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Dynorphin-containing axons directly innervate noradrenergic neurons in the rat nucleus locus coeruleus

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Abstract

Stress causes increased dynorphin (DYN) expression in limbic brain regions and antagonism of kappa-opioid receptors may offer therapeutic potential for the treatment of depression. A potential site of DYN action relevant to stress and related neuropsychiatric disorders is the locus coeruleus (LC), the primary source of forebrain norepinephrine. Therefore, using immunofluorescence and immunoelectron microscopic analyses, we characterized the cellular substrates for interactions between DYN and tyrosine hydroxylase (TH), a catecholamine synthesizing enzyme in single sections through the LC. Light microscopic analysis of DYN immunoreactivity indicated that DYN fibers are distributed within the core and peri-coerulear subregions of the LC. Using electron microscopy, immunoperoxidase labeling for DYN was primarily found in axon terminals, although in some cases was diffusely localized to somatodendritic processes. When DYN-containing axons formed synaptic contacts, they typically (89%) exhibited an asymmetric morphology. Almost a third (28%) of the postsynaptic targets of DYN-containing axons contained immunogold labeling for TH. These findings reveal some diversity as to the localization of DYN in the LC within axons that contact both TH and non-TH containing dendrites. However, the present data provide the first ultrastructural evidence that DYN-containing axon terminals directly innervate catecholaminergic LC dendrites. Moreover, DYN axon terminals targeting catecholaminergic LC dendrites via asymmetric synapses is consistent with localization within excitatory type afferents to the LC. Therefore, direct modulation

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of catecholaminergic LC neurons maybe an important site of action for DYN relevant to stress and stress-related disorders.

Previous studies have shown that dynorphin (DYN), a potent endogenous opioid peptide implicated in stress regulation, is widely distributed throughout the neuroaxis (Goldstein and Ghazarossian, 1980, Holtt et al., 1980, Khachaturian et al., 1982, Watson et al., 1982, Watson et al., 1983, Fallon and Leslie, 1986, Elde and Hokfelt, 1993). Radioimmunoassay experiments have revealed that DYN is detected in pontine regions (Goldstein and Ghazarossian, 1980, Holtt et al., 1980) specifically in the locus coeruleus (LC; Zamir et al., 1983). Immunohistochemical analysis revealed DYN cell bodies in several brain regions including the hypothalamus, central nucleus of the amygdala (CNA), dentate gyrus, hippocampus, bed nucleus of stria terminalis (BNST) and the nucleus of the solitary tract (NTS; Khachaturian et al., 1982, Watson et al., 1982, Watson et al., 1983, Fallon and Leslie, 1986) while DYN-containing fibers are distributed in multiple brain regions including the LC (Khachaturian et al., 1982, Fallon and Leslie, 1986, Elde and Hokfelt, 1993).

Although DYN peptides may interact with multiple opioid receptors (Mulder et al., 1989), DYN is considered a selective endogenous ligand of the kappa opioid receptor (Chavkin et al., 1982). In situ hybridization studies have demonstrated the presence of kappa-opioid receptors in the LC (Mansour et al., 1994; DePaoli et al., 1994) and kappa-opioid receptors are poised to mediate the physiological actions of DYN (Chavkin et al., 1982; Arvidsson et al., 1995). Intracellular physiological studies have shown that application of kappa-opioid receptor agonists (U50488) *in vitro* produces a concentration-dependent depression of the excitatory postsynaptic potential evoked by electrical stimulation of afferent inputs to LC neurons (McFadzean et al., 1987). These findings were extended by Pinnock 1992 who demonstrated that the kappa-opioid receptor agonist (CI-977) causes a depression of evoked postsynaptic potentials on LC neurons and the depression remained in the presence of a glutamate antagonist (Pinnock, 1992).

The LC is densely packed with approximately 1,600 norepinephrine-containing neurons located in the rostral dorsal pontine tegmentum (Swanson, 1976). As the largest cluster of noradrenergic neurons in the brain (Foote et al., 1983, Waterhouse et al., 1993), this pontine nucleus is known for its widespread divergence of noradrenergic terminals to all levels of the neuroaxis (Foote et al., 1983). Thus, LC neurons can simultaneously innervate various neural targets (Waterhouse et al., 1993). A role for the LC in regulating arousal and attention as well as adaptive behavioral responses has been shown in a number of electrophysiological and pharmacological studies (Aston-Jones and Bloom, 1981, Foote et al., 1983, Aston-Jones et al., 1994). In addition, LC neurons have been implicated in a wide variety of autonomic functions (Morilak et al., 1987, Murase et al., 1993), and are involved in stress related disorders such as anxiety, depression and post-traumatic stress disorder (Korf et al., 1973, Svensson, 1987, Valentino et al., 1998).

During stress and depression, it has been demonstrated that DYN levels are increased in limbic brain regions and that intracerebroventricular injection of nor-binaltorphimine dihydrochloride, a kappa-opioid antagonist, produces antidepressant-like effects in animal models of depression suggesting that a blockade of kappa-opioid receptors may offer a therapeutic potential for the treatment of depression (Shirayama et al., 2004).

Our previous ultrastructural studies show evidence that terminals containing opioids directly contact catecholaminergic dendrites in the LC. These include met-enkephalin (Van Bockstaele et al., 1995, Van Bockstaele and Chan, 1997a, Tjounmakaris et al., 2003), endomorphin-1 (Peoples et al., 2002) and proopiomelanocortin (POMC; Reyes et al., 2006). An anatomical substrate that may underlie the electrophysiological effects of kappa opioid receptor agonists is

the presynaptic modulation of excitatory afferents in the LC. Interestingly, our laboratory described the presence of vesicular glutamate transporters (V-Glut) in DYN afferents to the LC (Barr and Van Bockstaele, 2005). However, this study did not examine synaptic contacts formed by DYN afferents with noradrenergic somatodendritic processes. Moreover, although light microscopic analysis has previously described DYN varicose fibers and processes in the LC (Khachaturian et al., 1982, Fallon and Leslie, 1986, Elde and Hokfelt, 1993), the synaptic organization of DYN in the LC has not been elucidated. The present study was therefore designed to examine the cellular substrates underlying interactions of DYN with LC neurons using an immunofluorescence and immunoelectron microscopic dual-labeling procedure.

EXPERIMENTAL PROCEDURES

Tissue preparation

Five adult male Sprague-Dawley rats (250–300 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used for examining the cellular associations of DYN with TH-containing cellular profiles in the dorsal pontine tegmentum using immunofluorescence while six rats were used for examining cellular associations using electron microscopy. The rats were housed 2–3 per cage on a 12-h light schedule in a temperature controlled (20 °C) colony room. They were given standard rat chow and water. The procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and conformed to NIH guidelines. All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data and experiments were designed to minimize any animal distress.

Immunofluorescence

Five rats were deeply anesthetized with sodium pentobarbital (80 mg/kg) and transcardially perfused through the ascending aorta with 500 ml of 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were then removed and post fixed in 4% formaldehyde overnight at 4°C. Forty micrometer thick sections through the LC were cut with a Vibratome (Technical Product International, St Louis, MO, USA) and rinsed extensively in 0.1 M PB and 0.1 M tris-buffered saline (TBS; pH 7.6). Sections were placed for 30 min in 1% sodium borohydride in 0.1 M PB to reduce amine-aldehyde compounds thereby diminishing autofluorescence (Clancy and Cauller, 1998). The tissue sections were then incubated in 0.5% bovine serum albumin (BSA) and 0.25% Triton X-100 in 0.1M TBS for 30 min. Thorough rinses in 0.1 M TBS were done following incubation. Subsequently, sections were incubated in a cocktail containing guinea pig anti-preprodynorphin (ppDYN) at 1:16,000 (kindly provided by Dr. Robert Elde, University of Minnesota, MN) and mouse anti-TH (Immunostar Inc., Hudson, WI, USA) at 1:1,000 in 0.1% BSA and 0.25% Triton X-100 in 0.1M TBS. Some sections were incubated in the same antisera that had been preabsorbed with the cognate peptide (1 M). Incubation time was 15–18 h in a rotary shaker at room temperature. Sections were then washed in 0.1 M TBS and incubated in a secondary antibody cocktail containing fluorescein isothiocyanate (FITC) donkey-anti-guinea pig (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and tetramethyl rhodamine isothiocyanate (TRITC) donkey anti-mouse (1:200; Jackson ImmunoResearch) antibodies prepared in 0.1 % BSA and 0.25% Triton X-100 in 0.1 M TBS for 2 h in the dark on a rotary shaker.

Following incubation with the secondary antibodies, the tissue sections were washed thoroughly in 0.1 M TBS. The tissue sections were then mounted on slides and allowed to dry in complete darkness. The slides were dehydrated in a series of alcohols, soaked in xylene and coverslipped using DPX (Sigma-Aldrich Inc., St. Louis, MO, USA). Although the FITC fluorophore is known to photobleach quickly with DPX, we immediately coverslipped the tissue sections and examined using a Leica DMRBE microscope (Leica Microsystems, Wetzlar, Germany) equipped for fluorescence with rhodamine and fluorescein filters. Images

were captured using Spot Advance software (Diagnostic Instruments Inc., Sterling Heights, MI).

Electron microscopy

Six rats were deeply anesthetized with sodium pentobarbital (80 mg/kg) and perfused transcardially through the ascending aorta with 10 ml heparinized saline followed with 50 ml of 3.75% acrolein (Electron Microscopy Sciences, Fort Washington, PA, USA), and 200 ml of 2% formaldehyde in 0.1 M PB (pH 7.4). The brains were removed immediately after perfusion fixation, sectioned into 1–3 mm coronal slices and postfixed for 30 min and, if necessary, stored in 0.1 M PB for a short period prior to sectioning. The storage period was usually not more than 1–2 hours. Sections were cut in the coronal plane at a setting of 40 μ m using a Vibratome (Technical Product International, St. Louis, MO) and collected into 0.1 M PB.

Forty- μ m thick sections through the rostrocaudal extent of the LC were processed for electron microscopic analysis of DYN and TH in the same section. Sections containing the LC were processed following the protocol described earlier for immunofluorescence except that Triton X-100 was not added to the solution for antibody incubation. Tissue sections were incubated in primary antibody cocktail of guinea pig anti-ppDYN (1:16,000) and mouse anti-TH (1:1,000) for 15–18 h at room temperature. The following day, tissue sections were rinsed three times in 0.1 M TBS and incubated in biotinylated donkey anti-guinea pig (1:400; Vector Laboratories, Burlingame, CA) for 30 min followed by rinses in 0.1 M TBS. Subsequently, a 30-minute incubation of avidin-biotin complex (Vector Laboratories) followed. For all incubations and washes, sections were continuously agitated with a rotary shaker. DYN was visualized by a 4-minute reaction in 22 mg of 3,3'-diaminobenzidine (Sigma-Aldrich Inc., St. Louis, MO) and 10 μ l of 30% hydrogen peroxide in 100 ml of 0.1 M TBS. Some sections were collected, dehydrated and coverslipped for light microscopic analysis of DYN immunoreactivity.

For gold-silver localization of TH, sections were rinsed 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, USA) 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 time 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; Leranthe and Pickel, 1989). Thin sections of approximately 50–100 nm in thickness were cut with a diamond knife (Diatome-US, Fort Washington, PA, USA) using a Leica Ultracut (Leica Microsystems, Wetzlar, Germany). Sections were collected on copper mesh grids and counterstained stained with 5% uranyl acetate followed by Reynold's lead citrate. Captured images of selected sections were compared with captured light microscopic images of the block face before sectioning. Sections were examined with a Hitachi electron microscope. Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop.

Controls and Data analysis

Some sections were used for control experiments and were processed without exposure to the primary antibody. These sections were processed in parallel with tissue sections incubated in the primary antibody. To evaluate cross-reactivity of the primary antisera by secondary antisera, some sections were processed for dual labeling with omission of one of the primary antisera. Tissue sections from rats with good preservation of ultrastructural morphology and with both markers clearly apparent were used for the analysis. Immunolabeling for EM using pre-embedding techniques is subject to false-negatives due to the fact that the two antigens are not detected with the same sensitivity. We avoided this by requiring positive labeling to be present in the surrounding region before drawing any conclusions about the absence of double-labeling. If labeling for each of the antigens was present within a 10 μ m radius of a structure but the structure itself did not appear to be double-labeled, we concluded that it was not double-labeled. This approach resulted in 247 DYN-labeled profiles.

The cellular elements were identified based on the description of Peters and colleagues (Peters et al., 1991, 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 μ m in diameter. A terminal was considered to form a synapse if it showed synaptic vesicles near a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or associated with postsynaptic thickening. 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 postsynaptically. A non-synaptic apposition 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. All potential targets defined as synaptic or non-synaptic appositions of DYN-labeled axon terminals were included in the analysis.

Identification of immunogold-silver labeling in profiles

Selective immunogold-silver labeled profiles were identified by the presence, in single thin sections, of at least three immunogold-silver particles within a cellular compartment. Whenever possible, the more lightly labeled somatic and dendritic labeling for TH was confirmed by detection in at least two serial sections. Even if a profile contained immunogold particles in adjacent thin sections, it was designated as lacking detectable immunoreactivity if there were fewer than three particles in each section.

RESULTS

Sections processed in the absence of DYN or TH primary antibody did not exhibit any detectable immunoreactivity. In addition, all immunoreactivity was eliminated when the DYN antiserum was preabsorbed with the antigenic peptide.

Using light and darkfield microscopy, DYN immunoreactivity was prominently distributed in both the core of the LC (Figs. 1A and 1C) and rostralateral pericoerulear (peri-LC) areas (Fig. 1A–B). Anatomically, the core of the LC is defined as the region enriched with noradrenergic cell bodies (Bajic et al., 2000). The peri-LC is defined as the region of the dorsal pontine tegmentum medial and lateral to the LC that contains noradrenergic dendrites (Shipley et al., 1996, Van Bockstaele et al., 1996a). Within the core of the LC, DYN immunolabeling was detectable as highly beaded fibers (Figs. 1A and 2B). Labeling within cell bodies was not detectable. This may be due to the low level of DYN labeling within cell bodies in this region (see our EM data below) and the use of non-colchicine treated rats in this study. Within the peri-LC, DYN immunoreactivity was identified in fibers immediately medial and ventral to

the superior cerebellar peduncle, dorsolateral to the core of the LC that were slightly less beaded than those found in the core of the LC (Fig. 1B). Dual immunofluorescence labeling showed DYN and TH labeling in the LC (Fig. 2A–F). TH immunoreactivity revealed a dense cluster of neurons distributed in the core of the LC (Fig. 2A). Consistent with previous reports (Van Bockstaele et al., 1995, Shipley et al., 1996), TH-immunoreactive dendrites were evident in the peri-LC in the rostral pontine levels (Fig. 2E). These extended medially from the core of the LC containing TH-immunoreactive somata. Some dendritic processes were distributed ventral to the fourth ventricle and medial to the TH-containing cell bodies (not shown). DYN immunoreactive varicose processes overlapped TH-immunoreactive perikarya in the core of the LC (Figs. 2A–D) and numerous processes targeted TH-immunoreactive dendrites in the peri-LC area (Figs. 2E–F).

Ultrastructure of DYN-immunoreactivity in the LC

For ultrastructural analysis, the region selected included both the core and peri-LC. At the electron microscopic level, immunoperoxidase labeling for DYN and immunogold-silver labeling for TH were localized within single sections of the LC and were readily distinguishable in the neuropil (Fig. 3). DYN was primarily localized within axon terminals (Figs. 3–4), however in some cases diffuse peroxidase labeling indicative of DYN immunolabeling was detected within somatodendritic processes (not shown). Of the 207 TH-labeled dendrites, 6% (13/207) were dual-labeled for DYN. Within axons, dense DYN immunolabeling could be identified on the plasmalemma of labeled axon terminals and was also associated with large dense core vesicles that were apposed to neighboring astrocytic processes (Figs. 3B–C and 4A and 4C). Small clear vesicles were rimmed with peroxidase reaction product in the DYN-labeled axon terminals (Figs. 3A, 3C–D).

Semiquantitative analysis revealed that about 64% (159/247) DYN-labeled axon terminals synapsed on somatodendritic processes. This total included both TH-labeled (Figure 3–4) and unlabeled processes. Of the 247 axon terminals, 88 did not form a detectable synaptic contact in the plane of section analyzed. Of the 159 axon terminals sampled that formed detectable synaptic contacts, 89% (142/159) were of asymmetric type while only 11% (17/159) were of the symmetric type. Out of the 247 axon terminals sampled, 23% (56/247) formed synapses on TH-labeled dendrites while 5% (12/247) were non-synaptically apposed to TH-labeled dendrites. When synapses were distinguishable on dendrites containing immunogold silver for TH, they were predominantly asymmetric (Figs. 3D and 4), although some symmetric type synapses were detected (Figs. 3A, 3C).

Some DYN-labeled axon terminals were apposed to other axon terminals (Fig. 3A–B). These appositions lacked a detectable synapse on either side of the contact (Fig. 3B). The apposed axon terminals lacked immunoreactivity for either TH or DYN and formed either asymmetric- or symmetric-type synapses with somatodendritic processes (Fig. 3). In addition, DYN-immunoreactive axon terminals were sometimes enveloped by astrocytic processes (Figs. 3B–C, 4A and 4C).

DISCUSSION

The findings of this study demonstrate that DYN-containing axon terminals in the core and peri-LC frequently form asymmetric-type synaptic specializations with TH-labeled and unlabeled dendrites and are also apposed to unlabeled axon terminals and astrocytic processes. These data provide an anatomical substrate for modulatory actions of DYN with noradrenergic neurons in the LC.

Using immunocytochemistry, the characterization and specificity of the guinea pig antiserum against DYN has been described previously (Arvidsson et al., 1995). The guinea pig antibody

is directed against residues 235–248 (SQENPNTYSEDLDV) of rat preprodynorphin (ppDYN) and specifically recognized ppDYN in various areas of the brain and spinal cord (Arvidsson et al., 1995). The antibody also showed a distribution in the LC consistent with previous studies (Elde and Hokfelt, 1993). Preabsorption of ppDYN with the antigenic peptide at 1 M blocked the DYN immunoreactivity throughout the rat brain, which is in agreement with the report that the DYN staining in rat brain tissue was blocked at peptide concentrations of 0.1 to 1 M (Arvidsson et al., 1995). The mouse antiserum against TH specifically recognizes the catecholamine synthesizing enzyme and has been previously characterized for specificity (Van Bockstaele and Pickel, 1993). Pre-embedding dual immunolabeling is inherently subject to under-detection principally due to penetration of immuno-reagents through the tissue sections. To mitigate this effect we employed a strategy of sampling profiles only when both labels were present within 10 m of the surrounding neuropil.

The distribution of DYN immunoreactivity in the present study substantiate previous anatomical studies showing DYN-immunoreactive fibers and varicose processes within the LC (Khachaturian et al., 1982, Fallon and Leslie, 1986, Fallon and Leslie, 1988, Elde and Hokfelt, 1993). In addition, previous in situ hybridization studies have shown that DYN mRNA is expressed in the LC neurons (Mansour et al., 1994). However, immunolabeling of cell somata with DYN was not readily detected using immunofluorescence. This is consistent with the observation that it is often difficult to detect somatodendritic labeling of peptides without the use of colchicine, which disrupts microtubule-based transport of peptides into axons resulting in an accumulation of immunolabeling within soma. However, the previous in situ results are consistent with our occasional observation of DYN in somatodendritic processes in the LC using EM. Taken together these findings suggest that much of the DYN produced in the LC may be transported to axons in areas innervated by the LC.

Each of the endogenous opioid peptide families has a characteristic distribution pattern in the LC. The distribution of DYN immunoreactivity in the LC is similar to certain opioids such as enkephalin (ENK; Van Bockstaele et al., 1995) that are also dense dorsolaterally, but different from others such as endomorphins and POMC. We have recently shown that dense POMC-immunoreactive processes are distributed in the ventromedial aspect of the LC and few were found dorsolaterally (Reyes et al., 2006). Similarly, endomorphin-containing fibers are distributed within the ventromedial peri-LC but few extend into the core of the LC (Peoples et al., 2002). The unique topographic distribution pattern of each endogenous opioid suggests that they may function in distinct and complementary fashion to regulate LC activity.

Although DYN is considered a selective ligand for kappa opioid receptors it also has some affinity for mu opioid receptors (Mulder et al., 1989). Both mu and kappa opioid receptors are expressed by LC neurons (Mansour et al., 1994; DePaoli et al., 1994). This contrasts to delta opioid receptors, which appear confined to presynaptic boutons in the LC (Van Bockstaele et al., 1997b). Although the subcellular distribution of kappa receptors remains poorly understood, mu receptors are a predominant postsynaptic receptor in LC neurons (Van Bockstaele et al., 1996c). The distinct topographic distribution of DYN coupled with the additional higher affinity for kappa opioid receptors compared to other endogenous opioids, supports likelihood that DYN supports unique signaling functions in the LC.

We provide ultrastructural evidence that axon terminals exhibiting DYN form primarily asymmetric (89%) synaptic junctions with dendrites in the LC. Asymmetric synapses have been correlated with excitatory transmission whereas symmetric synapses have been correlated with inhibitory transmission (Gray, 1959, Peters et al., 1991, Peters and Palay, 1996). We have recently shown that V-Glut-1-labeled terminals also contain DYN in LC and the majority of these terminals form asymmetric synapses with unidentified dendrites (Barr and Van Bockstaele, 2005). Hence, DYN is likely a co-transmitter in glutamatergic axons that to some

extent directly synapses on catecholaminergic neurons in the LC. Previous studies have shown that kappa opioid agonists decrease evoked excitatory postsynaptic potentials in the LC (McFazean et al., 1987). Taken together, these observations raise the possibility that DYN has homosynaptic effects in modulating glutamatergic neurotransmission in the LC. However, future studies on the localization of kappa opioid receptors will help define the potential sites of action of DYN.

The nucleus paragigantocellularis (PGi; Ennis and Aston-Jones, 1988) is a likely source of glutamate and dynorphin containing axons in the LC (Khachaturian et al., 1983, Menetrey and Basbaum, 1987), and this pathway has been implicated in opiate withdrawal. However, there are several brain areas that both contain DYN expressing neurons and neurons that project to the LC that may be sources. These include the CNA, BNST, NTS and hypothalamic regions including the paraventricular nucleus (PVN; Khachaturian et al., 1982, Watson et al., 1983). In fact, we have previously shown using anterograde and retrograde tracing studies combined with electron microscopic analysis that 1) neurons in the NTS monosynaptically innervate TH-containing dendritic processes in the LC (Van Bockstaele et al., 1999a); 2) axon terminals originating from the BNST synapse with TH-labeled dendrites in the LC (Van Bockstaele et al., 1999b); 3) CNA axon terminals innervate TH-labeled dendrites in LC neurons (Van Bockstaele et al., 1996a); and 4) neurons from the PVN send projections to the LC neurons (Reyes et al., 2005). These nuclei that send efferents to the LC have been implicated in autonomic control (Harper et al., 1984, Reis et al., 1984, Maskati and Zbroayma, 1989, Martin et al., 1991, Ciriello and Janssen, 1993, Roder and Ciriello, 1993, Schlenker et al., 2001) and in stress responses (Borsook et al., 1994, Alheid et al., 1995, Dayas et al., 2004).

In addition to DYN, we have previously shown that CRF in the LC arises from similar sources including the CNA, BNST and PVN, suggesting that DYN may colocalize with CRF (Van Bockstaele et al., 1996b, Van Bockstaele et al., 1998, Reyes et al., 2006). Anatomical and physiological studies have shown that CRF-labeled terminals in the LC form synapses with TH-labeled dendrites (Van Bockstaele et al., 1998) and intracerebroventricular and intracoeular microinjection of CRF is known to activate noradrenergic LC neurons (Valentino and Foote, 1988, Curtis et al., 1997). Preliminary data from our laboratory shows that DYN and CRF may co-exist in the same axon terminals in the LC (Johnson and Van Bockstaele, 2004). In summary, DYN may act as a co-transmitter with glutamate and CRF in LC afferents and may be involved in regulation of stress responses.

This study is the first to demonstrate that DYN-containing axons directly synapse on catecholamine neurons in the LC. In addition, DYN-containing axons formed indirect interactions with non-catecholaminergic neurons, astrocytes and other axon terminals. This information reveals the cellular substrates of DYN actions in the LC may be critical for understanding how this endogenous opioid serves to modulate nociception (Tan-No et al., 2005; Petrie et al., 1982), autonomic functions (Rao et al., 2003; Zhou et al., 2006; Woo et al., 1983; Gilbeau et al., 1986), memory (Hiramatsu et al., 1995, 1997; Itoh et al., 1993) and stress responses (Morley et al., 1982; Guaza et al., 1986).

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Figure 1.

A. Photomicrograph using darkfield illumination showing immunoperoxidase labeling of DYN immunoreactivity in the locus coeruleus (LC). Dense fibers can be seen in the peri-LC (arrowheads) and punctate labeling can be seen in the core of the LC (straight white arrow). **B–C.** Brightfield photomicrographs showing peroxidase labeling for DYN in the peri-LC (Panel B) and the core of the LC (Panel C). Varicose fibers (arrowheads) are visible in the peri-LC as well as in the LC core. Arrows indicate dorsal (D) and medial (M) orientation of the tissue section. Scale bars, 100 μm. Cb, cerebellum; D, dorsal; IV, fourth ventricle; L, lateral; scp: superior cerebellar peduncle.

Figure 2.

A–F. Confocal fluorescence micrographs showing DYN-labeled processes are associated with TH-labeled dendrites in the core and peri-locus coeruleus. TH (straight white arrows) was detected using a rhodamine isothiocyanate-conjugated secondary antibody (red) and DYN (arrowheads) was detected using a fluorescein isothiocyanate-conjugated secondary antibody (green). TH-labeling (straight white arrows) was detected in perikarya (A–D) as well as smaller processes (E–F). DYN labeling appeared punctate throughout (arrowheads) and overlying TH-labeled dendrites and soma. Arrows indicate dorsal (D) and medial (M) orientation of the tissue section. IV, fourth ventricle. Scale bars, 100 μm.

Figure 3.

Electron micrographs showing peroxidase labeling for DYN and immunogold-silver labeling for TH in the locus coeruleus (LC). **A.** A peroxidase-labeled DYN terminal (DYN-t) forming a symmetric synapse (curved arrow) with an immunogold-labeled (arrowheads) TH dendrite (TH-d). The same DYN-t forms an axo-axonic contact (arrow) with an unlabeled axon terminal (at). Another TH-labeled dendrite can be seen nearby. Dense core vesicles (dcv) are present and filled with peroxidase reaction product in the DYN terminal. **B.** Electron micrograph of a TH-labeled dendrite (TH-d) and a peroxidase-labeled axon terminal (DYN-t) in the same field. DYN-labeled axon terminal is partially surrounded by an astrocytic process (asterisks). **C.** An immunogold-labeled (arrowheads) TH dendrite (TH-d) apposed (double arrow) to a

peroxidase-labeled DYN terminal (DYN-t). Dense core vesicles filled with peroxidase reaction product (dcv) can be seen in one DYN terminal. Another DYN-labeled axon terminal can be seen to form a symmetric synapse (curved arrow) with the same TH-labeled dendrite. Asterisks denote an astrocytic process. **D.** A peroxidase-labeled DYN terminal (DYN-t) forms an asymmetric synapse (arrows) with an immunogold-labeled (arrowheads) TH dendrite (TH-d). Scale bars, 0.5 μ m.

Figure 4.

Electron micrographs showing DYN axon terminals forming asymmetric synapses (arrows) with TH dendrites in the locus coeruleus. **A.** A DYN axon terminal forming an asymmetric synapse (arrow) with an immunogold-labeled (arrowheads) TH-dendrite. The DYN terminal contains dense core vesicles (dcv) and is also surrounded by an astrocytic process (asterisks). **B.** A peroxidase-labeled DYN terminal forms an asymmetric synapse with an immunogold-labeled (arrowheads) TH dendrite. Another TH-containing dendrites can be seen in the neuropil. **C.** A peroxidase-labeled DYN terminal forms an asymmetric synapse (arrow) with an immunogold-labeled (arrowheads) TH dendrite. Also seen in the neuropil are two other TH-

labeled dendrites. The DYN terminal is also surrounded by astrocytic processes (asterisks). Scale bars, 0.5m.