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Localization of the delta opioid receptor and corticotropin-releasing factor in the amygdalar complex: role in anxiety

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Abstract

It is well established that central nervous system norepinephrine (NE) and corticotropin releasing factor (CRF) systems are important mediators of behavioral responses to stressors. More recent studies have defined a role for delta opioid receptors (DOPR) in maintaining emotional valence including anxiety. The amygdala plays an important role in processing emotional stimuli, and has been implicated in the development of anxiety disorders. Activation of DOPR or inhibition of CRF in the amygdala reduces baseline and stress-induced anxiety-like responses. It is not known whether CRF- and DOPR-containing amygdalar neurons interact or whether they are regulated by NE afferents. Therefore, the present study sought to better define interactions between the CRF, DOPR and NE systems in the basolateral (BLA) and central nucleus of the amygdala (CeA) of the male rat using anatomical and functional approaches. Irrespective of the amygdalar subregion, dual immunofluorescence microscopy showed that DOPR was present in CRF-containing neurons. Immunoelectron microscopy confirmed that DOPR was localized to both dendritic processes and axon terminals in the BLA and CeA. Semi-quantitative dual immunoelectron microscopy analysis of gold-silver labeling for DOPR and immunoperoxidase labeling for CRF revealed that 55% of the CRF neurons analyzed contained DOPR in the BLA while 67% of the CRF neurons analyzed contained DOPR in the CeA. Furthermore, approximately 41% of DOPR-labeled axon terminals targeted BLA neurons that expressed CRF while 29% of DOPR-labeled axon terminals targeted CeA neurons that expressed CRF. Triple label immunofluorescence microscopy revealed that DOPR and CRF were co-localized in common cellular profiles that were in close proximity to NE-containing fibers in both subregions. These anatomical results indicate significant interactions between DOPR and CRF in this critical limbic region and reveal that NE is poised to regulate these peptidergic systems in the amygdala. Functional studies were performed to determine if activation of DOPR could inhibit the anxiety produced by elevation of NE in the amygdala using the pharmacological stressor yohimbine. Administration of the DOPR agonist, SNC80, significantly attenuated elevated anxiogenic behaviors produced by yohimbine as measured in the rat on the elevated zero maze. Taken together, results from this study demonstrate the convergence

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Conflict of Interest

The authors declare that they have no conflict of interest.

of three important systems, NE, CRF, and DOPR, in the amygdala and provide insight into their functional role in modulating stress and anxiety responses.

Keywords

amygdala; delta opioid receptor; corticotropin-releasing factor; norepinephrine

Introduction

The amygdala is composed of multiple subregions that play a critical role in the processing of emotional stimuli (LeDoux, 2000, McGaugh et al., 2002). Considered to be the gatekeeper of the amygdala, the lateral nucleus (LA) receives inputs from multiple sensory systems (Sah et al., 2003). From the LA, information is processed through the basolateral amygdala (BLA), which is involved in the consolidation of highly salient emotional stimuli (Sah and Lopez De Armentia, 2003) and receives dense noradrenergic projections (Asan, 1998). The central nucleus of the amygdala (CeA) acts as the amygdala's output station mediating behavioral and autonomic responses to emotionally arousing stimuli through its highly connected afferents to endocrine and autonomic centers in the hypothalamus and brainstem (Veening et al., 1984, Petrovich et al., 2001). Amygdalar dysfunction is seen in stress-induced anxiety as well as anxiety associated with substance abuse and withdrawal (Retson et al., 2014, Spanagel et al., 2014, Radley et al., 2015, Silberman and Winder, 2015).

Physiological and emotional responses to stress are tightly linked to the hypothalamic pituitary adrenal axis, and the neuropeptide corticotropin releasing factor (CRF) is integral to this response. CRF in the amygdala mediates the behavioral and emotional consequences of stress including anxiety (Heinrichs et al., 1994, Francis et al., 1999, Tovote et al., 2015) and CRF mRNA levels increased in the CeA following stress (Mamalaki et al., 1992). In fact, CRF or CRF receptor agonist administered directly into the amygdala produces an aversive state including anxiety-like behavior (Gray and Bingaman, 1996, Sajdyk et al., 2006). It has been shown that CRF in the amygdala is critical for the reconsolidation of contextual fear memories (Pitts et al., 2009), and application of a variety of acute stressors result in elevations of CRF expression in the central nucleus of the amygdala of the rat (Iwasaki-Sekino et al., 2009, Ciccocioppo et al., 2014). Norepinephrine (NE) is another neuromodulator in the amygdala that is also involved in behavioral and emotional responses to stressors (Koob, 1999). Stressors have been shown to alter NE activity. For example, stressors increase NE release in the amygdala (Tanaka et al., 1991a, Tanaka et al., 1991b, Galvez et al., 1996, Quirarte et al., 1998) and induce high density of NE transporter binding sites in the amygdala (Tejani-Butt et al., 1994). Clinical studies using functional magnetic resonance imaging and a strategy of pharmacologically potentiating NE neurotransmission in healthy individuals have demonstrated that elevated NE neurotransmission enhances basolateral amygdalar nuclear complex (BLA) responses to fear signals (Onur et al., 2009). Furthermore, NE system in the BLA is implicated in memory modulation by stress (McGaugh et al., 2002, Ferry and McGaugh, 2008, McGaugh and Roozendaal, 2009).

In addition to CRF, the delta opioid system has been implicated in the control of emotion-regulated behaviors including anxiety. The endogenous opioid peptides, met- and leu-enkephalin, reduce anxiety through activation of delta opioid receptors (DOPR) (Nieto et al., 2005). Selective DOPR agonists decrease anxiety-like behaviors in rodent models and have a modulatory effect on a variety of stress-induced responses (Nieto et al., 2005, Saitoh et al., 2005, Perrine et al., 2006, Perrine et al., 2008, Randall-Thompson et al., 2010). Conversely, genetic deletion of the DOPR gene (Filliol et al., 2000) or administration of selective DOPR antagonists (Saitoh et al., 2005, Perrine et al., 2006) increases anxiety-like responses, suggesting a tonic dampening of anxiety mediated by the delta opioid system. The amygdala appears to be an important site of action for the anxiolytic effects of DOPR agonists. Direct infusion of the selective DOPR agonist, DPDPE, into the amygdala reduces basal and stress-induced anxiety-like behaviors in a rat model (Randall-Thompson et al., 2010).

The present study focused on the amygdalar complex as the site of the anxiolytic effects DOPR agonists. Our previous results demonstrating that activation of DOPR specifically in the CeA can attenuate stress-induced anxiety (Randall-Thompson et al., 2010), suggest that DOPR may interact with CRF neurons in the amygdala. Although the role of amygdalar CRF in anxiety has been studied extensively (Heinrichs et al., 1992, Liang et al., 1992, Swiergiel et al., 1993, Merlo Pich et al., 1995, Rodriguez de Fonseca et al., 1997, Merali et al., 1998, Bruchas et al., 2009, Knoll et al., 2011, Lam and Gianoulakis, 2011, Andero et al., 2013, McCall et al., 2015), there is a gap in our knowledge regarding whether DOPR directly modulates CRF activity. Considering the importance of this stress-integrative circuitry, we sought to determine whether amygdalar neurons that express CRF were positioned to be modulated by opioids via DOPR. We also investigated the anatomical interactions between noradrenergic afferents and amygdalar DOPR- and CRF-containing neurons due to the established role of NE in anxiety and stress responses. Finally, this study tested the ability of a DOPR agonist to reduce anxiety-like behaviors produced by a noradrenergic pharmacological stressor.

Materials and Methods

All procedures used in the present study were approved by the Institutional Animal Care and Use Committees of Drexel University and Temple University and conformed to National Institute of Health's Guide for the Care and Use of Laboratory Animals. Rats were housed two – three per cage on a 12-h light schedule in a temperature controlled colony room (20 ºC). 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 the minimum number of animals necessary to produce reliable scientific data.

Experimental animals and brain preparation for anatomical analysis

Eleven adult male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 250–300g were used to examine the cellular associations of DOPR and CRF with norepinephrine transporter (NET) in the BLA and CeA. Out of 11 male rats, five rats were used for light microscopy and immunofluorescence experiments and six rats were used for electron microscopy. Of the six rats used for electron microscopy and ultrastructural

analysis, three rats received injections of colchicine, a microtubule inhibitor (50 μg; Sigma-Adrich, St. Louis, MO), into their lateral ventricles to elevate peptide levels in soma and dendrites. Three rats were anesthetized with cocktail of ketamine hydrochloride (100 mg/kg: Phoenix Pharmaceutical Inc., St. Joseph, MO) and xylazine hydrochloride (2 mg/kg; Phoenix Pharmaceutical Inc.), and placed in stereotaxic apparatus. Using a Picospritzer (General Valve Corporation, Fairfield, NJ), colchicine was injected at 24–26 psi, 10 ms duration and 0.2 Hz. Coordinates for the lateral ventricle infusions were taken from the rat brain atlas of Paxinos and Watson (1986) as follows: 0.26 mm posterior from the bregma, 1.2 mm medial/lateral and 3.6 mm ventral from the top of the skull. About eighteen hours after colchicine administration, rats were deeply anesthetized with sodium pentobarbital (Beuthanasia) at 60 mg/kg via an intraperitoneal route 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, cut in half and immersed in 2% formaldehyde overnight at 4°C.

Antibody characterization

DOPR antibody (Alomone Laboratories, Jerusalem, Israel) was raised in rabbit and directed against an extracellular epitope of the mouse DOPR. Previous studies using this DOPR antibody have demonstrated that DOPR immunoreactivity was abolished following preadsorption with antigenic peptide (Salman et al., 2013, Salman et al., 2014). In the present study to ensure antibody specificity, we performed control experiments and replicated these results with both immunohistochemistry and Western blotting technique.

The CRF antibody (Peninsula Laboratories, San Carlos, CA) in this study was used to identify CRF-containing amygdalar neurons. This CRF serum was raised in guinea pig and was generated against human/mouse/rat CRF peptide (SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII) (Peninsula Laboratories). The specificity of CRF immunolabeling was tested by lack of immunoreactivity in areas not known to expresss CRF. In addition, blocking with the antigenic CRF peptide via preabsorption abolished CRF immunoreactivity as previously conducted (Bachtell et al., 2003, Lim et al., 2007, Sawada et al., 2008).

NE transporter (NET) was used to identify NE 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, Kravets et al., 2015).

Dual immunofluorescence

Coronal tissue sections from five naïve rats were cut through the amygdala at 40 μm using a Vibratome (Technical Product International, St Louis, MO) 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. Tissue sections were incubated overnight at room temperature with a rabbit antibody directed against DOPR (1:1000; Alomone) and mouse anti-NET (1:1000; MAb Technologies) or guinea pig anti-CRF (1:2000, Peninsula Laboratories) 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 for 2h at room temperature the tissue sections were incubated in secondary antibodies, fluorescein isothiocyanate-conjugated donkey anti-rabbit (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for visualization of DOPR and rhodamine isothiocyanate-conjugated donkey anti-guinea pig (1:200; Jackson Immunoresearch) to visualize CRF. 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. Some mounted tissue sections were also incubated with 4′,6-diamidino-2-phenylindole (DAPI; EMD Millipore, Billerica, MA) at 1:10,000 for 5 minutes and washed 3 times with 0.05 M PB prior to dehydration in alcohols and xylene. The tissue sections were coverslipped using DPX mounting medium (Sigma-Aldrich, St. Louis, MO).

Triple immunofluorescence

The procedures used for dual immunofluorescence described earlier were also followed for triple immunofluorescence except that the tissue sections were incubated overnight at room temperature with a mouse antibody directed against NET (1:1000; MAb Technologies), rabbit anti-DOPR (1:1000; Alomone), and guinea pig anti-CRF (1:2000, Peninsula Laboratories). Moreover, the tissue sections were incubated in secondary antibodies, namely: rhodamine isothiocyanate-conjugated donkey anti-guinea pig (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) to visualize CRF; fluorescein isothiocyanate-conjugated donkey anti-rabbit (1:200; Jackson Immunoresearch) for visualization of DOPR, and cyanine dye Cy5-conjugated donkey anti-mouse (1:200, Jackson Immunoresearch) to visualize NET. 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, St. Louis, MO).

Dual immunolabeling: immunoperoxidase and immunogold-silver labeling

Using immunoelectron microscopy, CRF was labeled with immunoperoxidase while DOPR was detected using the immunogold-silver labeling. The brains were removed, cut in half and immersed in 2% formaldehyde overnight at 4°C. CRF 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 rabbit anti-DOPR (1:1000) and guinea pig anti-CRF (1:2000) with 0.1% BSA in 0.1 M TBS. CRF antibody was visualized using

immunoperoxidase detection by incubating the tissue sections in biotinylated goat anti-guinea pig 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 μL of 30% hydrogen peroxide in 100 mL of 0.1 M TBS for 10 min. After visualization of CRF, the tissue sections were processed for immunogold-silver labeling for DOPR as previously described (Reyes et al., 2008, Reyes et al., 2011, Kravets et al., 2015).

Gold-silver localization of DOPR was carried out by rinsing the tissue sections three times with 0.1 M TBS, followed by rinses with 0.1 M PB and 0.01 M phosphate buffered saline (PBS; pH 7.4). Sections were then incubated in a 0.2% gelatin-PBS and 0.8% BSA buffer for 10 min. This was followed by incubation in goat anti-mouse IgG conjugate in 1 nm gold particles (Amersham Bioscience Corp., Piscataway, NJ) at room temperature for 2 hours. Sections were then rinsed in buffer containing the same concentration of gelatin and BSA as above and subsequently rinsed with 0.01 M PBS. Sections were then incubated in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.01 M PBS for 10 min followed by washes in 0.01 M PBS and 0.2 M sodium citrate buffer (pH 7.4). A silver enhancement kit (Amersham Bioscience Corp.) was used for silver intensification of the gold particles. The optimal times for silver enhancement were determined by empirical observation for each experiment and ranged 7–8 min. Following intensification, tissue sections were rinsed in 0.2 M citrate buffer and 0.1 M PB, and incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M PB for 1 h, washed in 0.1 M PB, dehydrated in an ascending series of ethanol followed by propylene oxide and flat embedded in Epon 812 (Electron Microscopy Sciences; (Leranth and Pickel, 1989).

Ultrathin sections of approximately 70 nm were cut with a diamond knife (Diatome-US, Fort Washington, PA, USA) using a Leica Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were collected on copper mesh grids and counterstained with 5% aqueous uranyl acetate for 20 minutes followed by Reynold's lead citrate for 5–7minutes. Captured images of selected sections were compared with light microscopic images of the block-face before sectioning to verify that the area of interest was targeted/collected. Tissue sections were examined with a Morgagni 268 transmission electron microscope (Fei Company, Hillsboro, OR). Digital images were obtained using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA). The TEM has an accelerating voltage of 80kv and we used the magnification of 11,000 to 14,000 in a field size of 9.3 μm × 9.3 μm for 11000× and 7.3 μm × 7.3 μm for 14000×. Figures were adjusted in brightness and contrast using Adobe Photoshop CS4 software (Adobe Systems, Inc., San Jose, CA).

Controls and Data analysis

Tissue sections for electron microscopy were taken from rats with the best immunohistochemical labeling and preservation of ultrastructural morphology. The semi-quantitative approach used in the present study is well established and has been described previously (Van Bockstaele et al., 1996a, Van Bockstaele et al., 1996b, Reyes et al., 2006,

Reyes et al., 2007). Three rats used for semi-quantitative analysis were injected with colchicine. Colchicine has 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 neurochemical signature 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.

While acrolein fixation optimizes the preservation of ultrastructural morphology, the caveat of limited and or differential penetration of immunoreagents in thick tissue sections exists (Leranth and Pickel, 1989, Jensen et al., 2009). Consequently, the limited penetration of CRF and DOPR may result in an underestimation of the relative frequencies of their distribution. We mitigated this limitation by collecting the tissue sections exclusively near the tissue-Epon interface where penetration is maximal and profile were sampled only when all the markers were present in the surrounding neuropil included in the analysis.

The cellular elements were identified based on the description of Peters and colleagues (Peters and Palay, 1996). Somata contained a nucleus, Golgi apparatus and smooth endoplasmic reticulum. Proximal dendrites contained endoplasmic reticulum, were postsynaptic to axon terminals and were larger than 0.7 μm in diameter. A terminal was considered to form a synapse if it showed a junctional complex, a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or associated with postsynaptic thickening. A synaptic specialization was only limited to the profiles that form clear morphological characteristics of either Type I or Type II (Gray, 1959). Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I; (Gray, 1959). In contrast, symmetric synapses had thin densities (Gray's type II; (Gray, 1959) both pre- and post-synaptically. An undefined synapse was defined as an axon terminal plasma membrane juxtaposed to that of a dendrite or soma devoid of recognizable membrane specializations and no intervening glial processes.

Semi-quantitative analysis include only tissue sections that contained both DOPR and CRF immunoreactivities. In sections dually labeled for DOPR and CRF, the number of DOPR-labeled dendrites and axon terminals were grouped from randomly selected sections taken from four nonadjacent ultrathin sections from each animal (n = 5). At least five sections were examined per animal. From the surface of the individual epon block containing the tissue, at least four to eight ultrathin sections were collected. Fields of at least 11,000X magnification showing DOPR-labeled dendrites or axon terminals and CRF-labeled profiles were captured and classified. This approach resulted in total profiles of 577 and 475 DOPR-labeled dendrites from the BLA and CeA, respectively as well as total profiles of 198 and 147 DOPR-labeled axon terminals from the BLA and CeA, respectively. All potential neuronal targets of DOPR-labeled axon terminals throughout the analysis were examined by defining their total associations with other profiles regardless of whether a membrane specialization was seen within the plane of section. The postsynaptic targets considered included dendrites containing gold-silver labeling for CRF and dendrites lacking gold-silver labeling for CRF-labeled profiles forming clear synaptic specializations were classified as symmetric (Gray's Type II) or asymmetric (Gray's Type I). On the other hand, undefined

contacts were characterized by a junctional complex that was not clearly distinguishable to allow a classification as either symmetric or asymmetric in the plane of section analyzed.

Identification of immunogold-silver labeling in profiles

Selective immunogold-silver labeled profiles were identified by the presence, in single thin sections, of at least two immunogold-silver particles within a cellular compartment. As we previously reported (Reyes, et al., 2006), single spurious immunogold-silver labeling can contribute to false positive labeling and can be detected on blood vessels, myelin or nuclei. Although minimal spurious labeling was identi ed in the present study, the criteria for de ning a process as immunolabeled was therefore set at 2 immunogold-silver particles in a cellular pro le. Whenever possible, the more lightly labeled somatic and dendritic labeling for DOPR was confirmed by detection in at least two adjacent sections.

Quantification of DOPR distribution

The analysis of DOPR distribution in the BLA and CeA was carried out by calculating the ratio of cytoplasmic to total immunogold-silver particles for each singly immunolabeled dendritic profile in individual rats. Cytoplasmic immunogold-silver particles are defined by their localization within the cytoplasmic compartment and are not contacting the plasma membrane. The total immunogold-silver particles include particles that are located within the cytoplasmic compartment and those that are located along or in direct contact with the plasma membrane.

Anxiety measurement

Thirty-two adult male Sprague Dawley rats were tested on the elevated zero maze for anxiety-like behavior (Braun et al., 2011). The zero maze apparatus (San Diego Instruments) is a beige circular runway 4 inches wide with a 48-inch diameter outer wall and 40-inch diameter inner wall. The platform is elevated 30 inches above the ground and divided into equal quadrants. Two opposing quadrants are “closed” with 8-inch high walls, separated by “open” quandrants with a half-inch high ledge. The lighting conditions were adjusted to produce light levels of ~75 lux in the open sections and ~30 lux in the closed sections of the maze. Rats were allowed to acclimate in the testing room to the dim (~75lux) lighting for 1 hour prior to testing. Testing took place between 10:00 AM and 2:30 PM. Each rat was placed in the same orientation in the same closed section of the maze and behavior was video recorded for 10 minutes. The maze was cleaned and dried between rats. Anxiety-like behavior was assessed by the amount of time each rat spent in the open quadrants of the maze and reported as a percent of the total time on the maze. A rat was considered to be in an open quadrant when at least the head and both front legs of the rat moved into an open section of the maze.

In order to assess stress-induced anxiety, yohimbine (2 mg/kg sc) or vehicle (sterile water 1 ml/kg sc) was administered 30 minutes prior to testing on the elevated zero maze. The selective DOPR agonist SNC80 (10 mg/kg s.c.) or vehicle (sterile water at pH 5 with HCl, 1 ml/kg sc) was administered 60 minutes prior to testing (30 minutes prior to yohimbine). Data were analyzed by a two-way ANOVA with pretreatment and treatment main factors.

Results

Specificity of antibodies and control experiments

Control experiments were carried out in order to rule out any cross-reactivity of antibodies. The following conditions were processed 1) primary antisera were omitted while secondary antibodies were added, 2) one of the primary antisera was omitted; 3) one of the secondary antibodies was omitted; and 4) all secondary antibodies were omitted. Thus, control tissue sections processed in the absence of either DOPR or CRF primary antibodies did not exhibit detectable immunoreactivity. As we previously reported (Ambrose-Lanci et al., 2008, Reyes et al., 2008, Carvalho et al., 2010, Reyes et al., 2011, Kravets et al., 2015), there was no detectable immunolabeling with the primary antibody omitted. We also evaluated the cross-reactivity of the primary antibodies with secondary antibodies by processing tissue sections with omission of one of each of the primary antisera. Furthermore, processing of additional controls included incubation of each primary antibody with an inappropriate secondary antibody. This resulted in the absence of immunolabeling for the incorrectly paired primary-secondary antibody combination. Thus, control sections that were incubated with rabbit DOPR followed by goat anti-mouse secondary antibody did not exhibit any detectable DOPR immunolabeling.

Region of interest selected for DOPR-CRF analysis

Different investigators have grouped the amygdalar nuclei in various ways (Price et al., 1987; McDonald, 1998; Sah et al., 2003). We use the nomenclature introduced by Price and colleagues (Price et al., 1987) with some modifications (McDonald, 1998; Sah et al., 2003). In the present study, the basolateral nucleus of the amygdala (BLA; Figure 1) is defined as the area encompassing the basolateral group as described by Sah and colleagues (Sah et al., 2003), and comprises the lateral and anterior subdivisions of the BLA (Zhang et al., 2013) at bregma level −1.80 through −3.0 (Paxinos and Watson, 1986). 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).

Using light microscopy, DOPR immunoreactivity appeared to be more robust and dense in the BLA compared to the CeA as shown in Figure 1. This is supported by the confocal immunofluorescence images showing a more abundant distribution of DOPR in the BLA (Figure 2A, 3A, 6A) than in the CeA (Figure 2D, 3D). The distribution of CRF in the BLA (Figure 2B) is consistent with our previous report showing less CRF immunoreactivity in this subregion when compared to the CeA (Figure 2E) (Kravets et al., 2014, Reyes et al., 2008, 2011). DOPR and CRF co-localization was present in both the BLA (Figure 2C) and the CeA (Figure 2F). Figure 3 illustrates nuclei staining in CRF-containing neurons that co-localized with DOPR in the BLA and the CeA.

Localization of DOPR in the BLA and CeA

Using electron microscopy, DOPR was localized in dendrites and axon terminals in the BLA (Figure 4A–C) and CeA (Figure 4D–F). In the BLA, approximately 45% (237/528) and 55% (292/528) of DOPR was localized in axon terminals (Figure 4A, 5C) and dendrites (Figure 4B–C, 5A–B), respectively. In the CeA, approximately 31% (145/473) and 69% (328/473)

of DOPR was localized in axon terminals (Figure 4D, 5F) and dendrites (Figure 4E–F, 5D–E), respectively.

DOPR-immunogold silver particles were distributed within the cytoplasmic compartment while some were associated with the plasma membrane (Fig. 4–5). Semi-quantitative analysis showed that the ratio of cytoplasmic to total DOPR immunogold-silver particles in the BLA dendrites was 0.54 ± 0.01 (n=115 dendritic profiles per rat analyzed). The ratio of cytoplasmic to total DOPR immunogold-silver particles in the CeA was 0.56 ± 0.04 (n=100 dendritic profiles analyzed). Likewise, semi-quantitative analysis showed that the ratio of cytoplasmic to total DOPR-immunogold silver particles in the BLA and CeA axon terminals was 0.48 ± 0.03 (n=108 axon terminals analyzed) and 0.53 ± 0.05 (n=108 axon terminals analyzed).

DOPR co-localization with CRF in the BLA—Using dual immunolabeling where CRF was labeled with immunoperoxidase and DOPR was labeled with immunogold-silver particles, approximately 55% (133/242; Table 1) of DOPR was co-localized with CRF in BLA dendrites (Figure 5A–B). Semi-quantitative analysis revealed that, of 133 DOPR +CRF-labeled dendrites analyzed, 49% (65/133) received symmetric synapses (arrowheads in Figure 5A) from unlabeled terminals, 23% (30/133) received asymmetric synapses (zigzag arrows in Figure 5B) and 29% (38/133) received synaptic specializations that could not be unequivocally determined. DOPR-labeled terminals targeting CRF-dendrites were also analyzed. Of 198 DOPR axon terminals analyzed in the BLA, 41% (81/198) targeted CRF-labeled dendrites (Figure 5C). Semi-quantitative analysis showed that of the 81 DOPR-labeled axon terminals contacting CRF-labeled dendrites 41% (40/81) formed symmetric synapses, 22% (18/81) formed asymmetric synapses and 28% (23/81) formed undefined synaptic specializations with the CRF-labeled dendrites. Asymmetric synapses had enlarged post-synaptic densities, while symmetric synapses exhibited densities that were comparable across pre- and post-synaptic membranes. In addition to co-localization of DOPR and CRF in dendrites in the BLA, we also observed some co-localization of DOPR and CRF in axon terminals albeit infrequently (Figure 5D).

DOPR co-localization with CRF in the CeA—Using dual immunolabeling where CRF was labeled with peroxidase and DOPR was labeled with immunogold-silver particles, approximately 67% (212/315; Table 1) of DOPR was co-localized with CRF in CeA dendrites (Figure 5D–E). Semi-quantitative analysis revealed that, of 212 DOPR+CRF-labeled dendrites analyzed, 60% (127/212) received symmetric synapses (Figure 5E) from unlabeled terminals, 20% (43/212) received asymmetric synapses and 20% (42/212) received undefined synapses (Figure 5D). DOPR-labeled terminals targeting CRF-dendrites were also analyzed. Of the 147 DOPR axon terminals analyzed in the CeA, 29% (43/147) targeted CRF-labeled dendrites (Figure 5E). Semi-quantitative analysis showed that of the 43 DOPR-labeled axon terminals contacting CRF-labeled dendrites 46% (20/43) formed symmetric synapses, 28% (12/43) formed asymmetric synapses and 26% (11/43) formed undefined synaptic specializations with CRF-labeled dendrites. Infrequent co-localization of DOPR and CRF in axon terminals was observed (Figure 5H).

Anatomical substrates for NE innervation of CRF- and DOPR-containing amygdalar neurons

We examined the interaction between DOPR and NET. Consistent with our previous report (Kravets et al., 2014), NET is abundantly distributed in the BLA (Figure 6B–C, 6E–F 7C–D) with lesser density in the CeA (Figure 6H–I, 7G–H). DOPR-containing neurons in the BLA (Figure 6A–F, 7A, C–D) and CeA (Figure 6G–I, 7E, G–H) are in close proximity with the NET-immunolabeled fibers. Interestingly, some DOPR are co-localized with NET in the BLA (Figure 6C,F) and CeA (Figure 6I).

To determine if NE axon terminals are positioned to directly regulate CRF- and DOPR-containing neurons in the BLA and CeA, immunoreactivities for NET, CRF, and DOPR were detected in the same section of tissue using immunofluorescence microscopy. Triple label immunofluorescence showed that DOPR (green labeling) are co-localized with CRF (pseudocolored with blue) labeled neurons and were in close proximity to NET-immunolabeled fibers (pseudocolored with red) in the BLA (Figure 7A–D) and CeA (Figure 7E–H), respectively. Consistent to our previous report (Kravets et al., 2015), NET was localized to processes that were highly varicose and punctate in appearance (Figure 7C–D, G–H). Moreover, NET immunoreactivity was more dense in the BLA (Figure 7C–D) compared to the CeA (Figure 7G–H). Likewise, CRF perikarya were more abundant in the CeA (Figure 7F, H) compared to the BLA (Figure 7B, D). While NET and DOPR co-localized in the BLA and CeA, co-localization of NET with DOPR and CRF was not observed (Figure 7D, 7H). In addition, we observed that the co-localized NET and DOPR in the BLA and CeA show a close proximity interaction with a CRF-labeled neuron (Figure 7D, 7H).

Functional interactions of DOPR, NE and CRF in stress-induced anxiety

In order to study the functional interactions of DOPR, NE, and CRF, the ability of the selective DOPR agonist, SNC80, to block anxiety produced by the pharmacological adrenergic stressor, yohimbine, a α_2-adrenergic receptor antagonist, was tested. Increases in norepinephrine transmission result in heightened stress and anxiety responses in both humans and animal models (Moran Santa Maria et al., 2014; Cai L, et al., 2012; Kaplan JS et al., 2012; Braun AA et al., 2011). Anxiety-like behaviors of rats were measured using the elevated zero maze 30 minutes after an injection of yohimbine (2 mg/kg sc) or vehicle. The effect of the DOPR agonist on yohimbine-induced anxiety was tested; SNC80 (10 mg/kg sc) or its vehicle was administered 30 minutes prior to yohimbine (ie, 60 min prior to testing). The time spent in the open quadrants of the maze was expressed as a percentage of total time spent on the maze.

As shown in Figure 8, results indicate significant differences in open-arm times among treatment groups. Analysis of the data by two-way ANOVA revealed significant main effects of yohimbine stress ($F_{1,27}$= 8.415, P=0.0073) and treatment with SNC80 ($F_{1,27}$=47.03, P<0.0001). Bonferroni's multiple comparison post hoc analysis revealed significantly lower time on the open arms in rats given the yohimbine stressor versus the vehicle controls (vehicle/yohimbine vs vehicle/vehicle, P<0.01) indicating that yohimbine produced heightened anxiety. Treatment with SNC80 significantly blocked the increase in anxiety-like

behaviors produced by yohimbine, as indicated by significantly greater open arm time in SNC80 treated stressed rats versus yohimbine stress alone (SNC80/yohimbine vs vehicle/yohimbine, $P<0.001$). These results indicate that administration of SNC80 prevented the stress-induced anxiety produced by elevated NE transmission.

Discussion

The present study provides anatomical evidence for a mechanism by which activation of DOPR could regulate amygdalar CRF activity and modulate stress responses including anxiety-like behaviors. An increased understanding of the fine synaptic organization of the amygdalar complex may lead to better treatments for anxiety disorders that are often precipitated by stress (de Kloet et al. 2005; Arborelius et al. 1999). In the present study, we advance knowledge of the intrinsic organization of limbic circuitry by providing evidence for co-localization of CRF and DOPR in individual neurons of the BLA and CeA. Additionally, our results demonstrate that DOPR and CRF-containing neurons in the BLA and CeA are in close proximity with NE afferents suggesting that NE modulation may impact amygdalar opioid-CRF circuitry. Taken together, these results provide anatomical evidence for the interaction of DOPR and CRF systems and suggest a role for NE in modulating DOPR and CRF-containing neurons in the amygdala that may be critical for the regulation of anxiety-like behaviors.

Amygdalar DOPR and anxiety

The opioid system continues to be an important target in the development of new therapies for stress and anxiety disorders because of its role in the modulation of emotional state and regulation of arousal and stress responses (Drolet et al., 2001, Primeaux et al., 2006). A prominent role of DOPR in emotional processing has been revealed in genetic mouse studies. Genetic deletion of the DOPR gene results in a phenotype of heightened anxiety (Filliol et al., 2000). This is specific to the DOPR as anxiogenic behavior is not observed in mu or kappa opioid receptor knockout mice (Konig et al., 1996, Filliol et al., 2000). In agreement with the DOPR knockout mouse model, systemic administration of the selective DOPR antagonist, naltrindole, increases anxiety-like behaviors (Perrine et al., 2006; Saitoh et al., 2005), as do conditions that produce dysfunction of DOPRs such as withdrawal from chronic cocaine administration (Perrine et al., 2008). The converse is also observed; enhanced DOPR function leads to reduced anxiety-like behaviors. This can be seen by administration of DOPR agonists (Saitoh et al., 2004; Perrine et al., 2006 and 2008; Nieto et al., 2005; Randall-Thompson et al., 2010) or manipulations that increase endogenous opioid peptides that activate DOPR receptors (Kang et al., 2005, Nieto et al., 2005). Together, these data indicate that DOPR plays an important role in the homeostatic regulation of anxiety states.

Amygdalar DOPR and CRF: anatomical considerations

Previous pharmacological and anatomical studies have demonstrated significant interactions between endogenous opioid peptide systems and CRF in the amygdalar complex (Drolet et al., 2001, Reyes et al., 2008, Van Bockstaele et al., 2010, Reyes et al., 2011, Andero et al., 2013, Kravets et al., 2015). Although CRF, DOPR and enkephalin-containing neurons are

abundant in the amygdalar complex (Sharif and Hughes, 1989, Mansour et al., 1995, Criado and Morales, 2000, Randall-Thompson et al., 2010), there were no previous reports of localization of DOPR to amygdalar CRF neurons. Although it had been demonstrated that DOPR and CRF are co-localized in other neural circuits such as hippocampal interneurons and pyramidal cell dendrites (Williams et al., 2011, Williams and Milner, 2011), this is the first report demonstrating that DOPR are expressed in CRF-containing neurons in the amygdala. The present study is in agreement with previous reports showing that CRF-containing neurons are more abundant in the CeA compared to the BLA (Cassell et al., 1986, Sakanaka et al., 1986, Cassell and Gray, 1989, Van Bockstaele et al., 1998, Reyes et al., 2008, Iemolo et al., 2013). Interestingly, the present results reveal that DOPR exhibits a topographic distribution within the BLA and CeA where dense DOPR immunoreactivity was observed in the BLA compared to the CeA. The subcellular localization of DOPR within the BLA and CeA suggests that DOPR may function to inhibit the excitation of BLA and CeA neurons in the presence of a stressor (Herringa et al., 2004; Tye et al., 2011). The postsynaptic localization of DOPR is also supported by in situ hybridization and immunohistochemical data of enkephalin mRNA and immunoreactivity in the amygdalar complex including the BLA and CeA (Berube et al., 2013; Chang et al., 2014; Chieng et al., 2006; Zhang and McDonald, 2016).

The presence of DOPR and CRF postsynaptically in common dendrites suggests that the activation of DOPR can affect the postsynaptic excitability of CRF-containing neurons in both BLA and CeA. This in agreement with our previous reports indicating that endogenous opioids interact with CRF in an opposing fashion such that opioids may serve as a counter-regulatory mechanism to balance or limit excessive activation of the stress response (Van Bockstaele et al., 2010; Xu et al., 2004; Curtis et al., 2001). A distinct intracellular signaling pathway supports the functional opposition since opioids bind with G_i/G_o coupled DOPR to inhibit the cAMP pathway while CRF binds to the Gs-coupled CRF receptor to stimulate the cAMP pathway (McKenzie and Milligan, 1990; Levitt et al., 2011; Grammatopoulos et al., 2001). While we demonstrated co-localization of CRF and DOPR in BLA dendrites, and evidence suggests that BLA sends unidirectional projections to the CeA (McDonald, 1998, LeDoux, 2000, Pitkänen, 2000, Tye et al., 2011), we were not able to determine the precise trajectory of BLA CRF efferents in the current study. Further investigation is needed to address this issue.

We also demonstrated that DOPR and CRF are co-localized in single axon terminals in the BLA and CeA, albeit infrequently. The anatomical interaction of DOPR and CRF suggests that DOPR may modulate CRF release in the BLA and CeA. Previous studies reveal that CeA and BLA receive afferents from several distinct brain regions (McDonald, 1998; Pittaken, 2000; Sah et al., 2003). Tract tracing studies demonstrate that potential sources of opioidergic input to the CeA arise from forebrain, thalamic, hypothalamic and hindbrain nuclei (Pitkanen et al., 2000). In particular, retrograde tracing studies have demonstrated that enkephalinergic afferents to the CeA originate from the bed nucleus of the stria terminalis (BNST) and from other amygdalar subnuclei (Poulin et al., 2006). With regard to afferents from amygdalar subnuclei, the major enkephalinergic afferents to the CeA arise from the anterolateral BNST, the cortical nucleus of the amygdala, the medial amygdaloid nucleus and basomedial nucleus (Poulin et al., 2006). Additionally, the ventromedial nucleus of the

hypothalamus and the pontine parabrachial nucleus provide moderate enkephalinergic afferents to the CeA (Poulin et al., 2006). Intrinsic amygdalar enkephalinergic neurons, that are known to be distinct from amygdalar CRF neurons (Drolet et al., 2001; Reyes et al., 2008; Van Bockstaele et al., 2010; Reyes et al., 2011; Andero et al., 2013; Kravets et al., 2015), may play a role in the local circuit regulation of amygdalar neurons as there exists significant evidence describing such intrinsic connections in the CeA (Haubensak et al., 2010; Ciocci et al., 2010; Pomrenze et al., 2015). Potential opioidergic afferents to the BLA could also originate from local axonal arborizations of intrinsic enkephalinergic pyramidal neurons originating from the BLA (McDonald et al., 2005; Pitkanen et al., 2003). It is also likely that other sources of input could arise from the thalamus (Carlsen and Heimer, 1988; LeDoux et al., 1991) and cerebral cortex (Farb and Ledoux, 1999; Smith et al., 2000; Pinard et al., 2010). Since to our knowledge, this is the first report describing the ultrastructural analysis of DOPR in the BLA, future studies are necessary to define the source of opioidergic afferents in the BLA (Zhang and McDonald, 2016).

Noradrenergic modulation of amygdalar DOPR and CRF systems

Our semi-quantitative analysis indicates that DOPR and CRF-containing dendrites in the BLA and CeA primarily received symmetric synapses as compared to asymmetric synapses. Symmetric synapses are thought to be involved principally in inhibitory neurotransmission while asymmetric synapses are thought to be involved in excitatory neurotransmission (Peters et al., 1991, Peters and Palay, 1996). The preponderance of symmetric synapses onto DOPR and CRF neurons indicate that these dually labeled amygdalar neurons are potentially under inhibitory control (Gray, 1959). Future studies are needed to unequivocally establish this possibility as well as to investigate whether DOPR and CRF neurons are GABAergic themselves as there exists a large population of GABAergic neurons in the amygdala (Swanson and Petrovich, 1998, Ciocchi et al., 2010). Given that NET fibers targeted DOPR and CRF-containing neurons in our confocal results, it is possible that synaptic transmission occurs. In addition, the unlabeled axon terminals that target DOPR and CRF-containing dendrites may contain NET immunoreactivity. A triple EM can be utilized to address this possibility. There are reports of catecholaminergic neurotransmission occurring through conventional wired synaptic transmission and volume transmission, in which the neurotransmitter permeates through the extracellular space (Agnati et al., 1986). Wired synaptic transmission is associated with rapid responses to stress while volume transmission is associated with more long-term events (Perez de la Mora et al., 2008). Previous reports suggest that noradrenergic axon terminals exhibit a low frequency of synaptic contacts in the cortex and amygdala (Seguela et al., 1990, Asan, 1998). Our present results as well as studies from others have demonstrated that noradrenergic axon terminals more frequently form synaptic connections in both the BLA and CeA (Zhang et al., 2013, Kravets et al., 2015). It would be interesting to investigate whether CRF and NET are co-expressed at the ultrastructural level and whether they may contact DOPR-labeled dendrites or somata. Future investigations are needed to address this potential anatomical interaction using a triple immunolabeling electron microscopy approach (Reyes et al., 2011; Reyes et al., 2012; Kravets et al., 2013; Jaremko et al., 2013).

Consistent with our previous immunocytochemical studies (Rudoy and Van Bockstaele, 2005, Kravets et al., 2015) and those of Schroeter and colleagues (Schroeter et al., 2000), results from the present study show a moderate density of NET-ir fibers throughout the amygdalar complex with the BLA exhibiting a more robust distribution of NET-immunolabeling compared to the CeA. NET distribution and binding have demonstrated differential patterns of expression in the amygdalar complex. Quantitative autoradiography and in situ hybridization in rodents reveal a homogeneous binding pattern for the NET in the amygdalar complex, with higher binding in the BLA (Benmansour et al. 1992; Tejani-Butt 1992), whereas in non-human primates, NET has a heterogeneous distribution and moderate binding density in the CeA (Smith et al., 2006), and in humans, using spectrofluorimetric assay in postmortem brains, high concentrations of NE is found in the CeA (Farley and Hornykiewicz 1977).

NE neurotransmission in the in the BLA and CeA has been implicated in different segments of the stress response (Buffalari and Grace, 2007, Brown et al., 2011, Wenzel et al., 2014, Karkhanis et al., 2015, Rajbhandari et al., 2015). 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). NE modulation of afferents to the BLA and CeA containing DOPR could possibly take place via α_1- and β1-adrenergic receptors as NE could either excite or inhibit neuronal activities via these receptors (Johnson et al., 2011). NE acting through α_{1A}-adrenergic receptors facilitates GABAergic activity in the BLA and repeated stress impairs this response (Braga et al., 2004). Excessive NE release in the BLA due to repeated stress exposure may desensitize the α_1- adrenergic receptors, thus contributing to the hyper-excitability of the amygdala in stress-induced anxiety disorders (Braga et al., 2004). Considering that BLA sends GABAergic afferents to the CeA (Perez de la Mora et al., 2008), dysregulation of GABAergic transmission in the BLA may in turn adversely affect CeA activity. Similarly to BLA, α_1-adrenergic receptors are abundantly distributed in the CeA (Day et al., 1997, Domyancic and Morilak, 1997). NE exerts its effects in the CeA via the α_1-adrenergic receptors that are involved in the behavioral response to stressors (Pacak et al., 1993, Khoshbouei et al., 2002). The CeA is also enriched in α_2- adrenergic receptors which are involved in the autonomic response to stressful stimuli (Glass et al., 2002). Similar to α_1-adrenergic receptors, β-adrenergic receptors in the amygdala have been implicated in stress-induced behavioral response (Porterfield et al., 2012, Camp and Johnson, 2015). Although β-adrenergic receptors are not as abundant in the CeA, they have been found to be co-localized with CRF neurons and functionally related (Rudoy et al. 2009). Administration of a β_1-adrenergic receptor antagonist attenuates heightened CRF expression levels produced by cocaine withdrawal in a rat model of chronic cocaine exposure (Rudoy et al. 2009).

The results from the present anatomical study suggest that DOPR might reside on presynaptic NE afferents in the amygdala, particularly in the CeA. As DOPR activation has been shown to inhibit NE release in a variety of tissues and species including humans (degli Uberti et al., 1993, Yilmaz and Gilmore, 1999, Berger et al., 2006, Hudzik et al., 2011, Schulte et al., 2011), this mechanism could be an important contributor to the anxiolytic effects of DOPR agonists. This relationship was tested functionally in the present study by examining the effects of a DOPR agonist, SNC80, on anxiety produced by yohimbine.

Yohimbine is an α_2-adrenergic antagonist that is used as a pharmacological stressor. It increases noradrenergic transmission in the amygdala (Khoshbouei et al., 2002, Crespi, 2009) and increases anxiety-like behaviors (Park et al., 2013, Yeung et al., 2013). Yohimbine also increases CRF mRNA in the amygdala of the rat which contributes to its actions as a pharmacological stressor (Funk et al., 2006). It is shown here that SNC80 administered prior to yohimbine blocked the anxiogenic effects of yohimbine as measured on the elevated zero maze. It is possible that DOPR located on NE terminals inhibited NE release and thus reduced the pro-anxiety effects of yohimbine. An alternative or perhaps additional mechanism is the potential blockade of yohimbine-induced CRF induction by SNC80 which would dampen the stress response. Further studies are needed to address these hypotheses.

Functional implications

The present results suggest an interaction between limbic CRF, NE, and DOPR systems that may play a significant role in the modulation of stress response in the amygdala. The LC, a noradrenergic nucleus that projects to almost all levels of neuraxis (Foote et al., 1983; Aston-Jones, et al., 1984), receives CRF and dynorphin (DYN) afferents from the amygdala (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1996b; Van Bockstaele et al., 1998; Reyes et al., 2007; Reyes et al., 2008; Reyes et al., 2011). Furthermore, the CRF and DYN amygdalar efferents directly target LC noradrenergic dendrites (Van Bockstaele et al., 1996a; Reyes et al., 2008; Reyes et al., 2011). Given that limbic NE is critical in activating CRF neurons (Raber et al., 1995), the present results indicate that amygdalar CRF neurons are under noradrenergic control and are poised to regulate the LC noradrenergic system. In the presence of stressors, activation of the CRF, NE, and DOPR systems elicits physiological and behavioral responses allowing the human body to respond appropriately and cope with the challenge. The immediate response to stress is adaptive; however, chronic or prolonged stress exposure may exacerbate the system that could lead to maladaptive responses. NE released into the CeA would activate CRF neurons, which in turn activate LC neurons. However, the localization of DOPR on CRF neurons may reduce CRF activity and therefore preclude CeA activation that regulates LC neurons. In addition, the BLA sends GABAergic projections to the CeA which could modulate its activity (Perez de la Mora et al., 2008). The colocalization of CRF and DOPR in the CeA that are targeted by NE suggests noradrenergic modulation of the limbic CRF and DOPR system that could impact global NE release, as we previously showed that CRF forms asymmetric synapses onto LC noradrenergic neurons (Van Bockstaele et al., 1998, Reyes et al., 2008, Reyes et al., 2011). Anatomical and electrophysiological evidence suggest CRF as an excitatory neurotransmitter in the LC that translates to enhanced norepinephrine release that impact cortical targets (Curtis et al., 1997, Page and Abercrombie, 1999, Curtis et al., 2002). The amplification of CRF and NE release may disturb allostasis, an essential component of maintaining homeostasis (McEwen, 2006) which involves adaptation in the face of potentially stressful challenges that activates neural, neuroendocrine and neuroendocrine-immune mechanisms (Sterling and Eyer, 1988). Given the intricate interactions of CRF, NE and DOPR systems, dysregulation of one system may succumb to dysfunction in another.

In summary, the findings presented herein indicate the complex relationship between the CRF, NE, and DOPR systems in two regions of the amygdala that are critically important to

stress responses and emotional homeostasis. Recent reports indicate a rising number of stress-related psychiatric disorders that are resistant to standard pharmacological treatments (Jasovic-Gasic, 2015). Targeting multiple neuromodulatory systems (Hopkins, 2007) including CRF, NE, and DOPR systems may be a relevant therapeutic approach to restore altered neuronal activity and treat stress-related disorders.

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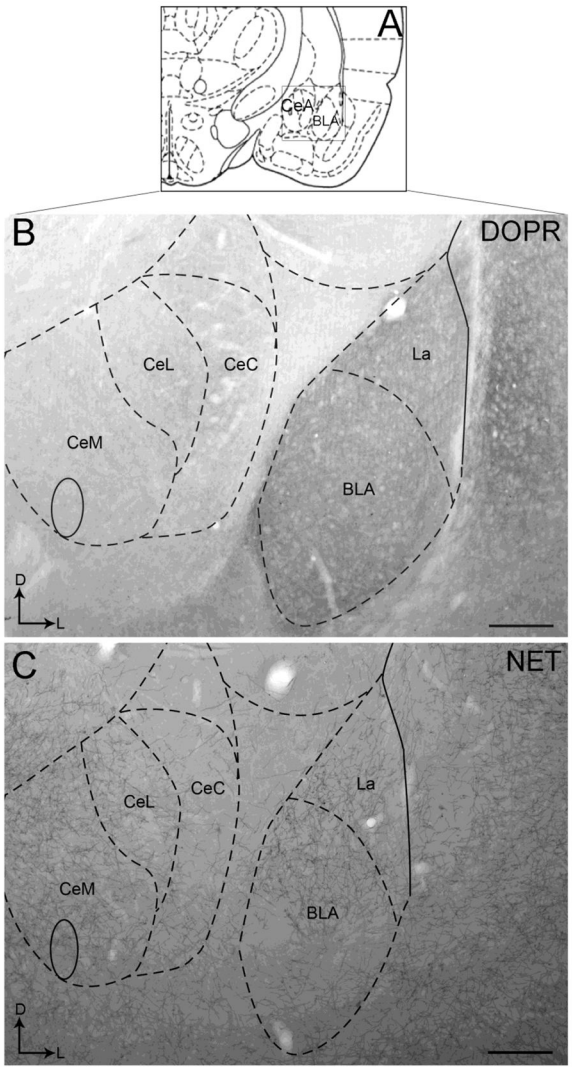

Figure 1.

Brightfield photomicrographs showing immunoperoxidase labeling of DOPR and NET immunoreactivities in the CeA and BLA. Dense DOPR immunoperoxidase labeling can be seen in the BLA and much less dense immunoperoxidase labeling is evident in the CeA as shown in panel B. Similar pattern of immunoperoxidase labeling is observed in NET immunoreactivity as shown in panel C. Panels B and C illustrate a representative section through the amygdalar region at bregma - 2.12. Arrows indicate dorsal (D) and medial (L) orientation of the tissue section. Schematic illustration adapted from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1997; Panel A) showing the anterior-posterior level from which the photomicrographs in panel B and C are taken. The region denoted by the box in panel A is shown in panels B and C. BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; CeC, capsular part of the CeA; CeL, lateral part of the CeA; CeM, medial division of the CeA La, lateral nucleus of the amygdala. Scale bars: 200 μm.

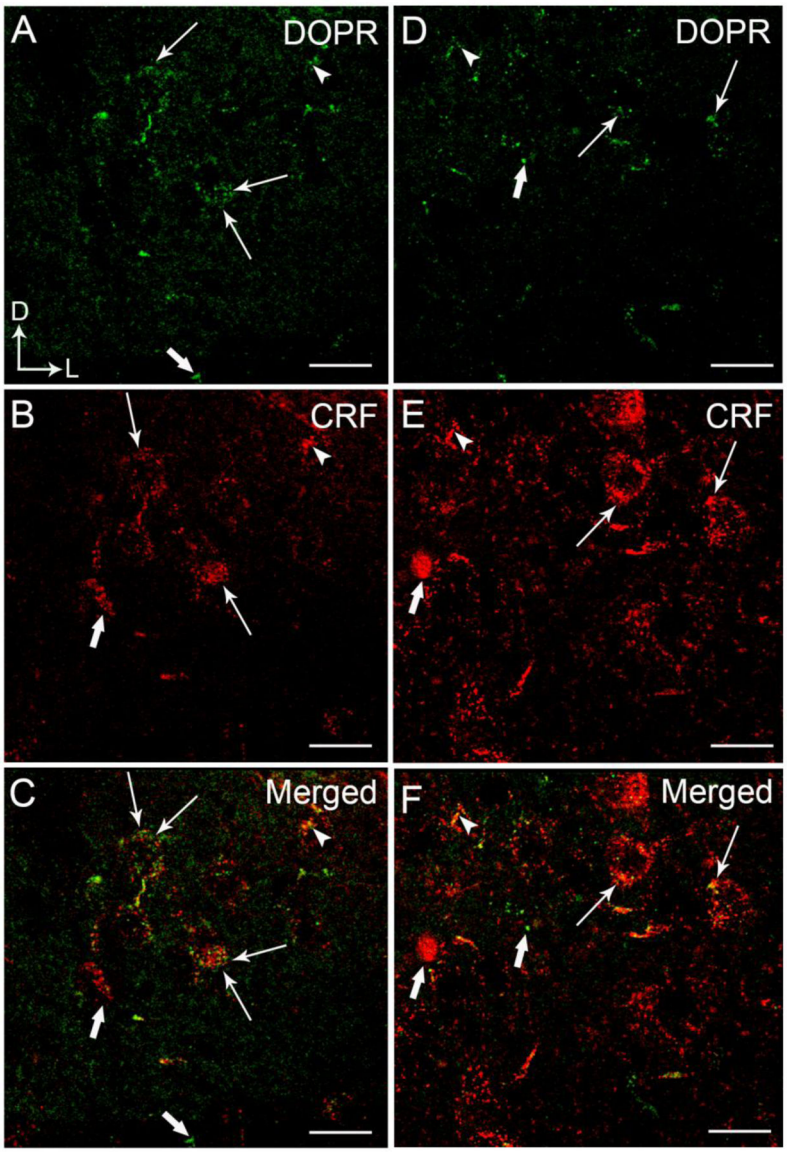

Figure 2.

High magnification confocal immunofluorescence photomicrographs showing dual-labeling for DOPR and CRF in a coronal section through the BLA (A–C) and CeA (D–F). DOPR immunoreactivity was labeled with fluorescein isothiocyanate (green) and CRF was labeled with rhodamine isothicyanate (red). **A–B.** Thin arrows depict dual labeling for DOPR (A) and CRF (B) while thick arrows depict singly labeled DOPR and CRF, respectively, in the BLA. **D–E.** Thin arrows depict dual labeling for DOPR (D) and CRF (E) in the CeA while thick arrows depict singly labeled DOPR and CRF. Panels C and F show merged image. Corner arrows indicate dorsal (D) and lateral (L) orientation. Scale bar, 25 μm.

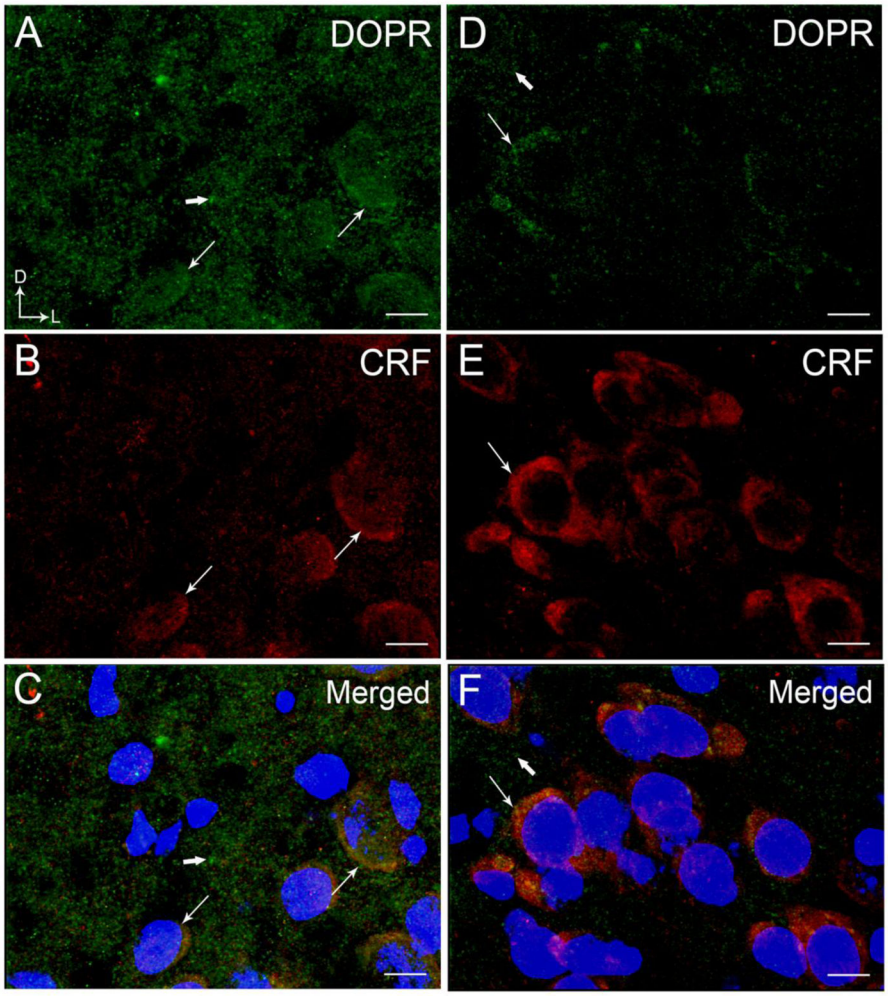

Figure 3.

High magnification confocal immunofluorescence photomicrographs showing labeling for DOPR and CRF in a coronal section through the BLA (A–C) and CeA (D–F). Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). DOPR immunoreactivity was labeled with fluorescein isothiocyanate (green), CRF was labeled with rhodamine isothicyanate (red) and DAPI in blue. **A–C.** Thin arrows depict labeling for DOPR (A) and CRF (B) with DAPI while thick arrows depict singly labeled DOPR and CRF, respectively, in the BLA. **D–E.** Thin arrows depict labeling for DOPR (D) and CRF (E) with DAPI in the CeA while thick arrows depict singly labeled DOPR and CRF. Panels C and F show merged image of DOPR, CRF and DAPI. Corner arrows indicate dorsal (D) and lateral (L) orientation. Scale bar, 25 μm.

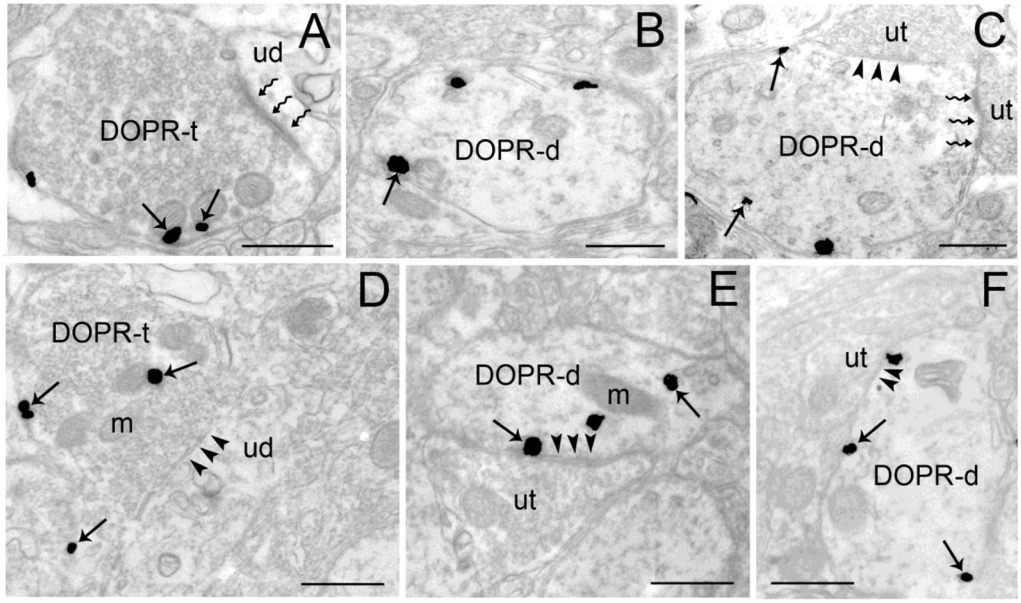

Figure 4.

Ultrastructural evidence for DOPR immunoreactivity in axon terminals and dendrites in the BLA and CeA. Electron photomicrographs showing immunogold-silver labeling for DOPR in the BLA (A–C) and CeA (D–F). **A.** Immunogold-silver labeling is present in a BLA axon terminal (DOPR-t) which forms an asymmetric synapse (zigzag arrows) with an unlabeled dendrite (ud). Arrows point to DOPR. **B–C.** DOPR-labeled dendrites (DOPR-d) in the BLA. Panel C shows a DOPR-d that receives an asymmetric (zigzag arrows) and symmetric synapses (arrowheads) from unlabeled terminals (ut). **D.** DOPR-immunogold-silver labeling can be seen in CeA axon terminal (DOPR-t) that forms a symmetric synapse (arrowheads) with unlabeled dendrite (ud). **E–F.** DOPR-containing dendrites in the CeA. Both DOPR-d receive a symmetric synapse (arrowheads) from an unlabeled terminal (ut). Arrows depict DOPR-immunogold silver particles. m, mitochondria. Scale bars, 0.50μm.

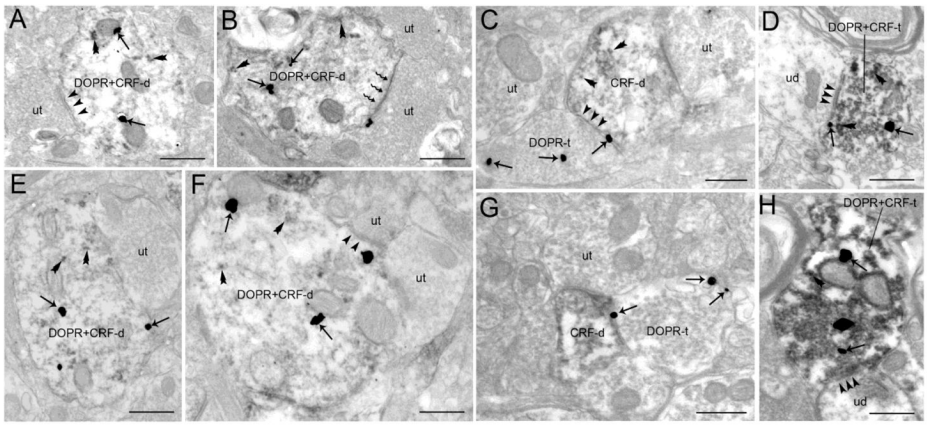

Figure 5.

Electron photomicrographs showing dual labeling for DOPR and CRF in the BLA and CeA. **A.** A DOPR and CRF-labeled dendrite (DOPR+CRF-d) receives a symmetric synaptic contact (arrowheads) from an unlabeled terminal (ut) in the BLA. **B.** A DOPR+CRF-d receives an asymmetric synapse (zigzag arrow) from an unlabeled terminal (ut) in the BLA. **C.** A DOPR-labeled axon terminal (DOPR-t) in the BLA forms a symmetric synapse (arrowheads) with a CRF-labeled dendrite (CRF-d). **D.** A DOPR and CRF-labeled axon terminal (DOPR+CRF-t) contacting (arrowheads) an unlabeled dendrite (ud) in the BLA. **E.** An unlabeled axon terminal (ut) is contacting a DOPR+CRF-d in the CeA. **F.** A DOPR +CRF-d receives a symmetric synapse (arrowheads) from an unlabeled terminal (ut). **G.** A DOPR-labeled axon terminal (DOPR-t) in the CeA contacting a CRF-d. **H.** A DOPR and CRF-labeled axon terminal (DOPR+CRF-t) forming a symmetric synapse (arrowheads) with an unlabeled dendrite (ud) in the CeA. Arrows point to DOPR-immunogold silver particles while double arrowheads point to peroxidase immunolabeling indicative of CRF. ut, unlabeled terminal. Scale bar, 0.50μm.

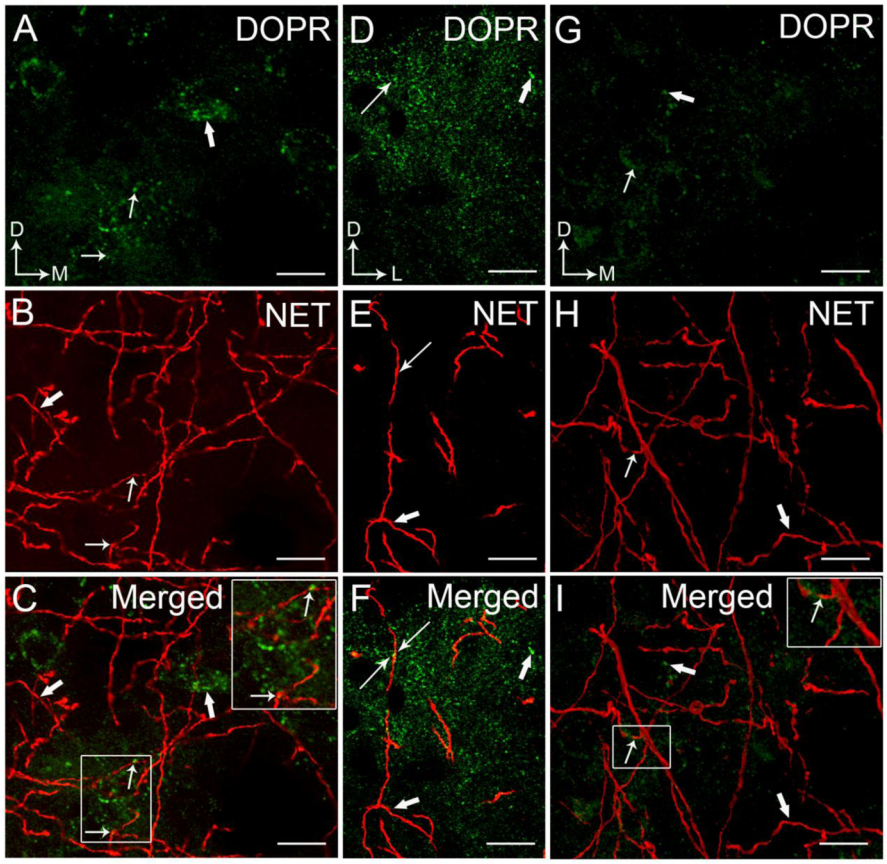

Figure 6.

High magnification confocal immunofluorescence photomicrographs showing dual-labeling for DOPR and NET in a coronal section through the BLA (A–F) and CeA (G–I). DOPR immunoreactivity was labeled with fluorescein isothiocyanate (green) and NET was labeled with rhodamine isothicyanate (red). Images of DOPR and CRF as collapsed Z-stack through the BLA (A–C) and CeA (G–I). Panels D–F are images of DOPR and CRF through the BLA, not as a collapsed Z-stack. Arrows depict site of dual labeling for DOPR (A,D) and NET (B,E) while thick arrow depicts singly labeled DOPR and NET, respectively, in the BLA. **G–H**. Arrows depict site of dual labeling for DOPR (G) and NET (H) while thick arrow depicts singly labeled DOPR and NET, respectively, in the CeA. Panels C, F, and I show merged images with dual labeling indicated by thin arrows. Arrows indicate dorsal (D) and lateral (L) orientation. Scale bar, 25 μm.

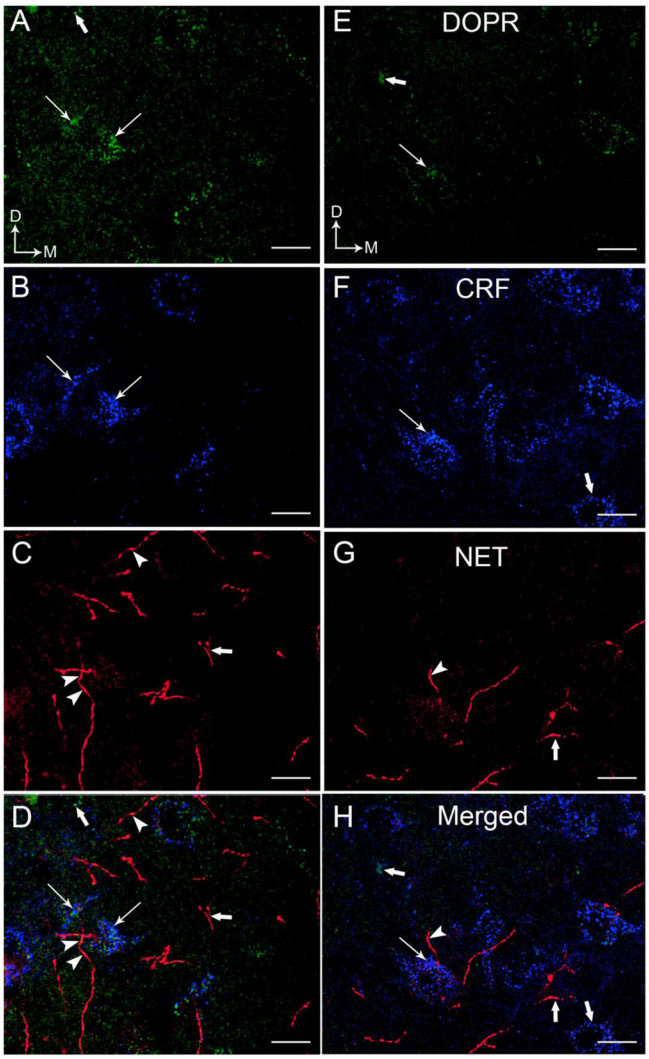

Figure 7.

High magnification confocal immunofluorescence photomicrographs demonstrating DOPR (A,E), CRF (B,F) and NET (C,G) immunoreactivities in the coronal section through the BLA (A–D) and CeA (E–H). DOPR immunoreactivity was labeled with fluorescein isothiocyanate (green), CRF was labeled with rhodamine isothiocyanate and pseudocolored with blue, and NET was labeled with cyanine dye 5 (Cy5) and pseudocolored with red. We pseudocolored NET with red to allow easier identification of co-localized DOPR and NET which produces yellow color (arrowheads). The merged images illustrate DOPR and CRF-containing neurons that are in close proximity to NET-labeled fibers (D,H). Arrows illustrate neurons that co-express DOPR and CRF.. Thick arrows indicate single labeled DOPR, CRF or NET. Arrows indicate dorsal (D) and lateral (L) orientation. Scale bar, 25 μm.

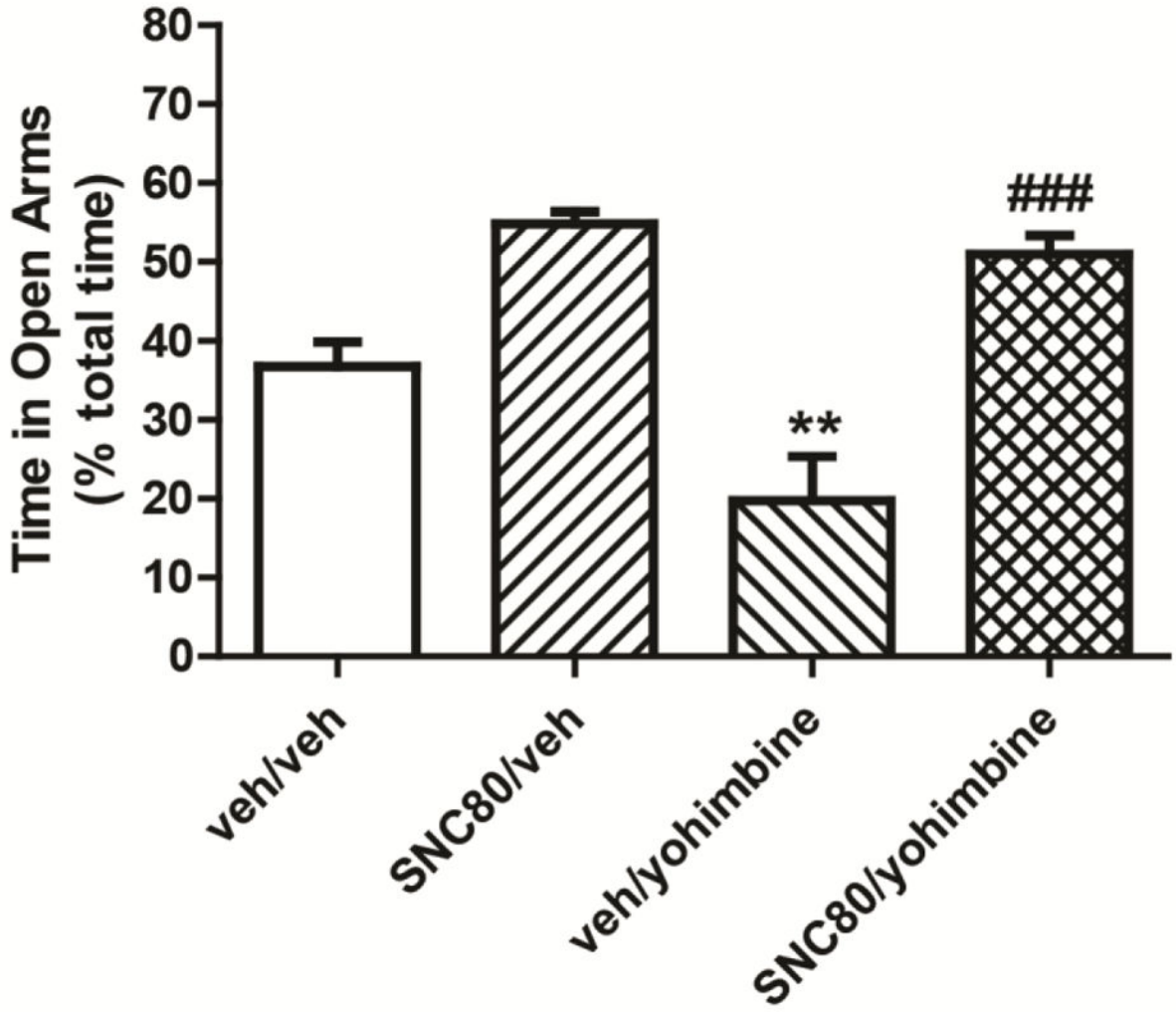

Figure 8.

The DOPR agonist, SNC80, attenuated the anxiogenic effects of yohimbine. Rats were pretreated with the selective DOPR agonist, SNC80 10 mg/kg, or vehicle 30 minutes prior to yohimbine administration. Anxiety-like behaviors were measured using the elevated zero maze. Time spent on the open quadrants of the zero maze was lower after yohimbine administration as compared with vehicle controls, indicating an anxiogenic response. The anxiogenic effects of yohimbine were significantly blocked by treatment with SNC80. Data are expressed as means + SEM. ** $P<0.01$ veh/yohimbine vs veh/veh; ### $P<0.001$ SNC80/yohimbine vs veh/yohimbine. N=8/group.

Table 1

Semi-quantitative analysis of DOPR and CRF interactions in the BLA and CeA

	BLA	CeA
Frequency of co-localization of CRF with DOPR in dendrites in different amygdalar subdivisions	55% (133/242) Asymmetric 23% (30/133) Symmetric 49% (65/133) Undefined 29% (38/133)	67% (212/315) Asymmetric 20% (43/212) Symmetric 60% (20/43) Undefined 20% (42/212)
Frequency of synapses formed by DOPR containing axon terminals with CRF containing dendrites in different amygdalar subdivisions	41% (81/198) Asymmetric 22% (18/81) Symmetric 41% (40/81) Undefined 28% (23/81)	29% (43/147) Asymmetric 28% (12/43) Symmetric 49% (65/133) Undefined 26% (11/43)