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Abstract

We previously demonstrated that the opioid peptide, enkephalin, and corticotropin-releasing factor (CRF) are occasionally co-localized in individual axon terminals but more frequently converge on common dendrites in the locus coeruleus (LC). To further examine potential opioid co-transmitters in CRF afferents, we investigated the distribution of proopiomelanocortin (POMC), the precursor that yields the potent bioactive peptide, β-endorphin, with respect to CRF immunoreactivity using immunofluorescence and immunoelectron microscopic analyses of the LC. Coronal sections were collected through the dorsal pontine tegmentum of rat brain and processed for immunocytochemical detection of POMC and CRF or tyrosine hydroxylase (TH). POMC-immunoreactive processes exhibited a distinct distribution within the LC as compared to the enkephalin family of opioid peptides. Specifically, POMC fibers were enriched in the ventromedial aspect of the LC with fewer fibers present dorsolaterally. Immunofluorescence microscopy showed frequent co-existence of POMC and CRF in varicose processes that overlapped TH-containing somatodendritic processes in the LC. Ultrastructural analysis showed POMC immunoreactivity in unmyelinated axons and axon terminals. Axon terminals containing POMC were filled with numerous large dense core vesicles. In sections processed for POMC and TH, approximately 29% of POMC-containing axon terminals (n = 405) targeted dendrites that exhibited immunogold-silver labeling for TH. Whereas, sections processed for POMC and CRF showed that 27% of POMC-labeled axon terminals (n = 657) also exhibited CRF immunoreactivity. Taken together, these data indicate that a subset of CRF afferents targeting the LC contain POMC and may be positioned to dually impact LC activity.

Introduction

We have previously demonstrated that corticotropin-releasing factor (CRF) and endogenous opioids interact in the locus coeruleus (LC) to modulate noradrenergic function (Valentino *et al.*, 1992; Van Bockstaele *et al.*, 1996a; Van Bockstaele *et al.*, 1996b; Xu *et al.*, 2004). Specifically, we showed that chronic morphine administration sensitized the LC neurons to CRF (Xu *et al.*, 2004). In an effort to further elucidate interactions between CRF and opioid associated with peptides that modulate mu-opioid receptors, we examined here the distribution of proopiomelanocortin (POMC), an opioid precursor, and CRF in this noradrenergic nucleus.

POMC is processed to yield the potent bioactive peptide, β-endorphin, which is associated with stress regulation (Baubet *et al.*, 1994). β-endorphin interacts with μ-opioid receptors to mediate stress-induced analgesia (Rubinstein *et al.*, 1996) and it has been implicated in nociception (Foley *et al.*, 1979; Yaksh *et al.*, 1982; Bertolini *et al.*, 1986; Baubet *et al.*, 1994). In the present study, the ultrastructural distribution of POMC and catecholamine-containing neurons in the LC that may likely underlie the effects of POMC were investigated using immunofluorescence and electron microscopy in the rat brain.

Previous studies suggest that the LC is densely innervated by processes exhibiting endogenous opioid peptides including leucine- and methionine-enkephalin (Drolet *et al.*, 1992; Van Bockstaele *et al.*, 1995; Van Bockstaele *et al.*, 1996c; Van Bockstaele & Chan, 1997; Van Bockstaele *et al.*, 2000), β-endorphin (Bloom et al., 1978a; Bloom et al., 1978b), and dynorphin (Fallon & Leslie, 1986). Likewise, LC neurons demonstrate a high concentration of opioid receptors including μ-opioid receptors (Tempel & Zukin,

1987; Mansour *et al.*, 1994; Mansour *et al.*, 1995; Van Bockstaele *et al.*, 1996a; Van Bockstaele *et al.*, 1996b).

Known for its population of noradrenergic neurons, the LC projects throughout all levels of the neuroaxis (Foote et al., 1983). Thus, LC neurons serve as the prime source of NE in the forebrain (Aston-Jones et al., 1985; McCormick et al., 1991). Electrophysiological and pharmacological studies showed a critical role of noradrenergic projections from the LC in the control of vigilance, attention and adaptive behavioral responses (Aston-Jones & Bloom, 1981b; a; Aston-Jones *et al.*, 1984; Aston-Jones *et al.*, 1994) . In fact, LC neurons exhibit the highest firing rates during waking and decreased activity during slow-wave sleep, and are nearly quiescent during paradoxical sleep (Aston-Jones & Bloom, 1981a). Moreover, LC neurons respond to autonomic influences (Svensson, 1987) and are implicated in multiple autonomic functions (Morilak *et al.*, 1987a; b; Miyawaki *et al.*, 1991). Afferents that innervate LC neurons can remarkably influence the neural circuitry involved in the diversity of functions ascribed to the LC (Aston-Jones *et al.*, 1991).

Corticotropin-releasing factor (CRF), the hypothalamic neurohormone that initiates the adrenocorticotropin release through its actions on corticotrophs of the adenohypophysis (Vale *et al.*, 1981; Cummings *et al.*, 1983; Swanson *et al.*, 1983), also acts as a neurotransmitter in extrahypophyseal circuits that mediate behavioral and autonomic responses to stressors. Anatomical studies have described CRF-immunoreactive fibers in noradrenergic LC neurons (Cummings *et al.*, 1983; Swanson *et al.*, 1983). Specifically, CRF-axon terminals target noradrenergic dendrites in the LC (Van Bockstaele *et al.*, 1996d; Van Bockstaele *et al.*, 1998b). In support of these data,

physiological studies have shown that CRF administration intracerebroventricularly or by microinfusion into the LC activated LC neurons (Valentino *et al.*, 1983; Curtis *et al.*, 1997). In addition, CRF central administration or microinfusion into the LC increases NE levels in prefrontal cortex, a region which derives NE solely from the LC (Lavicky & Dunn, 1993; Smagin *et al.*, 1995).

Anatomically, medullary afferents to the LC originate from the nucleus of the solitary tract (NTS; (Van Bockstaele *et al.*, 1998a; Van Bockstaele *et al.*, 1999) that provides monosynaptic input to noradrenergic dendrites in the LC (Van Bockstaele *et al.*, 1999) . Additionally, immunohistochemical and biochemical analyses have shown POMC (Schwartzberg & Nakane, 1982) and CRF (Herbert & Saper, 1990) containing cell bodies within the NTS. Taken together with the important role of these neuropeptides in stress regulation (Baubet *et al.*, 1994; Rubinstein *et al.*, 1996; Vale *et al.*, 1981; Cummings *et al.*, 1983; Swanson *et al.*, 1983), it is important to elucidate the role of POMC afferents that may express CRF as a co-transmitter in the regulation of noradrenergic function.

Materials and Methods

Tissue preparation

Ten adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 225-250 g were used for this study. Five rats were used for examining the cellular associations of POMC with tyrosine hydroxylase- (TH) or CRF-containing cellular profiles in the dorsal pontine tegmentum using immunofluorescence while five 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 ºC)

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 attempts were made to minimize animal suffering.

Immunofluorescence microscopy

Rats were deeply anesthetized with sodium pentobarbital (60 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 remove reactive aldehydes and incubated in 0.5% bovine serum albumin (BSA) in 0.1M TBS for 30 min. Subsequently, sections were then incubated in 0.5% bovine serum albumin (BSA) and 0.25% Triton X-100 in 0.1M TBS for 30 min and rinsed extensively in 0.1 M TBS. Subsequently, sections were processed for POMC and TH or POMC and CRF immunoreactivities.

Sections processed for POMC and TH were incubated in a cocktail containing rabbit anti-POMC (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) at 1:5,000 and mouse anti-TH (Immunostar Inc., Hudson, WI, USA) at 1:1,000. Incubation time was 15-18 h in a rotary shaker at room temperature. The antiserum to POMC (27-52 amino acid sequence) was generated in rabbits against porcine POMC. The specificity of the mouse

antiserum against TH was previously decribed (Van Bockstaele & Pickel, 1993) and it specifically recognizes the catecholamine synthesizing enzyme.

Sections were then washed in 0.1 M TBS and incubated in a secondary antibody cocktail containing flourescein isothiocyanate (FITC) goat-anti-rabbit (1:100; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and tetramethyl rhodamine isothiocyanate (TRITC) donkey anti-mouse (1:100; Jackson ImmunoResearch) 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.

Sections processed for POMC and CRF were incubated in a cocktail containing rabbit anti-POMC at 1:5,000 and guinea pig anti-CRF (Peninsula Pharmaceuticals Inc., Belmont, CA, USA) at 1:1,000. Incubation time was 15-18 h in a rotary shaker at room temperature. The antiserum to CRF was raised in guinea pig and recognizes human and rat CRF. Sections were then washed in 0.1 M TBS and incubated in a secondary antibody cocktail containing FITC goat-anti-rabbit (1:100; Jackson ImmunoResearch Laboratories Inc.) and TRITC donkey anti-guinea pig (1:100; Jackson ImmunoResearch Laboratories Inc.) 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 tissues were washed thoroughly in 0.1 M TBS. Afterwards, the tissues were mounted on slides and allowed to dry in complete darkness. The slides were then dehydrated in a series of alcohols, soaked in xylene and coverslipped using DPX (Sigma-Aldrich Inc., St. Louis, MO, USA). A confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY, USA) was

used to visualize the immunofluorescence labeling and digital images were obtained and imported using the LSM 5 image browser (Carl Zeiss Inc.).

Immunoelectron microscopy

The rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially through the ascending aorta with (1) 10 ml heparinized saline, (2) 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. Immediately after perfusion fixation, the brains were removed, sectioned into coronal slices and postfixed in the same fixative overnight at 4ºC.

Alternate 40-μm thick sections through the rostrocaudal extent of the LC were processed for electron microscopic analysis of POMC and TH or POMC and CRF in the same section. Immunoperoxidase labeling was used to identify POMC immunoreactivity in sections processed for POMC and TH while immunoperoxidase labeling was used to identify CRF immunoreactivity for sections processed for POMC and CRF. Sections containing the LC were placed for 30 min in 1% sodium brohydride in 0.1 M PB and collected into 0.1 M PB to remove reactive aldehydes. Then sections were rinsed extensively in 0.1 M PB and incubated in 0.5% BSA in 0.1 M TBS for 30 min followed by several rinses in 0.1 M TBS. Tissue sections were incubated in primary antibody cocktail of rabbit anti-POMC (1:5000) and mouse anti-TH (1:1,000) for 15-18 h at room temperature. The following day, sections were rinsed three times in 0.1 M TBS andincubated in biotinylated donkey anti-rabbit (1:400; Jackson ImmunoResearch Laboratories, Inc.) for 30 min followed by rinses in 0.1 M TBS. Subsequently, a 30-minute incubation of avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA)

followed. For all incubations and washes, sections were continuously agitated with a rotary shaker. POMC was visualized by a 9-minute reaction in 22 mg of 3,3'-diaminobenzidine (Sigma-Aldrich Inc.) and 10 µl of 30% hydrogen peroxide in 100 ml of 0.1 M TBS. Some sections were collected, dehydrated and coverslipped for light microscopic analysis of POMC immunoreactivity.

For gold-silver localization of TH (in sections processed for POMC and TH) and POMC (in sections processed for POMC and CRF), sections were rinsed three times with 0.1 M TBS, followed by rinses with 0.1 M PB and 0.01 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 either goat anti-mouse IgG conjugate in 1 nm gold particles (Amersham Bioscience Corp., Piscataway, NJ, USA) for TH or goat anti-rabbit IgG conjugate in 1 nm gold particles (Amersham Bioscience Corp.) at room temperature for 2 h. 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 10-15 min. Following intensification, tissues 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). 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, examined with an electron microscope (Morgagni, Fei Company, Hillsboro,OR, USA) and digital images were captured using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA, USA). Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop.

Control and data analysis

Tissue sections for electron microscopy were taken from rats with the best immunocytochemical labeling and preservation of ultrastructural morphology. The quantitative approach used in the present study is well established and has been described previously (Van Bockstaele *et al.*, 1996a; Van Bockstaele *et al.*, 1996d; Van Bockstaele *et al.*, 1998a). Control sections were run in parallel in which one of the primary antisera was omitted but the rest of the processing procedure was identical. Sections processed in the absence of primary antibody did not exhibit immunoreactivity. The region of the LC selected for electron microscopic analysis is shown in Figure 1A. For quantification of labeled profiles in 40 μm-thick sections immunolabeled before embedding for electron microscopy, we have observed that the collection of sections only from the surface of the section minimizes artifacts that may be associated with incomplete penetration of antisera. The analysis of tissue sections collected at the plastic-tissue interface ensured that both markers were detectable in all sections used for analysis (Chan *et al.*, 1990). The cellular elements were identified based on the description of Peters and colleagues (Peters & Palay, 1996; Peters *et al.*, 1991). 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. Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I; (Gray *et al.*, 1959), in contrast, symmetric synapses had thin densities (Gray's type II; (Gray *et al.*, 1959) both pre- and postsynaptically. A non-synaptic contact or 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.

In sections dually labeled for POMC and TH, the number of POMC axons and axon terminals was grouped from randomly selected sections from ultrathin sections taken from four nonadjacent sections from each animal (n = 5). At least four 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 POMC-labeled axons and axon terminals, and TH-labeled profiles were captured and classified. This approach resulted in 405 POMC-labeled profiles. All potential neuronal targets of POMC-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. These associations consisted of close appositions of neuronal plasma membranes not separated by glial processes. These profiles were considered since opioids as well as other peptides are released by exocytosis from nonsynaptic portions of plasmalemma (Thureson-Klein *et*

al., 1986; Zhu *et al.*, 1986; Karhunen *et al.*, 2001). Postsynaptic targets considered included dendrites containing gold-silver labeling for TH, dendrites lacking gold-silver labeling for TH, unlabeled terminals and glial processes. POMC-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 readily identifiable.

In sections dually labeled for POMC and CRF, POMC axons and axon terminals were studied from fields of at least 11,000X magnification found in at least four ultrathin sections taken from four sections from each animal (n = 5). Fields showing POMC-labeled axons and axon terminals and CRF-labeled neuropil were captured and classified. This approach resulted in 657 POMC-labeled profiles.

Results

By light microscopy, the LC was visualized using an antibody directed against POMC and appeared as dense immunoreactive processes distributed in the ventromedial aspect of the LC in the rostral pons (Fig. 1A). Fewer POMC-labeled fibers were found dorsolaterally. Immunofluorescence labeling for POMC and TH was visualized in coronal sections of the LC (Figs 1C-D). Immunocytochemical labeling for TH at rostral pontine levels showed considerable TH-immunoreactive dendrites extending from somata in the LC, medial and ventral to the fourth ventricle (Fig. 1B-D) consistent with previous studies (Van Bockstaele *et al.*, 1995; Shipley *et al.*, 1996). At caudal levels, TH-immunoreactive dendrites of the peri-LC area, immediately medial to the core of LC neurons, was evidently less dense than that of the rostral levels (not shown). Conversely, the core of the LC exhibited a dense population of TH-immunoreactive somata (Fig. 1B and D). Immunocytochemical labeling for POMC revealed varicose processes within the peri-LC at rostral levels (Figs 1B-D). Numerous POMC-immunoreactive processes extended into the core of the LC where the somata of LC neurons are found (Figs 1B and D). POMC-immunoreactive fibers were morphologically heterogeneous. Some fibers were thin and beaded while others were thicker and lacked varicosities (Figs 1B-D).

Ultrastructural analysis of POMC and TH

At the electron microscopic level, immunoperoxidase labeling for POMC and immunogold-silver labeling for TH were localized within single sections of the LC. POMC immunoreactivity was identified primarily in unmyelinated axon terminals (Fig. 2). POMC-labeled dense core vesicles could be identified adjacent to the plasmalemma most commonly apposed to astrocytic process (Fig. 2A). Morever, POMC-labeled axon

terminals contained abundant small clear spherical vesicles (Figs 2A-C). Often, peroxidase labeling for POMC rimmed the membranes of small clear spherical vesicles (Figs 2A-B). Large dense core vesicles were observed in POMC-labeled terminals (Figs 2 & D) and contained abundant peroxidase labeling. TH-immunoreactivity was primarily localized to somatodendritic processes (Figs 2C-D), although occasionally axon terminals exhibited gold-silver labeling for TH.

POMC-labeled axon terminals showed synaptic specializations with TH-labeled and -unlabeled dendrites (Figs 2B-C). Semiquantitative analysis revealed that POMC-labeled axon terminals frequently contacted TH-labeled dendrites (29%; n = 118). When synaptic specializations were identifiable, they were either of the symmetric, inhibitory type (Gray's type; Gray, 1959) or the asymmetric, excitatory type. Some of the POMC-labeled axon terminals did not form clearly defined synaptic specializations with postsynaptic targets in the single planes of section analyzed (Fig. 2C). Of the 118 POMC-labeled axon terminals that were in direct contact with TH-labeled dendrites, 39% (46/118) were of the symmetric type while 18% (21/118) were asymmetric. The remainder did not form well defined synapses in the plane of sections analyzed. Approximately, 15% of the POMC-labeled axon terminals were occasionally apposed to other axon terminals. Frequently, appositions with other terminals lacked the specialized densities characteristic of axo-axonic synapses.

Cellular interactions between POMC and CRF

Immunofluorescence labeling for POMC and CRF was visualized in coronal sections in the rostral pontine tegmentum (Figs 3A-C). Immunocytochemical labeling for POMC and CRF exhibited a punctate pattern of staining and densely labeled fibers (Figs

3A-C). POMC immunoreactivity was localized to CRF-labeled processes and this was more prominent in the peri-LC as compared to the core region (Fig. 3C).

Ultrastructural analysis of POMC and CRF

At the ultrastructural level, POMC immunolabeling showed a similar distribution as described above (Figs 4 and 5). CRF immunoreactivity was restricted to axons and axon terminals and was rarely seen in somata and dendrites consistent with previous findings (Van Bockstaele *et al.*, 1996d; Van Bockstaele *et al.*, 1998b). Of 657 POMC-labeled axon terminals, where CRF immunoreactivity was present in the neuropil, 27% (178/657) contained CRF immunoreactivity. Conversely, of 542 CRF-labeled terminals where POMC immunoreactivity was present in the neuropil, 33% (178/542) were dually labeled. The dually-labeled terminals contained several recognizable small clear vesicles and one or more mitochondria (Figs 4A-D). Some of these also contained large dense core vesicles (Figs 4A-C). The dually-labeled axon terminals contacted primarily medium to small sized dendrites. Some of these terminals did not form recognizable synaptic specializations in the single planes of section analyzed. However, when synaptic specializations were recognizable, they were more frequently of the asymmetric type (Figs 4A, 4C and 5A-B). Of 178 POMC-labeled axon terminals that contained CRF immunoreactivity, 29% and 21% formed asymmetric and symmetric synapses (Figs 4D and 5C), respectively. The remainder did not form identifiable synapses in sections examined.

Discussion

The present findings demonstrate the first ultrastructural evidence that POMC-immunoreactive axon terminals form synaptic specializations with TH-containing dendrites in the LC. In addition, results show that POMC and CRF co-exist in single axon terminals in the LC. These data provide a neuroanatomical substrate whereby CRF afferents containing POMC derived peptides, such as β-endorphin, are positioned to impact the LC-NE system. This substrate could underlie activation of the brain NE system by stress and may serve to integrate autonomic components of the stress response associated with functions of LC neurons.

The present results provide the first ultrastructural description of POMC-labeled fibers in the rat LC. POMC fibers have been demonstrated in the LC in aquatic toad (Tuinhof *et al.*, 1998). Our findings of the POMC distribution in the LC are consistent with that of β-endorphin, a peptide synthesized from POMC described in earlier studies, (Bloom *et al.*, 1978a) where fibers course toward the middle third of the LC in the ventromedial aspect but are scant at caudal levels. By using dual immunoelectron microscopy, we provide the first ultrastructural evidence that POMC fibers form synaptic specializations with catecholamine-containing dendrites in the LC. In addition, we demonstrate that POMC-labeled fibers form primarily symmetric synapses that are correlated with inhibitory transmission, with TH-labeled dendrites in the LC (Peters & Palay, 1996; Peters *et al.*, 1991).

β-endorphin influences nociception (Foley *et al.*, 1979; Yaksh *et al.*, 1982; Bertolini *et al.*, 1986; Baubet *et al.*, 1994), cardiovascular (Laubie *et al.*, 1977; Holaday, 1983), respiratory (Moss & Friedman, 1978) and stress responses (Baubet *et al.*, 1994).

As such, intracoerulear injections of β-endorphin suppressed long lasting posture asymmetry and movement (Bertolini *et al.*, 1986) and significantly elevated nociceptive threshold (Yaksh *et al.*, 1982) which were reversed by naloxone, an opioid antagonist. In addition, β-endorphin administration via the cisterna magna caused a transient increase in blood pressure and heart rate followed by a delayed hypotension and bradycardia (Laubie *et al.*, 1977). Loh and colleagues (Loh *et al.*, 1976) demonstrated that β-endorphin inhibits striatal dopamine release in vitro. Furthermore, in situ hybridization study showed that acute stress increased POMC mRNA expression in the mediobasal hypothalamus (Baubet *et al.*, 1994). Our present findings indicate that POMC modulates LC neurons directly in the dorsal pontine tegmentum. Hence, the central effects of β-endorphin described earlier may represent actions of this potent peptide on LC neurons as these neurons are engaged in similar activities.

Previous studies have shown that the arcuate nucleus (ARC) and NTS are enriched with POMC-immunoreactive perikarya (Schwartzberg & Nakane, 1982; Gee *et al.*, 1983). ARC and NTS are brain regions involved in a myriad of autonomic functions such as cardiovascular (Lu *et al.*, 2002; Weston *et al.*, 2003; Balan Junior *et al.*, 2004; Guo & Moazzami, 2004), nociception (Sun & Yu, 2005; Tonosaki *et al.*, 2005) and stress responses (Buller, 2003; Dayas *et al.*, 2004; Helmreich *et al.*, 2005; Kas *et al.*, 2005; Sergeyev *et al.*, 2005). Similarly, LC neurons have been implicated in autonomic functions including cardiovascular, respiratory and stress responses (Aston-Jones *et al.*, 1991; Allen & Cechetto, 1992; Cechetto & Chen, 1992; Reis & Golanov, 1997; Valentino *et al.*, 1998; Machado, 2001). POMC fibers in the LC may be derived from the ARC and NTS since anatomical studies using retrograde tracing revealed that neurons

from the ARC send projections to the LC (Luppi *et al.*, 1995). In addition, we have previously demonstrated that efferent projections from the NTS monosynaptically innervate dendrites exhibiting TH-immunoreactivity in the rostral LC area where we found abundant POMC fibers (Van Bockstaele *et al.*, 1999). The present study demonstrating dual-labeling for POMC-immunoreactive terminals and TH-immunoreactive dendrites show that majority of the synapses identified were of symmetric type. These suggest that the ARC and NTS may be possible sources of POMC afferent fibers projecting to the LC that could regulate LC activity.

In the present study, immunofluorescence showed prominent co-localization of POMC and CRF terminals in the ventromedial aspect of the LC. However, there was little evidence of POMC and CRF co-localization in the dorsolateral aspect of the LC. The anatomical distribution of POMC and CRF co-localization is in contrast to the distribution of fibers dually labeled for enkephalin and CRF in the LC (Tjoumakaris *et al.*, 2003) which was visualized more in the LC core and some observed dorsolaterally. This was confirmed at the ultrastructural level, where POMC and CRF dually labeled terminals were located primarily in the ventromedial aspect of the LC. With regard to the degree of co-localization with enkephalin (12% of enkephalin terminals; Tjoumakaris *et al.*, 2003), the percentage of POMC-labeled terminals that exhibited CRF immunoreactivity in this study was greater. Therefore, although many POMC and CRF terminals were singly labeled, this study also shows a distinct population of terminals that co-localized both peptides.

The NTS is a potential source of POMC and CRF dually labeled terminals in the LC because it contains numerous CRF (Herbert & Saper, 1990) and POMC

(Schwartzberg & Nakane, 1982) neurons. Taken with our previous demonstration that NTS efferents form synaptic specializations with catecholaminergic LC dendrites (Van Bockstaele *et al.*, 1999), it is tempting to speculate that CRF neurons in the NTS that co-express β-endorphin innervate LC neurons. Future studies would be useful to elucidate whether these terminals arise from the NTS.

Our findings also show that POMC/CRF afferents containing axon terminals form asymmetric (29%) and symmetric (21%) synapses with postsynaptic targets. Asymmetric synapses have been correlated with excitatory transmission whereas symmetric synapses have been correlated with inhibitory transmission (Gray 1959; Carlin *et al.*, 1980; Carlin *et al.*, 1981; Peters *et al.*, 1991; Peters et al., 1996). These data suggest that POMC/CRF afferents may originate from different afferent nuclei (ARC vs NTS) based on their morphological heterogeneity. In addition, the heterogeneous morphological differentiation of the synapses may indicate co-existence with either excitatory or inhibitory amino acid transmitter such as glutamate or gamma-aminobutyric acid (GABA). We have shown that CRF forms primarily asymmetric synapses with LC dendrites and that some of these contain glutamate (30%; Valentino *et al*., 2001). However, we also showed that some CRF terminals contain GABA (5%) suggesting that, in the present study POMC/CRF terminals may co-express distinct amino acid transmitters, and by the virtue of this, exhibit distinct synaptic specializations. Future studies are required to determine whether POMC/CRF terminals co-express different amino acid transmitters.

In conclusion, our present results suggest that POMC is localized to efferents that target LC neurons, a circuit that may facilitate modulation of autonomic functions

including nociceptive, cardiovascular and respiratory functions. In addition, afferents expressing both CRF and POMC as co-transmitters may be involved in regulation of stress responses known to be subserved by this region.

Abbreviations

CRF, corticotropin-releasing factor; LC, locus coeruleus; POMC, proopiomelanocortin; TH, tyrosine hydroxylase;

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Figure Legends

Figure 1. A. Brightfield photomicrographs of proopiomelanocortin (POMC) in the locus coeruleus (LC). Arrows indicate individual POMC-labeled processes. Note that many are highly beaded and thin. Inset in panel A shows a schematic diagram adapted from the rat brain atlas of Swanson (1992) depicting the region sampled. In the inset, arrows indicate dorsal (D) and medial (M) orientation of the sections illustrated. **B-D.** Confocal fluorescence photomicrographs of POMC (arrowheads) labeled with fluorescein isothiocyanate (green) and tyrosine hydroxylase (TH; arrows) labeled with rhodamine isothiocyanate (red) in the LC. Abundant POMC-labeled processes are associated with TH-labeled dendrites (C). This association extends into the LC somatic region (B and D). Scale bars, 100 μm. Abbreviations: scp, superior cerebellar peduncle; D, dorsal; IV, fourth ventricle; M, medial; mlf, medial longitudinal fasciculus; moV, motor root of the trigeminal nucleus; V, motor nucleus of the trigeminal nucleus.

Figure 2. Electron photomicrographs showing peroxidase labeling for proopiomelanocortin (POMC) and gold-silver labeling for tyrosine hydroxylase (TH) in the locus coeruleus (LC). **A.** Peroxidase labeling can be seen in a POMC-labeled terminal (POMC-t) that contains dense core vesicles (dcv). Several unlabeled terminals (ut) that contain unlabeled dense core vesicles (udcv) can be seen in the neuropil. **B.** A peroxidase-labeled POMC-t forms a symmetric synapse (arrow) with an unlabeled dendrite (ud). **C.** A peroxidase-labeled POMC-t that contains dcv is directly contacted (double arrow) with a TH-labeled dendrite (TH). Arrowheads indicate gold-silver labeling for TH. Arrows point to symmetric synapses formed by unlabeled terminals (ut).

D. Another peroxidase-labeled POMC-t forms a symmetric synapse (arrow) with a TH-d. Scale bars, 0.50 μm. ma, myelinated axon.

Figure 3. Confocal fluorescence photomicrographs showing co-localization of proopiomelanocortin (POMC) and corticotropin-releasing factor (CRF) in the locus coeruleus (LC). **A and A1.** CRF-labeling was detected by rhodamine isothiocyanate (red). **Arrows** point to individual CRF-labeled varicose processes that contain POMC. **Arrowheads** point to varicose processes that contain CRF. **B and B1.** POMC-labeling was detected by fluorescein isothiocyanate (green). **Arrows** point to individual POMC-labeled varicose processes that contain CRF. **Arrowheads** point to varicose processes that contain POMC. **C and C1.** Merged image. **Arrows** point to POMC- and CRF-dual labeled varicose processes. **Arrowheads** point to either POMC or CRF varicose processes. Scale bars, 100 μm.

Figure 4. Electron photomicrographs showing convergence and co-existence of proopiomelanocortin (POMC) and corticotropin-releasing factor (CRF) in the locus coeruleus (LC). **A.** A gold-silver labeled (arrowheads) POMC terminal (POMC-t) and peroxidase-labeled CRF axon terminal (CRF-t) synapse (curved arrows) with a common dendrite (d). Another peroxidase-labeled CRF-t can also be identified in the neuropil. **B.** Two axon terminals containing both peroxidase labeling for CRF and gold-silver labeling (arrowheads) for POMC contact (arrow) a dendrite (d). **C-D.** Axon terminals containing both peroxidase labeling for CRF and gold-silver labeling (arrowheads) for POMC (POMC + CRF-t) contact dendrites (d) in the LC. Scale bars, 0.5μm.

Figure 5. Electron photomicrographs showing synaptic specializations formed by proopiomelanocortin (POMC) and corticotropin-releasing factor (CRF) axon terminals in

the locus coeruleus (LC). **A-B.** Gold-silver labeling for POMC (arrowheads) and peroxidase-labeling for CRF (POMC+ CRF-t) can be found in axon terminal forming asymmetric synapses (arrows) with dendrites (d). **C.** A POMC+ CRF-t forms a symmetric synapse (curved arrow) with a dendrite (d). Scale bars, 0.5μm.