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IMMUNOREACTIVITY FOR THE NMDA NR1 SUBUNIT IN BULBOSPINAL CATECHOLAMINE AND SEROTONIN NEURONS OF RAT VENTRAL MEDULLA

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Abstract

Bulbospinal neurons in the ventral medulla play important roles in the regulation of sympathetic outflow. Physiological evidence suggests that these neurons are activated by N-methyl-D-aspartate (NMDA) and non-NMDA subtypes of glutamate receptors. In this study, we examined bulbospinal neurons in the ventral medulla for the presence of immunoreactivity for the NMDA NR1 subunit, which is essential for NMDA receptor function. Rats received bilateral injections of cholera toxin B into the tenth thoracic spinal segment to label bulbospinal neurons. Triple immunofluorescent labelling was used to detect cholera toxin B with a blue fluorophore, NR1 with a red fluorophore and either tyrosine hydroxylase or tryptophan hydroxylase with a green fluorophore. In the rostral ventrolateral medulla, NR1 occurred in all bulbospinal tyrosine hydroxylase-positive neurons and 96% of bulbospinal tyrosine hydroxylase-negative neurons, which were more common in sections containing the facial nucleus. In raphé pallidus, the parapyramidal region and the marginal layer, 98% of bulbospinal tryptophan hydroxylase-positive neurons contained NR1-immunoreactivity. NR1 was also present in all of the bulbospinal tryptophan hydroxylase-negative neurons, which comprised 20% of bulbospinal neurons in raphé pallidus and the parapyramidal region. These results show that virtually all bulbospinal tyrosine hydroxylase and non-tyrosine hydroxylase neurons in the rostral ventrolateral medulla and virtually all bulbospinal serotonin and non-serotonin neurons in raphé pallidus and the parapyramidal region express NR1, the obligatory subunit of the NMDA receptor. NMDA receptors on bulbospinal neurons in the rostral ventral medulla likely influence sympathoexcitation in normal and pathological conditions.

Keywords

Glutamate; autonomic; C1 neurons; tyrosine hydroxylase; tryptophan hydroxylase; medulla oblongata

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INTRODUCTION

The ventral medulla contains several important groups of central autonomic neurons. The influence of these neurons on autonomic function is dependent on many factors, including the type and amount of input they receive; the types of neurotransmitter receptors and enzymes that they express and where they send their axons. Two of the major autonomic cell groups in the ventral medulla are the bulbospinal catecholamine- and serotonin-containing neurons (Jeske and McKenna, 1992; Loewy and McKellar, 1981). Because glutamate is the primary fast-acting transmitter in the central nervous system, the number and distribution of glutamate receptor subtypes located on spinally-projecting catecholamine and serotonin neurons is likely to shape their function and how they contribute to various physiological and pathophysiological processes.

N-methyl-D-aspartate (NMDA) receptors are one of the major subtypes of ionotropic glutamate receptors involved in synaptic transmission (Dingledine et al., 1999) and play important roles in neuroplasticity (Rebola et al., 2010). The NMDA receptor complex is a multi-meric ion channel composed of subunits that include NR1, NR2A-D, and in some cases NR3 (Dingledine et al., 1999). The NR1 subunit is an obligatory subunit for a fully functional channel (Dingledine et al., 1999) and can thus serve as a general marker for NMDA receptors. To our knowledge, the presence of NMDA receptor on bulbospinal catecholamine and serotonin neurons has not yet been documented anatomically. Therefore, the purpose of this study was to determine the number of bulbospinal catecholamine and serotonin neurons that contained immunoreactivity for the NR1 subunit of the NMDA receptor. We accomplished this aim by combining retrograde tracing with cholera toxin B subunit (CTB) from the lower thoracic cord with triple immunofluorescent labelling for CTB, NR1 and either the catecholamine synthetic enzyme, tyrosine hydroxylase (TH), or the enzyme that synthesizes serotonin, tryptophan hydroxylase (TpTH).

MATERIALS AND METHODS

This study was carried out on ten male Sprague Dawley rats weighing 330–420 g. The rats were given unlimited access to food and water and were maintained on a 12 hour light:dark cycle. Approval for all animal protocols was obtained from the Institutional Animal Care and Use Committee at Wayne State University.

Retrograde Labelling and Perfusion

Bulbospinal neurons in the ventral medulla were retrogradely labelled with CTB (List Biological Laboratories; Campbell CA). Rats were anesthetized with isoflurane (3% for induction, 1.5–2% for maintenance). After removal of the spinous process of the T8 vertebra, a laminectomy was performed at T9. A small part of the rostral portion of the T10 vertebra was removed, as needed, to improve access to the spinal cord. The tip of a glass micropipette was targeted to the dorsal root entry zone (~0.5–1 mm lateral to midline), inserted to a depth of 1 mm and 3 × 60 nl of CTB was injected into each side of the cord (6 injections total). CTB injections were spaced between 0.2 and 1 mm apart in order to avoid damaging surface blood vessels. Three days after tracer injection, rats were anesthetized and perfused through the heart with DMEM/F12 tissue culture medium (D-8900; Sigma Chemical, St Louis, MO, USA) followed by 4% formaldehyde in 0.1M phosphate buffer, pH 7.4. Three untreated male Sprague-Dawley rats without CTB injections in T10 were also perfused. Brains and spinal cords in their vertebral columns were post-fixed in the same fixative for 3 days and washed in several changes of phosphate buffer.

Perfused and post-fixed brains and spinal cords in their vertebral columns were transferred to phosphate buffer containing 0.05% sodium azide for shipment to Flinders University.

Medullas were trimmed from the brains using a coronal precision brain slicer (Braintree Scientific, Braintree MA) so that sections would have a consistent orientation. Medullas were infiltrated with 20% then 30% sucrose and cut on a cryostat into 4 series of 30 μ m transverse sections. Sections were stored in a cryoprotectant solution at -20°C until use.

Spinal cords were removed from their vertebral columns and post-fixed for a further 2–3 days at room temperature in phosphate-buffered 4% formaldehyde so that the locations of CTB injection sites could be assessed in the perfused spinal cords. The portion of each lower thoracic spinal cord into which CTB had been injected was divided into segments on the basis of dorsal root entry zones. Thoracic (T) segments T9–T11 were embedded in albumin gelatine (Llewellyn-Smith et al., 2007), infiltrated with sucrose, sectioned transversely on a cryostat into 6 series of 25 μ m sections and stored in cryoprotectant.

Immunohistochemistry

Sections were removed from cryoprotectant, rinsed in phosphate buffer, washed 3×10 min in 10 mM Tris, 0.9% NaCl, 0.05% thimerosal in 10 mM phosphate buffer, pH 7.4 (TPBS) containing 0.3% Triton X-100 and exposed to 10% Gibco heat-inactivated normal horse serum (NHS; Life Technologies, Grand Island, NY, USA) in TPBS-Triton for at least 30 min. Primary antibodies were diluted with TPBS-Triton containing 10% NHS; secondary antibodies, with TPBS-Triton containing 1% NHS and avidin complexes, with TPBS-Triton. Incubations in primary antibodies lasted 2–3 days; incubations in secondary antibodies, overnight; and incubations in avidin complexes, for 4–6 hours. Dilutions of primary antibodies were determined by titration to give the maximum number of immunoreactive structures combined with the minimum level of background staining in formaldehyde-fixed tissue. Sections were washed 3×10 min in TPBS after each incubation in immunoreagents. All steps were done at room temperature with continuous agitation on a shaker.

Single Immunoperoxidase Labelling—To localize NR1-immunoreactivity in the medulla, one series of sections from each of the 3 untreated rats was incubated in 1:2,000 rabbit anti-NR1 (Upstate Biotechnology, Lake Placid NY; now owned by Millipore), then in 1:500 biotinylated anti-rabbit immunoglobulin (Ig) G (Jackson ImmunoResearch, West Grove PA USA) and finally in 1:1500 ExtrAvidin peroxidase (Sigma). To localize CTB at injection sites, one series of sections from each of the rats injected with CTB at T10 was incubated in 1:100,000 goat anti-CTB (List), in 1:500 biotinylated anti-goat IgG (Jackson ImmunoResearch) and finally in 1:1500 ExtrAvidin peroxidase (Sigma). Immunoreactivity in medulla and spinal cord sections was revealed with a nickel-intensified peroxidase reaction in which diaminobenzidine was the substrate and peroxide was generated by glucose oxidase (Llewellyn-Smith et al., 2005). Immunoperoxidase stained sections were mounted onto chrome alum-gelatine coated slides, dried, dehydrated and coverslipped with Permaslip mounting medium (Alban Scientific, St Louis, MO USA).

Triple Immunofluorescent Staining—Two series of medulla sections from each rat were placed in serial order using a dissecting microscope with dark field illumination. Triple immunofluorescent labelling for NR1, CTB and either TH or TpTH was done on 12 sections encompassing the rostral ventral medulla, including 7 sections caudal to the caudal pole of the facial nucleus (FN) to 4 sections rostral to the caudal pole (i.e., section numbers FN–7 to FN+4, with the first section containing the caudal pole of the FN numbered as FN0). From each rat, sections FN–7 to FN+4 from one series were stained for NR1+CTB+TH and sections FN–7 to FN+4 from the next series were stained for NR1+CTB+TpTH. A procedure that produced blue fluorescence in CTB-immunoreactive neurons, red fluorescence in NR1-immunoreactive neurons and green fluorescence in TH- or TpTH-immunoreactive neurons was then used to immunostain the sections.

After washing in TPBS-Triton and exposure to 10% NHS, the sections were incubated in primary antibodies. One series of FN-7 to FN+4 sections from each rat was exposed to 1:10,000 goat anti-CTB (List) plus 1:2,000 rabbit anti-NR1 (Upstate) plus 1:500 chicken anti-TH (Aves Labs Inc., Tigard, Oregon). The second series of sections from each rat was exposed to 1:10,000 goat anti-CTB plus 1:2,000 rabbit anti-NR1 plus 1:1,000 mouse monoclonal anti-TpTH (Sigma). The incubation in primary antibodies was followed by exposure to a solution containing secondary antibodies (all from Jackson ImmunoResearch). Sections stained for CTB+NR1+TH were incubated in 1:200 AMCA-labelled donkey anti-goat IgG plus 1:500 biotinylated donkey anti-rabbit IgG plus 1:200 FITC-labelled donkey anti-chicken IgY. In the secondary antibody solution used to stain sections for co-localization of CTB+NR1+TpTH, 1:200 FITC-labelled donkey anti-mouse IgG replaced the FITC-labelled anti-chicken IgY. Finally, the sections were incubated in 1:1000 Cy3-labelled streptavidin (Amersham Biosciences / GE Healthcare Bio-Sciences Pty. Ltd, Rydalmere NSW). Incubations in secondary antibodies and Cy3-streptavidin were done in the dark. After the final wash in TPBS, sections FN-7 to FN+4 were mounted in buffered glycerol in serial order from caudal to rostral, coverslipped, sealed with nail polish to prevent dehydration and stored in the dark at 4°C until examination.

Antibody Specificity—The Upstate Biotechnology rabbit anti-NR1 antiserum produces a single band when immunoblotted against protein samples extracted from rat (Upstate Product Information Sheet) and human (Kristiansen et al., 2006) brain. We have shown elimination of staining when the List goat anti-CTB antiserum is absorbed with CTB (Llewellyn-Smith et al., 1995). In rat brain and spinal cord, the Aves chicken anti-TH antibody stains cell bodies and axons that show the same distribution as with other anti-TH antisera. The Sigma mouse monoclonal anti-TpTH antibody labels medullary neurons that have the same distribution as cell bodies revealed by serotonin (5-HT)-immunoreactivity (Burman et al., 2003).

Data Collection and Analysis

Medulla sections immunofluorescently stained to reveal NR1, CTB and TH or TpTH were examined with an Olympus AX70 epifluorescence microscope fitted with a single-wavelength LED illumination system and emission filters that unequivocally distinguished the fluorescence of blue, green and red fluorophores without bleed-through (For AMCA, excitation = 365 nm; emission = 435–485 nm; for FITC: excitation = 490 nm; emission = 515–545 nm; for Cy3, excitation = 525 nm; emission = 575–615 nm). We analyzed sections FN-7 to FN+4 from the medullas of four rats with bilateral injections of CTB into T10. Although retrograde tracer was injected in seemingly identical locations in all of the rats used in this study, the retrograde labeling obtained in the medulla was variable. We chose to count neurons in the four rats that contained the maximum numbers of retrogradely labeled neurons in the ventral medulla. Neurons retrogradely labelled from T10 were first identified by the presence of blue immunofluorescence in their cytoplasm. Each CTB-immunofluorescent neuron that had a clearly defined, non-fluorescent nucleus was classified as positive or negative for green TH- or green TpTH-immunofluorescence. Finally, we assessed each neuron for the presence of red NR1-immunofluorescence. We excluded CTB-immunoreactive neurons lacking clearly defined nuclei from the counts. All CTB-immunoreactive neurons in the midline (i.e., raphe pallidus neurons) were counted whereas neurons in nuclei that occur bilaterally were counted on only one side of the medulla.

We used a similar strategy to assess the occurrence of NR1-immunoreactivity in **non-**bulbospinal neurons that were TH- or TpTH-immunoreactive. TH- and TpTH- positive cell bodies with clearly defined nuclei were first checked to confirm that they lacked CTB-immunoreactivity. Neurons in which CTB was absent were then assessed for the presence of

NR1-immunoreactivity. We did not count CTB-negative TpTH neurons in raphé magnus because they are more likely to play a role in controlling thermoregulatory rather than blood pressure-related circuitry at the spinal cord level (Morrison and Blessing, 2011; Zaretsky et al., 2003).

Cytoarchitectonic landmarks and neuronal morphology were used to categorize bulbospinal neurons that lacked immunoreactivity for TH or TpTH. In the series of **sections stained for NR1, CTB and TH**, we classified CTB-positive neurons in which TH-immunoreactivity was absent as being in the rostral ventrolateral medulla (RVLM) if they lay lateral to the pyramids and either ventral to nucleus ambiguus (sections FN-7 to FN-1) or ventral/ventromedial to FN (sections FN0 to FN+4). We excluded CTB-positive, TH-negative cell bodies that occurred immediately adjacent to the pyramids and had a predominantly dorsoventral orientation as RVLM neurons because serotonergic PPY neurons characteristically occur in this location and have this morphology. We also eliminated CTB+/TH- neurons in the marginal layer from the RVLM population because bulbospinal neurons that lay within the marginal layer were consistently immunoreactive for TpTH. In the series of sections stained for NR1+CTB+TH from two rats, section FN0 contained less than five large motor neurons in the region of the FN. These sections were therefore at or very close to the caudal pole of FN and allowed us to compare with confidence differences in the proportions of bulbospinal C1 and non-C1 neurons rostral versus caudal to the caudal pole of FN (i.e. FN-7 to FN0 versus. FN+1 to FN+4). In the **sections stained for NR1, CTB and TpTH**, CTB-positive neurons that lacked TpTH-immunoreactivity were included in the counts if they had cell bodies in the midline that were amongst the TpTH-positive neurons in raphé pallidus (RPa). In more caudal sections, bulbospinal TpTH-negative neurons were counted not only in RPa but also the ventral portion of raphé obscurus.

All quantitative data are expressed as mean $\pm$ standard error (SE).

Preparation of Figures

Immunoperoxidase-stained sections of the medulla and spinal cord were examined with a BH-2 Olympus brightfield microscope. Digital images were captured using a SPOT RT colour camera and SPOT RT software version 4.6 (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Images of immunofluorescent neurons were captured using an Orca high-resolution cooled CCD digital camera (Hamamatsu, Hamamatsu City, Japan) and analySIS ruler software (version 5; Soft Imaging Solutions, an affiliate of Olympus, Olympus Soft Imaging Solutions GmbH, Münster, Germany). Adobe PhotoShop was used to adjust the sharpness, brightness and contrast of TIFF files and to pseudo-color the images. Plates were also prepared using PhotoShop.

RESULTS

Distributions of NR1-Immunoreactive Neurons

Many neurons throughout all regions of the ventral medulla showed immunoreactivity for NR1 both in sections stained with immunofluorescence and in sections stained with immunoperoxidase (Figure 1). In many neurons, immunoreactivity for NR1 was moderate to intense, with the most heavily stained neurons occurring in the region of raphé magnus (RMg), in the ventral midline in the region of RPa, and in the marginal layer along the ventral medullary surface.

Placement of Spinal CTB Injections

A very sensitive three-layer immunoperoxidase method was used to assess the locations of injection sites and the spread of retrograde tracer around them. Placement of the bilateral injections of CTB was consistent in the 7 rats used to study the occurrence of NR1 in bulbospinal monoamine neurons in the ventral medulla. The injection sites were always centered rostrocaudally within the T10 segment and dorsoventrally at the IML or slightly ventral to it in lamina VII. At the centres of the injection sites, very heavy deposits of peroxidase reaction product obscured tissue architecture and moderate CTB-immunoreactivity surrounded the tracks made by the glass micropipettes used for tracer injection (Figure 2). There were also very dense deposits of peroxidase reaction product in the ventrolateral, lateral and ventral portion of the dorsolateral funiculus of the white matter. CTB staining that was sufficiently intense to prevent the structure of underlying tissue to be discerned occurred over approximately the length of one segment and was presumed to represent the area over which neurons could take up the retrograde tracer. In T9 and T11 sections that were most distant from the centers of the injection sites, CTB reaction product was prominent in the IML, ventral lamina V and dorsal lamina VII. In contrast to sections at the injection sites, these sections showed immunoreactivity in somata that had retrogradely transported CTB and in punctate structures that were probably anterogradely-labelled terminals. This staining pattern was presumed to indicate sections that were outside the region of the cord to which injected CTB had spread.

Distribution of bulbospinal and monoamine neurons

Triple immunofluorescent labelling for NR1, CTB and either TH or TpTH was done on 12 sections encompassing the rostral ventral medulla. The sections were selected from 1:4 series of 30 μ m transverse sections through perfused medullas from 7 male Sprague Dawley rats. The 12 sections for immunostaining included 7 sections caudal to the caudal pole of the facial nucleus (FN) to 4 sections rostral to the caudal pole (i.e., FN-7 to FN+4, with the first section containing FN numbered as FN0). From each rat, sections FN-7 to FN+4 from one series were stained for NR1+CTB+TH and sections FN-7 to FN+4 from the next series were stained for NR1+CTB+TpTH.

All sections from FN-7 to FN +4 contained neurons that had retrogradely transported CTB from spinal cord segment T10. CTB-labelled neurons were scattered throughout the ventral medulla. There were many CTB-immunoreactive cell bodies in the rostral ventrolateral medulla (RVLM). CTB-positive somata occurred in raphé pallidus (RPa) and raphé obscurus and in the parapyramidal region (PPY), which lay immediately lateral to the pyramids adjacent to the ventral surface of the medulla. Occasional neurons in the marginal layer on the ventral medullary surface (see Weston et al, 2004, for anatomy of this region) also showed CTB-immunoreactivity. The raphé magnus region just dorsal to the pyramids, contained many CTB-immunoreactive perikarya.

TH-immunoreactive neurons also occurred in all sections of the rostral ventral medulla and lay lateral to the pyramids in the ventrolateral medulla. In rostral sections (i.e., FN+1 to FN +4), TH-positive neurons were also located ventral, medial and ventromedial to FN.

TpTH-immunoreactive neurons occurred in RPa, raphé obscurus and raphé magnus and in PPY. The marginal layer on the ventral surface of the medulla also contained a few TpTH-positive neurons.

Occurrence of NR1-immunoreactivity in Bulbospinal Neurons

In RVLM, TH-immunoreactive neurons retrogradely labelled with CTB from T10 contained immunoreactivity for NR1 (Figures 3A–F). In almost all of these bulbospinal C1 neurons,

immunoreactivity for NR1 was moderate to intense. In RVLM, TH-negative neurons retrogradely labelled with CTB from T10 also contained immunoreactivity for NR1 (Figures 3D–F). In many of these spinally-projecting non-C1 neurons, immunoreactivity for NR1 was intense. The CTB+/TH– neurons with NR1-immunoreactivity appeared to be more concentrated rostral to FN0 than caudal to FN0.

In rostral ventral medullary regions containing serotonin neurons, many bulbospinal neurons were immunoreactive for the NMDA R1 subunit. In RPa (Figures 4A–C) and PPY (Figures 4D–F), both TpTH-positive and TpTH-negative neurons that had retrogradely transported CTB from T10 contained immunoreactivity for NR1. In many of these bulbospinal neurons, immunoreactivity for NR1 was very intense. Occasional CTB-labelled neurons also occurred in the marginal layer at the ventral surface of the medulla (Figures 4G–I). These marginal layer neurons also showed immunoreactivity for NR1, which was often intense.

Quantification of Immunoreactive Bulbospinal Neurons

The proportion of bulbospinal rostral ventral medullary neurons that contained immunoreactivity for NR1 was quantified in sections stained for CTB, NR1 and TH (Table 1) and in sections stained for CTB, NR1 and TpTH (Table 2) from 4 rats. In TH-stained sections, only CTB-positive neurons that lay (1) lateral to the cluster of bulbospinal neurons in PPY and (2) either ventral to the nucleus ambiguus or ventral/medial/ventromedial to FN were counted. In TpTH-stained sections, counts were done on CTB-positive neurons in RPa, PPY, ventral raphé obscurus, the marginal layer and RMg.

Sections stained for NR1, CTB and TH—NR1-immunoreactivity occurred in $97.9 \pm 0.2\%$ (mean $\pm$ standard error; $n = 4$) of the total population of RVLM neurons retrogradely labelled with CTB from T10 (i.e., bulbospinal C1 + bulbospinal non-C1). All of the CTB+/TH+ (bulbospinal C1) neurons were NR1-immunoreactive and $95.6 \pm 0.5\%$ ($n = 4$) of the CTB+/TH– (bulbospinal non-C1) neurons showed NR1-immunoreactivity.

In sections FN–7 to FN+4, $51.7 \pm 2.5\%$ ($n = 4$) of the neurons containing CTB were immunoreactive for TH and $48.3 \pm 2.5\%$ lacked TH-immunoreactivity (neurons that were positive for CTB and NR1 plus neurons that were positive for CTB only, see Table 1). As indicated in Methods, section FN0 from two rats contained only a very few large facial motor neurons in the area of FN, indicating that these sections were very close to the caudal pole of FN. It was therefore possible in these two rats to make a confident comparison between the proportion of CTB-positive neurons containing TH-immunoreactivity that lay caudal to and at the level of the FN (i.e. FN–7 to FN0) with the proportion of CTB-positive neurons containing TH-immunoreactivity that lay rostral to the caudal pole of FN (i.e. FN+1 to FN+4). In one rat, 64.9% (48/74) of the CTB-positive neurons were TH-immunoreactive in sections FN–7 to FN0 whereas 43.2% (19/44) of the CTB-positive neurons were TH-immunoreactive in sections FN+1 to FN+4. Similarly, in the second rat, 61.5% (48/78) of the CTB+ neurons were TH-immunoreactive in sections FN–7 to FN0 whereas 46.2% (24/52) of the CTB+ neurons were TH-positive in sections FN+1 to FN+4.

NR1-immunoreactivity was also assessed in CTB-negative, TH-positive neurons in FN–7 to FN–1. These neurons are functionally distinct from the bulbospinal C1 neurons and project rostrally to innervate the hypothalamus (Verberne et al., 1999). In these sections, $95.6 \pm 1.1\%$ ($n=4$) of the TH+/CTB– neurons were immunoreactive for NR1 (Table 1).

Sections stained for NR1, CTB and TpTH—NR1-immunoreactivity occurred in $98.1 \pm 0.01\%$ ($n = 4$) of the total population of bulbospinal CTB-positive neurons in regions of the rostral ventral medulla that contain serotonin neurons. In RPa, $97.7 \pm 1.1\%$ ($n = 4$) of the CTB-positive neurons with TpTH-immunoreactivity contained the NR1 subunit and NR1

was present in all of the CTB-immunoreactive neurons that lacked TpTH-immunoreactivity. The data were virtually the same for neurons in PPY, with $97.9 \pm 0.9\%$ of CTB+/TpTH+ neurons and all of the CTB+/TpTH- neurons showing NR1 immunoreactivity. In the marginal layer, all of the bulbospinal neurons were immunoreactive for NR1.

Of the total population of CTB-immunoreactive neurons in sections stained for NR1+CTB+TpTH, $80.2 \pm 3.0\%$ ($n = 4$) were immunoreactive for TpTH and $19.8 \pm 3.0\%$ were not. In RPa, $79.8 \pm 4.1\%$ ($n = 4$) of the CTB-positive neurons contained immunoreactivity for TpTH (neurons that were positive for CTB and NR1 plus neurons that were positive for CTB and TpTH only, see Table 2) and $20.2 \pm 4.1\%$ did not. In PPY, the proportions of CTB-labelled neurons with and without immunoreactivity for TpTH were almost identical ($79.2 \pm 3.6\%$ and $20.8 \pm 3.6\%$, respectively, $n = 4$, Table 2). In the marginal layer, $95.2 \pm 2.8\%$ ($n = 4$) of the CTB-labelled neurons contained immunoreactivity for TpTH (Table 2).

RMg neurons containing TpTH- and CTB-immunoreactivity in sections FN-1 to FN+4 were analyzed for their content of NR1. In this region of the ventral medulla, only 9 TpTH+/CTB+ RMg neurons were found in 4 rats and 8 of these neurons contained immunoreactivity for NR1.

NR1-immunoreactivity was also assessed in CTB-negative, TpTH-positive neurons in sections FN-7 to FN-1. In these sections, $93.2 \pm 2.1\%$ ($n=4$) of the TpTH+/CTB- neurons in RPa, $91.9 \pm 2.5\%$ in PPY and $95.0 \pm 5.0\%$ in the marginal layer were immunoreactive for NR1 (Table 2).

DISCUSSION

Our primary aim in this study was to determine the proportion of bulbospinal catecholamine and serotonin neurons that contain immunoreactivity for the NR1 subunit of the NMDA receptor in autonomic regions of the rat rostral ventral medulla. Our present data provide four important new observations. First, the NR1 subunit of the NMDA receptor occurs in spinally-projecting neurons throughout the rostrocaudal extent of the rostral ventral medulla. Second, NR1-immunoreactivity occurs in both bulbospinal catecholamine (i.e., C1) and bulbospinal non-catecholamine (i.e., non-C1) neurons in the RVLM. Third, in raphe pallidus and the parapyramidal region, both bulbospinal serotonergic and bulbospinal non-serotonergic neurons show immunoreactivity for the NR1 subunit. Finally, around 98% of all bulbospinal neurons in the rostral ventral medulla contain immunoreactivity for the NR1 subunit, regardless of whether or not they contain a monoamine, and NR1 is also present in more than 90% of the non-bulbospinal monoamine neurons in ventral medullary autonomic regions. These results provide the anatomical basis for previous pharmacological studies showing that NMDA receptors play a variety of functional roles in the ventral medulla and support the conclusion that NMDA receptors are important for neurotransmission in diverse types of autonomic neurons within this brain region.

In the present study, we used an antibody directed against the NR1 subunit to localize NMDA receptors on neurons that were also retrogradely labelled from the spinal cord and stained to show immunoreactivity for either TH or TpTH. The NR1 subunit is required for fully functional NMDA channels (Dingledine et al., 1999), which contain two NR1 subunits plus either two NR2 subunits or an NR2 and an NR3 subunit (Traynelis et al., 2010). Because we did not stain retrogradely labeled TH or TpTH neurons for NR2 or NR3 subunits, we cannot exclude the possibility that a group of neurons in the ventral medulla express the NR1 subunit but do not produce functional NMDA channels, as is the case for Purkinje cells of the cerebellum (Petralia et al., 1994). Nevertheless, many physiological studies have demonstrated the functional importance of NMDA receptors in the ventral

medulla (Bardgett et al., 2010; Lin et al., 2006; Mao and Abdel-Rahman, 1995; Sun and Reis, 1995); and we have shown here that virtually all of the bulbospinal catecholamine and serotonin neurons in the ventral medulla express the NR1 subunit. Although we used a very sensitive three-layer immunofluorescent procedure to assess the presence of NR1, it is possible that some neurons contained undetectable levels of this NMDA receptor subunit. If that is the case, then all monoamine neurons in the ventral medulla express NR1. Taken together, our anatomical observations and the published physiological data are consistent with the expression of fully functional NMDA channels on a significant and functionally relevant proportion of bulbospinal ventral medullary monoamine neurons.

We chose to study spinally-projecting monoamine neurons because they are well known to be important for sympathetic control of the cardiovascular system (Guyenet, 2006; Schreihofer and Sved, 2011). Our spinal CTB injections targeted the intermediolateral cell column (IML) and were routinely placed within the T10 segment. Analysis of injection sites with a very sensitive three-layer immunoperoxidase procedure indicated that our CTB injections were centered on an area of the spinal grey matter that included not only the IML but also the ventral portion of lamina V and the dorsal portion of lamina VII over approximately one segment's length. Despite the limited injection sites and differences in quantification methods, the retrograde labeling of spinally-projecting C1 and non-C1 neurons that we obtained is as robust as in previous studies (Carrive and Gorissen, 2008; Minson et al., 1990; Zhang et al., 2000). Because of the consistent placement of our injections, we can conclude that our study population contains a high proportion of ventral medullary neurons that project to the IML but few neurons that project to the dorsal or ventral horns. The scarcity of TpTH+/CTB+ neurons in RMg is consistent with a meager representation from neurons projecting to the dorsal horn amongst the bulbospinal neurons that we studied here. We cannot comment on the NR1 content of bulbospinal monoamine neurons that project to higher levels of the thoracic cord because T10 was the only segment into which we injected CTB.

Bulbospinal Catecholamine Neurons in the Rostral Ventral Medulla

Spinally projecting neurons in the ventral medulla subserve a variety of important autonomic functions. Of particular importance is the C1 group of bulbospinal RVLM neurons, which is involved in blood pressure regulation (Schreihofer and Sved, 2011). Along with a population of bulbospinal non-catecholamine (i.e. non-C1) neurons, the C1 neurons contribute to both resting and reflex regulation of sympathetic outflow (Guyenet, 2006; Jeske and McKenna, 1992; Ross et al., 1981; Ross et al., 1984; Schreihofer and Sved, 2011). Both C1 and non-C1 neurons are excitatory because they contain mRNA for vesicular glutamate transporter (VGLUT) 2 (Stornetta et al., 2002a; Stornetta et al., 2002b). Via their projections to the IML, these two groups of medullary neurons directly activate sympathetic preganglionic neurons in pathways regulating a variety of target organs (Schreihofer and Sved, 2011). On the basis of their location relative to the facial nucleus, virtually all of the bulbospinal TH-positive and TH-negative neurons identified here in the RVLM likely belong to the C1 and non-C1 cell groups, respectively.

In the present work, we focused on neurons in the rostral ventral medulla that directly innervated the lower thoracic spinal cord. We labeled these neurons by injecting the retrograde tracer CTB into the spinal cord at the T10 level. In addition to sympathoadrenal neurons, spinal segment T10 in rats contains many sympathetic preganglionic neurons that project to the coeliac, superior mesenteric and aortico-renal ganglia (Strack et al., 1988). The coeliac and superior mesenteric ganglia contain almost all of the sympathetic postganglionic neurons that provide innervation to the gastrointestinal tract from the stomach to the distal colon (Quinson et al., 2001; Trudrung et al., 1994) and to mesenteric blood vessels (Hsieh et

al., 2000). Hence, by injecting at T10, we targeted a functionally mixed population of medullary neurons involved in controlling either the abdominal organs and their vasculature or the adrenal medulla. Jeske and McKenna (1992) reported that more C1 neurons marked by immunoreactivity for phenylethanolamine N-methyltransferase (PNMT) projected to the upper thoracic cord than to lower thoracic segments. In contrast, they found that roughly equal numbers of PNMT-negative (i.e. non-C1) neurons projected to most levels of the spinal cord, including T9 and T11. In the present study, we were not able to compare ventral medullary monoamine neurons that projected to the caudal thoracic cord with those that supply rostral thoracic regions because we injected retrograde tracer only into the T10 segment.

By retrogradely labelling from T10, we would have specifically excluded from our study population bulbospinal medullary neurons that are part of circuits controlling sympathetically-innervated organs in the head and neck, such as the iris and salivary glands, and in the thoracic cavity, including the heart. Sympathetic drive for these organs comes from segments rostral to T10 (Strack et al., 1988). As well as lacking neurons in pathways that regulate structures in the head and neck, the neurons that we studied here were also unlikely to include a significant number of medullary neurons in circuits controlling pelvic organs. Sympathetic preganglionic neurons caudal to T10 are the source of sympathetic outflow to the pelvic viscera (e.g., Llewellyn-Smith et al, 2006).

Our current results indicate that NMDA receptors are located on virtually all bulbospinal C1 and non-C1 neurons. This anatomical finding underpins previous functional studies demonstrating that NMDA and alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) types of ionotropic glutamate receptors are important in the RVLM in control of sympathetic outflow and blood pressure regulation under normal and pathophysiological conditions (Bardgett et al., 2010; Lin et al., 2006; Mao and Abdel-Rahman, 1995; Mischel and Mueller, 2011; Sun and Reis, 1995).

Bulbospinal Serotonin Neurons in Rostral Ventral Medulla

The RPa and PPY regions of the ventral medulla as well as the marginal layer along its ventral surface also contain significant populations of spinally-projecting neurons, each of which has been identified in the present study. Many serotonergic and non-serotonergic neurons also send axons to the spinal cord from RMg. However, we found very limited labelling of raphespinal serotonergic neurons in this region, possibly because of the precise targeting and limited spread of our retrograde tracer in T10. Since RMg extends much more rostrally than the region of the ventral medulla that was the focus of our study, spinally-projecting serotonergic RMg neurons may occur mainly rostral to the area covered by the sections that we quantified. In this regard, injection of a replication-defective recombinant virus into RPa or RMg at levels that are at the rostral edge of the region examined here anterogradely labels axons in both upper (T1–T4) and lower (T10–T13) thoracic segments of the spinal cord (Nakamura et al., 2004a).

Bulbospinal neurons in RPa, PPY and the marginal layer show a variety of neurochemical phenotypes. Many but not all use serotonin as a neurotransmitter (Loewy and McKellar, 1981) and subsets of the serotonin and non-serotonin neurons in these regions express VGLUT3 and the GABA synthesizing enzyme, GAD67 (Nakamura et al., 2004a; Nakamura et al., 2004b; Stornetta et al., 2004; Stornetta et al., 2005). Some of the non-serotonin neurons in raphe nuclear regions contain mRNA for VGLUT2 (Stornetta et al., 2002a) and there are also VGLUT2-expressing neurons in the marginal layer at the level of FN (Weston et al., 2004). Labelling with pseudorabies virus indicates that at least some of the GAD67-expressing serotonin neurons in RPa and PPY are in circuits that control the adrenal gland

(Stornetta et al., 2004). Since we retrogradely labelled from spinal segments containing many sympathetic preganglionic neurons that supply the adrenal medulla, a significant fraction of the NR1-immunoreactive serotonin neurons identified here are likely to be capable of synthesizing GABA.

We observed intense immunoreactivity for the NR1 subunit of the NMDA receptor in virtually all RPa and PPY neurons projecting to the lower thoracic cord. Overall, the medullary raphé and parapyramidal regions are important in a wide variety of physiological processes, including thermoregulation (Morrison and Blessing, 2011) and changes in heart rate (Horiuchi et al., 2004; Zaretsky et al., 2003). However, because our spinal cord injections were in the lower thoracic region (i.e., T10), it is likely the NMDA receptors identified here are an anatomical substrate for regulation of organs controlled by lower thoracic sympathetic outflow, including the adrenal glands, kidneys and gut (Cano et al., 2004; Hsieh et al., 2000; Quinson et al., 2001; Stornetta et al., 2004; Trudrung et al., 1994). If the regulation of these organ systems involves glutamatergic neurotransmission in the RPa or PPY, then it could be mediated by the NR-1 immunoreactive TpTH-positive and TpTH-negative neurons identified here.

Although it has been established that marginal layer neurons in the retrotrapezoid nucleus are glutamatergic, chemosensory and project to respiratory neurons in the medulla (Guyenet et al., 2010; Weston et al., 2004), relatively little is known about the function(s) of the more medial marginal layer neurons that project to the spinal cord. While it would be difficult to specifically target these neurons with microinjections of drugs in order to assess their physiological role(s), anatomical studies on rats have produced some insight into their functions. Some of the bulbospinal marginal layer neurons are likely to be in circuits that control the adrenal medulla because they are transynaptically labelled after injections of pseudorabies virus into the adrenal gland (Stornetta et al., 2004). In unpublished work, we have found that neurons in the marginal layer, like neurons in RPa, express Fos in response to hypotension, suggesting that marginal layer neurons may share autonomic roles with RPa neurons. Hypercapnia also evokes Fos in some marginal layer neurons (Okada et al., 2002; Sato et al., 1992) so at least a subset of these neurons are likely to be involved in controlling respiration (Hodges and Richerson, 2010a; Hodges and Richerson, 2010b).

Non-Monoaminergic Bulbospinal Neurons in Autonomic Regions of the Rostral Ventral Medulla

The barosensitive C1 and non-C1 vasomotor presympathetic neurons lie within the RVLM, which is often defined as the area of the rostral ventral medulla that occupies the 500–600 μm immediately caudal to the caudal pole of FN. However, there is evidence to suggest that the cell bodies of these two types of neurons also occur more rostrally, extending medial and ventromedial to FN for several hundred micrometers (Schreihofer and Sved, 2011). In the present study, we found TH-positive and TH-negative neurons that had retrogradely transported CTB from T10 in sections as far as 500 micrometers rostral to the caudal boundary of FN. In 2 rats in which section FN0 contained the most caudally-located facial motor neurons, we also compared counts of bulbospinal C1 and non-C1 neurons in sections in which FN was absent versus sections in which FN was present. We found that very close to half of the CTB-labelled non-C1 neurons had cell bodies that lay medial or ventromedial to FN. This observation indicates that bulbospinal non-C1 neurons are concentrated in a region that many researchers do not consider when studying the RVLM. These data also suggest that pharmacological or electrophysiological studies aimed at dissecting out the roles in autonomic control of bulbospinal non-C1 neurons might be more efficient and effective if they focused on areas medial/ventromedial to FN rather than caudal to it.

In the present study, we also assessed the contribution of serotonin and non-serotonin neurons to the midline and parapyramidal projections to the lower thoracic spinal cord. We found that a relatively high proportion of the neurons that showed retrograde labelling from T10 are probably not capable of producing serotonin. Immunoreactivity for the serotonin synthesizing enzyme, TpTH, was absent from 20% of the CTB-immunoreactive neurons in both RPa and PPY. In contrast, 30 out of 32 spinally projecting neurons in the marginal layer were positive for TpTH. Since uptake of our retrograde tracer was restricted to ventral lamina V, the IML and dorsal lamina VII, these observations might indicate that non-serotonergic neurons in the midline and parapyramidal region have an underappreciated influence on the control of sympathetic outflow.

In summary, the results of the present study show that the NR1 subunit of the NMDA receptor is ubiquitously expressed on neurons of the ventral medulla, which are critical for the control of a variety of autonomic functions. There is increasing evidence to suggest that medullary autonomic circuits exhibit significant neuroplasticity in response to various physiological and pathophysiological challenges and it is intriguing to speculate that alterations in NMDA-mediated neurotransmission in the RVLM may contribute to pathophysiological states. Certainly, in other cardiovascular brain regions, such as the paraventricular nucleus of the hypothalamus, the function and expression of NMDA receptors change in diseases such as heart failure (e.g., (Kleiber et al., 2008)). Our present findings should form the basis for future studies on plastic changes in the expression of medullary NMDA receptors in response to both normal physiological and pathophysiological challenges.

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FIGURE 1. NR1-immunoreactivity in the ventral medulla

Immunoperoxidase staining for NR1 in a section through the rat ventral medulla just caudal to the caudal pole of the facial nucleus. The micrograph shows anatomical landmarks and regions in which NR1-immunoreactive neurons were quantified. Neurons showing intense staining for NR1 occur in the rostral ventrolateral medulla (RVLM), raphé pallidus (RPa), raphé obscurus (ROb), raphé magnus (RMg) and the parapyramidal region, (PPY). NAc, compact formation of the nucleus ambiguus. Scale bar, 0.5 mm.

FIGURE 2. Bilateral injections of retrograde tracer into the lower thoracic spinal cord
A, Immunoperoxidase staining for CTB in the T10 segment of a rat that received bilateral CTB injections targeted at the intermediolateral cell column (IML, stars). The centers of the injections lie just below the IML in dorsal lamina VII and pipette tracks showing moderate staining for CTB (arrows) travel toward the center of the injection sites through the lateral edge of the dorsal horn (DH). cc, central canal; VH, ventral horn. Scale bar, 0.5 mm. **B**, Diagram of segment T10 showing spinal cord laminae (I–X). py, pyramidal tract. Modified from Paxinos and Watson, 1998.

FIGURE 3. NR1-immunoreactivity in bulbospinal C1 and non-C1 neurons

Triple immunofluorescent labelling for CTB (blue, AMCA; Figs. 1A and 1D), TH (green, FITC; Figs. 1B and 1E) and NR1 (red, Cy3; Figs. 1C and 1F). **A–C**, Two CTB-positive/TH-positive neurons that lie just caudal to the caudal pole of FN contain intense immunoreactivity for NR1. **D–F**, Four CTB-positive neurons (1–4) lying approximately 250 μ m rostral to the caudal pole of FN (section FN+2) contain NR1-immunoreactivity. Only one of these neurons (1) is TH-immunoreactive. Scale bars, 25 μ m.

FIGURE 4. NR1-immunoreactivity in bulbospinal neurons of the ventromedial medulla and marginal layer

Triple immunofluorescent labelling for CTB (blue, AMCA; Figs. 2A, 2D and 2G), TH (green, FITC; Figs. 2B, 2E and 2H) and NR1 (red, Cy3; Figs. 2C, 2F and 2I). **A–C**, In PPY, eight CTB-positive neurons (1–8) contain NR1-immunoreactivity. Seven of the neurons are TpTH-positive but neuron 2 is not. **D–F**, In RPa, ten CTB-positive neurons (1–10) contain NR1-immunoreactivity. Nine of the neurons are TpTH-positive but neuron 2 is not. **G–I**, In the marginal layer, a CTB-positive neuron is immunoreactive for NR1 and TpTH. Scale bars, 25 μ m.

TABLE 1

Quantification of Bulbospinal and Non-Bulbospinal Catecholamine Neurons Immunoreactive for NR1

Bulbospinal neurons (FN-7 to FN+4)					
	Total Bulbospinal Neurons	CTB/TH/NR1 Neurons	CTB/NR1 Neurons	CTB/TH Neurons	CTB only Neurons
Total Number	475	247	218	0	10
Mean Number	118.8 ± 6.2	61.8 ± 5.7	54.5 ± 1.9	0	2.5 ± 0.3
Mean %		51.7 ± 2.5%	46.2% ± 2.5%	0%	2.1% ± 0.2%

Non-bulbospinal neurons (FN-7 to FN-1)			
	Total TH Neurons	TH/NR1 Neurons	TH only Neurons
Total Number	334	319	15
Mean Number	83.5 ± 4.5	79.8 ± 3.8	3.8 ± 1.0
Mean %		95.6% ± 1.1%	4.6% ± 1.2%

All data are presented as mean ± standard error.

n = 4 rats for all data

TABLE 2

Quantification of Bulbospinal and Non-bulbospinal Serotonin Neurons Immunoreactive for NR1

Bulbospinal neurons (FN-7 to FN+4)					
	Total Bulbospinal Neurons	CTB/TpTH/NR1 Neurons	CTB/NR1 Neurons	CTB/TpTH Neurons	CTB only Neurons
RPa					
Total Number	359	279	73	7	0
Mean Number	89.8 ± 6.0	69.8 ± 4.8	18.3 ± 4.0	1.8 ± 0.9	0
Mean %		77.8 ± 3.2%	20.2% ± 4.1%	1.9 ± 1.0%	0%
PPY					
Total Number	247	192	50	5	0
Mean Number	61.8 ± 9.1	48.0 ± 7.8	12.5 ± 2.4	1.3 ± 0.6	0
Mean %		77.5 ± 3.4%	20.8% ± 3.6%	1.7 ± 0.8%	0%
Marginal Layer					
Total Number	32	30	2	0	0
Mean Number	8.0 ± 1.5	7.5 ± 1.2	0.5 ± 0.3	0	0
Mean %		95.2 ± 2.8%	4.8% ± 2.8%	0%	0%

Non-bulbospinal neurons (FN-7 to FN-1)			
	Total TpTH neurons	TpTH+/NR1+ Neurons	TpTH only Neurons
RPa			
Total Number	239	223	16
Mean Number	59.8 ± 2.7	55.8 ± 3.3	4.0 ± 1.1
Mean %		93.2 ± 2.1%	6.8% ± 2.1%
PPY			
Total Number	166	153	13
Mean Number	41.5 ± 4.0	38.3 ± 4.3	3.3 ± 0.9
Mean %		91.9 ± 2.5%	8.1% ± 2.5%
Marginal Layer			
Total Number	16	14	2
Mean Number	4.0 ± 2.0	3.5 ± 1.6	0.5 ± 0.5
Mean %		95.0 ± 5.0%	5.0% ± 5.0%

All data are presented as mean ± standard error.

n = 4 rats for all data