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Direct targeting of peptidergic amygdalar neurons by noradrenergic afferents: linking stress-integrative circuitry

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Abstract

Amygdalar norepinephrine (NE) plays a key role in regulating neural responses to emotionally arousing stimuli and is involved in memory consolidation of emotionally charged events. Corticotropin-releasing factor (CRF) and dynorphin (DYN), two neuropeptides that mediate the physiological and behavioral responses to stress, are abundant in the central nucleus of the amygdala (CeA), and directly innervate brainstem noradrenergic locus coeruleus (LC) neurons. Whether the CRF- and DYN-containing amygdalar neurons receive direct noradrenergic innervation has not yet been elucidated. The present study sought to define cellular substrates underlying noradrenergic modulation of CRF- and DYN-containing neurons in the CeA using immunohistochemistry and electron microscopy. Ultrastructural analysis revealed that NE-labeled axon terminals form synapses with CRF- and DYN-containing neurons in the CeA. Semi-quantitative analysis showed that approximately 31% of NET-labeled axon terminals targeted CeA neurons that co-expressed DYN and CRF. As a major source of CRF innervation to the LC, it is also not known whether CRF-containing CeA neurons are directly targeted by noradrenergic afferents. To test this, retrograde tract-tracing using FluoroGold (FG) from the LC was combined with immunocytochemical detection of CRF and NET in the CeA. Our results revealed a population of LC-projecting CRF-containing CeA neurons that are directly innervated by NE afferents. Analysis showed that approximately 34% of NET-labeled axon terminals targeted LC-projecting CeA neurons that contain CRF. Taken together, these results indicate significant interactions between NE, CRF, and DYN in this critical limbic region and reveal direct synaptic interactions of NE with amygdalar CRF that influence the LC-NE arousal system.

Keywords

amygdala; norepinephrine; corticotropin-releasing factor; dynorphin; locus coeruleus

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Introduction

As the most prevalent class of mental disorders in the general population, anxiety disorders are a major global health issue impacting millions of lives each year (Kessler et al. 2009; Somers et al. 2006; Wittchen and Jacobi 2005). The noradrenergic system continues to be an important target in the development of new therapies for anxiety disorders because of its critical role in the modulation of emotional state and regulation of arousal and stress responses (Charney and Egnor 1989; Ballenger 2000; Carrasco and Van de Kar 2003). Upon exposure to emotionally arousing stimuli, norepinephrine (NE) is released into the amygdalar complex where it elicits behavioral responses (Williams et al. 1998; Quirarte et al. 1998). As a heterogeneous telencephalic nuclear complex, the amygdala, plays a critical role in the processing of emotional stimuli (Le Doux 2000; McGaugh et al. 2002). It is composed of multiple subregions with diverse functions (Sah et al. 2003). In particular, the central nucleus of the amygdala (CeA) mediates behavioral and autonomic responses to emotionally arousing stimuli through its highly connected afferents to endocrine and autonomic centers in the hypothalamus and brainstem (Petrovich et al. 2001; Veening et al. 1984). The CeA is a major extra-hypothalamic source of the stress-related peptide, corticotropin-releasing factor (CRF) to many brain areas (Swanson et al. 1983; Sakanaka et al. 1986; Erb et al. 2001) including brainstem catecholaminergic nuclei (Van Bockstaele et al. 1998). The CeA is also enriched with the opioid peptide, dynorphin (DYN) (Merchenthaler et al. 1997).

As important mediators of the stress response, previous pharmacological and anatomical studies have demonstrated significant interactions between CRF and endogenous opioid peptide systems in the amygdalar complex (Van Bockstaele et al. 2010; Andero et al. 2013; Chaijale et al. 2013) and in several aspects of the addiction cycle (Knoll et al. 2011; Wittmann et al. 2009; Land et al. 2008; Gray 1993; Bruchas et al. 2009; Lam and Gianoulakis 2011). Lesions of the CeA block CRF-induced enhancement of the acoustic startle response (Liang et al. 1992). Administration of the CRF antagonist, α -helical CRF, directly into the CeA attenuates stress-induced freezing (Swiergiel et al. 1993) and increases exploratory behavior in the plus-maze (Heinrichs et al. 1992). Acute stress and drug withdrawal have been shown to increase amygdalar CRF expression levels (Merali et al. 1998; Pich et al. 1992; Merlo-Pich et al. 1995; Rodriguez de Fonseca et al. 1997). Dynorphin also has a role in the stress response (Fallon and Leslie 1986). It has been shown that DYN produces aversive dysphoric-like effects (Shippenberg et al. 2007; Wee and Koob 2010). DYN preferentially binds to κ -opioid receptors (κ -OR) (Chavkin et al. 1982), and antagonism of the κ -OR has been shown to increase exploration in the elevated plus-maze and to attenuate fear-potentiated startle (Knoll et al. 2007).

Anatomical studies have shown that CRF and DYN co-localize to a large extent in the CeA (Marchant et al. 2007; Reyes et al. 2008). Several studies have indicated that DYN and CRF may interact to regulate each other's expression levels and synaptic release (Buckingham and Cooper 1986; Nikolarakis et al. 1986; Land et al. 2008; McLaughlin et al. 2003). Retrograde tract tracing and immunocytochemistry experiments have shown that DYN and CRF are co-expressed in perikarya and dendritic processes of the CeA that send projections to the locus coeruleus (LC) (Reyes et al. 2008; (Reyes et al. 2011), the major norepinephrine

(NE)-containing nucleus that serves as the primary source of NE to the forebrain and to the majority of the neural axis (Foote et al. 1983; Aston-Jones et al. 1991). Approximately 42% of the LC-projecting CeA neurons contain both CRF and DYN (Reyes et al. 2011). Electrolytic lesion of the CeA results in a reduction of CRF and DYN immunoreactivities in the LC (Koeigler-Muly et al. 1993; Reyes et al. 2008). These anatomical and lesion studies suggest that the CeA is a major source of DYN and CRF innervation of the LC-NE system. The CRF/DYN afferents regulate LC activity that, in turn, affects the output of NE to the forebrain and consequently has global effects on behavior.

Stress activates both the CRF and NE systems, and previous studies have suggested an interaction between these two systems. The β -adrenergic antagonist, propranolol, attenuates anxiety-related behaviors induced by centrally administered CRF (Dunn and Berridge 1990; Cole and Koob 1988). Inactivation of CRF receptors also prevents the behavioral changes induced by phenylephrine, an α_1 -adrenergic receptor agonist (Dunn and Berridge 1990). A feed-forward mechanism has been proposed whereby NE and CRF mutually excite each other. In the paraventricular nucleus of hypothalamus (PVN), NE excites CRF-expressing neurons (Plotsky et al. 1989; al-Damluji 1988), and in turn CRF-containing PVN neurons project to noradrenergic LC neurons (Reyes et al. 2005). NE and CRF feed-forward mechanisms are thought to occur in other brain regions as well, including the CeA (Koob et al. 2013; Ronan and Summers 2011; Dunn et al. 2004). However, there is a lack of anatomical evidence to support this hypothesis.

Although the neurochemical substrates of CRF amygdalar projections to the LC-NE system have been characterized (Van Bockstaele et al. 1998; Reyes et al. 2011), there is a gap in our knowledge regarding whether amygdalar neurons projecting to the LC are themselves regulated by noradrenergic afferents. Considering the importance of this stress-integrative circuitry (amygdala-LC), we sought to determine whether amygdalar neurons that express CRF and DYN are regulated directly by NE. We also investigated the noradrenergic innervation of amygdalar CRF-containing neurons that project to LC by using high-resolution neuroanatomical approaches and retrograde tract tracing.

Material and Methods

All procedures conformed to Thomas Jefferson University's Institutional Animal Care and Use Committee and *with National Institute of Health's Guide for the Care and Use of Laboratory Animals*. Rats were housed three per cage on a 12-h light schedule in a temperature controlled colony room. They were allowed access to standard rat chow and water ad libitum and were acclimated to the housing facility for several days prior to handling. All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data and attempts were made to minimize any animal distress.

Experimental animals

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 250-300g were used in this study. Of the ten rats that underwent surgery, four rats with successful placement of FluoroGold (FG) into the LC were used in this study.

Antibody characterization

NE transporter (NET) was used to identify NE labeled terminals. The monoclonal antibody directed against NET (MAb Technologies, Stone Mountain, GA) was generated using a peptide (amino acids 5-17) of a mouse and rat NET coupled to a keyhole limpet hemocyanin by the addition of a C-terminal cysteine. We have previously tested the specificity of NET antibody by preabsorption of this antibody with the antigenic peptide (MAb Technologies). This resulted in the absence of immunoreactivity in rat tissues that were expected to show NET immunolabeling including nucleus accumbens and amygdala (Carvalho et al. 2010).

The monoclonal antibody against dopamine β -hydroxylase (D β H) was generated in mouse (Chemicon, Millipore, Billerica, MA). Validation by preabsorption with the antigenic peptide blocked immunoreactivity for D β H (Alpha Diagnostics, San Antonio, TX).

The CRF antiserum (kindly provided by Dr. Vale of the Salk Institute) was raised in rabbit and was generated against human/rat CRF peptide (SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEIINH₂), conjugated to human α -globulins. Radioimmunoassay determined that this antibody recognizes the NH₂ terminus (residues 4-20) of ovine hypothalamic CRF (Rivier et al. 1983). Immunoreactivity is eliminated by prior absorption with its immunogen at 1 mg/mL and 10 mg/mL (Sawchenko et al. 1984; Van Bockstaele et al. 1996).

Another CRF antibody (Peninsula Laboratories, San Carlos, CA) was used in the retrograde tract-tracing study. This CRF serum was raised in guinea pig and was generated against human/mouse/rat CRF peptide (SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEI) (Peninsula Laboratories). Blocking with the antigenic CRF peptide abolished CRF immunoreactivity (Sawada et al. 2008).

The rat preprodynorphin (ppDYN) antiserum (Neuromics, Edina, MN) raised against residues 235-248 (SQENPTYSEDLDV) was generated in guinea pig. Preabsorption with its antigenic peptide blocked immunoreactivity for ppDYN (Arvidsson et al. 1995; Reyes et al. 2007).

Retrograde transport

For retrograde tract-tracing, a burr hole was drilled through the skull of each rat using the coordinates for the LC obtained from the rat brain atlas of Paxinos and Watson (1997): 10.04 mm posterior from bregma and 1.4 mm medial/lateral from the midline. A glass micropipette was filled with the retrograde tracer, FG. The tip of the glass micropipette was lowered 7.1 mm ventral from the top of the skull for deposit of FG into the LC. Seven to ten days later, rats received injections of colchicine (50 μ g; Sigma-Adrich, St. Louis, MO) into each lateral ventricle. Colchicine was injected using a Picospritzer (General Valve Corporation, Fairfield, NJ) at 24-26 psi, 10 ms duration and 0.2 Hz, while the rat was placed in a plane of anesthesia [cocktail of ketamine hydrochloride (100 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and xylazine (2mg/kg; Phoenix Pharmaceutical, Inc.) in saline intraperitoneally]. Injection of colchicine was done bilaterally into the lateral ventricle of each animal using coordinates obtained from the rat brain atlas of Paxinos and

Watson (1986): 0.26 mm posterior from the bregma, 1.2 mm medial/lateral and 3.6 mm ventral from the top of the skull. Glass micropipettes with tip diameters of 15–20 μm were filled with colchicine. The tips of the glass micropipettes were lowered to the appropriate area for deposit of colchicine into the lateral ventricle. About twenty hours after colchicine administration, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg; intraperitoneal injection) and transcardially perfused through the ascending aorta with (1) 10 mL heparin, (2) 50 mL of 3.75% acrolein (Electron Microscopy Sciences, Fort Washington, PA), and (3) 2% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were removed, blocked and immersed in 2% formaldehyde overnight at 4°C.

Immunofluorescence

Coronal tissue sections from three naïve rats were cut through the amygdala at 40 μm using a Vibratome (Technical Product International, St Louis, MO, USA) and collected in 0.1 M PB. Free-floating tissue sections were treated with 1% sodium borohydride in 0.1 M PB for 30 minutes. They were then washed in 0.1 M PB followed by 0.1 M Tris buffered saline (TBS, pH 7.6). The tissue sections were blocked in 0.5% bovine serum albumin (BSA) in 0.1 M TBS for 30 minutes and washed in 0.1 M TBS twice for 5 minutes. The tissue sections were incubated overnight at room temperature with a mouse antibody directed against D β H (1:1000; Chemicon, Millipore), guinea pig anti-ppDYN (1:2000; Neuromics), and rabbit anti-CRF (1:8000, kindly provided by Dr. Vale of the Salk Institute). The primary antibody incubation was in 0.1% BSA with 0.25% Triton X in 0.1 M TBS. The tissue sections were washed in 0.1 M TBS for 10 min, 3 times. Then the tissue sections were incubated in secondary antibodies for 2 h at room temperature. Rhodamine isothiocyanate-conjugated donkey anti-guinea pig (1:200; Jackson ImmunoResearch) was used to visualize ppDYN, cyanine dye Cy5-conjugated donkey anti-rabbit (1:200; Jackson ImmunoResearch) for visualization of CRF, and D β H antibody was detected with fluorescein isothiocyanate-conjugated donkey anti-mouse (1:200, Jackson ImmunoResearch) to visualize D β H. The tissue sections were washed 3 times for 10 min each in 0.1 M TBS followed by a wash for 10 min. in 0.1 M PB and then 0.05 M PB. The tissue sections were mounted on gelatin coated slides and dehydrated in ascending series of alcohols and xylene. The tissue sections were coverslipped using DPX mounting medium (Sigma-Aldrich).

Triple immunolabeling: immunoperoxidase and sequential immunogold-silver labeling

Triple immunolabeling where NET was labeled with immunoperoxidase while DYN and CRF were labeled using the sequential immunogold-silver labeling was carried out for electron microscopy. NET, DYN, and CRF were visualized in coronal tissue sections through the amygdala from three naïve rats that were perfused with 3.75% acrolein and 2% formaldehyde (Electron Microscopy Sciences) in 0.1 M PB. Twenty hours prior to transcardial perfusion, rats received injections of colchicine (50 μg ; Sigma-Aldrich) into each lateral ventricle. The brains were removed, blocked and immersed in 2% formaldehyde overnight at 4°C. NET was visualized by immunohistochemical reaction. The tissue sections were processed following the protocol described for immunofluorescence, but without the use of Triton-X 100 in the antibody incubation solutions. The tissue sections were incubated overnight at room temperature in primary antibody solution containing mouse anti-NET (1:1000), rabbit anti-CRF (1:8000), and guinea pig anti-ppDYN (1:2000) with 0.1% BSA in

0.1 M TBS. NET antibody was visualized using immunoperoxidase detection by incubating the tissue sections in biotinylated goat anti-mouse IgG (1:400, Jackson ImmunoResearch Laboratories) for 30 min followed by a 30 min incubation in avidin-biotin complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA). The tissue sections were washed in 0.1 M TBS three times for 10 min each. The tissue sections were then immersed in 22 mg of 3-3'-diaminobenzidine (DAB, Sigma-Aldrich) with 10 μ L of 30% hydrogen peroxide in 100 mL of 0.1 M TBS for 10 min. After visualization of NET, the tissue sections were processed for dual immunogold-silver labeling for CRF and DYN as previously described (Reyes et al. 2012, Reyes et al. 2011; Jin et al. 2010). Sequential immunogold-silver labeling was conducted by incubating with one ultra-small gold conjugate, followed by silver enhancement, and then incubating with the second ultra-small gold conjugate, followed by a second silver enhancement (Yi et al. 2001). This procedure yields two distinct groups of silver-enhanced particles: smaller particles that were enhanced once and larger particles that underwent two enhancements. The tissue sections were washed in 0.1 M TBS three times and once in 0.01 M PBS. Tissue sections were blocked with washing incubation buffer for 10 min. Tissue sections were incubated in goat anti-rabbit IgG ultrasmall conjugate (1:50; Amersham Bioscience Corp., Piscataway, NJ) at room temperature for 8 h. Tissue sections were washed in washing incubation buffer for 5 min once, in 0.01 M PBS 6 times at 5 min each, and washed in 0.1 M PB twice for 5 min each and stored overnight. Pre-enhancement washings were done in Enhancement Conditioning Solution (ECS; Amersham Bioscience Corp.) followed by the first silver enhancement (R-Gent SE-EM enhancement mixture; Amersham Bioscience Corp.) for 90 minutes and then tissues sections were washed in 0.2 M citrate buffer, twice in 0.1 M PB at five min each, and 0.01 M PBS once. The tissue sections were then incubated for 8 h in goat anti-guinea pig IgG ultrasmall conjugate (1:50, Amersham Bioscience Corp.). Tissue sections were washed in washing incubation buffer six times for five min per wash and then in 0.1 M PB twice at five min each and stored overnight in 0.1 M PB. The tissue sections were incubated in 2% glutaraldehyde (EM grade; Electron Microscopy Sciences) for 10 min and washed in 0.1 M PB twice at five min each and then in distilled water four times at five min each. The tissue sections underwent a second silver enhancement (R-Gent SE-EM enhancement mixture; Amersham Bioscience Corp.) for 60 min. Tissue sections were washed with distilled water and 0.1 M PB. The tissue sections were incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M PB for 1 h and washed in 0.1 M PB, and dehydrated in an ascending series of ethanol followed by propylene oxide. The propylene oxide was replaced with a 1:1 solution of Epon and propylene oxide and incubated overnight. Tissue sections were rotated in 100% Epon for 2 h and flat embedded in between two sheets of aclar fluorohalocarbon film. Sections of about 50-100 nm in thickness were cut with a diamond knife (Diatome-US, Fort Washington, PA) using a Leica Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany). Tissue sections were collected on copper mesh grids and examined with an electron microscope (Morgagni, Fei Company, Hillsboro, OR). Digital images were obtained using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA). Figures were adjusted in brightness and contrast using Adobe Photoshop CS4 software (Adobe Systems, Inc., San Jose, CA).

Retrograde tract tracing: triple immunolabeling (immunoperoxidase and sequential immunogold-silver labeling)

NET, CRF, and FG were visualized in amygdala coronal sections in four rats that received FG injections in the LC. Processing of these tissues for electron microscopy was described above. NET (1:1000) was visualized using immunoperoxidase. After visualization of NET, the tissue sections were processed for dual immunogold-silver labeling for FG and CRF. Tissue sections were incubated in goat anti-rabbit IgG ultrasmall conjugate (1:50; Amersham Bioscience Corp.) at room temperature for 8 h. Tissue sections were washed in washing incubation buffer for five min once, in 0.01 M PBS six times at five min each, and washed in 0.1 M PB twice for five min each and stored overnight. Pre-enhancement washings were done in Enhancement Conditioning Solution (ECS; Amersham Bioscience Corp.) followed by the first silver enhancement (R-Gent SE-EM enhancement mixture; Amersham Bioscience Corp.) for 90 minutes and then tissue sections were washed in 0.2 M citrate buffer, twice in 0.1 M PB at five min each, and 0.01 M PBS once. The tissue sections were then incubated for 8 h in goat anti-guinea pig IgG ultrasmall conjugate (1:50, Amersham Bioscience Corp.) The tissue sections were then processed as previously described.

Control and data analysis

Control tissue sections lacking exposure to primary antibodies were run in parallel with those that were processed with primary antibodies. To evaluate cross-reactivity of the primary antisera by secondary antisera, some sections were incubated in primary antisera (NET, CRF and DYN) with omission of one of the primary antisera (NET, CRF and DYN). In addition, some tissue sections were incubated with primary antisera, using inappropriate secondary antibodies. For example, some tissue sections incubated in primary antibody solution containing guinea pig anti-ppDYN (1:2000) were subsequently incubated with goat anti-rabbit IgG ultrasmall conjugate (1:50; Amersham Bioscience Corp.).

To ensure the reliability of the single and dual silver-enhancement exposure, tissue sections were incubated in a solution containing guinea pig anti-ppDYN (1:2000) and silver-enhanced either once or twice. Analysis revealed that sections silver-enhanced once exhibited gold-silver particles less than 40 ± 2 nm in diameter while tissue sections silver-enhanced twice exhibited gold-silver particles greater than 47 ± 3 nm. For the measurement of immunogold-silver particle size and to allow for distinction of different sized gold particles, immunolabeled profiles exhibiting ultrastructural morphology consistent with dendritic profiles were randomly selected from ultrathin sections where all immunolabels were clearly present in the neuropil. A total of 317 particles within dendrites were measured across an area of 0.05 m. The diameter was calculated and particle diameters were plotted resulting in a distinct segregation between two groups of gold-silver particles.

To ensure that the embedded tissue sections had optimal antibody penetration, only tissue sections from the area just beneath the tissue-Epon interface were used for quantification. At least 10 grids containing 4-7 ultrathin sections each were obtained from a Vibratome tissue section. Three vibratome tissue sections per rat were collected. Images of axon terminals containing immunoperoxidase labeling for NET where dual immunogold-silver labeling was

also present in the fields of at least 11,000x were obtained for quantification. In the retrograde tract tracing experiment, FG was labeled with large immunogold-silver particles and CRF was labeled with small immunogold-silver particles.

Characteristic of neuronal perikarya is the presence of a nucleus while proximal dendrites have endoplasmic reticulum and are located postsynaptically to axon terminals. Proximal dendrites were larger than 0.7 μm . An axon terminal was considered to make a synaptic connection if it showed a junctional complex. Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I) and symmetric (Gray's type II) if it had a thin pre- and postsynaptic density (Gray et al. 1984).

Results

Control experiments and distinguishing gold-silver particle sizes

Control sections processed in the absence of either NET, CRF or DYN antibodies did not exhibit any detectable immunoreactivity. To evaluate cross-reactivity of any of the primary antibodies used with secondary antisera, tissue sections were processed for triple labeling (NET, CRF and DYN) with the omission of one of each of the primary antisera. There was no detectable immunoreactivity with the primary antiserum that was omitted. Additional controls included incubating each of the primary antisera with an inappropriate secondary antibody, which revealed a lack of immunoreactivity for the incorrectly paired primary-secondary antibody combination. For example, tissue sections that were incubated with guinea pig anti-ppDYN followed by goat anti-rabbit secondary IgG did not exhibit any detectable immunoreactivity. Finally, to ensure reliability of the silver enhancement procedure, some sections were processed where the primary antibody (ppDYN) was enhanced either once or twice in parallel sections and analysis of resulting particle sizes revealed differentially sized gold particles (see Methods).

Consistency of silver enhancement is critical for producing particle size segregation. With any enhancement procedure, some variability is expected. However, to ensure reliability in the analysis of silver-intensified particles for assessing the co-localization DYN and CRF, we measured the diameter of differentially-sized immunogold-silver enhanced particles to determine the extent to which large and small gold silver particles indicative of the antigen of interest overlap (Figure 4E). In so doing, a range of diameters were collected as shown in Figure 4F. When the distribution of randomly selected gold-silver particles was plotted, a clear segregation in differently sized gold particles was revealed and a very small overlap was evident (Figure 4F). Therefore, gold particles that were only enhanced once, considered small, ranged in size from 5-42 nm in diameter. Gold particles that were enhanced twice, considered large, ranged in size from 52-125 nm in diameter. Considering the importance of homogeneity in particle size for our analysis, we sought to apply certain criteria in our analysis so that the larger the size difference between the two groups of particles, the lower the likelihood of cross-reactivity. Therefore, immunogold-silver particles with a diameter ranging between 42-52 nm were excluded from the analysis as this represented a range where the confidence level was not high for discriminating one label from the other.

Anatomical substrates for NE innervation of CRF- and DYN-containing amygdalar neurons using light and immunofluorescence

Coronal tissue sections through the amygdalar complex were processed for Nissl staining to delineate regional boundaries and were matched at rostrocaudal levels with tissue sections processed for NET (Figure 1). The basolateral nucleus of the amygdala (BLA) is located between the external capsule and longitudinal association bundle (Krettek and Price 1978). The CeA is located medially to the BLA and can be further subdivided based on nuclear density and shape (McDonald 1982; Cassell et al. 1986). Utilizing light microscopy and an antibody directed against NET (a marker for noradrenergic fibers), immunoperoxidase labeling revealed NET in varicose processes in the CeA and the BLA (Figure 1A,B). The distribution of NET in these two amygdalar subregions is consistent with the distribution of NE-labeled fibers described in previous studies (Asan 1998; Rudoy and Van Bockstaele 2005). Within coronal sections, NET immunoreactivity was densest in the BLA with sparser labeling in the lateral nucleus (LA) along the rostrocaudal extent of the amygdalar complex, consistent with previous findings (Asan 1998). Within the CeA, NET-ir fibers were observed in the capsular (CeC), lateral (CeL), and medial (CeM) subdivisions. There were sparse NET-labeled fibers in the CeL comparable to prior published studies of D β H-labeling (Asan 1998).

Figure 2 shows immunoperoxidase labeling for CRF and DYN in the CeA. Consistent with our previous observations (Reyes et al. 2008; Reyes et al. 2011), the CeA contains abundant CRF- and DYN-containing neurons. Peroxidase labeling for CRF (Figure 2A-C) and DYN (Figure 2D-F) was identified in perikarya and dendritic processes in the CeA. CRF and DYN immunoreactivity were most abundant in the mid-rostrocaudal level of the amygdalar complex (Figure 2B, 3B). Consistent with previous reports, the lateral subdivisions contained abundant expression of CRF and DYN (Marchant et al. 2007; Retson and Van Bockstaele 2013).

To determine if NE axon terminals are positioned to directly regulate CRF- and DYN-containing neurons in the CeA, antibodies directed against D β H or NET, CRF, and DYN were localized in the same section of tissue and identified using immunofluorescence microscopy. Triple label immunofluorescence showed that fibers exhibiting immunolabeling for D β H or NET (green labeling) were in close proximity to CRF (blue labeling) and DYN (red labeling) labeled neurons (Figure 3). This interaction was further analyzed using immuno-electron microscopy.

Ultrastructural analysis of NE, CRF and DYN in the CeA

Using electron microscopy, peroxidase labeling was combined with sequential dual immunogold-silver labeling where the immunogold-silver particles were differentiated based on their size (Figures 4, 5). Sequential immunogold-silver labeling was conducted by incubating with one ultra-small gold conjugate, followed by silver enhancement, and then incubating with the second ultra-small gold conjugate, followed by a second silver enhancement (Yi et al. 2001). This procedure yielded two distinct groups of silver-enhanced particles: smaller particles that were enhanced once and larger particles that underwent two enhancements (Yi et al. 2001). Immunoperoxidase labeling for NET and dual immunogold-

silver labeling (large and small gold-silver particles, for CRF and DYN, respectively) were localized in the same tissue section in the CeA (Figure 4, 5). Gold-silver labeling for CRF and DYN were readily distinguishable from each other and were localized to the appropriate cellular structures. CRF and DYN-immunolabeling was localized in dendrites and perikarya. In very few cases, CRF and DYN appeared in axon terminals. Immunogold-silver labeling for CRF and DYN was also clearly distinguishable from the immunoperoxidase reaction product. Peroxidase labeling for NET was localized to axon terminals as indicated by a diffuse reaction product (Figure 4). Consistent with previous findings, we found that CRF and DYN were localized to common dendrites in the CeA (Reyes et al 2011; Marchant et al 2007) although it was beyond the scope of the present study to determine the frequency of co-existence of CRF and DYN in the entire extent of the CeA. Our analysis was centered on quantifying targets of NET-labeled axon terminals in the CeA. We found that NET-labeled axon terminals formed direct synaptic associations with dendrites that exhibited both CRF and DYN immunoreactivities in the CeA (Figures 4C, 5A,C-C', D). Semi-quantitative analysis revealed that, of 360 NET-labeled axon terminals analyzed, 31% (112/360) were in direct synaptic contact with dendrites that contained CRF- and DYN in the CeA; 12% (43/360) were in direct synaptic contact with singly labeled CRF-containing dendrites and 24% (85/360) contacted singly labeled DYN-containing dendrites. The remaining NET-labeled axons and axon terminals targeted dendrites that lacked any detectable immunoreactivity for CRF or DYN. We also report the associations with respect to the number of DYN only, CRF only and CRF+DYN profiles in similar portions of the neuropil that were either directly contacted by NET-labeled axon terminals or lacked direct innervation by NET-labeled axon terminals in the region sampled for analysis. Of 502 profiles, 8% (43/502) of CRF-only labeled dendrites, 17% (85/502) of DYN-only labeled dendrites and 22% (112/502) of CRF + DYN-only labeled dendrites received direct innervation from NET-labeled axon terminals. We also quantified the number of DYN only, CRF only and CRF+DYN profiles that lacked direct contact by NET-labeled axon terminals in the region sampled for analysis. The following represents the percentage of DYN only, CRF only and CRF+DYN profiles (n=502) that did not receive direct innervation from NET-labeled axon terminals: 11% (57/502) were CRF-labeled only, 27% (137/502) were DYN-labeled only and 14% (68/502) were CRF + DYN-labeled only. Future studies are required to determine whether the fraction of NET axon terminals targeting CRF-DYN neurons in the entire volume of the CeA represents a specific targeting of this population of co-expressing neurons with respect to all CeA neuronal profiles.

Although many synaptic specializations could not be determined, effort was made to follow individual synapses in adjacent sections. An example of a two adjacent sections is shown in Figure 5C-C'. With this approach, analysis showed that the NET-labeled axon terminals formed both asymmetric (Type I) and symmetric (Type II) type synapses with CeA neurons. Asymmetric synapses had enlarged post-synaptic densities, while symmetric synapses exhibited densities that were comparable across pre- and post-synaptic membranes. Of the 360 direct synaptic contacts observed, 24% (86/360) formed asymmetric-type synapses with CRF- and/or DYN-labeled dendrites (Figure 4C, 5D) while 12% (43/360) formed symmetric-type synapses (Figure 4C, 5C-C'). The remaining NET-labeled contacts were unidentifiable.

LC-projecting amygdalar neurons expressing CRF are directly targeted by NE afferents

Retrograde tract tracing and immunoelectron microscopy was employed to determine whether LC-projecting CeA neurons are directly regulated by NE-containing axon terminals. Figure 6A shows a representative brightfield photomicrograph depicting a FG injection site into the LC. Only cases in which FG was appropriately localized to the LC were used for the electron microscopy study. As well documented in prior studies, FG injections into the LC yielded prominent retrograde transport in the CeA (Figure 6B).

Immunoperoxidase labeling combined with sequential dual immunogold-silver labeling indicated that LC-projecting amygdalar neurons are directly targeted by NE afferents. Immunoperoxidase-labeling for NET and dual immunogold-silver labeling (large and small gold-silver particles, for FG and CRF, respectively) were localized in the same tissue section in the CeA (Figure 6C-D). Of the NET-labeled axon terminals analyzed, approximately 34% (118/343) were in direct synaptic contact with CRF-containing CeA neurons that projected to the LC, as evidenced by the presence of dual gold-silver labeling for FG and CRF in dendrites. Of the NET-labeled axon terminals forming synapses with postsynaptic dendrites, approximately 66% of the NET terminals contacted CRF-labeled neurons, and 38% of the NET terminals contacted FG-labeled neurons.

Discussion

Amygdalar NE plays an important role in emotional processing and, as the primary output station of the amygdala, the CeA is responsible for mediating autonomic and endocrine responses to highly salient emotional stimuli (Sah et al. 2003). Dysfunction of the amygdala has been implicated in the pathophysiology of anxiety disorders (Keen-Rhinehart et al. 2009; Lopez and Watson 1999) and an increased understanding of the fine synaptic organization of this area may lead to better treatments for these disorders that are often precipitated by chronic stress (de Kloet et al. 2005; Arborelius et al. 1999). Anatomical studies have shown that the LC receives direct amygdalar CRF and DYN afferents, two neuropeptides that are key mediators in the physiological and behavioral response to stress-related stimuli (Van Bockstaele et al. 1996; Reyes et al. 2008, 2011). Here, we confirm co-expression of CRF and DYN in the CeA but also extend knowledge of the intrinsic organization of limbic circuitry by providing ultrastructural evidence for direct noradrenergic innervation of CRF and DYN neurons in the CeA. As the CeA is a major source of CRF and DYN afferents to the LC modulating noradrenergic activity (Van Bockstaele et al. 1998; Kreibich et al. 2008; Curtis et al. 2002), the present study also demonstrates that NE afferents directly innervate CRF-containing CeA neurons suggesting a feed-forward circuit linking these stress-integrative brain regions. Therefore, in response to stress, activation of CRF- and DYN-containing amygdalar neurons by noradrenergic afferents may directly regulate global NE release via projections to the LC, further implicating the amygdalar-LC circuit in contributing to the pathophysiology of stress-induced anxiety disorders.

Methodological considerations

The pre-embedding immunocytochemical technique combined with electron microscopy has several advantages. This approach maintains morphological preservation while preserving discrete subcellular localization of the antigen of interest. In combination with peroxidase detection, which appears as a dense homogeneous precipitate within cellular compartments, the addition of dual sequential immunogold-silver labeling allows for identifying synaptic circuitry onto defined populations of neurons. However, as with all experimental approaches, some limitations exist. Acrolein fixation optimizes the preservation of ultrastructural morphology; however, it produces only limited penetration of immunoreagents in thick tissue sections (Leranth and Pickel 1989; Chan et al. 1990). As limited penetration of reagents may result in underestimation of the relative frequencies of their distribution, we minimized this caveat by collecting tissue sections near the tissue-Epon interface where penetration is optimal and sampling profiles only when all the markers were present in the surrounding neuropil. D β H is a vesicular-bound enzyme, which makes it more difficult to detect without using enhancement methods (such as increased detergents i.e. Triton X-100). Such permeabilization agents compromise the ultrastructure preservation of the neuropil. Therefore, we opted to use an antibody directed against the NET as a marker of noradrenergic axon terminals for the electron microscopy studies. Previous studies have examined the dual localization of D β H and NET and found extensive co-existence between the two (Zhang et al. 2013; Carvalho et al. 2010).

Using immunohistochemical techniques, it is difficult to detect robust levels of neuropeptides in dendrites without the use of colchicine, a microtubule inhibitor that is commonly used to increase the amount of peptide in somatodendritic processes. However, colchicine has also been shown to induce stress-like responses and increased mRNA expression levels for certain neuropeptides (Swanson et al. 1983; Moga et al. 1990). Since the goal of the present study was to use antibodies to determine the phenotype of neurons rather than to quantify their expression levels, the use of colchicine in the present studies should not impact the interpretation of our electron microscopy results.

Retrograde tract-tracing studies pose certain technical caveats that must be taken into consideration when interpreting results. The tracer may spread at the injection site as well as be taken up by fibers of passage in brain areas adjacent to the site of injection. In the present study, only cases where FG injections were circumscribed to the LC were selected for analysis (Figure 6A). In addition, the retrograde labeling within the CeA was comparable to the anatomical distribution observed in previous reports (Van Bockstaele et al. 1998; Reyes et al. 2011).

The ultrastructural features of NE-containing terminals have been well described in many brain regions (Seguela et al. 1990; Asan 1998; Farb et al. 2010). Some studies support the notion that NE axon terminals do not always form traditional synaptic specializations (Descarries and Mechawar 2000; Zhang et al. 2013). It was previously thought that the majority of NE varicosities do not make synaptic contacts in the cortex (Seguela et al. 1990) or in the amygdala (Asan 1998). However, a recent study showed that noradrenergic terminals form more synaptic connections in the BLA than previously reported (Zhang et al. 2013). Catecholaminergic neurotransmission occurs via wired synaptic transmission and

volume transmission, where the neurotransmitter diffuses through the extracellular space (Agnati et al. 1986). While wired transmission can mediate rapid responses to stress, volume transmission may mediate more long-term events (Perez de la Mora et al. 2008). Wired and volume transmission are characterized by differences in specificity and spatiotemporal aspects (Agnati et al. 1995). Although we identified approximately 34% of the NE-labeled terminals as forming direct synapses with CeA neurons, many of these could not be unequivocally established as Type I or Type II synapses because of the lack of clear morphological differentiation of the synaptic specialization. Therefore, although the present data supports direct NE targeting of CRF and DYN, the concept of volume transmission cannot be ruled out. Previous reports have shown that the CeA robustly expresses α_1 -adrenergic receptors (Day et al. 1997; Domyancic and Morilak 1997). NE acts on α_1 -adrenergic receptors in the CeA to mediate behavioral responses to immobilization stress (Pacak et al. 1993; Khoshbouei et al. 2002; Cecchi et al. 2002). Furthermore, α_2 -adrenergic receptors are found in the CeA and are involved in the autonomic response to stressful stimuli (Glass et al. 2002). Although not abundant β -adrenergic receptors have been found to be co-localized with CRF neurons in the CeA (Rudoy et al. 2009). Identifying the subcellular distribution of adrenergic receptors with respect to noradrenergic afferents and peptidergic content will contribute to further testing the hypothesis of whether volume transmission from noradrenergic terminals impacts DYN, CRF or neurons that co-express both DYN and CRF in the CeA.

NE innervation of the amygdala: anatomy and functional implications

Our data are consistent with other immunocytochemical studies showing a moderate density of NET-ir fibers throughout the amygdalar complex, but with the BLA exhibiting a more robust distribution of immuno-labeling (Schroeter et al. 2000; Rudoy and Van Bockstaele 2005). In rodents, a homogeneous binding pattern has been reported for the NET in the amygdalar complex, with increased binding in the BLA (Benmansour et al. 1992; Tejani-Butt 1992). In non-human primates, the CeA exhibits robust NET binding (Smith et al., 2006), and in humans there is a high degree of NE content in the CeA (Farley and Hornykiewicz 1977). Stress-induced NE release occurs in both the BLA and CeA (Williams et al. 1998; Quirarte et al. 1998; Pacak et al. 1993; McIntyre et al. 2002) where the NE influence on the amygdala is considered to be excitatory in nature. Direct application of NE into the amygdala enhances neuronal activity (Stone et al. 1997), and α_1 - and β -adrenergic receptor antagonists block this response (Duncan et al. 1996).

Both α_1 - and β -adrenergic receptors are expressed in the BLA and CeA and NE transmission in these regions is thought to regulate different aspects of the stress response (Talley et al. 1996; Rosin et al. 1996; Day et al. 1997; Ferry et al. 1997; Buffalari and Grace 2007; Abraham et al. 2008; Farb et al. 2010). Excessive NE release in the BLA may desensitize the α_1 -adrenergic receptors, which act on GABAergic transmission, contributing to the hyper-excitability of the amygdala in stress-induced anxiety disorders (Braga et al. 2004). Alteration of GABAergic transmission in the BLA in turn may affect CeA activity since the BLA sends GABAergic projections to the CeA (Perez de la Mora et al. 2008). The BLA β -adrenergic receptors have an important role in memory encoding and consolidation and antagonism of these receptors impairs memory consolidation (Hatfield and McGaugh 1999).

CRF, DYN, and NE interactions in the CeA

Pharmacological targeting of NE, DYN, or CRF systems in the CeA has been shown to attenuate behavioral responses to fear- and anxiety-related stimuli (Liang et al. 1992; Swiergiel et al. 1993; Knoll et al. 2007; Dunn and Berridge 1990). Additionally, an interaction of these systems is supported by pharmacological and electrophysiological studies (Valentino et al. 1993; Raber et al. 1995; Van Bockstaele et al. 1996; Dunn et al. 2004). Our data provide an anatomical basis for the interpretation of these physiological and behavioral studies examining the role of NE, DYN, and CRF in the amygdala complex by providing anatomical evidence that NE-labeled axon terminals form direct synaptic connections with CRF- and DYN-containing CeA neurons.

It is not yet fully understood how these two neuropeptides interact, but several studies suggest their co-release and activity are dependent on each other (Buckingham and Cooper 1986; Land et al. 2008). In preprodynorphin knock-out mice, CRF expression levels are reduced in the CeA suggesting that these neuropeptides may also interact to regulate expression levels (Kastenberger et al. 2012). Noradrenergic receptor signaling can alter CRF gene expression in response to stress. CeA neurons co-express CRF and β_1 -adrenergic receptors, and antagonism of this adrenergic receptor attenuates CRF expression levels after cocaine withdrawal (Rudoy et al. 2009). It has been previously shown that NE stimulates CRF neurons in the CeA (Raber et al. 1995). Taken together, it is tempting to speculate that NE may have an excitatory influence on CRF and DYN expressing amygdalar neurons, although future studies are required to address this fully.

Potential sources of noradrenergic input to the CeA arise from the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM) (Zardetto-Smith and Gray 1990; Roder and Ciriello 1993). The majority of the CeA-projecting neurons originating from the A2 region of the NTS provide the bulk of catecholaminergic innervation to the CeA (Zardetto-Smith and Gray 1990; Reyes and Van Bockstaele 2007). Likewise, combined retrograde and anterograde tracing studies have demonstrated that VLM neurons provide catecholaminergic innervation to the CeA (Roder and Ciriello 1993). NE innervation from these medullary brain regions would implicate the amygdala as a key integrator of the physiological and emotional aspects of the stress response. In the present study, we did not investigate the source of NE innervation to the CeA and this will require further investigation.

Stress-related neural circuitry: Linking limbic NE-CRF to the LC

Amygdalar CRF is important for the behavioral effects of stress and with its afferents to the LC, it is hypothesized that it also has a role in the arousal responses to stress. Previous research has shown that the CeA sends CRF and DYN afferents to the LC (Reyes et al. 2008, 2011; Van Bockstaele et al. 1996). Electrophysiological studies have shown that direct CRF application into the LC activates LC noradrenergic neurons resulting in an increase in neuronal discharge (Aston-Jones and Bloom 1981; Curtis et al. 1997; Page and Abercrombie 1999). This change in LC activity is associated with scanning of the environment, going off task and increased behavioral flexibility (Aston-Jones and Bloom 1981; Berridge and Waterhouse 2003), and is correlated with an increase in cortical NE levels (Curtis et al. 1997; Page and Abercrombie 1999; Zhang et al. 1998). The shift towards a state of scanning

the environment and behavioral flexibility may be adaptive in a dynamic environment with life-threatening stimuli. However, if LC neurons were persistently in this state or if they were in this state inappropriately, this would be pathological.

Limbic NE plays a role in activating CRF neurons (Raber et al. 1995). The present study demonstrated that CRF-containing amygdalar neurons are under noradrenergic control and are poised to regulate the LC-NE system. Using high-resolution ultrastructural analysis, it was previously reported that DYN- and CRF-containing axon terminals directly target LC noradrenergic neurons (Reyes et al. 2007) and that a major source of DYN and CRF afferents to the LC is the CeA (Reyes et al. 2011). We were not able to identify whether LC-projecting CRF neurons in the CeA also co-express DYN due to the limitation of identifying four distinct electron dense markers in the same tissue section. However, it is tempting to speculate that NE targets a population of CeA neurons that co-express CRF and DYN and that these project to the LC.

Functional implications: A feed-forward mechanism linking stress-responsive circuits

CRF and NE systems rapidly mobilize the body for behavioral responses to stressors. The NE/CRF interactions may play an important role in activating the LC-NE system in response to stress so that an individual can respond appropriately to a stressor. However, in stress-related anxiety disorders this pathway may be maladaptive. In a feed-forward mechanism (Figure 7), medullary NE released into the amygdala could activate CRF neurons that in turn release CRF into the LC, thereby increasing NE release in the forebrain. The LC-NE may also in turn target the BLA. The BLA sends GABAergic projections to the CeA, which could modulate its activity (Perez de la Mora et al. 2008). Such a feed-forward mechanism may be vulnerable to dysfunction, particularly following repeated exposure to stressors. One possible effect may be the escalation of NE and CRF release. This may alter the allostatic state, as defined by Koob and Le Moal (2001) as the state of chronic deviation of the regulatory system from its normal operating level. Chronic stress may lead to changes to the NE-CRF systems, potentially leading to enhanced activity above the normal stress response.

The present study suggests that limbic CRF and DYN circuitry may be significantly impacted by NE modulation. In a feed forward mechanism, limbic NE could regulate global NE release. This may occur through the action of CRF as it has been previously shown that CRF makes asymmetric or excitatory synapses onto LC noradrenergic neurons (Valentino et al. 2001; Reyes et al. 2007). CRF release into the LC is correlated with an increase in NE cortical levels (Curtis et al. 1997; Page and Abercrombie 1999). It has been proposed that NE-CRF feed forward loops need to be tightly regulated as mutual excitation of these systems could lead to an exaggerated stress response as exhibited in various disorders such as panic (Dunn and Sweigert 2008; Ronan and Summers 2011).

Stress-related anxiety disorders are managed by psychotherapeutic and pharmacological interventions, but many patients fail to respond to treatments (Schneier 2011). There is still a need for improved treatment for these disorders. Due to the complex interactions of the NE, CRF, and DYN systems, dysfunction in one may lead to alterations in another. Combined therapeutic approaches using drugs that target multiple neuromodulatory systems (Hopkins 2007) may be necessary in order to restore aberrant network activity. Knowledge of stress-

related circuitry is a crucial step in understanding the maladaptive responses that underlie stress-related psychopathologies and developing better therapeutics.

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Figure 1.

Brightfield photomicrographs illustrate norepinephrine transporter (NET) immunoreactive fibers and Nissl staining in the amygdalar complex. Both panels feature overlays depicting boundaries for subdivisions in the amygdalar complex adapted from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). Panel A and A' depict a section through the rostral aspect of the amygdalar complex (at bregma -1.80) while panel B and B' illustrate a representative section from a more caudal division of the amygdala (at bregma -2.30). For purposes of clarity, labeling of CeA subdivisions is omitted in the photomicrographs showing NET-ir. Arrows indicate dorsal (D) and medial (M) orientation of the section. La, lateral nucleus of the amygdala; BLa, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala. Scale bars = 50 μ m

Figure 2.

Brightfield photomicrographs showing immunoperoxidase labeling of corticotropin-releasing factor (CRF) and dynorphin (DYN) immunoreactivities in the central nucleus of the amygdala (CeA). All panels feature overlays of the CeA morphology adapted from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). For purposes of clarity, labeling of CeA subdivisions is omitted in the photomicrographs showing CRF- and DYN-ir. Panels A and D are at bregma -1.80, panels B and E at bregma -2.30, and panels C and F are at bregma -2.80. Arrows indicate dorsal (D) and medial (M) orientation. CeA, central nucleus of the amygdala; CeC, capsular subdivision; CeL, central nucleus of the amygdala

lateral subdivision; CeM, central nucleus of the amygdala medial division. Scale bars = 50 μm

Figure 3.

High magnification photomicrographs illustrating CRF-immunoreactive (A, E), DYN-ir (B, F), and DβH- or NET-ir (C, G) in CeA neurons. The merged image shows CRF- and DYN-containing CeA neurons that are in close proximity to NE-labeled fibers (D, H). Arrowheads point to individual CRF-ir (A) and DYN-ir (B) neurons, whereas arrows denote neurons that co-express CRF and DYN. DβH or NET-labeled fibers are indicated by double arrowheads (C, D, G, H). Arrows indicate dorsal (D) and medial (M) orientation. Scale bar = 100 μm

Figure 4.

A. Electron photomicrographs showing immunoperoxidase labeling for norepinephrine transporter (NET) and dual immunogold-silver labeling for corticotropin-releasing factor (CRF; large gold) and dynorphin (DYN; small gold) in the central nucleus of the amygdala (CeA). Dense peroxidase labeling can be seen in a NET-labeled axon terminal (NET-t) in close proximity to a dendrite containing large gold-silver grains that indicate labeling for CRF (CRF-d) and a dendrite containing small gold-silver labeling (arrowheads) that indicate labeling for DYN (DYN-d). **B.** In a separate profile, a NET-labeled axon terminal (NET-t)

can be seen in close proximity to a dendrite containing small (arrowheads) and large (arrows) gold-silver grains that indicate labeling for DYN and CRF (DYN+CRF-d), respectively. An unlabeled terminal (ut) can also be seen in the neuropil contacting a dendrite lacking any detectable immunoreactivity. **C.** NET-labeled terminals (NET-t) converge on a common dendrite containing DYN and CRF immunoreactivities (DYN+CRF-d); one forms a symmetric synapse (curved arrow) and another forms an asymmetric synapse (zigzag arrow). **D.** A NET-labeled terminal (NET-t) is in direct synaptic contact with a perikaryon containing small (arrowheads) and large (arrows) gold-silver grains that indicate labeling for DYN and CRF (DYN+CRF-s), respectively. **E.** Two DYN-labeled dendrites (DYN-d) containing small gold-silver grains (arrowheads) are in close proximity with each other. A CRF-labeled dendrite (CRF-d) containing large gold-silver grains (arrows) is located nearby. Peroxidase labeled NET-containing axon terminals (NET-t) are also seen in the neuropil. A rectangle surrounds a gold-silver particle whose size is depicted by an arrowhead in Panel F representing a particle classified as small (28 nm). Another gold-silver particle (97 nm) is indicated by a circle whose size is depicted by an arrow in Panel F representing a particle classified as large. **F.** Immunogold-silver particle size distribution. Circles represent once-enhanced immunogold-silver gold particles (mean = 260.14 nm; n = 188) while squares represent twice-enhanced immunogold-silver particles (mean = 79.50 1.80 nm; n = 188) in CRF and DYN-labeled dendrites. Scale bar = 0.50 μ m

Figure 5.

A. Electron photomicrographs showing immunoperoxidase labeling for norepinephrine transporter (NET) and dual immunogold-silver labeling for corticotropin-releasing factor (CRF; large gold-silver grains) and dynorphin (DYN; small gold-silver grains) in the central nucleus of the amygdala (CeA). **A-B.** A NET-labeled axon terminal (NET-t) contacting a DYN and CRF-containing dendrite (DYN+CRF-d). **C-C'.** Two adjacent sections showing a NET-labeled axon terminal (NET-t) that forms a symmetric synapse (curved arrow) with a dendrite containing DYN and CRF (DYN+CRF-d). DYN-d is labeled with small gold-silver particles (arrowheads) while CRF-d is labeled with large gold-silver particles (arrows). **D.** A NET-labeled axon terminal (NET-t) forms an asymmetric synapse (zigzag arrow) with a DYN and CRF-labeled dendrite (DYN+CRF-d). Arrowheads point to small immunogold-silver particles that indicate DYN immunoreactivity. Arrows point to large immunogold-silver particles that denote CRF immunoreactivity. Scale bars = 0.05 μ m.

Figure 6.

A. Low magnification brightfield photomicrograph of a representative FluoroGold (FG) injection into the locus coeruleus (LC) in the rat brain. Arrows indicate neurons exhibiting peroxidase labeling for FG at the injection site. **B.** High magnification brightfield photomicrograph of a representative retrograde labeling in the central nucleus of the amygdala (CeA) following FG injection into the LC. Schematic diagrams adapted from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986) showing the anterior posterior level of the region of the LC (A) targeted for injection placement and of the CeA (B) for the retrogradely labeled neurons. Arrows indicate dorsal (D) and medial (M) orientation. **C.** Electron photomicrograph showing immunoperoxidase labeling for norepinephrine transporter (NET) and dual immunogold-silver labeling for FG (large gold-silver grains) and corticotropin-releasing factor (CRF; small gold-silver grains) in the CeA. Dense peroxidase labeling can be seen in a NET-labeled axon terminal (NET-t) contacting a FG (arrows) and CRF (arrowheads)-containing dendrite (FG+CRF-d). **D.** A NET-labeled

axon terminal (NET-t) forms a symmetric synapse (curved arrow) with a FG+CRF-d). ut, unlabeled terminal. CeA, central nucleus of the amygdala; LC, locus coeruleus; 4V, fourth ventricle; scp, superior cerebellar peduncle. Scale bar for panels A-B = 50 μm . Scale bar for panels C-D = 0.50 μm

Figure 7.

Schematic diagram illustrating a proposed NE-CRF feed-forward mechanism. Medullary brain regions, nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM), are sources of NE to the CeA. Upon NE release into the CeA, the CRF neurons are activated, which in turn activate LC-NE neurons. The LC provides NE to the forebrain and BLA. The BLA projects to the CeA, whereby it can influence its activity. Dashed lines represent NE projections and solid lines represent CRF projections. CeA, central nucleus of the amygdala; LC, locus coeruleus; BLA, basolateral nucleus of the amygdala.