odor elicits equally high levels of ACTH in lactating and virgin rats (Deschamps et al., 2003). Further, the presence of pups (compared with removal for 2.5 hr) coincides with an elevation of ACTH in lactating females in response to a stressor. Given that elements of the stress response (such as increased heart rate and elevated glucose mobilization) would be important for launching an attack, maintaining the responsiveness of certain components of the stress response (e.g., ACTH release) during lactation, especially with recent contact with pups, would be valuable. Our work suggests, though, that behavioral aspects of the stress response (including the central actions of CRF on the CNS that affect fear and anxiety) are likely reduced to allow for the production of maternal defense behavior. In support of this idea, restraint stress in rats (which would result in increased central CRF release) increases Fos in a number of regions, but in lactating females, those increases are suppressed in LS and mAMY (da Costa et al., 1996). This finding suggests that a critical modification of neurotransmission occurs during lactation in brain regions common to the stress response, CRF release, and maternal aggression. Further, we do not believe that maternal aggression is related to stress-induced aggression because icv CRF (0.1 and 0.01 $\mu$g) elevates that form of aggression in rats (Tazi et al., 1987), whereas icv CRF decreased maternal aggression in our study.

### *Summary*

In this study, we present evidence for the first time that reduced CRF is necessary for the elevation of maternal aggression that occurs during lactation. Our findings indicate that CRF may directly alter neurotransmission within brain regions involved in maternal aggression circuitry. The fact that the CRF antagonist did not increase maternal aggression suggests that decreases in CRF action (and corresponding decreases in fear and anxiety), though

necessary, are not sufficient for the production of maternal aggression. We speculate that other neuromodulators play a role in direct activation of the behavior. Finally, because CRF can reflect general environmental stressors, its negative control of maternal aggression may be extremely adaptive in that certain high-risk reproductive behaviors can be terminated if the environment does not favor reproduction.

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