Published in final edited form as:

Synapse. 2013 February ; 67(2): 94–108. doi:10.1002/syn.21624.

Columnar Distribution of Catecholaminergic Neurons in the Ventrolateral Periaqueductal Gray (vlPAG) and Their Relationship to Efferent Pathways

Shelby K. Suckow¹, Emily L. Deichsel¹, Susan L. Ingram², Michael M. Morgan³, and Sue A. Aicher¹

¹Oregon Health & Science University, Department of Physiology and Pharmacology, Portland, OR 97223, USA

²Oregon Health & Science University, Department of Neurological Surgery, Portland, OR 97223, USA

³Washington State University Vancouver, Department of Psychology, Vancouver, WA 98686, USA

Abstract

The periaqueductal gray (PAG) is a critical brain region involved in opioid analgesia and provides efferents to descending pathways that modulate nociception. In addition, the PAG contains ascending pathways to regions involved in the regulation of reward, including the substantia nigra (SN) and the ventral tegmental area (VTA). SN and VTA contain dopaminergic neurons that are critical for the maintenance of positive reinforcement. Interestingly, the PAG is also reported to contain a population of dopaminergic neurons. In this study, the distribution of catecholaminergic neurons within the ventrolateral (vl) PAG was examined using immunocytochemical methods. In addition, the catecholaminergic PAG neurons were examined to determine whether these neurons are integrated into ascending (VTA, SN) and descending (RVM) efferent pathways from this region. The immunocytochemical analysis determined that catecholaminergic neurons in the PAG are both dopaminergic and noradrenergic and these neurons have a distinct rostrocaudal distribution within the ventrolateral column of PAG. Dopaminergic neurons were concentrated rostrally and were significantly smaller than noradrenergic neurons. Combined immunocytochemistry and tract tracing methods revealed that catecholaminergic neurons are distinct from, but closely associated with, both ascending and descending efferent projection neurons. Finally, by electron microscopy, catecholaminergic neurons showed close dendritic appositions with other neurons in PAG, suggesting a possible non-synaptic mechanism for regulation of PAG output by these neurons. In conclusion, our data indicate that there are two populations of catecholaminergic neurons in the vlPAG that form dendritic associations with both ascending and descending efferents suggesting a possible non-synaptic modulation of vlPAG neurons.

Corresponding author: Sue A. Aicher, Ph.D., Department of Physiology and Pharmacology, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239-3098, Phone: 503-418-2550, aichers@ohsu.edu.

Conflict of Interest Statement:

None of the authors of the manuscript has declared any conflict of interest within the last three years which may arise from being named as an author on the manuscript.

Keywords

dopamine; tyrosine hydroxylase; immunocytochemistry; confocal microscopy; electron microscopy

Introduction

The periaqueductal gray (PAG) is involved in the modulation of a variety of brain functions including cardiovascular regulation, sleep, and nociception (Carrive et al., 1987; Carrive et al., 1989; Lovick, 1985; Lovick, 1993; Watkins et al., 1993). The well-defined columnar organization of the ventrolateral PAG (vlPAG) gives rise to several major efferent and afferent pathways connecting the PAG to other nuclei in the brainstem and more distant targets such as the spinal cord, amygdala and thalamus (Bajic and Proudfit, 1999; Cameron et al., 1995a; Cameron et al., 1995b; Carrive and Morgan, 2012; Rizvi et al., 1991; Van Bockstaele et al., 1991). Descending pathways from the vlPAG that innervate the rostral ventral medulla (RVM) are involved in antinociception. In addition to this well-studied descending pathway, anatomical studies have revealed an ascending afferent pathway from the vlPAG to regions involved in reward circuits, including the ventral tegmental area (VTA) and the substantia nigra (SN) (Cameron et al., 1995a). Recent studies have demonstrated synaptic connections between PAG efferents and specific groups of VTA neurons, including dopaminergic neurons (Omelchenko and Sesack, 2010), identifying this pathway as a possible substrate for the rewarding effects of opioids.

The vlPAG contains a population of catecholaminergic neurons (Arsenault et al., 1988) that appear to contribute to opioid antinociception (Flores et al., 2004; Flores et al., 2006; Hasue and Shammah-Lagnado, 2002; Meyer et al., 2009). Inhibition of the dopamine network within PAG, either by dopamine depletion or dopamine receptor antagonism, attenuates morphine antinociception (Flores et al., 2004). However, the contributions of intrinsic and vlPAG projection neurons to the dopaminergic effects observed in the vlPAG are still unknown.

The present study was designed to examine catecholaminergic neurons in the vlPAG to determine if they are part of the descending nociceptive pathways or ascending pathways involved in the reward system. Using retrograde tracers we mapped vlPAG neurons that project to RVM, VTA, and SN to determine their relationship to catecholaminergic neurons within the vlPAG. Furthermore, electron microscopy was used to examine the cellular interactions of catecholaminergic neurons in the vlPAG. Using immunocytochemistry combined with confocal and electron microscopy, we demonstrate that the catecholaminergic neurons of the vlPAG are not all dopaminergic, but in fact the column transitions from a noradrenergic phenotype caudally to a dopaminergic phenotype rostrally. The two populations are also distinct with regard to cell size. While the catecholaminergic neurons do not send efferents to RVM, VTA or SN, they do form close dendritic associations with these neurons. The abundance of dendro-dendritic appositions suggests a potential non-synaptic role for PAG catecholaminergic neurons in the modulation of nociceptive and reward systems.

Material and Methods

Experimental animals

All protocols adhered to the 2011 Eighth edition NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Experiments were performed on male

Sprague-Dawley rats (total n=19) weighing 250-350g (Charles River, Wilmington, MA). Animals were initially anesthetized in a Plexiglas chamber with 5% isoflurane. The head was shaved, and the rat was placed in a stereotaxic frame with a nose cone for anesthesia delivery (3% in oxygen). For all survival surgeries, incisions were closed with sutures and the animal was kept on a warming blanket and carefully monitored during recovery from anesthesia.

Retrograde tract tracing techniques

The retrograde tracer *FluoroGold* (FG, 2% in saline; Fluorochrome, Inc.) was used to identify neurons in vlPAG that project to the RVM. In addition, *Rhodamine microbeads* (undiluted; Lumafluor) were used as a retrograde tracer to identify neurons in vlPAG that project to the VTA and SN. FG and Rhodamine were pressure injected (60-100 nl) into the RVM (midline; 2.3 mm caudal, 11.6 mm ventral), left VTA (: 4.5 mm caudal, 0.5 lateral, 8.5 mm ventral) or left SN (4.5 mm caudal, 2.2 mm lateral, 8.7 mm ventral), respectively.

Perfusion and tissue preparation

Rats were perfused 7 days following injection of FG and Rhodamine into the RVM and/or VTA respectively. Rats that received Rhodamine into the SN were perfused 3 days post injection. Rats were given a lethal dose of sodium pentobarbital (150 mg/kg, i.p.) and perfused transcardially through the ascending aorta with 10 mL of heparinized saline (1000 U/mL), followed by 50 mL of 3.8% acrolein in 2% paraformaldehyde followed by 200 mL of 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brain was removed, and blocks of tissue containing the PAG, RVM, VTA and SN were placed in the final fixative for 30 min before transferring to 0.1 M PB. Tissue was sectioned on a vibrating microtome (Leica, Malvern, PA) at 40 μ m. Free-floating tissue sections were placed in 1% NaBH₄ (Sigma-Aldrich; St. Louis, MO) for 30 min to bind remaining free aldehydes. Correct placement of retrograde injections was verified in tissue sections that contained the RVM, VTA and SN using an Olympus BX51 epifluorescent microscope.

Immunofluorescent Immunocytochemistry

In the first experiment, tissue sections from PAG were incubated for 48 h at 4°C with the primary antibody mouse anti-TH (1:10,000; ImmunoStar) and rabbit anti-dopamine beta hydroxylase (DBH; 1:4000; AbCam). The primary antibody cocktail contained 0.25% Triton and 0.1% BSA. The secondary antibody cocktail contained Alexa Fluor® 647 donkey anti-mouse IgG (1:800; Invitrogen) and Alexa Fluor® 488 donkey anti-rabbit IgG (1:800; Invitrogen) in 0.1% BSA. Tissue sections were incubated for 2 hours at RT in secondary antibody cocktail. In a second experiment, tissue sections from PAG were incubated for 48 h at 4°C with the primary antibody mouse anti-TH (1:10,000; ImmunoStar) and rabbit anti-FG (1:15,000; Fluorochrome, LLC). The primary antibody cocktail contained 0.25% Triton and 0.1% BSA. The secondary antibody cocktail contained Alexa Fluor® 488 goat anti-mouse IgG (1:800; Invitrogen) and Alexa Fluor® 647 goat anti-rabbit IgG (1:800; Invitrogen) in 0.1% BSA. Tissue sections containing Rhodamine labeling were incubated in the Nissl stain neurotrace® 640/660 (1:250; Invitrogen) for 20 minutes. All primary and secondary antibodies were diluted in 0.1 M Tris (TS; pH 7.6). Tissue was rinsed in 0.05 M PB, mounted on gelatin-coated slides, cover slipped with Prolong Gold™ Antifade and stored at -20°C to preserve labeling.

Antibody Characterization

The mouse anti-TH antibody recognizes an epitope in the conserved midportion of the TH molecule and the antibody bound to a corresponding band of protein at the molecular weight of TH (60 kDa; manufacturer's technical specifications). The specificity of the antibody was

determined by preabsorption with the TH control peptide (1 μ M) and omission of the primary antibody abolished any detectable immunoreactivity (Reyes et al., 2007). Immunopositive labeling in the current study was similar to other studies using rats (Chan et al., 1990; Reyes et al., 2007; Reyes et al., 2008). The rabbit anti-DBH antibody recognizes the full length native protein from cow adrenal medulla and bound to a corresponding band of protein at the approximate molecular weight of DBH (72 kDa; manufacturer's technical specifications). Immunopositive labeling in the current study was similar to other studies (Fortune and Lurie, 2009; Nosjean et al., 2002)

Microscopy and analysis

Fluorescent markers were visualized using a Zeiss LSM 510 META confocal microscope. Antibodies typically show limited penetration into Vibratome sections; therefore, Z-stacks bounded by the vertical extent of labeling into the tissue were collected (typically 10-20 μ m). The single pass, multi-tracking format was utilized to allow multiple tracers to be individually excited with different lasers, and the emitted spectra were collected separately to minimize overlap. AlexaFluor 488 was excited with a 488 nm (Argon/2) laser, and emissions passed through a 500-550 nm band pass filter. Rhodamine was excited with a 543 nm laser (HeNe1) and emitted light passed through a 565-615 nm band pass filter. AlexaFluor 647 and neurotrace® 640/660 was excited with a 633 nm laser (HeNe2) and emitted light passed through a 650-710 nm band pass filter. Images were collected using an oil immersion objective (40x/1.3 NA EC Plan-NEOFLUAR). Sections for analysis were chosen based on anatomical landmarks visible under darkfield illumination, as well as the presence of FG and Rhodamine-labeled neurons. For PAG cell counts, cells were counted per section using the ImageJ program (NIH, Bethesda, MD) where FG, Rhodamine and TH-labeled cells were counted and marked; double labeled cells were determined by superimposing the marked image with the labels being investigated. Confocal micrographs are projections of several optical sections. The cross-sectional diameter of TH-ir and DBH-ir neurons was measured using the measurement function in the Zeiss LSM 510 META software. In addition, Zeiss LSM 510 META software was used to adjust for optimal brightness and contrast of confocal micrographs.

Dual-label immunocytochemistry for electron microscopy

Tissue sections to be used for electron microscopic analysis were processed using combined immunoperoxidase and colloidal gold immunocytochemical methods (Aicher et al., 2003; Chan et al., 1990; Zeng et al., 2006). The freeze-thaw method was used to increase antibody penetration of the tissue, and the sections were then incubated in 0.1% sodium borohydride in 0.1 M Tris (TS) and 0.5% BSA in 0.1 M TS. In one set of experiments, tissue sections were then incubated in a cocktail of the primary antibodies directed against TH (1:5000; Immunostar) and FG (1:15,000; Fluorochrome, LLC) for 48 h at 4 °C. Bound FG primary antibody was visualized by incubating tissue sections in biotinylated goat anti-rabbit IgG secondary antibody (1:400; Jackson ImmunoResearch) followed by incubation in Avidin-Biotin (Elite Vectastain ABC kit) and diaminobenzidine-hydrogen peroxidase (DAB-H₂O₂) solution. To visualize the TH primary antibody, the tissue was incubated in colloidal gold-conjugated goat anti-mouse IgG secondary antibody (1:50; Electron Microscopy Sciences (EMS), Hatfield, PA) for 2 h. In another set of experiments, the tissue was stained with immunoperoxidase for TH antibody labeling. In this case, the primary antibody concentration was 1: 10,000. The TH primary antibody was visualized with a biotinylated goat anti-mouse IgG secondary antibody (1:400; Jackson ImmunoResearch) as described above. In both sets of experiments, the tissue sections were then fixed in 2% EM grade glutaraldehyde (EMS) and the gold particles were enhanced with the Amersham IntenSE™ M silver enhancement kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Tissue sections were osmicated (1 h; 2% osmium tetroxide), dehydrated through ethanol and

propylene oxide and then incubated in a 1:1 mixture of propylene oxide and EMBed 812 (EMS) overnight. The following day, the sections were embedded in EMBed 812 between two sheets of Aclar fluoroalocarbon plastic film (Ted Pella, Redding, CA) and then placed in an oven for 48 h at 60 °C. Regions of tissue containing both TH and FG labeling were selected from the ventrolateral region of the PAG and were glued onto Beem capsules (Ted Pella), sectioned at 75 nm on a Leica Microsystems ultramicrotome, collected onto 400 mesh copper grids (EMS) and counterstained with uranyl acetate and Reynold's lead citrate.

Data analysis for electron microscopy

Ultrathin sections were examined on a Tecnai 12 electron microscope (FEI, Hillsboro, OR) and images were captured digitally on an Advanced Microscopy Techniques (AMT, Danvers, MA) camera. The selection of ultrathin sections for EM analysis was based on optimal preservation of morphological details and maximal detection of the labeling (Peters et al., 1991). Sections were selected from an area just below the surface of the tissue, at the tissue/plastic interface, where the penetration of antibodies was optimal in order to avoid under detection of the immunogold antigen (Aicher et al., 2003; Chan et al., 1990). Analysis was focused on the ventrolateral PAG (vIPAG) in which TH-labeled profiles were found. Regions of tissue had to contain both immunogold and immunoperoxidase-labeled profiles in order to be included in this study (Aicher et al., 2003).

Images were assessed for the type of profile (i.e., somata, dendrites, terminals, or axons) labeled with either immunoperoxidase, immunogold or both (Peters et al., 1991). Images were also assessed for the type of synaptic input to labeled somata or dendrites (symmetric or asymmetric) or the type of synapse formed by labeled terminals (Peters et al., 1991). In order for a profile to be considered positive for immunogold labeling, two or more gold particles had to be present in somata, dendrites, terminals, or axons.

Photomicrograph production

Confocal photomicrographs are projections of several optical sections. Zeiss LSM 510 META software was used to adjust for optimal brightness and contrast whereas Adobe Illustrator was used in the completion of the figure. Adobe Photoshop was used to adjust for optimal brightness and contrast for electron microscopy photomicrographs and were completed in Adobe Illustrator.

Results

Characteristics of catecholaminergic neurons in the vIPAG

Previous studies have demonstrated that catecholaminergic neurons are present within the periaqueductal gray (PAG) (Arsenault et al., 1988; Flores et al., 2004; Flores et al., 2006; Grzanna and Molliver, 1980; Hasue and Shammah-Lagnado, 2002; Meyer et al., 2009). These neurons have been described as either dopaminergic or noradrenergic, depending on which synthetic enzymes were detected. Tyrosine hydroxylase (TH) is present in all catecholaminergic neurons, and the presence of additional synthetic enzymes can indicate the ability of the neuron to synthesize other catecholamines. The present studies used antibodies against the catecholamine synthetic enzymes TH and dopamine beta hydroxylase (DBH). Noradrenergic cells contain both enzymes while dopaminergic cells contain only TH. We validated our methods (Figure 1) by confirming that the catecholaminergic neurons of the locus coeruleus (LC) are exclusively noradrenergic (Figure 1A) (Versteeg et al., 1976) while neurons in the ventral tegmental area (VTA) and substantia nigra (SN) were dopaminergic (Figure 1B,C). The rostrocaudal distribution of dopaminergic and noradrenergic labeling in the ventrolateral PAG (vIPAG) was examined in more detail. Three separate regions of PAG were examined (Figure 2). In caudal regions of the vIPAG

(Figure 2A), neurons contained both TH and DBH suggesting these neurons are noradrenergic (Herbert and Saper, 1992). Mid-vIPAG regions exhibited DBH and TH co-localization as well as a population of neurons that were only TH-ir (Figure 2B); more rostral vIPAG regions contained TH-ir neurons but no DBH-ir was present (Figure 2C). These data indicate that the majority of the dopaminergic vIPAG neurons are located at more rostral regions.

There was a wide distribution in the size of catecholaminergic cells in vIPAG. Previous studies (Flores et al., 2004) found two different populations of TH-ir neurons: large neurons with multipolar morphology and small rounded neurons that were primarily located near the aqueduct. Based on our observations of both noradrenergic and dopaminergic neurons in PAG, we evaluated whether there was a size difference between catecholaminergic populations. In the current study, cells in vIPAG with TH and DBH-ir were significantly larger (cell diameter of 15-25 μ m) than neurons containing TH-ir but not DBH-ir (cell diameter of 5-15 μ m) (Figure 3). These results suggest the two populations of vIPAG TH-containing neurons represent different catecholamine cell groups.

Ultrastructural analysis revealed the cellular interactions of catecholaminergic neurons in the vIPAG

The objective of this experiment was to determine the ultrastructural features of catecholaminergic neurons in vIPAG as well as their interactions with other neurons. Electron microscopy revealed TH-ir in both pre- and postsynaptic structures (Fig. 4), including somata (Fig. 4A) and axon terminals (Fig. 4B). TH-ir was observed most frequently in dendrites (Fig. 3C) and axon terminals (Table 1). TH-ir dendrites were typically contacted by unlabeled axon terminals (60%, n=36; Table 1; Fig. 4C). Unlabeled axon terminals formed appositions (52%, n=31) as well as asymmetric synapses onto TH-ir dendrites (25%, n=15). No symmetric synapses were observed onto TH-ir dendrites from unlabeled axon terminals and 25% (n=15) of TH-ir dendrites did not receive any contacts. TH-ir axon terminals apposed unlabeled postsynaptic profiles (64%, n=23) as well as formed asymmetric synapses (17%, n=6). TH-ir axon terminals did not form symmetric synapses and 19% (n=7) had no contacts. TH-ir dendrites were also in direct contact with unlabeled dendritic profiles (17%, n=10; Table 1; Fig. 4D). To determine if TH-ir dendro-dendritic contacts with unlabeled profiles were a specialization of TH-ir profiles or vIPAG profiles in general, unlabeled dendritic profiles in the vIPAG also were examined. We found that 24% (n=23; Table 1) of vIPAG unlabeled dendritic profiles contacted other unlabeled dendritic profiles (Fig. 4E). These findings suggest that dendro-dendritic appositions are a common feature in vIPAG that is not unique to TH-ir neurons and suggests that these contacts may represent a non-synaptic form of cellular interaction between neurons in this brain region.

RVM, VTA, and SN-projecting neurons are differentially distributed in the vIPAG and lack TH

The relationship between catecholaminergic vIPAG neurons and several efferent pathways from this brain region were examined next. Given the differences in the distribution of TH-ir cells in vIPAG, three distinct rostrocaudal levels of the vIPAG were examined. FG injections into the RVM (n=6) included the raphe magnus and gigantocellular reticular nucleus (Figure 5A). Rhodamine injections into the VTA (n=4) encompassed the left VTA and interpeduncular nucleus (Figure 5B). Rhodamine injections in the left SN included all subregions, reticular, compact, and lateral (Figure 5C).

Injections of FG into the RVM resulted in retrogradely labeled neurons throughout the vIPAG (Figure 6). Immunocytochemical analysis revealed that FG-labeled RVM-projecting

neurons do not contain TH-ir. However, at all regions of vlPAG (caudal to rostral), RVM-projecting neurons came within close proximity of TH-ir neurons (Figure 6A, B). FG-ir neurons were found preferentially in both caudal and midlevel vlPAG regions (50 ± 6 and 48 ± 5 neurons per section, respectively) as compared to rostral sections of the vlPAG (27 ± 2 neurons per section) (Figure 6C).

Injections of Rhodamine into the ventral tegmental area (VTA) resulted in retrograde labeling of neurons throughout the vlPAG (Figure 7) and in the dorsal raphe nucleus (Figure 1D), in agreement with previously published data (Vertes, 1991). Immunocytochemistry demonstrated that VTA-projecting neurons do not co-localize with TH-ir neurons in the vlPAG but are in close proximity to them (Figure 7A, B). However, VTA-projecting neurons did contain TH-ir in dorsal raphe neurons (Figure 1D). Similar numbers of VTA-projecting neurons were found in all three regions of the vlPAG (caudal: 23 ± 3 ; midlevel: 24 ± 6 ; rostral: 29 ± 2 ; Figure 7C).

Rhodamine labeled SN-projecting neurons were present throughout the vlPAG (Figure 8). SN-projecting neurons within the vlPAG are immunonegative for TH but are also found in interspersed with TH-ir neurons (Figure 8A, B). A similar distribution of VTA-projecting and SN-projecting neurons was evident with an even distribution of SN-projecting neurons across rostrocaudal vlPAG levels (caudal: 16 ± 3 ; midlevel: 15 ± 2 ; rostral 19 ± 2 ; Figure 8C).

In addition to examining the number of projection neurons in the various vlPAG regions, we also counted the number of TH-ir neurons. Immunocytochemical analysis revealed a consistent number of TH neurons present in each region of vlPAG in sections that included projection neurons from the RVM (caudal: 12 ± 3 ; midlevel: 14 ± 3 ; rostral: 21 ± 2 ; Figure 6C), VTA (caudal: 14 ± 6 ; midlevel: 11 ± 1 ; rostral: 15 ± 1 ; Figure 7C) and SN (caudal: 10 ± 3 ; midlevel: 10 ± 3 ; rostral: 13 ± 2 ; Figure 8C).

The vlPAG has distinct projections to the RVM and VTA

Given the abundance of both RVM- and VTA-projecting neurons in the vlPAG, dual retrograde tract tracing was undertaken to determine whether these neurons originate from the same group of cells. FG and rhodamine injections into the RVM and VTA resulted in retrograde labeling in the vlPAG. The distribution of RVM- and VTA-projecting neurons was comparable to what we found with single tract tracer injections mentioned previously (Figure 6, 7). Dual tract tracing demonstrated that RVM- and VTA-projecting neurons do not co-localize in the vlPAG (Figure 9) indicating that these projection neurons do not send collaterals to both regions. As shown above, these labeled neurons were immunonegative for TH but were in close proximity to TH-ir neurons within the vlPAG (Figure 9).

Ultrastructural analysis revealed the localization of catecholaminergic and RVM-projection neurons in the vlPAG

Although catecholamines were not localized within projection neurons, close appositions between these groups of neurons were common. Electron microscopic analyses were conducted to determine whether these close appositions represent direct neuronal contact. FG-immunoreactivity was found exclusively in postsynaptic profiles, specifically somata (45%, $n=34$; Table 2; Fig. 10A) and dendrites (53%, $n=40$) (Figure 10B; Table 2). In addition, FG-ir dendrites contacted unlabeled dendrites (25%, $n=10$; Table 2). TH-ir cells were also in close proximity to FG-ir profiles (Fig. 10C). Ultrastructural analysis revealed that 3% of TH-ir profiles apposed FG-ir dendrites ($n=2$). In addition, 7% of TH-ir profiles apposed FG-ir somatas ($n=5$; Table 2). Ultrastructural analysis did not reveal any synaptic specializations between TH and FG-ir profiles (Figure 10C).

Discussion

These studies report three distinct but related findings. First, we find that both dopaminergic and noradrenergic neurons are found in the vlPAG and appear to have a distinct columnar organization. Second, we find that although these catecholaminergic neurons are located in close proximity to neurons that send projects to efferent targets of vlPAG, such as RVM, SN, and VTA, those projection neurons do not contain catecholaminergic markers. Third, we find that catecholaminergic neurons located at a mid-level region of vlPAG, which are likely dopaminergic neurons, form dendro-dendritic contacts with neurons that project to the RVM, as well as other neurons in the vlPAG, suggesting a non-synaptic mechanism for modulation of PAG activity.

Columnar organization of catecholaminergic neurons in PAG

The current study affirms the presence of catecholaminergic neurons within the ventrolateral periaqueductal gray (vlPAG); where our analysis of both tyrosine hydroxylase (TH) and dopamine-beta hydroxylase (DBH) reveals two distinct populations of catecholaminergic neurons within the vlPAG. More specifically, noradrenergic neurons have a multipolar morphology and are located caudally, whereas dopaminergic neurons are much smaller, less complex, and located rostrally. This columnar distribution of catecholaminergic neurons with distinct phenotypes between rostral and caudal groups is reminiscent of the catecholaminergic cell groups in the ventrolateral medulla which transition from a caudal noradrenergic group (A1) to a rostral epinephrine group (C1) (Armstrong et al., 1982; Dahlstroem and Fuxe, 1964b; Kalia et al., 1985). Previous studies determined that in rostral areas of the PAG catecholaminergic neurons do not contain DBH suggesting that these neurons are dopaminergic. Whereas these previous findings are in agreement with our data, they did not include caudal and/or mid level PAG sections in their analysis. Grzanna and Moliver (1980) were the first to demonstrate that noradrenergic neurons are present in the caudal vlPAG region. Therefore, our studies in conjunction with these previous studies (Flores et al. 2004; Grzanna and Moliver, 1980), show a mixed population of noradrenergic and dopaminergic neurons in mid level vlPAG, a dopaminergic population in the rostral vlPAG and a noradrenergic population contained in more caudal levels of vlPAG. In addition, noradrenergic neurons of the vlPAG are the largest DBH-containing neurons in the midbrain (Grzanna and Molliver, 1980) which is in agreement with our finding that noradrenergic neurons are larger than dopaminergic neurons of the vlPAG. Therefore, our observations are consistent with previous studies, but provide a more complete integration of the seemingly disparate reports regarding the anatomical and morphological characterization of noradrenergic and dopaminergic neurons in the vlPAG. Future studies will explore whether these two distinct catecholaminergic groups have additional distinctions other than their neurochemical and morphological differences.

Catecholaminergic neurons are distinct from efferent projection neurons of PAG

Because of our interest in the role of vlPAG neurons in ascending and descending modulation of nociception, we determined if the vlPAG catecholaminergic neurons are a part of several PAG efferent pathways. Our retrograde tracing studies showed that projections to RVM were most dense from caudal regions of vlPAG, while projections to SN and VTA were similar density at all of the rostrocaudal levels examined. With regard to co-localization of catecholamines in these projection neurons, we were not able to detect TH in neurons that project to any of the targets we examined (RVM, SN, VTA). To confirm that these results were not simply due to technical limitations, we verified the presence of dual-labeled neurons in other regions. Immunocytochemical analysis revealed dual-labeled neurons in the dorsal raphe following retrograde tracer injections into the VTA which is in agreement with Vertes (1991). The present data suggest that catecholaminergic neurons in

PAG do not send projections to these regions associated with nociception or reward. Omelchenko and Sesack (2010) investigated vIPAG efferent terminals in the VTA. Their study found a small population of rostral vIPAG efferent terminals in the VTA that contain TH. The difference between our results (0%) and their results (3%) may partially be due to the specific region of the PAG examined in each study (i.e. caudal vs. rostral regions of the vIPAG) since Omelchenko and Sesack (2010) examined VTA terminals that originated from an area of vIPAG that is more rostral than where our data was acquired; or may also be due to differences in the sensitivities of anterograde and retrograde tracing methods. Even though the pathway from vIPAG to VTA is largely non-catecholaminergic, based on our confocal analysis, there are catecholaminergic neurons that may interact with VTA efferents.

Based on our findings and those of Grzanna and Molliver (1980), the catecholaminergic neurons described in the most caudal regions of vIPAG are likely noradrenergic rather than dopaminergic. Recent studies would suggest that these neurons project to the central extended amygdala (Hasue et al. 2002). Following retrograde tracing, it was determined that the catecholaminergic neurons of the vIPAG are located primarily in the periventricular zone of PAG, as well within the dorsal raphe (Hause et al. 2002). Based on these findings, it is unlikely that the catecholaminergic neurons in our study are a part of the amygdala efferent pathway. However, further investigation is needed to determine whether amygdala-projecting neurons at different rostrocaudal levels of PAG are dopaminergic or noradrenergic. Given the lack of robust extrinsic projections detected for PAG catecholaminergic neurons in our study it is possible that some of these catecholaminergic neurons may be intrinsic to the vIPAG but this would be difficult to prove conclusively since a number of other brain regions send catecholaminergic projections to the PAG (Dahlstroem and Fuxe, 1964a; Herbert and Saper, 1992; Kwiat and Basbaum, 1990).

Catecholaminergic neurons form dendro-dendritic appositions with other PAG neurons

The mechanisms for modulation of vIPAG neurons by these putative intrinsic neurons are not clear. It is possible that these neurons send local projections that synapse on other neurons in PAG such as inhibitory GABAergic neurons. Another intriguing possibility is that these neurons and other cells in the PAG form gap junctions (Buma et al., 1992). Previous studies of PAG ultrastructure have reported an abundance of dendro-dendritic appositions that may represent gap junctions (Buma et al., 1992). This previous study examined the PAG neuropil in general and did not determine if these dendritic appositions were between projection neurons or interneurons. Our data show both putative intrinsic catecholaminergic neurons and RVM-projecting neurons form these appositions with other PAG neurons. Definitive gap junctions were difficult to verify due to the extensive immunocytochemical processing of the tissue, but these findings suggest that non-synaptic modulation of PAG output neurons may be a distinct mechanism for modulation of PAG efferent projections. It is also possible that conditions that alter the coupling of PAG neurons may alter the efficacy of this non-synaptic modulation.

In conclusion, our data indicate that there are two populations of catecholaminergic neurons in the vIPAG which can be differentiated by both location and size. Furthermore, catecholaminergic neurons form dendritic associations with both ascending and descending efferents suggesting a possible non-synaptic modulation of vIPAG neurons, including output neurons. Further work is needed to determine the exact role of vIPAG catecholaminergic neurons in nociception, reward and other PAG functions.

Acknowledgments

The authors would like to thank Dr. Deborah M. Hegarty and Sam Hermes for their technical assistance.

This work was supported by grants from the NIH: DA02765 (M.M.M, S.L.I., S.A.A) RR016858 (confocal microscope), P30 NS061800(S.A.A.)

Reference List

- Aicher SA, Mitchell JL, Swanson KC, Zadina JE. Endomorphin-2 axon terminals contact mu-opioid receptor-containing dendrites in trigeminal dorsal horn. *Brain Res.* 2003; 977:190–198. [PubMed: 12834879]
- Armstrong DM, Ross CA, Pickel VM, Joh TH, Reis DJ. Distribution of dopamine-, noradrenaline-, and adrenaline-containing cell bodies in the rat medulla oblongata: demonstrated by the immunocytochemical localization of catecholamine biosynthetic enzymes. *J Comp Neurol.* 1982; 212:173–187. [PubMed: 6142061]
- Arsenault MY, Parent A, Seguela P, Descarries L. Distribution and morphological characteristics of dopamine-immunoreactive neurons in the midbrain of the squirrel monkey (*Saimiri sciureus*). *J Comp Neurol.* 1988; 267:489–506. [PubMed: 3346372]
- Bajic D, Proudfit HK. Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception. *J Comp Neurol.* 1999; 405:359–379. [PubMed: 10076931]
- Buma P, Veening J, Hafmans T, Joosten H, Nieuwenhuys R. Ultrastructure of the periaqueductal grey matter of the rat: an electron microscopical and horseradish peroxidase study. *J Comp Neurol.* 1992; 319:519–535. [PubMed: 1619043]
- Cameron AA, Khan IA, Westlund KN, Cliffer KD, Willis WD. The efferent projections of the periaqueductal gray in the rat: a *Phaseolus vulgaris*-leucoagglutinin study. I. Ascending projections. *J Comp Neurol.* 1995a; 351:568–584. [PubMed: 7721984]
- Cameron AA, Khan IA, Westlund KN, Willis WD. The efferent projections of the periaqueductal gray in the rat: a *Phaseolus vulgaris*-leucoagglutinin study. II. Descending projections. *J Comp Neurol.* 1995b; 351:585–601. [PubMed: 7721985]
- Carrive P.; Morgan, MM. Periaqueductal Gray. In: Mai, Juergen K.; Paxinos, George, editors. *The Human Nervous System*. Elsevier; San Diego: 2012. p. 367-400.
- Carrive P, Bandler R, Dampney RA. Somatic and autonomic integration in the midbrain of the unanesthetized decerebrate cat: a distinctive pattern evoked by excitation of neurones in the subtentorial portion of the midbrain periaqueductal grey. *Brain Res.* 1989; 483:251–258. [PubMed: 2706518]
- Carrive P, Dampney RA, Bandler R. Excitation of neurones in a restricted portion of the midbrain periaqueductal grey elicits both behavioural and cardiovascular components of the defence reaction in the unanaesthetised decerebrate cat. *Neurosci Lett.* 1987; 81:273–278. [PubMed: 3431744]
- Chan J, Aoki C, Pickel VM. Optimization of differential immunogold-silver and peroxidase labeling with maintenance of ultrastructure in brain sections before plastic embedding. *J Neurosci Methods.* 1990; 33:113–127. [PubMed: 1977960]
- Dahlstroem A, Fuxe K. A method for the demonstration of monoamine-containing nerve fibres in the central nervous system. *Acta Physiol Scand.* 1964a; 60:293–294. [PubMed: 14131843]
- Dahlstroem A, Fuxe K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol Scand Suppl.* 1964b; (SUPPL-55)
- Flores JA, El BF, Galan-Rodriguez B, Fernandez-Espejo E. Opiate anti-nociception is attenuated following lesion of large dopamine neurons of the periaqueductal grey: critical role for D1 (not D2) dopamine receptors. *Pain.* 2004; 110:205–214. [PubMed: 15275769]
- Flores JA, Galan-Rodriguez B, Ramiro-Fuentes S, Fernandez-Espejo E. Role for dopamine neurons of the rostral linear nucleus and periaqueductal gray in the rewarding and sensitizing properties of heroin. *Neuropsychopharmacology.* 2006; 31:1475–1488. [PubMed: 16292327]
- Fortune T, Lurie DI. Chronic low-level lead exposure affects the monoaminergic system in the mouse superior olivary complex. *J Comp Neurol.* 2009; 513:542–558. [PubMed: 19226511]
- Grzanna R, Molliver ME. The locus coeruleus in the rat: an immunohistochemical delineation. *Neuroscience.* 1980; 5:21–40. [PubMed: 6988734]

- Hasue RH, Shammah-Lagnado SJ. Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *J Comp Neurol.* 2002; 454:15–33. [PubMed: 12410615]
- Herbert H, Saper CB. Organization of medullary adrenergic and noradrenergic projections to the periaqueductal gray matter in the rat. *J Comp Neurol.* 1992; 315:34–52. [PubMed: 1371780]
- Kalia M, Fuxe K, Goldstein M. Rat medulla oblongata. II. Dopaminergic, noradrenergic (A1 and A2) and adrenergic neurons, nerve fibers, and presumptive terminal processes. *J Comp Neurol.* 1985; 233:308–332. [PubMed: 2858497]
- Kwiat GC, Basbaum AI. Organization of tyrosine hydroxylase- and serotonin-immunoreactive brainstem neurons with axon collaterals to the periaqueductal gray and the spinal cord in the rat. *Brain Res.* 1990; 528:83–94. [PubMed: 1978796]
- Lovick TA. Ventrolateral medullary lesions block the antinociceptive and cardiovascular responses elicited by stimulating the dorsal periaqueductal grey matter in rats. *Pain.* 1985; 21:241–252. [PubMed: 3991230]
- Lovick TA. Integrated activity of cardiovascular and pain regulatory systems: role in adaptive behavioural responses. *Prog Neurobiol.* 1993; 40:631–644. [PubMed: 8484005]
- Meyer PJ, Morgan MM, Kozell LB, Ingram SL. Contribution of dopamine receptors to periaqueductal gray-mediated antinociception. *Psychopharmacology (Berl).* 2009; 204:531–540. [PubMed: 19225762]
- Nosjean A, Hamon M, Darmon M. 5-HT_{2A} receptors are expressed by catecholaminergic neurons in the rat nucleus tractus solitarii. *Neuroreport.* 2002; 13:2365–2369. [PubMed: 12488828]
- Omelchenko N, Sesack SR. Periaqueductal gray afferents synapse onto dopamine and GABA neurons in the rat ventral tegmental area. *J Neurosci Res.* 2010; 88:981–991. [PubMed: 19885830]
- Peters, A.; Palay, S.L.; Webster, H.D. *The Fine Structure of the Nervous System: Neurons and Their Supporting Cells.* Oxford; New York: 1991.
- Paxinos, G.; Watson, S.J. *The rat brain, in stereotaxic coordinates.* Academic Press; Sydney: 2005.
- Reyes BA, Drolet G, Van Bockstaele EJ. Dynorphin and stress-related peptides in rat locus coeruleus: contribution of amygdalar efferents. *J Comp Neurol.* 2008; 508:663–675. [PubMed: 18381633]
- Reyes BA, Johnson AD, Glaser JD, Commons KG, Van Bockstaele EJ. Dynorphin-containing axons directly innervate noradrenergic neurons in the rat nucleus locus coeruleus. *Neuroscience.* 2007; 145:1077–1086. [PubMed: 17289275]
- Rizvi TA, Ennis M, Behbehani MM, Shipley MT. Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. *J Comp Neurol.* 1991; 303:121–131. [PubMed: 1706363]
- Van Bockstaele EJ, Ston-Jones G, Pieribone VA, Ennis M, Shipley MT. Subregions of the periaqueductal gray topographically innervate the rostral ventral medulla in the rat. *J Comp Neurol.* 1991; 309:305–327. [PubMed: 1717516]
- Versteeg DH, Van Der GJ, De JW, Palkovits M. Regional concentrations of noradrenaline and dopamine in rat brain. *Brain Res.* 1976; 113:563–574. [PubMed: 953752]
- Vertes RP. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J Comp Neurol.* 1991; 313:643–668. [PubMed: 1783685]
- Watkins L, Sherwood A, Goldstein DS, Maixner W. Mechanisms underlying cardiovascular defense reaction evoked by dorsal periaqueductal gray stimulation. *Am J Physiol.* 1993; 265:R1155–R1161. [PubMed: 8238618]
- Zeng J, Thomson LM, Aicher SA, Terman GW. Primary afferent NMDA receptors increase dorsal horn excitation and mediate opiate tolerance in neonatal rats. *J Neurosci.* 2006; 26:12033–12042. [PubMed: 17108177]

Figure 1. Confocal photomicrographs verifying the dual labeling methods

Noradrenergic neurons that are TH-ir (magenta) and DBH-ir (green) are present in the locus coeruleus (arrowheads) (A). Dopaminergic neurons that are TH-ir (magenta) but not DBH-ir (green) are present in the ventral tegmental area (VTA) and substantia nigra (SN) (B,C). VTA-projecting neurons (green) in the dorsal raphe are TH-ir (magenta; arrowheads) (D). Scale bar = 50µm.

Figure 2. Confocal photomicrographs demonstrating the localization of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) neurons in PAG
 Noradrenergic neurons that are TH-ir (magenta) and DBH-ir (green) are present in the caudal regions of the vlPAG (**A**). Dopaminergic neurons that are TH-ir but not DBH-ir are found in some vlPAG regions (triangles) (**B**). In rostral vlPAG regions, TH-ir neurons do not have DBH-ir (arrowheads) (**C**). Scale bar = 50μm.

Figure 3. Catecholaminergic neurons that are tyrosine hydroxylase (TH)-ir but not dopamine β -hydroxylase (DBH)-ir have a smaller cell diameter than cells that are both TH- and DBH-ir
 The average size of TH-ir but not DBH-ir cells is $10\mu\text{m} \pm 2\mu\text{m}$ (black bars) whereas the average size of TH- and DBH-ir cells is $19\mu\text{m} \pm 3\mu\text{m}$ (grey bars) ($P < 0.001$; Mann-Whitney rank sum test). Thus, the size difference of TH-ir neurons represents two populations of catecholaminergic neurons in the vIPAG.

Figure 4. Electron microscopy demonstrating localization of tyrosine hydroxylase (TH) in the ventrolateral periaqueductal gray (vlPAG)

A-C TH-ir was found in somata (A), terminals (B) and dendrites (C). **(A)** A TH-ir soma is apposed (arrowhead) to an unlabeled axon terminal (ut). **(B)** A TH-ir axon terminal (TH-t) apposed to (arrowhead) an unlabeled dendrite (ud) that also receives an apposition (arrowhead) from a different unlabeled axon terminal (ut). **(C)** A TH-ir dendrite (TH-d) apposed to an unlabeled axon terminal (ut). **(D)** TH-ir dendrite (TH-d) apposed to an unlabeled dendrite (ud; arrowhead). **(E)** Two unlabeled dendrites (ud) form a potential gap junction (arrowhead). Photomicrographs taken at vlPAG level Bregma: -8.00 ; Scale bar = 500nm

Figure 5. Composite summary of retrograde tracer injections

Fluorogold (FG) injections in rostral ventral medulla (RVM) (A) ventral tegmental area (VTA) (B), and substantia nigra (SN) (C). Injection sites are collapsed onto one rostrocaudal plane for clarity. Successful injections into the RVM (n=6), VTA (n=4) and SN(n=4) are indicated by grey circles.

Figure 6. Rostral ventral medulla (RVM)-projecting neurons are abundant in caudal region of the ventrolateral periaqueductal gray (vIPAG) and do not contain catecholamines
(A) Representative drawings demonstrating localization of RVM-projecting neurons in the caudal (−8.7mm from Bregma), mid-level (−8.0mm from Bregma) and rostral (−7.8 from Bregma) vIPAG regions. RVM-projecting neurons (open circles) do not co-localize with TH-ir neurons (stars) in any of the vIPAG regions. **(B)** Confocal photomicrographs of FG-labeled RVM-projecting neurons (green) and TH-ir neurons (magenta) in the vIPAG. **(C)** RVM projecting neurons are differentially distributed in the vIPAG with larger populations of projection neurons in caudal and mid-level regions and a smaller population in rostral vIPAG regions (50±10; 48±5; 27±1 per region respectively). Scale bar = 50µm.

Figure 7. Ventral tegmental area (VTA)-projecting neurons are present in the ventrolateral periaqueductal gray (vlPAG) and do not contain catecholamines

(A) Representative drawings demonstrating localization of VTA-projecting neurons in the caudal (−8.7mm from Bregma), mid-level (−8.0mm from Bregma), and rostral (−7.8mm from Bregma) vlPAG regions. VTA-projecting neurons (open circles) do not co-localize with TH-ir neurons (stars) in any of the vlPAG regions. (B) Confocal photomicrographs of rhodamine labeled VTA-projecting neurons (triangle; green), Nissl stain (blue), and TH-ir neurons (magenta) in the vlPAG. VTA-projecting neurons do not co-localize with TH in all three regions of the vlPAG. (C) Cell counts demonstrate that there is no significant difference in the number of VTA-projecting neurons in any of the vlPAG regions (23 ± 3 ; 24 ± 6 ; 29 ± 2 per region respectively). Scale bar = $50 \mu\text{m}$.

Figure 8. Substantia Nigra (SN)-projecting neurons are present in the ventrolateral periaqueductal gray (vlPAG)

(A) Representative drawings demonstrating localization of SN-projecting neurons in the caudal (−8.7mm from Bregma), mid-level (−8.0mm from Bregma), and rostral (−7.8mm from Bregma) vlPAG regions. (B) Confocal photomicrographs of rhodamine labeled SN-projecting neurons (triangle; green), Nissl stain (blue), and TH-ir neurons (magenta) in the vlPAG. SN-projecting neurons do not co-localize with TH in any of the regions of the vlPAG. (C) Cell counts show there is no significant difference in the number of SN-projecting neurons at different rostrocaudal levels of vlPAG regions (16±3; 15±2; 19±2 per region respectively). Scale bar = 50µm.

Figure 9. Rostral ventral medulla (RVM)- and ventral tegmental area (VTA)-projecting neurons do not collateralize in the ventrolateral periaqueductal gray (vlPAG)

(A) Representative drawings demonstrating localization of dual retrograde tracing of RVM- and VTA-projecting neurons. Both populations of neurons are within close proximity of each other but are distinct populations of neurons. (B) Confocal photomicrographs demonstrate that RVM-projecting neurons (arrowheads; green) do not co-localize with TH-ir neurons (left panel; magenta) and VTA-projecting neurons (triangles; cyan) also do not co-localize with TH-ir neurons (middle panel; magenta). Merged photomicrographs demonstrate that RVM-(arrowheads; green) and VTA-projecting neurons (triangles; cyan) are distinct. Scale bar = 20 μ m.

Figure 10. Electron microscopy demonstrating localization of Rostral ventral medulla (RVM)-projecting neurons in the ventrolateral periaqueductal gray (vlPAG)

Fluorogold (FG)-ir was found in somata dendrites (A) and dendrites (B,C). (A) A FG-ir somata (FG-soma) in the vlPAG (B) A FG-ir dendrite (FG-d) is apposed (arrowheads) to two unlabeled axon terminals (ut). (C) A FG-ir dendrite (FG-d) is apposed to a TH-ir dendrite (TH-d; arrowhead). Photomicrographs taken at vlPAG level Bregma: -8.00; Scale bar = 500nm

\$watermark-text

\$watermark-text

\$watermark-text

Classification of neuronal profiles containing tyrosine hydroxylase (TH) immunoreactivity (ir) in the vIPAG and the cellular contacts made by TH-ir dendrites

Table 1

Antigen	N	Somata	Dendrites	Axons	Terminals	
TH	122	5 (4%)	60 (50%)	20 (16%)	36 (30%)	
Antigen	N (dendrites)	No Contacts	Somata	Dendrites	Axons	Terminals
TH	60	14 (23%)	0 (0%)	10 (17%)	0 (0%)	36 (60%)
Unlabeled	96	6 (6%)	0 (0%)	23 (24%)	8 (8%)	59 (61%)

* cellular contacts of unlabeled profiles in the same field are included for comparison.

\$watermark-text

\$watermark-text

\$watermark-text

Table 2
Classification of tyrosine hydroxylase (TH) and Flurogold (FG) immunoreactive profiles in the vIPAG and the neuronal contacts made by immunoreactive dendrites

Antigen	N	Somata	Dendrites	Axons	Terminals
TH	68	12 (18%)	32 (45%)	9 (13%)	15 (22%)
FG	75	34 (45%)	40 (53%)	1 (1%)	0 (0%)
Antigen	N(dendrites)	No Contacts	Somata	Dendrites	Terminals
TH	32	11 (35%)	2 (6%)	9 (28%)	10 (31%)
FG	40	12 (30%)	0 (0%)	10 (25%)	18 (45%)
Antigen	N	FG Somata	FG Dendrites	Axons	Terminals
TH	68	5 (7%)	2 (3%)	0 (0%)	0 (0%)