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Activation of corticotropin releasing factor-containing neurons in the rat central amygdala and bed nucleus of the stria terminalis following exposure to two different anxiogenic stressors

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Abstract

Rats exposed to the odor of a predator or to the elevated plus maze (EPM) express unique unconditioned fear behaviors. The extended amygdala has previously been demonstrated to mediate the response to both predator odor and the EPM. We seek to determine if divergent amygdalar microcircuits are associated with the different behavioral responses. The current experiments compared activation of corticotropin-releasing factor (CRF)-containing neuronal populations in the central amygdala and bed nucleus of the stria terminalis (BNST) of rats exposed to either the EPM (5 minutes) versus home cage controls, or predator ferret odor versus butyric acid, or no odor (30 minutes). Sections of the brains were prepared for dual-labeled immunohistochemistry and counts of c-Fos co-localized with CRF were made in the centrolateral and centromedial amygdala (CLA and CMA) as well as the dorsolateral (dl), dorsomedial (dm), and ventral (v) BNST. Ferret odor-exposed rats displayed an increase in duration and a decrease in latency of defensive burying versus control rats. Exposure to both predator stress and EPM induced neuronal activation in the BNST, but not the central amygdala, and similar levels of neuronal activation were seen in both the high and low anxiety groups in the BNST after EPM exposure. Dual-labeled immunohistochemistry showed a significant increase in the percentage of CRF/c-Fos co-localization in the vBNST of ferret odor-exposed rats compared to control and butyric acid-exposed groups as well as EPM-exposed rats compared to home cage controls. In addition, an increase in the percentage of CRF-containing neurons co-localized with c-Fos was observed in the dmBNST after EPM exposure. No changes in co-localization of CRF with c-Fos was observed with these treatments in either the CLA or CMA. These results suggest that predator odor and EPM exposure activates CRF neurons in the BNST to a much greater extent than CRF

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neurons of the central amygdala, and indicates unconditioned anxiogenic stimuli may activate unique anatomical circuits in the extended amygdala.

Keywords

predator odor; EPM; CRF; amygdala; BNST

1. Introduction

The behavioral response to different unconditioned fear stimuli can vary greatly. Rodents exposed to a predator threat often exhibit behaviors ranging from an escape or burying response to freezing, depending on the testing conditions [1–4]. In contrast, rodents in a novel, aversive environment, such as an elevated plus maze (EPM), often exhibit avoidance of open arms [5]. Despite the diversity of unconditioned fear behaviors to different stimuli these responses are often presumed to be mediated by similar neural circuits that include the extended amygdala.

The extended central amygdala is comprised of numerous subnuclei which extend from the anterior portion of the BNST to the posterior portion of the central amygdala [6–11]. Within the extended amygdala are numerous neuronal populations which can be differentiated based on their neuropeptide-containing phenotype [10, 12]. The extended amygdala mediates the behavioral response to various unconditioned and conditioned stimuli [13–16]. However, the role of microcircuits of the extended amygdala that are involved in the different behavioral responses to these stimuli needs clarification. In the central amygdala and BNST, nearly all neurons are inhibitory (GABAergic) including those which contain the neuropeptide corticotropin-releasing factor (CRF) [17, 18]. CRF-containing neurons in these regions predominantly project outside their subdivision to downstream brain regions [19]. In numerous species, CRF mediates threat recognition responses [20, 21]. This has been demonstrated with pharmacological intervention of the CRF response to both predator odor [22] and the EPM [23]. We have demonstrated that the pattern of activation of phenotypically-distinct neurons in the central amygdala is different following exposure to noxious odor, predator odor, or the elevated plus maze [1, 2]. A recent study by De Francesco et al. [24] demonstrated increased co-localization of the immediate early gene *c-Fos* in CRF neurons of the central amygdala following acute social defeat stress. However, no studies to date have directly compared the activation of CRF-containing neurons in the central amygdala and BNST following predator odor and EPM exposure.

In this study, we combined behavioral analysis with dual-label immunohistochemistry to directly compare the pattern of activation of CRF-containing neuronal populations of the central amygdala and BNST following exposure to predator odor and the EPM. We hypothesize that CRF-containing populations in the central amygdala and BNST will show distinct patterns of activation based on differing behavioral responses to predator odor exposure and the EPM.

2. Experimental Procedures

2.1 Animals

Male Long-Evans rats (Harlan Laboratories, Indianapolis, IN), weighing approximately 250–300g were single-housed in an environmentally controlled animal facility on a 12:12 h light: dark cycle with lights on at 0700 hours. Rat chow and water were available *ad libitum*. All experiments were conducted during the light phase, beginning at least 2 hours after light phase onset and concluding at least 2 hours before transition to the dark phase. Animals were housed in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Animal care and use procedures were carried out in accordance with protocols written under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

2.2 Predator Odor Exposure

The defensive burying test was performed as described previously [1, 2, 25]. Rats were habituated to the novel arena for 2 consecutive days prior to testing. The habituation phase lasted for 30 minutes and was recorded onto videotape. On the test day, rats were exposed to either a piece of ferret-scented towel, an untreated piece of gauze or a piece of gauze treated with butyric acid as a noxious, but non-threatening, odor control, and behavior was recorded onto videotape for a duration of 30 minutes. A Plexiglas chamber (45 × 30 × 44 cm) filled up to a depth of 5 cm with fresh pine bedding with either a piece of untreated gauze (control), gauze with 100 µL butyric acid, or a piece of ferret-scented towel (5 cm²) placed 2 cm above bedding, was used for this analysis. The ferret towels were a generous donation from Dr. John Hines (Yale University) that he prepared by placing a clean (unscented) towel in the cage with three ferrets (Pumpkin, Zooey, Valentine) for at least one month. The ferret-scented towels were shipped frozen, cut into 5 cm² pieces, and stored at –80°C until use. Each towel piece was used only once. Immediately after the 30 minute odor exposure trial, rats were placed back into the home cage for 1.5 hours.

2.2.1 Predator odor-exposure behavior assessment—A trained rater blind to the experimental conditions assessed behavior using the Observer[®] XT 9.0 software package (Noldus Information Technology, Wageningen, The Netherlands). Behaviors were measured from videotapes for the duration of the 30 min test. Aversive behaviors measured included latency to bury, duration of burying (defined as spraying bedding toward the gauze/towel), duration of tunneling (defined as the act of burrowing under the bedding), duration of freezing behavior (defined as the cessation of all movement except that needed for respiration), the number of flat-backed approaches (defined as elongation of the body while moving slowly to the source odor) and the number of escape attempts (defined as jumping towards the top of the chamber) to the ferret-scented cloth. In the absence of burying, animals were assigned a latency to bury of 30 min and duration of burying of zero. In addition, general exploratory/locomotor activity was measured including the duration of walking, grooming, sniffing, and the number and duration of rears (defined as number of times the animal lifted both forelimbs).

2.3 Elevated Plus Maze

The elevated plus maze (EPM) was comprised of two open ($56 \times 10 \times 1$ cm) and two closed arms ($56 \times 10 \times 40$ cm) made of black Plexiglas. The maze was elevated 50 cm above the floor. One week after arrival in the colony, animals receiving EPM exposure (N=12) were individually transported into the testing room. Rats were placed immediately into the center of the EPM facing an open arm and their behavior recorded for five minutes. Immediately after testing, rats were returned to their home cages. Controls (N=6) remained in their home cages without testing. The maze was cleaned with a 7% ammonium hydroxide solution between subjects.

Analysis of time spent in open and closed arms, entries into open and closed arms, and overall distance traveled was conducted using the Ethovision software program (Noldus Information Technology, The Netherlands). Zones for each arm were drawn such that all four paws must enter the arm in order for the rat to be considered in an open or closed arm of the maze. Percent open arm entries was calculated as the number of open arm entries divided by total entries (open arm entries plus closed arm entries). Percent open arm time was calculated as the amount of time spent in open arms divided by the total amount of time spent in both open and closed arms. Distance traveled (cm) was used as the measure of activity. Animals were divided into high and low anxiety groups for analysis based on a median split of open arm time in the EPM, for comparison to home cage controls.

2.4 Perfusion and immunohistochemistry

Two hours from the beginning of the odor exposure and EPM tests, rats were placed under deep isoflurane anesthesia and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The two hour time point was chosen because that is within the 1–3 hour post-exposure time frame in which c-Fos is maximally expressed [26] and to maintain consistency with our previous studies [1, 2]. The brains were removed, post-fixed overnight and transferred to 30% sucrose solution in 0.1 M phosphate buffer for cryoprotection. Serial coronal sections throughout the rostral – caudal extent of the CEA and BNST were cut on a microtome at 50 microns and stored at -20°C in anti-freezing solution (30% sucrose and 30% ethylene glycol in 0.1M phosphate buffer) until they were processed for immunohistochemistry. Dual-labeled immunohistochemistry (c-Fos and CRF) was performed on multiple sections in both the anterior and posterior portions of the central amygdala (from Bregma: -2.12 mm to -2.80 mm) and BNST (from Bregma: $+0.20$ mm to -0.4 mm) (Figure 1). Immunohistochemistry from the groups of control and EPM-exposed rats or the control, butyric acid-exposed and ferret odor-exposed rats were processed at the same time. Briefly, all sections were initially incubated with goat (1:1000) anti-c-Fos (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody in Tris-buffered saline (TBS) with 4% normal horse serum and 0.2% Triton X-100 for 48 h at 4°C . This was followed by incubation with a biotinylated donkey anti-goat secondary antibody (1:1000) for 1.5 h at room temperature (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and then horseradish peroxidase-conjugated streptavidin for 1 h at room temperature (1:1600; Jackson ImmunoResearch Laboratories Inc.). c-Fos immunoreactivity was visualized by developing the sections in a nickel-cobalt intensified diaminobenzidine solution with 0.3 % hydrogen peroxide, yielding a blue-black reaction

product confined to the nucleus of c-Fos-positive cells. The immunolabeling continued to determine co-localization of c-Fos and CRF. After c-Fos staining, sections were incubated in rabbit primary antisera directed against CRF [rabbit anti-CRF primary antibody (antiserum #PBL rC70) was a generous gift from the laboratory of Dr. Wylie Vale (Salk Institute, La Jolla, CA) and was used at a 1:4000 dilution] at 4°C for 48 h, followed by incubation with unlabeled donkey anti-rabbit secondary antibodies (1:100; Jackson ImmunoResearch Laboratories Inc.) for 2 hours at room temperature and finally with rabbit peroxidase-anti-peroxidase (1:250; Covance, Florida). Immunoreactivity for CRF was visualized by developing the sections in plain diaminobenzidine solution with 3.0 % hydrogen peroxide, yielding a brown reaction product confined to the cytoplasm of immunoreactive cells with these markers. After standard mounting, dehydration and coverslipping procedures, total numbers of neurons labeled with c-Fos or CRF were counted, as well as numbers of dual-labeled neurons showing c-Fos positive nuclei and cytoplasm stained with CRF using a Nikon E600 microscope at 20x magnification and counts were obtained using the Neurolucida software program (Version 7, MBF Bioscience, 2006). The centrolateral and centromedial subdivisions of the central amygdala in the left and right hemispheres (Figure 1A) were counted as well as the dorsomedial, dorsolateral, and ventral BNST (Figure 1B). This allowed analysis of hemispheric and rostro-caudal differences in activation (see analysis description below). No hemispheric differences were seen, so data from left and right regions were combined to enhance consistency and numbers of neurons counted per region. Neuronal counts were done by investigators blinded to the treatment condition of the sections. The total number of neurons from single and double-stained tissue was divided by the area of the subregion to determine the density of the immunoreactive cells per mm². Photomicrographic images were captured with a Photometrics CoolSnap digital camera (Roper Scientific, Trenton, NJ, USA) linked to a computer equipped with IPLab software using a Nikon E600 microscope. Images were collected and imported into Adobe Photoshop where minor adjustments for contrast and brightness were made.

2.5 Data analysis

Animals exposed to the EPM were divided into two groups (N=6; Low anxiety and high anxiety) based on a median split of open arm time as previously described (Primeaux et al. 2005, 2006), and behavioral endpoints were compared using a t-test. The distribution of all the behavioral and immunohistochemistry data sets following odor exposure were tested for normality using Shapiro-Wilk's. For normally distributed data (Shapiro-Wilk's normality test $P > 0.05$), behavioral endpoints in the three groups for odor exposure (control, butyric acid, ferret odor) were compared using a one-way analysis of variance (ANOVA) for behavioral parameters. The source of significant ($P < 0.05$) main effects was determined by post-hoc Student-Newman-Keuls (SNK) comparisons. For data which was not normally distributed (Shapiro-Wilk's normality test $P > 0.05$), Kruskal-Wallis test was performed to determine an overall effect followed by Dunn's multiple comparisons test for between group differences.

Immunohistochemistry data were expressed both as a percentage of c-Fos-positive neurons with CRF [(total number of double-labeled neurons / total number of c-Fos-labeled neurons) * 100] and the percentage of CRF-labeled neurons with c-Fos-positive nuclei [(total

number of double-labeled neurons / total number of CRF-labeled neurons) * 100]] in each subdivision of the central amygdala and BNST. Average cell counts (density) for single-labeled and double-labeled neurons with each marker were also compared. Cell counts were added from the left and right hemispheres at both anterior and posterior levels because no differences in hemispheric and rostro-caudal neuronal activation were measured (data not shown). If any of the left/right, anterior/posterior portions of central amygdala and BNST were damaged the counts from that subject were not included in the final data set. Predator odor groups and EPM groups were measured as separate groups with distinct one-way ANOVAs. The source of significant ($P < 0.05$) main effects was determined by post-hoc Student-Newman-Keuls (SNK) comparisons. Data were analyzed using GraphPad Prism software (v. 5.03; La Jolla, CA) with significance level set at $\alpha = 0.05$.

3. Results

3.1 Exposure to ferret odor elicits fear behaviors

Rats expressed innate fear-related behaviors upon initial exposure to ferret odor. Kruskal-Wallis analysis of variance (ANOVA) revealed a significant effect of odor exposure on the duration of burying (Kruskal-Wallis statistic = 15.55; $P < 0.0001$) (Figure 2A), latency to begin burying (Kruskal-Wallis statistic = 11.42; $P = 0.0009$) (Figure 2B), and the total number of escape attempts (Kruskal-Wallis statistic = 10.08; $P = 0.0030$) (Figure 2C). In addition, Dunn's multiple comparison tests revealed that exposure to ferret odor resulted in the expression of robust innate aversive behaviors as indicated by an increase in the total duration of defensive burying ($P < 0.0001$) compared to no odor control rats, a decrease in the latency to bury compared to control ($P < 0.01$) and butyric acid-exposed ($P < 0.05$) rats, and a significant ($P < 0.01$) increase in total number of escape attempts compared to butyric acid-exposed rats. No overall effect of odor exposure was measured on the duration of tunneling, freezing, walking, number of flat-back approaches, and the total number or duration of rears (data not shown).

3.2. Behavior of low and high anxiety rats after exposure to the EPM

Animals exposed to novelty, such as the EPM, demonstrate individual differences in anxiety-related behavioral responses to these tasks [27–29]. Therefore, animals were divided into high and low anxiety groups based on a median split of the percent time spent in the open arms. This resulted in significant difference in percent open arm time ($t_{10} = 2.258$; $P = 0.0475$, Figure 3A) and percent open arm entries ($t_{10} = 3.384$; $P = 0.007$, Figure 3B) between the groups. There were no differences in distance traveled ($t_{10} = 1.071$; $P = 0.3093$, see Table 1). Rats spent an average of $13 \pm 6\%$ (range of 3 to 35%; low anxiety) and $0 \pm 0\%$ (range of 0 to 1%; high anxiety) in the open arms of the EPM (Table 1).

3.3. Neither odor nor EPM exposure altered CRF-positive neuronal activation in the central amygdala

In the CEA, no significant effect of either odor exposure (CLA: $F_{2,21} = 1.658$; $P = 0.21$. CMA: $F_{2,21} = 2.72$; $P = 0.092$; Table 2) or EPM exposure (CLA: $F_{2,17} = 0.82$; $P = 0.46$. CMA: $F_{2,17} = 0.93$; $P = 0.41$) (Table 2) was seen on the density of c-Fos, suggesting neither predator stress or novelty stress induced significant neuronal activation in this region. In addition,

one-way ANOVA revealed no significant effect of either odor exposure (CLA: $F_{2,19}=1.104$; $P=0.352$. CMA: $F_{2,19}=1.385$; $P=0.2744$) (Figure 4A) or EPM exposure (CLA: $F_{2,13}=1.007$; $P=0.392$. CMA: $F_{2,13}=0.5254$; $P=0.6034$) (Figure 4B) on the percentage of co-localization of CRF with c-Fos in any subdivision of the CEA. Similarly, no effect odor or EPM exposure on c-Fos density, CRF density, density of double-labeled CRF/c-Fos neurons, or percentage of c-Fos neurons co-localized with CRF was observed (Table 2).

3.4. Different activation patterns of CRF-positive neurons in the BNST following odor or EPM exposure

The BNST is crucial for the expression of unconditioned anxiety [16, 30]. Here, we demonstrate that noxious odor, predator odor and EPM-exposure differentially modify activation of CRF neurons of the BNST. Predator odor exposure induced neuronal activation in the BNST, and ANOVA indicated a significant main effect of odor treatment group on c-Fos density in the dorsomedial BNST (dmBNST, $F_{2,21}=6.548$; $P<0.007$, Table 3) and ventral BNST (vBNST; $F_{2,21}=16.53$; $P<0.0001$, Table 3), but not the dorsolateral BNST (dlBNST; $F_{2,21}=1.675$; $P=0.21$). One-way ANOVA also revealed a significant effect of odor exposure on the percentage of CRF neurons co-localized with c-Fos in the ventral portion of the BNST (vBNST; $F_{2,18}=22.85$; $P<0.0001$) (Figure 5A). Post-hoc test revealed that exposure to butyric acid resulted in a reduction in co-localization of CRF with c-Fos in the vBNST ($P<0.05$) compared to controls, while predator odor exposure resulted in a significant increase in co-localization of CRF with c-Fos in the vBNST ($P<0.001$) compared to butyric acid- and non-odor-exposed rats (Figure 5A).

Exposure to the EPM similarly induced neuronal activation in all the subregions of the BNST, including the dlBNST. As seen in Table 3, a significant main effect of EPM exposure on c-Fos density was seen in the dmBNST ($F_{2,17}=20.08$; $P<0.0001$), vBNST ($F_{2,17}=8.98$; $P<0.003$), and the dlBNST ($F_{2,17}=3.710$; $P=0.049$). Post-hoc analysis indicated that both high and low anxiety groups differed from home cage control groups after EPM exposure but there was no difference between high and low anxiety groups in the level of neuronal activation in the BNST. Similar to odor exposure, EPM exposure also significantly increased the percentage of CRF neurons co-localized with c-Fos in the vBNST ($F_{2,13}=5.375$; $P=0.0199$) (Figure 5B). In addition, there was also a significant effect of EPM exposure in the dorsomedial portion of the BNST (dmBNST; $F_{2,13}=8.754$; $P=0.0039$) (Figure 5B). Post-hoc analysis demonstrated a significant co-localization of CRF with c-Fos in the vBNST ($P<0.05$) and dmBNST ($P<0.01$) of both low and high anxiety rats compared to home cage controls (Figure 5B). No significant effects of either predator odor or EPM exposure on the percentage of c-Fos positive neurons containing CRF, or the density of CRF neurons were observed in any subdivision of the BNST (Table 3).

4. Discussion

We compared the pattern of activation of CRF-positive neuronal populations in the central amygdala and BNST of rats following exposure to noxious odor (butyric acid), predator odor (ferret), and, in separate groups of rats, exposure to the EPM. As we reported previously [1], our results indicate that exposure of rats to a predator odor (ferret) elicits aversive behaviors such as increased defensive burying, a decrease in the latency to bury,

and an increase in the number of escape attempts. In addition, exposure to the EPM shows individual differences in open arm behaviors, and rats were divided into high and low anxiety groups based on open arm time [27–29]. First, exposure to both predator stress and EPM induced neuronal activation in the BNST, but not the central amygdala, and similar levels of neuronal activation were seen in both the high and low anxiety groups in the BNST after EPM exposure. CRF-positive neurons in the CEA project to regions such as the periaqueductal gray [18, 19] and have recently been demonstrated to be activated following acute social defeat stress [24]. Dual-labeled immunohistochemistry showed that rats which were exposed to butyric acid, ferret odor, or exposure to the EPM displayed no change in co-localization of CRF with c-Fos in any subdivision of the CEA compared to controls. In the ventral BNST, however, rats exposed to ferret odor as well as rats which were exposed to the EPM displayed an increase in co-localization of CRF with c-Fos. Interestingly, exposure to a noxious but non-threatening odor (butyric acid) resulted in a slight decrease in neuronal activation of CRF neurons in the vBNST compared to no-odor controls. In the dmBNST, EPM-exposed rats displayed an increase in CRF co-localization with c-Fos compared to controls, whereas ferret or butyric acid odor exposure resulted in no change compared to controls. There were no differences in CRF neuronal activation of the CEA or BNST between rats showing different levels of anxiety-like behaviors (percent open arm time or entries) in the plus maze.

An understanding of the different roles of subdivisions of the BNST, as well as the phenotypic properties of neuronal populations in these subregions, is important to understanding their distinct functions in conditioned and unconditioned anxiety. For example, van Dijk et al. [31] found that stimulating the dorsal subdivisions of the BNST of rats resulted in no change in the percent time spent in the open arms of the EPM. Similarly, lentiviral upregulation of CRF or infusion of calcitonin gene-related peptide in the dBNST did not alter the expression of unconditioned anxiety following the EPM test or basal acoustic startle, but did demonstrate a role for the dBNST in conditioned fear [32, 33]. Studies have also demonstrated that serotonergic neurotransmission in the BNST mediates EPM-induced anxiety [34] and that individual differences in the anxiogenic response to the EPM is a function of the neurotransmission of the BNST [35]. Our data, while correlative, suggests that selective inhibition of CRF neurons in the vBNST would be a viable future direction for attenuating the anxiogenic effects of novelty in the EPM. Similarly, exposure to predator ferret odor dramatically increased the co-expression of c-Fos with CRF in the vBNST compared to control odor and butyric acid-exposed rats. Exposure to 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a component of fox feces [36–38], as well as exposure to cat urine [39] have both been shown activate vBNST or dBNST circuits using several immediate early genes, and TMT specifically acts through norepinephrine-dependent mechanisms in the vBNST to modify threat behaviors [40]. Moreover, exposure to TMT increases expression of *crh* mRNA in the ventral, but not dorsal, BNST [38]. Interestingly, butyric acid exposure decreased the co-expression of c-Fos with CRF in the vBNST compared to controls. This might suggest that GABAergic inhibitory mechanisms in the vBNST are activated with a non-threatening noxious odor, whereas glutamatergic projections into the vBNST are activated with predator odor. This supports the notion that noxious odors may have distinct neural circuitry compared to the threatening aspects of

predator odors [37]. Clearly, the vBNST represents a target for more mechanistic studies aimed at elucidating its role in responses to predator stress that would include approaches using pharmacogenetics [41] and optogenetics [42] to selectively silence phenotypically-distinct populations, such as vBNST CRF-containing neurons. This study as well as several others have produced evidence supporting the idea of selective targeting of BNST populations in an effort to attenuate anxiety [for review see 30]. While EPM and predator odor exposure-induced anxiety both utilize the BNST, divergence occurs in the dmBNST where EPM exposure activates CRF neurons but predator odor and butyric acid do not. This would indicate that engagement of excitatory circuits to the dmBNST, possibly from regions such as the ventral tegmental area or even the vBNST, are activated with mild environmental stress, whereas intense anxiety with life-threatening consequences (predator encounters) engages different circuits. The EPM is also regarded as a test of trait anxiety-like responses, and studies suggest CRF and/or CRF receptor expression seem to correlate with trait anxiety [43]. It is also possible that trait anxiety also modulates predator odor-induced fear through CRF mechanisms, perhaps within the BNST, though further studies are needed to confirm this hypothesis.

The precise role of the central amygdala in unconditioned stress and anxiety responses is not clear. Originally, it was suggested that there was preferential role of the BNST over the central amygdala in unconditioned fear responses [15, 44]. However, recent studies have demonstrated that the central amygdala does mediate unconditioned anxiety to the EPM [45]. In addition, CRF-containing neurons of the central amygdala displayed increased co-localization with c-Fos in a number of acute behavior paradigms including acute social defeat stress, a fasting/feeding paradigm, and lipopolysaccharide administration [24]. Furthermore, a previous study determined that the CEA mediates unconditioned anxiety in the EPM test through enkephalin-dependent mechanisms [46] which supported our own previous studies which demonstrated a role of opioids and the μ -opioid receptor in the CEA in mediating anxiety-related behaviors and the actions of anxiolytics in the EPM and defensive burying task [25, 47–49]. In contrast, our current study showed no increase in c-Fos expression in CRF-containing neurons with EPM exposure. Several prior studies which investigated the effect of lesions to the CEA showed no role in the mediation of unconditioned anxiety to predator odor [50–52]. This supports our own data which demonstrated no activation of CRF-containing neurons in subdivisions of the CEA following predator odor stress. Other studies using dissection of amygdala also demonstrated no increase in c-Fos expression following TMT exposure [53]. In contrast, several studies have demonstrated activation of the CEA using following exposure to TMT [37, 38, 54], as well as an increase in *crh* mRNA expression in this region [38]. Ferret exposure also increased CRF immunoreactivity in punches of the CEA in a subpopulation of fast-seizing mice [55]. It is possible that other neuronal phenotypes in this region are activated in the CEA and/or that CRF projections into the CEA are activated by ferret exposure to enhance the release and/or expression of CRF in this area. Alternatively, as described in the review by Rosen and colleagues [37], exposure to kairomones in the fur or urine may activate distinct neuroanatomical circuitry compared to that induced by specific molecular components of predator odors, such as TMT which is found in the feces. Different aspects of such signals from fur, feces or urine activate distinct olfactory receptors as well,

potentially activating unique downstream brain areas to initiate appropriate behavioral responses [37]. Finally, the animals in our study were analyzed following behavioral responses associated with defensive burying, as opposed to freezing behavior, which may have also activated a distinct set of neural structures compared to prior studies with TMT. Therefore, the role of central amygdala, and specifically CRF-containing neurons, on unconditioned stress behavior appears to depend on the type of stressor, including the complexity of the predator odor stimulus. In addition, it is possible that unconditioned stress engages opioidergic mechanisms in this region, which represent a distinct neuronal population from CRF containing neurons [12]. Since these enkephalin-containing neurons are thought to be inhibitory interneurons, they may serve to inhibit CRF containing cells. Future studies will be needed to determine if the distinct CRF and enkephalinergic populations of the CEA display converging or opposing roles in regulating unconditioned anxiety-like responses. One caveat, of course, is that it is difficult to see decreases in neuronal activation using analysis of c-Fos, so this lack of activation of the CRF containing population does not eliminate the role of the central amygdala (or these neurons) in these behavioral tasks.

The study presented here is the first to compare the activation of CRF-containing populations of the central amygdala and BNST following exposure to predator odor and the EPM. Therefore, the data enhances our knowledge of the circuitry of the central amygdala and BNST involved in the expression of innate fear induced by the odor of a natural predator or to an environmental stressor and the role of CRF-containing neurons in passive and active avoidance to unconditioned stressors. We show that distinct neuronal populations in discrete subdivisions of the BNST are activated by different stressors/anxiogenic stimuli, suggesting that these stimuli activated unique neuronal circuits through this brain area. Such knowledge is important for the fundamental understanding of fear and anxiety and could be used to determine viable targets for anxiety-related disorders. Furthermore, it supports the idea that CRF-containing populations would be a viable target for future studies examining the circuitry of innate anxiety-related responses.

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Research highlights

- Predator stress and exposure to the elevated plus maze (EPM) both induced neuronal activation of the BNST
- Predator stress induced activation of CRF-containing neurons in the ventral BNST
- EPM exposure induced activation of CRF-containing neurons of the ventral and medial BNST
- Neither predator stress nor EPM exposure induced activation CRF-containing neurons of the central amygdala

Figure 1.

Depiction of the anatomical levels of the (A) central amygdala and (B) bed nucleus of the stria terminalis (BNST) analyzed in this study. Separate counts were made in anterior and posterior portions of the centrolateral and centromedial subdivisions of the central amygdala (from Bregma: -2.12 mm to -2.80 mm) and the dorsolateral, dorsomedial, and ventral subdivisions of the BNST (from Bregma: $+0.20$ mm to -0.4 mm) [56].

Figure 2.

Anxiety measures in the defensive burying task after exposure to butyric acid and ferret odor. The total duration of odor exposure was 30 minutes. Ferret odor exposure increased the duration of defensive burying (A), while butyric acid and ferret odor exposure decreased the latency to begin burying (B). Ferret odor exposure also increased the (C) total number of escape attempts. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. non-odor controls; ## $P < 0.01$ vs. butyric acid exposed rats ($N = 6-8$).

Figure 3.

Low and high anxiety measures in the elevated plus maze (EPM). The total duration of EPM exposure was 5 minutes. High anxiety rats expressed significant decreases in both the percentage of open arm time (A) and the percentage of open arm entries (B) compared to low anxiety rats. Distance traveled was not different between groups (not shown) ** $P < 0.01$, * $P < 0.05$ vs. low anxiety rats. Data represent mean $\pm$ S.E.M (N=6 per group).

Figure 4.

Changes in c-Fos expression of CRF+ neurons of the centrolateral (CLA) and centromedial (CMA) amygdala (CEA) induced by (A) Odor exposure and (B) elevated plus maze exposure. (Top) Representative photomicrograph with CRF (brown) and c-Fos (blue/black) labeling in butyric acid (left) and ferret odor-exposed (right) rats. (Middle) Representative photomicrograph with CRF (brown) and c-Fos (blue/black) labeling in home cage control (left) and EPM-exposed (right) rats. Exposure to butyric acid, ferret odor, or EPM resulted in no significant change in the percentage of CRF-positive neurons with c-Fos in the CLA and CMA compared to respective controls (N=6). Percent CRF with c-Fos neurons represent: $(\text{the total number of double-labeled neurons} / \text{total CRF-labeled neurons}) * 100$. Pictures are at 20x magnification. Scale bar represents approximately 100 microns. The solid arrows indicate double-labeled neurons (blue/black = c-Fos and brown = CRF), and the dotted arrows indicate single-labeled CRF neurons.

Figure 5.

Changes in c-Fos expression of CRF+ neurons of the dorsolateral (dl), dorsomedial (dm) and ventral (v) BNST induced by (A) Odor exposure and (B) EPM exposure. (Top) Representative photomicrograph with CRF (brown) and c-Fos (blue/black) labeling in butyric acid (left) and ferret odor-exposed (right) rats. (Middle) Representative photomicrograph with CRF (brown) and c-Fos (blue/black) labeling in home cage control (left) and EPM-exposed (right) rats. Exposure to ferret odor increased the percentage of CRF-positive neurons with c-Fos in the vBNST (Bottom, A) compared to control odor and butyric acid. EPM exposure resulted in an increase in the percentage of CRF-positive neurons with c-Fos in the dmBNST and vBNST (Bottom, B) compared to home cage controls, but there was no difference between high and low anxiety groups. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ ($N = 5-7$). Percent CRF with c-Fos neurons represent: (the total number of double-labeled neurons / total CRF-labeled neurons) * 100. Pictures are at 20x magnification. Scale bar represents approximately 100 microns. The solid arrows indicate double-labeled neurons (blue/black = c-Fos and brown = CRF), and the dotted arrows indicate single-labeled CRF neurons.

Table 1

Behavior assessment of low and high anxiety rats upon exposure to the elevated plus maze. Data represent group means $\pm$ SEM.

	Low Anxiety (N=6)	High Anxiety (N=6)
Distance travelled (cm)	1542 $\pm$ 111	1390 $\pm$ 88
Open Arm Time (seconds)	26 $\pm$ 11	1 $\pm$ 0
Closed Arm Time (seconds)	168 $\pm$ 10	210 $\pm$ 8
Total Arm Time (seconds)	194 $\pm$ 7	211 $\pm$ 8
Open Arm Entries	4 $\pm$ 1	1 $\pm$ 0
Closed Arm Entries	14 $\pm$ 1	13 $\pm$ 2
Total Entries	17 $\pm$ 2	13 $\pm$ 2
Center Time (seconds)	99 $\pm$ 6	84 $\pm$ 8

c-Fos⁺, corticotropin releasing factor-positive, double-labeled neuronal density, and percentage c-Fos with corticotropin releasing factor in central amygdalar subdivisions of odor-exposed and elevated plus maze-exposed rats.

Table 2

Test	Treatment	c-Fos density		CRF density		CRF+c-Fos density		% c-Fos with CRF	
		CLA	CMA	CLA	CMA	CLA	CMA	CLA	CMA
Defensive Burying	No odor	30±8	21±7	270±50	497±41	14±6	12±5	34±10	47±11
	BA	16±4	6±2	266±44	465±44	7±4	4±2	43±12	65±16
	Ferret	21±4	14±4	218±45	440±51	11±3	8±3	46±11	47±11
Elevated Plus Maze	Home Cage	25±11	18±7	79±25	384±89	4±3	5±2	10±6	35±12
	EPM (LA)	28±9	16±5	121±14	399±28	4±1	6±2	17±5	35±8
	EPM (HA)	41±8	26±4	168±39	310±50	9±2	7±2	21±7	25±5

Density of neurons in subregions of the central amygdala (CEA) which are immunoreactive for c-Fos and corticotropin releasing factor (CRF) following exposure to no odor, butyric acid, ferret odor, home cage, or elevated plus maze (EPM). The data is presented as the density of labeled neurons in the subregion (per mm²). Percent c-Fos with CRF neurons represent: (the total number of double-labeled neurons / total c-Fos-labeled neurons) X 100. Data are means ± SEM (N=6–8 for Defensive Burying, N=4–5 for EPM). •P<0.05 vs. Ferret; CLA = lateral division of the CEA, CMA = medial division of the CEA, BA = butyric acid, LA = low anxiety, HA = high anxiety.

Table 3

c-Fos⁺, CRF-positive, double-labeled neuronal density, and percentage c-Fos with CRF in BNST subdivisions of odor-exposed and elevated plus maze-exposed rats.

Test	Treatment	c-Fos density			CRF density			CRF+c-Fos density			% c-Fos with CRF		
		dlBNST	dmBNST	vBNST	dlBNST	dmBNST	vBNST	dlBNST	dmBNST	vBNST	dlBNST	dmBNST	vBNST
Defensive Burying	No odor	20±4	28±5	55±9	460±30	431±45	453±58	13±3	17±4	36±6	60±8	63±13	65±7
	BA	12±3	14±2 ^{••}	20±5 ^{••••}	450±61	491±96	357±40	7±2	9±2	15±4 ^{••••}	55±9	65±5	72±5
	Ferret	20±4	41±8	180±36 ^{•••••}	478±62	400±44	612±94	11±3	25±6	115±23 ^{•••}	44±10	58±6	64±3
Elevated Plus Maze	Home Cage	48±7	25±3 ^{###}	73±12	366±70	871±90	540±53	25±7 [#]	19±3 [#]	51±10	49±12	74±7	71±9
	EPM (LA)	23±7	62±7	110±15 [•]	302±29	617±51	503±31	8±2	42±7	80±14	30±9	67±7	71±6
	EPM (HA)	47±8	77±7 ^{••••}	145±8 ^{•••}	340±29	751±103	538±52	17±4	54±7 [*]	92±10	35±1	70±6	63±5

Density of neurons in subregions of the BNST which are immunoreactive for c-Fos and CRF following exposure to no odor, butyric acid, ferret odor, home cage, or elevated plus maze (EPM). The data is presented as the density of labeled neurons in the subregion (per mm²). Percent c-Fos with CRF neurons represent: (the total number of double-labeled neurons / total c-Fos-labeled neurons) X 100. Data are means ± SEM (N=6–8 for Defensive Burying, N=4–5 for EPM).

*** P<0.001,

** P<0.01,

* P<0.05 vs. control;

P<0.01,

P<0.05 vs. butyric acid/LA;

••• P<0.001,

•• P<0.01,

• P<0.05 vs. ferret/HA.

BA = butyric acid, dlBNST = dorsolateral BNST, dmBNST = dorsomedial BNST, vBNST = ventral BNST, LA = low anxiety, HA = high anxiety.