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Cellular profile of the dorsal raphe lateral wing sub-region: Relationship to the lateral dorsal tegmental nucleus

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ABSTRACT

As one of the main serotonergic (5HT) projections to the forebrain, the dorsal raphe nucleus (DRN) has been implicated in disorders of anxiety and depression. Although the nucleus contains the densest population of 5HT neurons in the brain, at least 50% of cells within this structure are non-serotonergic, including a large population of nitric oxide synthase (NOS) containing neurons. The DRN has a unique topographical efferent organization and can also be divided into sub-regions based on rostro-caudal and medio-lateral dimensions. NOS is co-localized with 5HT in the midline DRN but NOS-positive cells in the lateral wing (LW) of the nucleus do not express 5HT. Interestingly, the NOS LW neuronal population is immediately rostral to and in line with the cholinergic lateral dorsal tegmental nucleus (LDT). We used immunohistochemical methods to investigate the potential serotonergic regulation of NOS LW neurons and also the association of this cell grouping to the LDT. Our results indicate that >75% of NOS LW neurons express the inhibitory $5HT_{1A}$ receptor and are cholinergic (>90%). The findings suggest this assembly of cells is a rostral extension of the LDT, one that it is subject to regulation by 5HT release. As such the present study suggests a link between 5HT signaling, activation of cholinergic/NOS neurons, and the stress response including the pathophysiology underlying anxiety and depression.

1. Introduction

The dorsal raphe nucleus is one of the main sources of serotonin (5HT) to the forebrain (O'Hearn and Molliver, 1984; Vertes, 1991) and has been implicated in both depression and anxiety disorders (Chaouloff, 1993; Graeff et al., 1996; Lowry, 2002; Lowry et al., 2005; Underwood et al., 1999). Although the nucleus contains the densest concentration of 5HT neurons in the brain (Palkovits et al., 1974), at least 50% of the cells within this structure are non-serotonergic (non-5HT; Descarries et al., 1982; Steinbusch et al., 1980). One of the more prominent non-5HT phenotypes present are cells that express neuronal nitric oxide synthase (NOS; Johnson and Ma, 1993; Wotherspoon et al., 1994), the enzyme responsible for the synthesis of the gaseous transmitter nitric oxide (NO). Neurons containing NOS have a unique topographical distribution and co-localization pattern across the DRN (see Vasudeva et al., 2011 for review).

Both NOS and tryptophan hydroxylase (TrpH, the enzyme that synthesizes 5HT), co-localize across the midline (ML) DRN in approximately 75% of 5HT cells, but neurons expressing both enzymes are never found in the lateral wing (LW) subdivision of the nucleus (Okere and Waterhouse, 2006a,b). NOS-positive cells increase in number along the rostrocaudal extent of the nucleus, beginning with little to no expression in the rostral DRN and increasing numbers of NOS-positive cells concentrated in the caudal end of the nucleus, specifically within a region designated in previous studies as the LW (Johnson and Ma, 1993; Lu et al., 2010; Okere and Waterhouse, 2006a,b). Interestingly, the number of NOS-positive cells in this region of the brainstem and the intensity of immunohistochemical staining in these neurons is increased following restraint (Okere and Waterhouse, 2006a,b), thus linking these nitrergic cells to the stress response.

Because of their morphology and location within the brainstem, caudal and lateral NOS-expressing cells are typically associated with the LW sub-region of the DRN (Okere and Waterhouse, 2006a,b; Vasudeva et al., 2011). However, it is important to note

Abbreviations: 5HT, serotonin; $5HT_{1A}$, serotonin-1A receptor; ACh, acetylcholine; cAMP, cyclic adenosine monophosphate; ChAT, choline acetyl transferase; DRN, dorsal raphe nucleus; GABA, gamma aminobutyric acid; GAD, glutamic acid decarboxylase; LDT, lateral dorsal tegmental nucleus; LW, lateral wing; ML, midline; NO, nitric oxide; NOS, nitric oxide synthase; TrpH, tryptophan hydroxylase; VAChT, vesicular acetylcholine transferase.

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the juxtaposition of the LW to the lateral dorsal tegmental nucleus (LDT). The LW is in line with and immediately rostral to the LDT (Paxinos and Watson, 1998), and forms a continuous column of cells that, in Nissl stained coronal sections, exhibit morphology that is indistinguishable from the LDT (Paxinos and Watson, 1998; Vasudeva et al., 2011). The LDT consists mainly of neurons that express both NOS and choline acetyl transferase (ChAT), the enzyme responsible for acetylcholine synthesis (Vincent et al., 1986), but the literature is not clear about the expression of ChAT within the DRN (Houser et al., 1983; Satoh et al., 1983; Wang et al., 2000; Woolf and Butcher, 1982). Given the well-established role of 5HT and the DRN in anxiety and stress responding, and the more recent findings of NOS-cell involvement in the DRN stress response (Okere and Waterhouse, 2006a,b), it is of interest to further investigate the receptor and neurochemical phenotype of this nitrergic (NOS-containing) cell population.

Historically, neurons have been assigned to the DRN based on their serotonergic content and/or their localization with respect to other 5HT-containing cell groups within this structure. Similarly, neurons have been assigned to the LDT based on their nitrergic or cholinergic content. Because of the juxtaposition of NOS-positive and 5HT cells in the border region between the caudal LW and rostral LDT, we postulated that NOS-containing cells in this area are also cholinergic and may be subject to regulation by 5HT release. Serotonergic regulation of NOS cells within this region could influence local circuit operations in response to stressor exposure. Prior investigations have demonstrated weak expression of the excitatory $5HT_{2A}$ receptor on cells of the LDT (Fay and Kubin, 2000), but beyond this study the 5HT receptor complement of this structure is not clear (Bonnavion et al., 2010).

Long considered a defining feature of 5HT cells, the $5HT_{1A}$ receptor is found on the soma and dendrites of 5HT neurons but is also expressed post-synaptically by non-5HT and/or cholinergic cells in DRN terminal fields (Kia et al., 1996a,b). The receptor has also been demonstrated on non-5HT cells within the DRN (Day et al., 2004; Kirby et al., 2003). Although $5HT_{1A}$ receptors have yet to be identified on NOS neurons associated with the caudal LW, the presence of this receptor on both 5HT-NOS co-localized cells and non-5HT cells of the midline (Kirby et al., 2003; Okere and Waterhouse, 2006a) suggests the possibility that NOS only cells within the LW may also express $5HT_{1A}$ receptors. Clark and colleagues (2006) have in fact demonstrated the presence of $5HT_{1A}$ receptor protein within the DRN LW and extending into the LDT (termed "extra DRN wings" in their study), providing support for the possibility that neurons within this structure express the $5HT_{1A}$ receptor. Serotonin has also been shown to inhibit LDT cells in vivo, indicating the presence of an inhibitory 5HT receptor (Koyama and Kayama, 1993).

The goals of the present study were to: (1) define the anatomical relationship between NOS-containing cells and cholinergic neurons within the caudal LW-LDT transition zone and (2) determine if NOS-positive neurons in this region express the $5HT_{1A}$ receptor. Further characterization of the NOS-containing cell group associated with the DRN will provide insight regarding the role of this class of neurons in the operation of the DRN circuitry as it relates to the stress response.

2. Methods

Animals. Male Long Evans rats ranging in weight from 300 to 450 g (Charles River Laboratories, Wilmington, MA) were group housed in standard facilities and maintained on a 12:12 light-dark schedule with access to rat chow and water *ad libitum*. All animals used in these studies were treated in accordance with the published National Institute of Health *Guide for Care and Use of Laboratory Animals*. All protocols and procedures were approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee.

Immunohistochemical controls. For all immunohistochemical procedures, control sections of brain were processed as described below but with elimination of the primary and/or secondary antibody. Elimination of either of these antibodies would reveal any potential non-specific staining or fluorescence. When available, control peptides were included with the primary antibody incubation to verify specificity.

2.1. NOS, TrpH, GAD, and VAChT expression

Perfusion and DRN sectioning. Animals ($n = 3$) were overdosed with 5% isoflurane and perfused through the left ventricle with 0.1 M phosphate buffer (PB, pH 7.3–7.4), followed by 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4). Brains were removed and post fixed in 4% PFA overnight, then transferred to 30% sucrose solution in 0.1 M PB for 3–5 days. Dorsal raphe sections 30 μm thick were collected in 0.1 M phosphate buffered saline (PBS), and sets of sections at 180 μm intervals across the DRN and LDT were processed for all immunohistochemical investigations.

Immunohistochemistry. The cholinergic profile of cells within the DRN and LDT was assessed with an antibody against the vesicular acetylcholine transporter (VAChT,), a marker for cholinergic cells ($n = 3$; Ichikawa et al., 1997). Serotonergic phenotype was determined by tryptophan hydroxylase-2 (TrpH2) staining, the rate limiting enzyme necessary for synthesis of 5HT. In addition to investigating the cholinergic content of NOS neurons, the cells were assayed for GABA decarboxylase (GAD, the enzyme responsible for the synthesis of GABA) to confirm that NOS LW neurons did not co-localize this inhibitory neurotransmitter as described in the literature for the LDT (Boucetta and Jones, 2009; Wang et al., 1997). All sections were washed in PBS (5 × 5 min) followed by PBS + 0.03% Triton-X 100 (PBS-T), except for VAChT. Tissue was blocked in 10% donkey serum before incubating in the following primary antibodies for 36–48 h at 4° Celsius followed by a two-hour incubation period in corresponding sets of secondary antibodies at room temperature:

- NOS-GAD
  - 1:1000 mouse anti-nNOS (Sigma Inc., St. Louis, MO) + 1:500 rabbit anti-GAD65 (Sigma Inc., St. Louis, MO) or 1:500 rabbit anti-GAD67 (ImmunoStar Inc., Hudson, WI) in PBS-T.
  - 1:500 FITC donkey anti-mouse (green) + 1:500 AlexaFluor 594 donkey anti-rabbit (red, Invitrogen Corporation, Carlsbad, CA) in PBS.
- NOS-TrpH-VAChT
  - 1:1000 rabbit anti-nNOS (ImmunoStar Inc.) + 1:1000 mouse anti-tryptophan hydroxylase 2 (Millipore, Billerica, MA) + 1:500 goat anti-VAChT (ImmunoStar Inc.) in PBS.
  - 1:500 AlexaFluor 594 donkey anti-rabbit (red) + 1:500 AlexaFluor 488 donkey anti-mouse (green) + 1:250 AlexaFluor 350 donkey anti-goat (blue) in PBS.

At the completion of the secondary antibody incubation, all sections were washed in PBS (5 × 5 min), mounted on gelatin-coated microscope slides, dehydrated overnight, and cover slipped with Slowfade Gold (Invitrogen Corporation).

An additional set of horizontal sections ($n = 1$) was stained for NOS (red) and TrpH2 (green) following the procedure as described above in order to demonstrate the interaction of these neurochemicals in another perspective across the lateral wing.

2.2. $5HT_{1A}$ and NOS expression

Perfusion and sectioning. Naïve animals ($n = 3$) were overdosed with 5% isoflurane gas anesthesia and perfused through the left ventricle with 0.1 M PB (pH 7.3) followed by 4% PFA in 0.1 M PB with 0.05% glutaraldehyde (pH 7.4). Brains were post-fixed in 4% PFA for 12–24 h before being immersed in 30% sucrose in 0.1 M PB (pH 7.4; without sodium azide) for 3–7 days. Sections 20 μm thick were collected in 0.01 M PBS (pH 7.4), and sets of sections 120 μm apart were processed for immunohistochemistry.

Immunohistochemistry. Due to the sensitivity of the $5HT_{1A}$ receptor antibody, the concentration and pH of buffer solutions were carefully monitored and the use of detergents such as Triton-X 100 was avoided as described by the manufacturer (ImmunoStar Inc.). Brain sections were washed in 0.01 M PBS (pH 7.4; 5 × 5 min) before blocking for one hour at room temperature with 10% goat serum. Sections were then incubated with 1:500 rabbit anti-$5HT_{1A}$ receptor (ImmunoStar Inc.) and 1:1000 mouse anti-nNOS (Sigma Inc.) for 48 h at 4 °C. The tissue was then washed with 0.01 M PBS (5 × 5 min) before incubating in 1:500 AlexaFluor Fa′B fragment 594 goat anti-rabbit (red) and 1:1000 AlexaFluor 488 goat anti-mouse (green; Invitrogen Corporation) at room temperature for two hours. The sections were then washed in 0.01 M PBS (5 × 5 min), mounted on gelatin-coated microscope slides, dehydrated overnight, and cover slipped using Slowfade Gold (Invitrogen Corporation).

2.3. Imaging and quantification

Imaging. All fluorescent immunohistochemical images were collected with a Leica DM RBE microscope affixed with an MTI 3CCD camera and MicroColor image converting system (model RGB-MS-C, CRI, Inc., Boston, MA). Images were generated