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## SOCIAL DEFEAT STRESS ACTIVATES MEDIAL AMYGDALA CELLS THAT EXPRESS TYPE 2 CORTICOTROPIN-RELEASING FACTOR RECEPTOR mRNA

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### Abstract

Defeat is a social stressor involving subordination by a threatening conspecific. Type 2 corticotropin-releasing factor receptors (CRF<sub>2</sub>) are abundant in brain regions implicated in defeat responses and are putative stress-related molecules. The present study sought to determine whether neuroactivation and CRF<sub>2</sub> expression co-occurred at brain region or cellular levels following acute defeat. Male “intruder” Wistar rats were placed into the cage of an aggressive “resident” Long-Evans rat ( $n=6$ ). Upon defeat, intruders ( $n=6$ ) were placed in a wire-mesh chamber and were returned to the resident’s cage for an additional 75 min. Controls ( $n=6$ ) were handled and returned to their home cage for the same duration. Coronal brain sections were stained for an immediate early gene product, Fos, as a neuronal activation marker. Combined immunohistochemistry with *in situ* hybridization was performed on a subset of brain sections from defeated intruders to visualize Fos immunoreactivity and CRF<sub>2</sub> mRNA jointly. Defeated rats had fivefold, sevenfold, and 10-fold more Fos-positive cells than controls in the arcuate, ventromedial nucleus of the hypothalamus, and medial amygdala post-defeat. Significant colocalization of CRF<sub>2</sub> mRNA and Fos-positive cells was observed in the posterior medial amygdala but not in the arcuate nucleus or ventromedial hypothalamus. The results indicate CRF<sub>2</sub> receptor-positive neurons in the posterior medial amygdala are involved in the neural response to social defeat.

### Keywords

corticotropin-releasing factor; CRF<sub>2</sub> receptor; ventromedial or paraventricular hypothalamus; medial nucleus of the amygdala; lateral septum; social defeat stress or resident-intruder test

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Defeat is a social stressor involving subordination by a threatening conspecific. In many species, a single defeat results in lasting autonomic, reproductive, and behavioral changes (Tornatzky and Miczek, 1993; Koolhaas et al., 1997; Blanchard et al., 2001; Korte and De Boer, 2003; Sapolsky, 2005). Victors develop increased offensive behaviors, whereas losers develop increased defensive behaviors and less territorial behavior (Blanchard et al., 1995; Tamashiro et al., 2004; Huhman, 2006; Shimozuru et al., 2006). Rodent subordinates show decreased testosterone production and decreased mounting behavior during chronic social conflict, whereas dominants exhibit normal or elevated testosterone levels (Blanchard et al., 1995; Tamashiro et al., 2004; Huhman, 2006). Even brief exposure to territorial, dominant males leads to weight loss, anorexia, and hyperthermia (Marini et al., 2006); reduced reproduction-relevant social behaviors such as territorial aggression, marking, and mating; and stress-like changes in hypothalamic–pituitary–adrenocortical and gonadal axis activity (Shively and Kaplan, 1984; Yoshimura and Kimura, 1991; Huhman, 2006).

Fos, the protein product of the immediate-early gene *c-fos*, has been used as a cellular marker of brain regions activated by defeat (Miczek et al., 2004). In rats, increased Fos protein or *c-fos* mRNA expression has been reported in the ventral lateral septum (LS), central and medial nuclei of the amygdala (CeA, MeA), bed nucleus of the stria terminalis (BNST), lateral hypothalamus, paraventricular nucleus of the hypothalamus (PVN), dorsal raphe, and hippocampus following acute defeat (Martinez et al., 1998; Gardner et al., 2005; Funk et al., 2006; Calfa et al., 2007). Defeated mice likewise showed higher *c-fos* mRNA in the septum, preoptic area, lateral hypothalamus, amygdala, and dorsal raphe (Stork et al., 1997). Hamsters showed high *c-fos* mRNA expression in the CeA, MeA, BNST, LS, and arcuate nucleus of the hypothalamus (ARH) following one defeat and also in the ventromedial hypothalamus (VMH) after repeated defeat (Kollack-Walker et al., 1997, 1999).

Corticotropin-releasing factor (CRF) family peptides are key mediators of behavioral, autonomic, and neuroendocrine responses to stress (Vale et al., 1981). CRF is distributed in the PVN, from which it elicits pituitary adrenocorticotrophic hormone release following secretion from the median eminence (Sawchenko et al., 1993), and in extrahypothalamic brain regions where it subserves non-neuroendocrine, stress-related functions (Zorrilla and Koob, 2004). For example, acute defeat elevates CRF mRNA in the hippocampus (Marini et al., 2006), ventral BNST, and CeA (Funk et al., 2006).

Two genes encoding G-protein-coupled CRF receptors have been identified (CRF<sub>1</sub> and CRF<sub>2</sub>) (Chen et al., 1993; Lovenberg et al., 1995). Localization studies show distinct distributions of these receptor subtypes, suggesting functional diversity (Fekete and Zorrilla, 2007). The CRF<sub>1</sub> receptor, expressed throughout the brain including the cortex and cerebellum, mediates activational and anxiogenic-like components of stress-related behaviors (Zorrilla and Koob, 2004). CRF<sub>2</sub> receptors are expressed in discrete brain areas, including the hypothalamus and limbic system, with CRF<sub>2</sub> receptors in the hypothalamus and hindbrain regulating food intake and gastric motility (Fekete and Zorrilla, 2007). However, whether and which CRF<sub>2</sub> receptors participate in endogenous responses to stress remain less clear, with data suggesting roles in the LS (Henry et al., 2006) and dorsal raphe (Hammack et al., 2003; Staub et al., 2005; Cooper and Huhman, 2007). Interestingly, CRF<sub>2</sub> receptor mRNA is also present at medium-to-high levels in other brain regions where defeat induces *c-fos* gene expression, including the BNST, MeA, PVN, ARH, and VMH (Van Pett et al., 2000), raising the possibility that CRF<sub>2</sub> neurons may mediate responses to social defeat.

Therefore, the present study tested the hypothesis that CRF<sub>2</sub>-containing neurons are activated by social defeat. Fos was used as a marker for neuronal activation to determine semiquantitatively whether social defeat activates neurons in areas that express CRF<sub>2</sub>. Combined immunohistochemistry and *in situ* hybridization was then performed to visualize

Fos immunoreactivity and CRF<sub>2</sub> mRNA simultaneously to identify neurons that express both Fos and CRF<sub>2</sub>.

## EXPERIMENTAL PROCEDURES

### Subjects

Adult male Wistar rats ( $n=12$ ; 275–300 g on arrival; Charles River, Raleigh, NC, USA) were “intruders” in the resident–intruder defeat model of the present studies. Subjects were single-housed in wire-topped, plastic cages (48×27×20 cm) in a 12-h light/dark (08:00 h lights off), humidity- (60%) and temperature-controlled (22 °C) vivarium. Larger, adult male Long-Evans rats ( $n=6$ , 450–500 g on arrival) were housed in enclosures (48×69×50 cm) with sawdust-covered, stainless steel floors and were territorial “residents” in the defeat model. Each resident was stably housed with an adult female Wistar rat ( $n=6$ ) that had received electrocauterization of the uterine coils under isoflurane anesthesia (1%–3% in oxygen) to prevent pregnancy. Food and water were available *ad libitum* unless stated otherwise. Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1996) and the “Principles of laboratory animal care” (<http://www.nap.edu/readin-groom/bookslabrats>) and were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute. All efforts were made to minimize the number of animals used and their suffering.

### Resident-intruder social defeat procedure

Each resident ( $n=6$ ) was housed with a sexually mature Wistar female for 1 month to promote territorial behavior. The female mate was removed from the cage before placing the intruder within the enclosure. Long-Evans rats were used as residents following the precedent of Miczek and colleagues (Miczek, 1979; Tornatzky and Miczek, 1993, 1994), who developed and characterized the resident–intruder model of social defeat. Long-Evans rats are used as residents in the model because of the strain’s propensity for territoriality and dominance behavior across the lifespan (Blanchard et al., 1984). Strains that show somewhat less social agonistic behavior are used as intruders in this model; here, intruders were from the less aggressive Wistar strain (Scholtens and Van de Poll, 1987; Schuster et al., 1993), also a well-studied model in neuroscience research. To potentiate dominance behavior, residents were exposed to “training” intruders—post-pubertal, smaller (200–225 g) male Wistar rats—for 16 days before experimental studies. Residents were exposed to different training intruders twice per day, every other day, with exposure lasting until the intruder submitted or, if submission did not occur, 5 min. Intruders were removed from the home cage immediately after defeat, and females were returned to the cage. The criterion for defeat was adoption of a submissive, supine posture by the intruder rat, as defined by Miczek and colleagues (Miczek, 1979; Tornatzky and Miczek, 1993). This training reduces the mean latency by which residents later achieve submissions over experimental intruders to <90 s and thereby reduces the duration of physical conflict that residents require to attain defeats. Potential residents that injured intruders during training, did not achieve 3 consecutive days of defeat, or had mean defeat latencies >120 s were excluded from the study. Training was conducted during the animals’ dark cycle under red lighting.

For testing, each experimental intruder ( $n=6$ ) was placed inside the resident’s home cage until defeat. Upon submission, intruders were placed inside a protective wire-mesh enclosure (20×20×32 cm) that was then placed within the resident’s home cage. Intruders remained in the enclosure for 75 min to allow for Fos protein expression and to provide further psychosocial threat. The wire enclosure prevented injurious physical interactions but allowed auditory, olfactory, visual and limited physical contact (mouth/nose). Control rats ( $n=6$ ) were picked up, briefly handled and returned to their home cage for 75 min.

## Perfusion and tissue sectioning

After the 75-min post-defeat interval (within the protective wire-mesh enclosure for intruders or home cage for controls), subjects were anesthetized with an overdose of chloral hydrate (1 g/kg body weight, i.p.) and perfused transcardially with 150 ml of saline followed by 350 ml of 4% paraformaldehyde in borate buffer (pH 9.5). The whole brain was post-fixed in 25% sucrose in 4% paraformaldehyde at 4 °C for 24 h. Brains were stored at -80 °C until use. Coronal sections (25 µm) were cut on a sliding microtome and collected in a one-in-12 series. The tissue sections were stored until use at -20 °C in multiwell tissue culture plates containing cryoprotectant. The first two wells were used for immunocytochemistry. Sections from one defeated rat were not useable for counting Fos-labeled cells in the LS, MeA, ARC or BNST; sections from one control rats were not useable for the ARC or BNST.

## Immunocytochemistry

Tissue sections were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% NaBH<sub>4</sub>-KPBS solution (Sigma, St. Louis, MO, USA). Sections were incubated with Fos protein antibodies raised in rabbit (1:60,000, EMD Biosciences, San Diego, CA, USA) in KPBS with 0.4% Triton X-100 at room temperature for 1 h, followed by 4 °C for 48 h. After incubation, sections were rinsed in KPBS and incubated in biotinylated donkey anti-rabbit IgG (1:600, Vector Laboratories, Burlingame, CA, USA) in KPBS with 0.4% Triton X-100 for 1 h at room temperature, followed by a 1-h incubation at room temperature in avidin-biotin complex solution (4.5 µl of A and B each per ml of KPBS-0.4% Triton X-100; Vectastain ABC Elite Kit, Vector Laboratories). The antibody-peroxidase complex was visualized with a mixture of 3,3-diaminobenzidine (0.2 mg/ml) and 3% H<sub>2</sub>O<sub>2</sub> (0.83 µl/ml) in 0.05 M Tris buffer-saline solution. Following the staining, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. For the Fos protein/CRF<sub>2</sub> mRNA double-labeling study, brain sections were processed for Fos staining as described above, with the exception that all solutions were treated with diethylpyrocarbonate to prevent RNase contamination. Following Fos staining, brain sections were processed for *in situ* hybridization.

## *In situ* hybridization

To determine whether some cells that expressed Fos protein following defeat were CRF<sub>2</sub> receptor-expressing cells, immunocytochemistry and *in situ* hybridization were serially combined to visualize Fos protein and CRF<sub>2</sub> mRNA simultaneously. All solutions were treated with diethylpyrocarbonate to protect brain sections from RNase contamination. For *in situ* hybridization, a [<sup>33</sup>P]uridine triphosphate-labeled (PerkinElmer, Boston, MA, USA) CRF<sub>2</sub> complementary RNA (cRNA) probe was transcribed from a 460 base-pair 5'-region of the CRF<sub>2</sub> cDNA linearized with XbaI (Li et al., 2002). The riboprobes were directed against the 5' region of rat CRF<sub>2</sub> receptors, covering the sequence up to the third presumed transmembrane region (Chalmers et al., 1995). The specific activity of the probe was ~5×10<sup>5</sup> cpm/ml of hybridization buffer. Following Fos immunostaining, brain sections were washed with KPBS, treated with acetic anhydride and exposed to the CRF<sub>2</sub> cRNA probe overnight in moist chambers at 55 °C. Sections were then washed two times in 4× sodium chloride/sodium citrate (SSC) buffer, in RNase A (30 µg/ml final concentration, Sigma), one time in 2× SSC and finally in 0.1× SSC at 60 °C, and mounted on gelatin-coated slides that were then dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA). The slides were exposed for 15 days at 4 °C and developed.

## Imaging

Slides were examined using a Nikon (E600) light microscope (Lake Forest, CA, USA). The numerical apertures of the 4×, 10× and 40× lens were 0.2, 0.45 and 0.95, respectively. Four brain sections from each region of interest were analyzed, relative to bregma (anterior/

posterior), as follows: from +1.2 to 0.6 for dorsal part of the lateral septum (dLS), from +0.48 to 1.3 for BNST, from -1.80 to -3.60 for MeA, from -1.0 to -2.12 for PVN, from -2.1 to -3.30 for VMH and from -1.80 to -3.60 mm for ARH per a rat brain atlas (Paxinos and Watson, 1998). Each coordinate range was further subdivided into 600  $\mu\text{m}$  segments (two segments for PVN and VMH and three segments for dLS, BNST, MeA and ARH). One to two sections were analyzed from each 600  $\mu\text{m}$  segment for data collection. Images were captured by a digital camera (Photometrics Cool-snap CF, Tucson, AZ, USA). Fos-positive cells in captured images were analyzed and counted using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). CRF<sub>2</sub> positive cells were identified from the same regions that were analyzed for Fos immunoreactivity as clusters of silver grains, indicating [<sup>33</sup>P]uridine triphosphate-labeled CRF<sub>2</sub> cRNA probe signals, in dark field photomicrographs. The images were cropped and adjusted to balance brightness and contrast in Adobe Photo-shop 8.0 (Adobe Systems, San Jose, CA, USA) before importing the images into Canvas 8.0 (Deneba Systems, Miami, FL, USA) for assembly into plates. The brain plates were then imported into Canvas for reordering.

### Statistical analysis

For each rat, Fos-positive cells were counted bilaterally in four brain sections and averaged, with all sections from a given brain region evaluated by a single treatment-naive rater. Data were subjected to Student's *t*-test for comparisons between the control and defeated groups or Welch's *t*-test when variance significantly differed between groups. The software package used was InStat 3.0 (GraphPad, San Diego, CA, USA).

## RESULTS

### Distribution of Fos-positive cells in selected rat forebrain areas after social defeat

Intruders were rapidly defeated by residents (range: 31–126 s). An initial survey of the forebrain found that defeated rats showed more Fos-positive cells compared with control rats 75 min post-defeat in several regions in the limbic system and the hypothalamus (Table 1). In limbic structures, Fos-positive cells were observed in the dLS, MeA, and anterior cortical nuclei of the amygdala. A few Fos-positive cells were observed in the BNST of defeated subjects, but not more than in controls.

In the hypothalamus, Fos-positive cells were consistently observed in the ARH and VMH (Table 1). The supraoptic nucleus and lateral hypothalamus showed few, but slightly increased numbers of Fos-positive cells after defeat. The PVN did not show Fos-positive cells 75 min post-defeat.

### Quantification of Fos protein expression following acute social defeat

Fos-positive neurons were counted in selected brain regions 75 min after defeat. As shown in Figs. 1 and 2, compared with control rats, socially defeated rats showed significantly more Fos-positive cells in the MeA (Welch's  $t_4=2.97$ ,  $P\leq 0.05$ ) (Fig. 1D, E, F), especially the posterior part of the MeA. In the hypothalamus, defeat induced significantly more Fos-positive cells in the VMH (Welch's  $t_5=2.80$ ,  $P\leq 0.05$ ) (Fig. 2A, B, C) and ARH (Student's  $t_8=2.38$ ,  $P\leq 0.05$ ) (Fig. 2D, E, F). The majority of Fos immunostaining was evident in the ventrolateral part of the VMH and medial part of the ARH (Fig. 2B, E). No significant difference in Fos-positive cell counts was observed between control vs. defeated rats within the dLS (Fig. 1A, B, C), anterior BNST (defeated:  $7.4\pm 3.9$ , control:  $7.2\pm 2.8$  cells), posterior BNST (defeated:  $3.3\pm 2.0$ , control:  $9.5\pm 4.8$  cells), or PVN (defeated:  $8.0\pm 2.9$ , control:  $11.4\pm 5.5$  cells).

### Partial colocalization of Fos protein and CRF<sub>2</sub> mRNA in the MeA

Similar to previous reports (Van Pett et al., 2000; Li et al., 2002), high levels of CRF<sub>2</sub> receptor mRNA were observed in the intermediate part of the LS (Supplementary Fig. 1A) and dorsomedial VMH (Supplementary Fig. 1C). Moderate CRF<sub>2</sub> mRNA density was observed in the dLS (Supplementary Fig. 1A), anterior and posterior MeA (Supplementary Fig. 1B), basomedial amygdala, and mainly in the posterior region of the BNST (not shown). Low levels of CRF<sub>2</sub> mRNA were also evident in the hippocampal formation (not shown), ARH (Supplementary Fig. 1D), magno-cellular division of the PVN, and supraoptic nucleus (see Supplementary Table 1). The pattern of CRF<sub>2</sub> expression significantly overlapped with that of defeat-induced Fos expression, suggesting that social defeat might activate CRF<sub>2</sub>-positive neurons in these areas. Therefore, dual-labeling of CRF<sub>2</sub> mRNA and Fos immunoreactivity was performed to determine whether some of the Fos-positive cells in these areas were also CRF<sub>2</sub>-positive. As shown in Fig. 3D, E, and F, high degrees of colocalization of signals for Fos protein and CRF<sub>2</sub> mRNA were found in the MeA, in which 49.7% of Fos-positive cells expressed CRF<sub>2</sub> mRNA. Only moderate numbers (12.7%) of Fos-positive cells in the dLS were found to colocalize with CRF<sub>2</sub> mRNA (Fig. 3A, B, C). Negligible or no colocalization of CRF<sub>2</sub> mRNA with Fos protein was seen in the VMH (Fig. 3G, H, I; 14.2%) or ARH (Fig. 3J, K, L; 0.0%), respectively.

### DISCUSSION

The present results indicate that CRF<sub>2</sub> receptor-expressing neurons in the MeA are involved in the response to acute defeat, a model of antagonistic social stress relevant to several affective and reproductive disorders (Luiten et al., 1985; Kollack-Walker and Newman, 1995; Martinez et al., 1998; Blanchard et al., 2001; Dominguez et al., 2001). Confirming previous findings that used *c-fos* mRNA or Fos protein expression as markers of neuronal activation, several brain areas were activated following social defeat (Cullinan et al., 1995). Fos protein expression 75 min post-defeat was evident in the MeA, LS, ARH, and VMH, structures which all express high levels of urocortin-CRF<sub>2</sub> system molecules (Van Pett et al., 2000; Li et al., 2002; reviewed in Fekete and Zorrilla, 2007). Combined *in situ* hybridization/immunocytochemistry demonstrated that a large proportion of Fos immunoreactive cells in the MeA, but not in the dLS, ARH, or VMH, were also positive for CRF<sub>2</sub> mRNA.

The MeA, which receives input from the vomeronasal and olfactory systems via the cortical nucleus of the amygdala (Newman, 1999), projects to brain regions involved in stress- and sociosexual reproduction-related functions, including other amygdala divisions, extended amygdala (e.g. BNST), hippocampus (Canteras et al., 1995), and hypothalamic nuclei that modulate neuroendocrine systems and sexual and agonist behaviors (e.g. medial preoptic area, VMH and ventral premammillary nucleus; Paredes and Baum, 1997). Accordingly, the MeA has a role in several social behavioral responses, including behavioral arousal (Kollack-Walker and Newman, 1995), acquisition and expression of conditioned defeat (Markham and Huhman, 2008), social learning and memory processes (Luiten et al., 1985), and fear and anxiety-like behavior (Chen et al., 2006). Relevant to defeat, MeA lesions prevent males from avoiding conspecifics that recently defeated them (Luiten et al., 1985) and severely impair mating, parental behavior (Sheehan et al., 2001), and other reproductive functions (Dominguez et al., 2001). Lesions of the MeA also reduce defensive responses to predator odor and escape responses to noxious stimuli (Blanchard et al., 2005). Additionally, the MeA is a proposed regulator of hypothalamic–pituitary–adrenal axis activity (Dayas et al., 1999), with MeA stimulation increasing plasma glucocorticoid and adrenocorticotrophic hormone levels (Herman et al., 1996). The results raise the hypothesis that CRF<sub>2</sub>-synthesizing neurons in the MeA may participate in consequences of, or counter-regulatory responses to, defeat.

As a structure subserving sociosexual and agonistic behavior, the MeA is structurally and functionally sexually dimorphic (Cooke and Woolley, 2005; Cooke, 2006), with the posterodorsal MeA ~50% larger in males than in females (Hines et al., 1992) in relation to circulating androgen levels. The MeA has many neurons that contain high levels of estrogen and/or testosterone and their receptors (Greco et al., 1998), a finding specifically true of CRF<sub>2</sub>-expressing neurons in the posterior MeA (Van Pett et al., 2000). Stressors, including defeat, suppress testosterone secretion in both rodents and primates, including man (Armario and Castellanos, 1984; Sapolsky, 1985; Schultheiss et al., 2005). Perhaps the defeat-induced changes in CRF<sub>2</sub>-expressing MeA circuitry are associated with the suppression of circulating testosterone.

A candidate natural ligand for MeA CRF<sub>2</sub> receptors is urocortin 3. Many urocortin 3-positive neurons and fibers are present in the MeA, where its expression is increased by stress (Jamieson et al., 2006), and in the cortical nucleus of the amygdala, from which the MeA receives extensive projections (Li et al., 2002). Several studies suggest an anxiolytic-like role for CRF<sub>2</sub> receptors. For example, administration of urocortin into the adjacent CeA reduced anxiety-like behavior during ethanol withdrawal (Valdez et al., 2004). I.c.v. urocortin 3 administration also reduced anxiety-like behavior in some, but not all, rodent models of anxiety-like behavior (Zhao et al., 2007). Some, but not all (Coste et al., 2000), studies of CRF<sub>2</sub> knockout mice observed an anxiogenic-like phenotype of mice following stressor exposure (e.g. in the elevated plus maze [Bale et al., 2000; Kishimoto et al., 2000], emergence, open field [Bale et al., 2000], or light/dark box tests [Henry et al., 2006]). Conversely, some findings (Pelleymounter et al., 2004) support an alternate hypothesis that CRF<sub>2</sub> receptor activation has anxiogenic-like effects, with lateral septal (Henry et al., 2006) or dorsal raphe (Hammack et al., 2003) CRF<sub>2</sub> activation promoting defensive responses to uncontrollable stressors. However, to our knowledge, no studies have evaluated the behavioral effects of intra-MeA CRF<sub>2</sub> agonist infusion. Thus, the specific anxiety-related or other behavioral role of CRF<sub>2</sub> receptors in the MeA remains to be determined.

Defeat also increased the number of Fos-positive cells in the VMH, another androgen receptor-expressing nucleus that is larger in males than in females (Dugger et al., 2007). However, induction of Fos immunoreactivity was observed mostly in the ventrolateral VMH, whereas CRF<sub>2</sub> mRNA predominated in the dorsomedial VMH, similar to previous studies (Lovenberg et al., 1995). Functional segregation of the dorsomedial vs. ventrolateral VMH has been proposed previously. For example, in males, the dorsomedial VMH is critical for ultrasonic vocalization and scent marking behaviors (Harding and McGinnis, 2005), whereas the ventrolateral VMH subserves mounting behavior (Pfaff and Sakuma, 1979). The dorsomedial VMH also putatively differentially subserves energy homeostasis (Flanagan-Cato, 2003), defensive aggression (Canteras et al., 1994; Canteras, 2002), and innate affective reactions to pain (Borszcz, 2006). The two VMH subregions receive innervation from topographically distinct aspects of the MeA (Canteras et al., 1995). The ventrolateral VMH also more closely innervates other hypothalamic regions that express gonadal steroid hormone receptors, including the medial preoptic, tuberal, and ventral premammillary nuclei, whereas the dorsomedial VMH differentially projects to anterior hypothalamic and dorsal premammillary nuclei (Canteras et al., 1994). Finally, the androgen-dependent sexual dimorphism of VMH volume is seen selectively in the ventrolateral, but not dorsomedial, subdivision (Dugger et al., 2007). The results suggest that defeat-induced Fos protein expression in the VMH may involve a subpopulation of cells distinct from those that express CRF<sub>2</sub> receptors.

Defeat also induced Fos protein expression in a sub-population of cells in the medial ARH distinct from those that synthesize CRF<sub>2</sub> receptors. Neurons in the medial ARH have a role in regulatory inhibition of the hypothalamic-pituitary-adrenal axis (Kollack-Walker et al., 1997) and of prolactin secretion via tuberoinfundibular dopamine projections to the median

eminence (Freeman et al., 2000). Lesions of the ARH increased basal glucocorticoid levels and enhanced adrenocortical activity in response to stress (Cullinan et al., 1995). A separate population of medio-basal ARH neurons has recognized roles in energy homeostasis and reproductive functions via parallel ascending projections of orexigenic neuropeptide Y/agouti-related protein-expressing neurons and anorexigenic proopiomelanocortin/cocaine and amphetamine-regulated transcript-expressing neurons to other hypothalamic nuclei (Hill et al., 2008). Arcuate neurons also are implicated in opioid-mediated stress-induced analgesia (Wang et al., 1990) following defeat (Miczek et al., 1985). Identification of the phenotypes of Fos-positive cells in the ARH may provide further insight into the physiological role and function of ARH neurons in social defeat stress.

The absence of PVN or BNST Fos protein expression following defeat stress was somewhat unexpected (Herman et al., 1996; Nail-Boucherie et al., 1998). The time course of analysis may be relevant, with PVN and BNST Fos expression perhaps occurring transiently and earlier in time. Indeed, in separate studies, increased Fos protein expression was observed in both the PVN and BNST 30 min post-defeat (ÉM Fekete, Y Zhao, C Li, V Sabino, WW Vale, EP Zorrilla, unpublished observations). Nonetheless, the present findings are consistent with reports that hypothalamic–pituitary–adrenal axis activation did not require PVN Fos expression (Brown and Sawchenko, 1997; Figueiredo et al., 2003) and that *c-fos* mRNA was not elevated in the LS, PVN, or BNST following defeat, while it was seen after foot shock or restraint (Funk et al., 2006). The functional significance of early BNST activation after defeat is similarly unclear. On the one hand, the BNST has been associated with fear-related behavior (Lee and Davis, 1997; Onaka and Yagi, 1998), and injection of antisauvagine-30 (a preferential CRF<sub>2</sub> antagonist) into the BNST reduced conditioned defeat behavior (Cooper and Huhman, 2005). On the other hand, the number of threats received by an intruder correlated negatively with *c-Fos* expression in the BNST in a previous study (Martinez et al., 1998).

Importantly, *c-fos* activation does not provide a complete map of all neurons activated during a given stimulus (Robertson et al., 1989). Some activated neurons that express Fos protein may lead to downstream inhibition, and Fos protein is not known as a good marker of inhibition. Thus, induction of Fos immunoreactivity provides positive identification of brain areas activated during social conflict, but the absence of such labeling does not necessarily equal lack of involvement. Studies with other functional markers may help further delineate brain activation and inhibition patterns associated with social defeat. The present findings show, however, that CRF<sub>2</sub>-synthesizing neurons in the posterior MeA are part of the neural response to social defeat.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

### ARH

arcuate nucleus of the hypothalamus



<b>BNST</b>	bed nucleus of the stria terminalis
<b>CeA</b>	central nucleus of the amygdala
<b>CRF</b>	corticotropin-releasing factor
<b>CRF<sub>1</sub></b>	type 1 corticotropin-releasing factor receptor
<b>CRF<sub>2</sub></b>	type 2 corticotropin-releasing factor receptor
<b>cRNA</b>	complementary RNA
<b>dLS</b>	dorsal part of the lateral septum
<b>KPBS</b>	potassium phosphate-buffered saline
<b>LS</b>	lateral septum
<b>MeA</b>	medial nucleus of the amygdala
<b>PVN</b>	paraventricular nucleus of the hypothalamus
<b>SSC</b>	sodium chloride/sodium citrate
<b>VMH</b>	ventromedial hypothalamus

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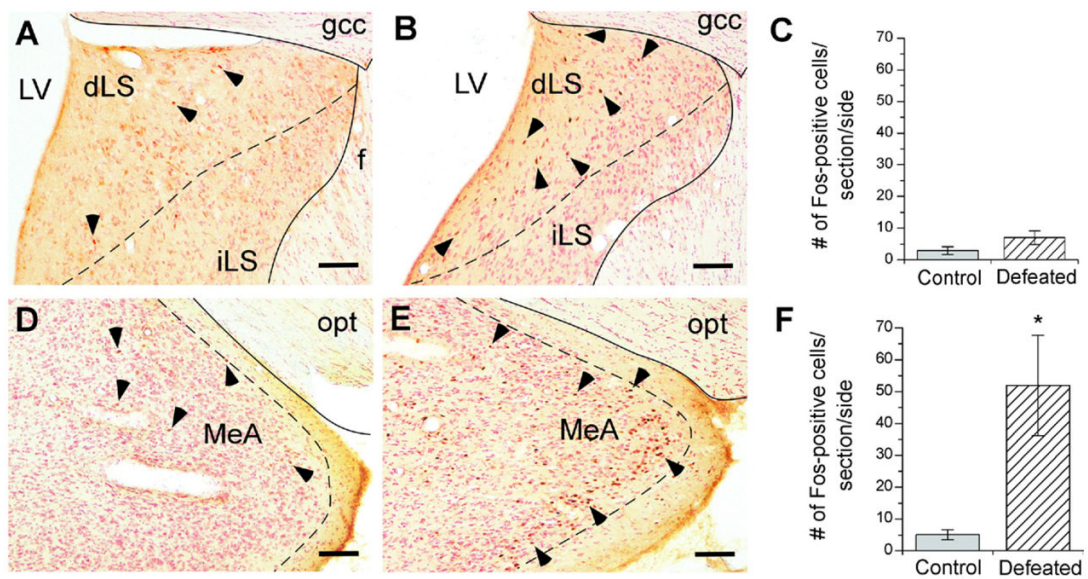
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## Appendix

### APPENDIX

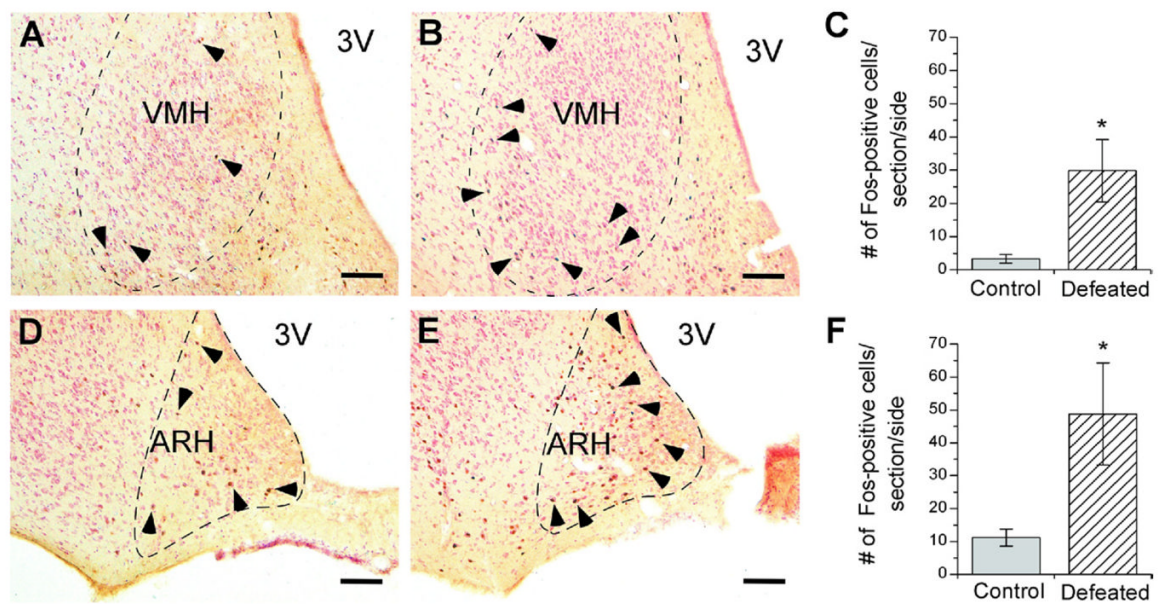
#### Supplementary data

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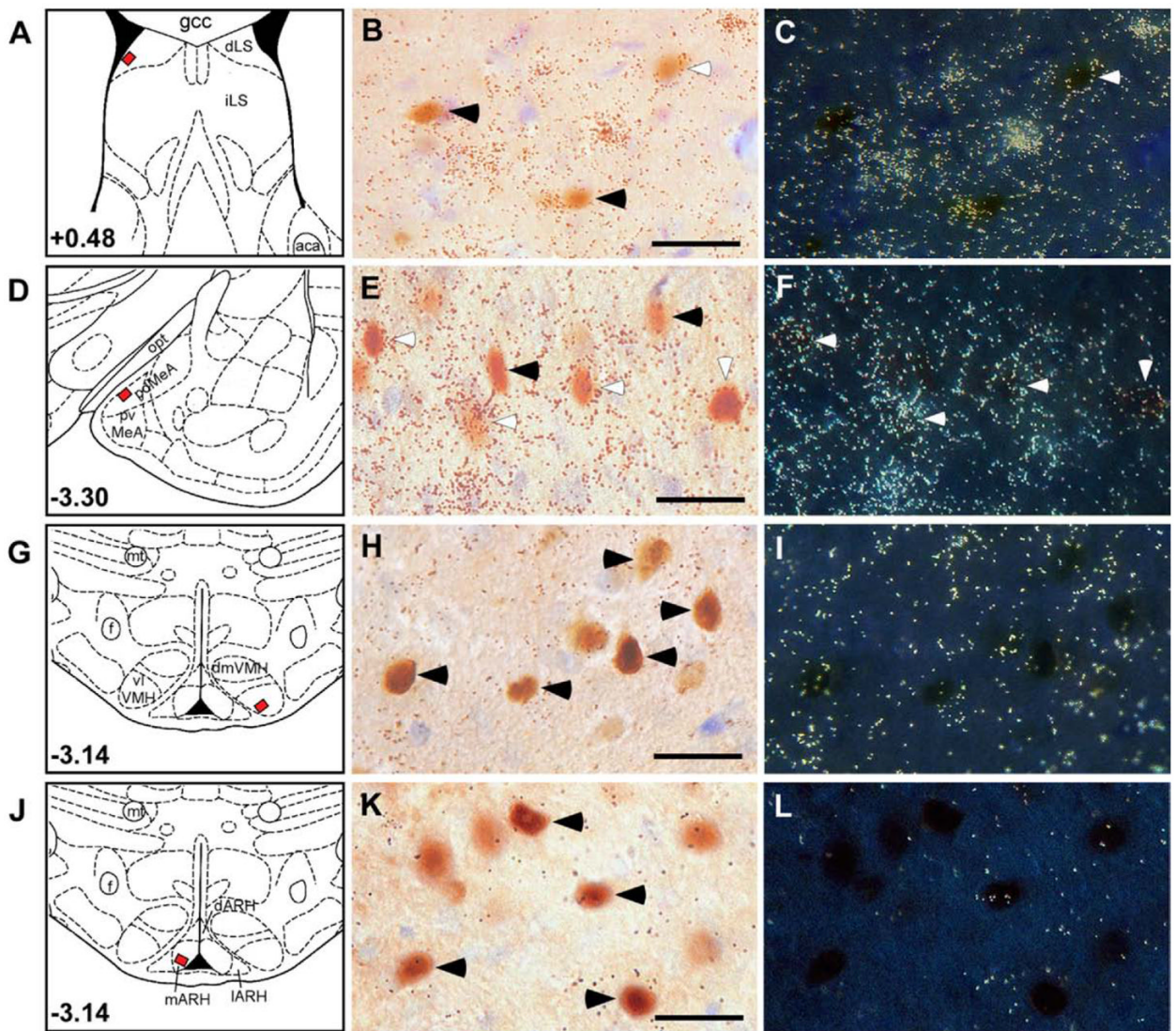


**Fig. 1.**

Fos immunoreactivity in the dorsal lateral septum (A, B), and medial amygdala (D, E) 75 min after an acute defeat (B,  $n=5$ ; E,  $n=5$ ) or control (A,  $n=6$ ; D,  $n=6$ ) procedure. Dorsal LS and MeA panels are in the range of  $-0.26$  to  $-0.30$  and  $-2.80$  to  $-3.14$  mm from bregma, respectively. Bar graphs represent the mean ( $\pm$ SEM) number of Fos-positive cells per section per side in the dorsal lateral septum (C) and medial nucleus of the amygdala (F) after control (grey bars) or acute defeat (stripped bars) conditions. Selected Fos-positive cells are indicated with a black arrowhead. Symbols indicate significant difference between control and defeated rats: \*  $P < 0.05$ . Scale bar =  $100 \mu\text{m}$ . LV, lateral ventricle; gcc, genu of corpus callosum; iLS, intermediate part of the lateral septum; opt, optic tract.



**Fig. 2.** Fos immunoreactivity in the ventromedial (A, B) and arcuate nuclei of the hypothalamus (D, E) 75 min after an acute defeat (B,  $n=6$ ; E,  $n=5$ ) or control (A,  $n=6$ ; D,  $n=5$ ) procedure. VMH and ARH panels are in the range of  $-2.30$  to  $-3.14$  and  $-2.30$  to  $-2.56$  mm (anterior/posterior) from bregma, respectively. Bar graphs represent the mean ( $\pm$ SEM) number of Fos-positive cells per section per side in the VMH (C) and the arcuate nucleus (F) after control (grey bars) or acute defeat (striped bars) conditions. Selected Fos-positive cells are indicated with a black arrowhead. Symbols indicate significant differences between control and defeated rats: \*  $P < 0.05$ . Scale bar =  $100 \mu\text{m}$ . 3V, Third ventricle.



**Fig. 3.**

Acute defeat induces Fos protein expression at sites of CRF<sub>2</sub> receptor mRNA expression in the rat brain. Schematic panels showing the bregma level (A, D, G, J) with the small black square filled with red within them indicating the region photographed at higher magnification and shown in B, C, E, F, H, I, K, and L. Brightfield photomicrographs of sections through the dorsal lateral septal (B), posterior medial amygdala (E), ventrolateral ventromedial hypothalamus (H), and medial arcuate nuclei (K) of the hypothalamus showing cells with acute defeat-induced nuclear Fos immunoreactivity and highlighted with silver grain clusters (dark dots), indicating a positive CRF<sub>2</sub> receptor mRNA signal (white arrowheads). Darkfield photomicrographs of adjacent sections (C, F, I, L) compare the distribution of CRF<sub>2</sub> mRNA (bright small dots, as clusters of silver grains, indicating [<sup>33</sup>P]uridine triphosphate-labeled CRF<sub>2</sub> cRNA probe signals). Cells labeled singly for Fos (black arrowheads) or the CRF<sub>2</sub> transcript colocalizing with Fos-labeled cells (white arrowhead) are shown for contrast. gcc, Genu of corpus callosum; iLS, intermediate lateral septum; aca, anterior part of anterior commissure; opt, optic tract; pd or pvMeA, posterodorsal or posteromedial medial amygdala; mt, mammillothalamic tract; f, fornix; vl or dmVMH, ventrolateral or dorsomedial



ventromedial nuclei of hypothalamus; d or m or IARH, dorsal or medial or lateral arcuate nuclei of the hypothalamus. Scale bar=50  $\mu$ m.

**Table 1**  
Semi-quantitative distribution of Fos-positive cells in selected areas in rat brain 75 min after acute defeat relative to control

Forebrain region	Density of Fos-immunoreactive cells
Septum	
Dorsolateral nucleus	+
Intermediate nucleus medial nucleus	-/+
	-/+
Amygdala	
Anterior cortical nucleus	+
Posterior cortical nucleus	-/+
Basolateral nucleus	-/+
Basomedial nucleus	-/+
Central nucleus	-/+
Medial nucleus	+++
BNST	
Rostral region	-
Posterior region	-
Hypothalamus	
Arcuate nucleus	++/+++
PVN	
Parvocellular	-
Magnocellular	-/+
Supraoptic nucleus	+
VMH	++/+++
Lateral hypothalamus	+

Semi-quantitative ratings reflect the density of Fos-positive cells relative to controls, with (-) representing control-like levels of Fos-labeled cells, and plus symbols indicating slightly (+), moderately (++), or highly elevated (+++) numbers of Fos-labeled cells compared with controls in a given cell group or field. The (-/+) symbol indicates control-like or slightly elevated numbers of Fos-labeled cells, varying across intruders.