Published in final edited form as:

Neuroscience. 2016 June 2; 324: 430–445. doi:10.1016/j.neuroscience.2016.02.066.

Distribution of Catecholaminergic Presympathetic-Premotor Neurons in the Rat Lower Brainstem

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Abstract

We previously characterized the organization of presympathetic-premotor neurons (PSPMN), which send descending poly-synaptic projections with collaterals to skeletal muscle and the adrenal gland. Such neurons may play a role in shaping integrated adaptive responses, and many of them were found within well-characterized regions of noradrenergic cell populations suggesting that some of the PSPMNs are catecholaminergic. To address this issue, we used retrograde trans-synaptic tract-tracing with attenuated pseudorabies virus (PRV) recombinants combined with multi-label immunofluorescence to identify PSPMNs expressing tyrosine hydroxylase (TH). Our findings indicate that TH-immunoreactive (ir) PSPMNs are present throughout the brainstem within multiple cell populations, including the A1, C1, C2, C3, A5 and A7 cell groups along with the locus coeruleus (LC) and the nucleus subcoeruleus (SubC). The largest numbers of TH-ir PSPMNs were located within the LC and SubC. Within SubC and the A7 cell group, about 70% of TH-ir neurons were PSPMNs, which was a significantly greater fraction of neurons than in the other brain regions we examined. These findings indicate that TH-ir neurons near the pontomesencephalic junction that are distributed across the LC, SubC, and the A7 may play a prominent role in somatomotor-sympathetic integration, and that the major functional role of the A7 and SubC noradrenergic cell groups maybe in the coordination of concomitant activation of somatomotor and sympathetic outflows. These neurons may participate in mediating homeostatic adaptations that require simultaneous activation of sympathetic and somatomotor nerves in the periphery.

Keywords

norepinephrine; tyrosine hydroxylase; pseudorabies; brainstem; autonomic; motor

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Role of authors

Both of the authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: IAK. Acquisition of data: HN, IAK. Analysis and interpretation of data: HN, IAK. Writing of the manuscript: HN, IAK. Statistical analysis: HN. Obtained funding: IAK.

1. Introduction

Numerous stress-elicited behaviors require simultaneous activation of somatomotor and autonomic circuits (Hilton, 1982, Jordan, 1990, Waldrop et al., 1996). Such coordination might be mediated by multiple descending projections from specific brain regions that integrate physiological functions. Our previous work utilized a retrograde trans-synaptic tract-tracing approach using attenuated pseudorabies virus (PRV) to identify neurons in the brain that send poly-synaptic collaterals to skeletal muscle and the adrenal gland. These cells, termed presympathetic-premotor neurons (PSPMN), are located within multiple sites throughout the brainstem and hypothalamus (Kerman et al., 2003, Kerman et al., 2006a, Kerman et al., 2006b, Kerman et al., 2007, Kerman, 2008, Shah et al., 2013). Distinct populations of PSPMNs are distributed within brain regions that regulate specific aspects of homeostasis, and synthesize transmitters that integrate autonomic, motor, and behavioral aspects of adaptive behaviors (Kerman, 2008). For example, we previously defined a dense population of serotonergic PSPMNs in the ventromedial medulla within the gigantocellular nucleus pars α and nucleus raphe magnus (Kerman et al., 2006b). Given the well-documented role of this region in coordinating motor, sensory, and autonomic responses to painful stimuli (Morgan and Whitney, 2000, Mason, 2001), as well as integrated motor and sympathetic responses as part of the cold defense (Nason and Mason, 2004, Morrison, 2011), it is possible that such neurons play multiple functional roles in homeostatic adaptations. Similarly, PSPMNs that express melanin-concentrating hormone or orexins in the lateral hypothalamus (Kerman et al., 2007) could participate in the passive vs. active coping strategies as part of the fight-or-flight response to stress (Marsh et al., 2002, Kayaba et al., 2003, Johnson et al., 2010).

Together these observations suggest that PSPMNs may mediate somatomotor-autonomic adaptive responses to a variety of stressors. Given the important role of central catecholamine circuits in broad stress integration (Sabban, 2010), we aimed to determine whether subpopulations of brainstem PSPMNs may be catecholaminergic. In our previous studies we detected a considerable number of PSPMNs within the locus coeruleus (LC), nucleus subcoeruleus (SubC), and the A5 cell group (Kerman et al., 2003, Kerman et al., 2006a), which have been classically described as part of the descending norepinephrine system (Kvetnansky et al., 2009). Other brain regions with significant noradrenergic cell populations containing PSPMNs included the Kölliker-Fuse nucleus, which overlaps with the A7 noradrenergic cell group (Lyons and Grzanna, 1988), nucleus tractus solitarius (NTS), which contains the A2 noradrenergic and the C2 adrenergic cell groups (Minson et al., 1990, Rinaman, 2011), and the ventrolateral medulla, which contains C1 adrenergic and A1 noradrenergic cell groups (Kerman et al., 2003, Card et al., 2006).

Previous studies have documented projections from these catecholaminergic brainstem areas to both the intermediolateral cell column (IML) and the ventral horn of the spinal cord, suggesting existence of reticulospinal neurons that collateralize to innervate sympathetic preganglionic neurons and motoneurons. Studies utilizing monosynaptic anterograde and retrograde tracers have demonstrated that noradrenergic neurons within the A5 cell group, LC, SubC, and the A7 cell group send descending projections that terminate at different rostro-caudal levels of the spinal cord (Westlund et al., 1982, 1983, Clark and Proudfit,

1991a, b). Similarly, adrenergic neurons from within C1, C2, and C3 adrenergic cell groups project to the spinal cord and terminate within the IML (Minson et al., 1990). Tract-tracing with viral vectors containing PRS2, a noradrenaline-specific regulatory element that is activated by Phox2 transcription factor (Hwang et al., 2001), which preferentially infect noradrenergic and adrenergic neurons and are transported anterogradely have extended these observations. Bruinstroop et al. used this methodology to demonstrate that noradrenergic neurons within the LC, SubC pars α , and the A7 cell send projections that terminate in the IML and the ventral horn (Bruinstroop et al., 2012). Similarly, adrenergic neurons within the C1 and C3 cell groups send dense projections to the spinal cord, which terminate within laminae IX and X (Card et al., 2006, Seigny et al., 2012). Previous studies that utilized PRV as a retrograde trans-synaptic tract-tracer have demonstrated the presence of TH-immunoreactive (ir) presympathetic neurons in the ventrolateral medulla, A5, LC, and SubC following injections of multiple sympathetically-innervated organs, including skeletal muscle, adrenal gland, the pancreas, and brown fat (Strack et al., 1989, Jansen et al., 1997, Xiang et al., 2014).

Taken together, these observations suggest the existence of catecholaminergic neurons in the brainstem with poly-synaptic collaterals to skeletal muscle and sympathetically-innervated peripheral organs. However, it is not clear whether any of these TH-ir cell groups contain PSPMNs. In this study we demonstrate the existence of TH-ir PSPMNs within multiple catecholaminergic populations of the lower brainstem. The greatest numbers of such TH-ir PSPMNs were found within the LC and SubC. Within the SubC and the A7 a large majority of TH-ir neurons are PSPMNs, suggesting that these cell groups are dedicated to somatomotor-sympathetic integration.

2. Experimental Procedures

2.1. Animals

All of the procedures regarding animal use in this study were consistent with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (1996, National Academy of Sciences) and were approved by the local Institutional Animal Care and Use Committee. Trans-synaptic tract-tracing was performed in male Sprague-Dawley rats (n = 12; Charles River Laboratories, Wilmington, MA).

2.2 Viral tracing

We previously observed a negative correlation between subject weight and the rate of motoneuron infection, with an optimal weight of approximately 200 g (Kerman et al., 2003). In light of this finding, we used rats that weighed between 163 g and 300 g, with an average weight of 205 ± 12 g (mean $\pm$ SEM; weighed at the time of sympathectomy; see below).

Animals were anesthetized with 5% isoflurane vaporized in 1.0–1.5 L/min of O₂ and were maintained at 1.5–2.5%. Surgical plane of anesthesia was achieved such that there was no spontaneous movement and no withdrawal responses to tail and/or foot pinch. Prior to PRV injections, surgical sympathectomy was performed as previously described (Kerman et al., 2003, Kerman et al., 2006a) to remove sympathetic innervation of the hindlimb musculature.

Briefly, a ventral laparotomy was performed and a segment of the lumbar sympathetic nerve from the level of the renal artery to the aortic bifurcation was extirpated. Neural plexuses along the abdominal aorta were stripped off under microscopic observation using fine forceps, and the aorta was swabbed with a 10% phenol solution.

Following a 2–10 day recovery period, animals were injected with PRV. We used recombinant strains of PRV that express unique reporter proteins, with PRV-152 expressing enhanced green fluorescent protein (EGFP) and PRV-BaBlu transcribing β -galactosidase (Billig et al., 2000). Both of these viral strains were derived from the attenuated strain PRV-Bartha, which is not infectious to humans but has been demonstrated to have the capability of simultaneous neuronal coinfection in rats (Standish et al., 1995, Billig et al., 2000). Viral stocks were harvested from pig kidney cell cultures at a titer of $10^8 - 10^9$ pfu/mL, aliquoted into 50 μ L volumes, and stored at -80°C until the time of inoculations when they were rapidly thawed in a 37°C water bath. PRV injections were performed as previously described (Kerman et al., 2003, Kerman et al., 2006b). Briefly, PRV-152 was injected throughout the lateral head of the gastrocnemius muscle in 1 μ L volumes (totaling 30 μ L) using a 10 μ L glass syringe (Hamilton Company, Reno, NV). PRV-BaBlu was similarly injected using a Hamilton syringe with a glass pipette attached to the tip with wax. A total of 2–4 μ L of PRV-BaBlu was injected into the ipsilateral adrenal gland.

In some animals ($n = 3$) gastrocnemius muscle and the adrenal gland were injected on the same day, while in others ($n = 9$) the adrenal gland was injected 24–25 or 32–33 h after the gastrocnemius injections to improve temporal matching of the infection from the two organs (Kerman et al., 2003). Rats were allowed to survive approximately: 96, 120, 132, or 144 h after initial PRV injections (Table 1). At the end of their survival period, animals were deeply anesthetized with sodium pentobarbital (150 mg/kg) and were transcardially perfused with 100–150 mL of physiological saline (0.9% NaCl) followed by 400–500 mL of paraformaldehyde L-lysine sodium metaperiodate (PLP) fixative (McLean and Nakane, 1974).

2.3. Tissue processing

Following perfusions, brains and spinal cords were extracted, post-fixed overnight, and immersed in 30% sucrose overnight on the following day. Brains were sectioned coronally on a freezing microtome at a thickness of 40 μ m and collected into six different bins, which were stored at -20°C in cryoprotectant (30% ethylene glycol, 1% polyvinyl-pyrrolidone, 30% sucrose in 0.1M sodium phosphate buffer (Watson et al., 1986)) until immunohistochemical processing. Spinal cords were extracted in three segments: T1–T7, T8–T13, and L1–L6. They were postfixed and cyroprotected as described above, and then sectioned horizontally at a thickness of 40 μ m collected into three bins and stored in cryoprotectant.

Brains from PRV-injected animals were processed for triple immunofluorescent detection of: EGFP, β -galactosidase, and TH. Free-floating brain sections were rinsed with 0.1 M phosphate buffer (PB; pH 7.4) several times at room temperature and then incubated for 1 hour in blocking buffer containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton X-100 (TX-100) in 0.1 M PB. Sections were then reacted with a

cocktail of primary antibodies: chicken anti-GFP IgY (13970, Abcam, Cambridge, MA) at 1:2,000, mouse anti- β -galactosidase IgG (G4644, Sigma-Aldrich, St. Louis, MO) at 1:1,000, and rabbit anti-TH (AB152, EMD Millipore, Billerica, MA) at 1:500 in a solution containing 1% NGS, 1% BSA, and 0.3% TX-100. Following overnight incubation at 4 °C, the tissue was rinsed with 0.1 M PB and reacted with a secondary antibody cocktail of Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA), AlexaFluor 488-conjugated goat anti-chicken IgG (1:200; Molecular Probes, Eugene, OR), and AlexaFluor 647-conjugated goat anti-rabbit IgG (1:200; Molecular Probes), dissolved in 1% NGS, 1% BSA, and 0.3% TX-100 in 0.1 M PB. Spinal cord sections from some of the animals were processed for GFP immunofluorescence using the same protocol as above, excluding antibodies for the detection of β -galactosidase and TH, to verify effectiveness of the surgical sympathectomy. Following processing, tissue sections were mounted on glass slides (SuperFrost slides, Fisher Scientific, Waltham, MA) then coverslipped with Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA).

2.4. Antibody Characterization

The chicken anti-GFP antibody (13970, Abcam, Cambridge, MA) was raised against recombinant full-length protein. This antibody yields a single band on Western blot and detects GFP in transgenic mice expressing GFP in lamina II of the spinal cord (manufacturer's technical information). Mouse anti- β -galactosidase antibody (G4644, Sigma-Aldrich, St. Louis, MO) was developed in mouse peritoneal cavities using β -galactosidase purified from *E. coli* as the immunogen. Using Western blot, this antibody was shown to be specific for β -gal in its native form (116 kD), and it reacts only with β -galactosidase from *E. coli* (manufacturer's technical information). Specificity of this antibody in immunofluorescent experiments has been previously documented (Kerman et al., 2003, Kerman et al., 2006b). The rabbit anti-TH antibody (AB152, EMD Millipore, Billerica, MA) was raised against denatured TH from rat pheochromocytoma of the adrenal medulla (denatured by sodium sulfate). By Western blot this antibody selectively labels a single band at 62kDa, and does not detect proteins from liver cells (manufacturer's technical information).

2.5. Tissue analysis

Tissue was examined using an Olympus BX61 microscope (Olympus America, Center Valley, PA) outfitted with motorized stage (96S100LE; Ludl Electronic Products, Hawthorne, NY) and a cooled mono CCD camera (Orca R2; Hamamatsu Corporation, Middlesex, NJ). Regions of interest were digitized under 4x and 20x objectives using fluorophore-specific filter sets (excitation and emission spectra): AlexaFluor 488–482/35, 536/40; Cy3–531/40, 593/40; AlexaFluor 647–628/40, 692/40. Images were acquired in z-stacks of optically-sectioned images within distinct focal planes, which were stitched together to visualize regions of interest and pseudocolored using CellSens Dimension software (Olympus America).

Neurons expressing different combinations of signals and located within different catecholaminergic nuclei were manually counted from the digitized images using Neurolucida 11 (MBF Bioscience, Williston, VT). Neurons were quantified bilaterally from

nine regions located throughout the medulla, pons, and the caudal midbrain at multiple rostro-caudal levels. Cell counts were expressed as an average number of neurons per section. These values were then averaged across all animals.

Anatomical analyses were guided by the Paxinos and Watson atlas 6th edition (Paxinos and Watson, 2007). Each area was defined by the boundary drawn around the cluster of TH-positive cell populations. For most of the areas, we analyzed sections spaced 240 μ m apart; for the LC, SubC, and A7 sections spaced every 120 μ m were quantified in animals in the long survival analysis group. The A1 and A2 noradrenergic cell groups were located within the caudal medulla, where we analyzed 3 consecutive sections spaced every 240 μ m from approximately -14.76 mm to -14.28 mm relative to bregma. The A1 cell group was located within the caudal ventrolateral medulla bordered by the pyramidal decussation caudally and extending rostrally to the opening of the fourth ventricle with TH-ir neurons distributed dorsally to the lateral reticular nucleus. The A2 noradrenergic cells were located within the dorsolateral medulla in the NTS. These cells extended dorsally to and laterally from the central canal and were ventral to area postrema. For analysis of the A2 cells, we excluded the dopaminergic neurons located nearby within the dorsal motor nucleus of the vagus. These neurons were TH-ir, but did not stain for dopamine beta-hydroxylase in our preliminary studies and were readily identified by their size and location apart from the A2 neurons (data not shown).

The C1, C2, and C3 adrenergic cell groups were located within the rostral medulla, at levels rostral to the opening of the fourth ventricle. The C1 cells were located within the rostral ventrolateral medulla ventral to the nucleus ambiguus, medial to the edge of the inferior olive, and lateral to the edge of the tissue. We analyzed C1 neurons from four sections spaced 240 μ m apart approximately -13.44 mm to -12.48 relative to bregma. The C2 neurons were located close to the borders of the fourth ventricle and were arranged along a ventrolateral extent from the ventricle within the rostral NTS approximately -13.44 mm to -13.20 mm relative to bregma. Two sections spaced 240 μ m apart were quantified for the C2 cell group. The C3 neurons were located more rostrally to the C2 cell group and were concentrated along the midline immediately ventral to the fourth ventricle at approximately -12.72 mm to -12.24 mm relative to bregma. Two sections spaced 240 μ m apart were analyzed for the C3 cell group. While we were limited in that we only analyzed TH-ir material, rather than also staining for dopamine beta hydroxylase and phenylethanolamine n-methyltransferase to fully characterize the extent of noradrenergic and adrenergic cell populations, the anatomical landmarks and the rostro-caudal levels that we chose for our analyses agree with previous investigations that have mapped the distribution of the noradrenergic and adrenergic cell populations in the medulla (Card et al., 2006, Seigny et al., 2012, Guyenet et al., 2013).

The LC, which corresponds to the A6 cell group (Moore and Bloom, 1979), was defined by a region densely populated by TH-ir neurons located in close proximity to the fourth ventricle at the medullopontine junction. We analyzed the LC at levels approximately -10.08 mm to -9.60 mm relative to bregma; these analyses were performed in sections spaced every 240 μ m (in animals from the short survival analysis group; total of two sections per animal) or 120 μ m (long analysis survival group; total of four sections per animal). The A5

noradrenergic cell group was located within the ventrolateral pons from approximately –9.84 mm to –9.36 mm relative to bregma. These neurons were located dorsolaterally to the superior olivary complex and ventromedially to the facial nerve within their caudal extent. At more rostral levels, the A5 cells were distributed dorsolaterally to the superior olive, ventromedially to the sensory trigeminal complex, and ventrally to the motor trigeminal complex. Neurons within the A5 cell group were analyzed on sections spaced every 240 μm (in animals from the short survival analysis group; total of two sections per animal) or 120 μm (long analysis survival group; total of four sections per animal).

The SubC was located at the pontomedullary junction ventral to the rostral pole of the LC. These scattered TH-ir neurons were located along a ventrolaterally oriented axis extending from the central gray toward the ventrolateral edge of the brainstem. The dorsal tip of this cluster of neurons was located medial to superior cerebellar peduncle and ventral to the central gray. At their lateral extent these neurons bordered the lateral border of the motor trigeminal complex; their ventral extent was dorsomedial to the rubrospinal tract and medial to the lateral lemniscus. We analyzed the SubC from approximately –9.36 mm to –8.88 mm relative to bregma; these neurons were analyzed on sections spaced every 240 μm (in animals from the short survival analysis group; total of two sections per animal) or 120 μm (long analysis survival group; total of four sections per animal). The A7 cell group was defined as a cluster of TH-ir neurons distributed medially to the middle cerebellar peduncle, ventrolaterally to the superior cerebellar peduncle, and dorsally to the principal sensory trigeminal nucleus. Caudally these neurons intermixed within the fibers of the lateral lemniscus, while more rostrally they formed a cluster medial to the lateral lemniscus. The A7 neurons were analyzed on sections spaced either every 240 μm (in animals from the short survival analysis group; total of two sections per animal) or 120 μm (in animals from the long analysis survival group; total of four sections per animal); these analyses were conducted in a region that extended from approximately –8.88 to –8.40 mm relative to bregma.

For presentation purposes, z-stacked images were projected onto a single image per channel using enhanced focal imaging in CellSens. Adobe Photoshop CS5.1 (Adobe Systems, San Jose, CA) was used to optimize brightness and contrast of the exported images. Figures were prepared using Adobe Photoshop and Illustrator CS5.1 (Adobe Systems).

2.6. Statistical analyses

Data were analyzed with IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY) and Prism 6.0 (GraphPad Software, San Diego, CA). The effects of survival time across multiple brain regions were analyzed via two-way ANOVA. The effects of survival time within a single brain region, or the effects of anatomical location within the long survival time group were analyzed with a one-way ANOVA. Bonferroni multiple comparison tests were conducted *post-hoc* where indicated. For the effects of anatomical location within the long survival time group, the non-parametric Kruskal-Wallis test was used when the dependent variables showed significant heterogeneity of variance on the Levene's test of homogeneity. In this case, multiple Mann-Whitney U tests with Bonferroni corrections were conducted for

post-hoc where indicated. Data are presented as mean $\pm$ SEM; significance was set at $p < 0.05$.

3. Results

3.1. Distribution of PSPMNs

Effectiveness of the surgical sympathectomy was validated in total of four animals from Group 1 (Table 1; i.e. survival of 96 h following PRV injections into adrenal and gastrocnemius – $n = 3$) and Group 2 (Table 1; i.e. survival of 96 h following adrenal injection and 120 h after gastrocnemius injection – $n = 1$). Spinal cords from these cases were horizontally-sectioned at 40 μ m and immunofluorescently-stained to detect GFP. In all cases we observed GFP expression within the lumbar motoneurons, but not within the IML, indicating selective infection of the somatic motor efferents following PRV injections into the gastrocnemius muscle. These observations are consistent with our previous reports, indicating effectiveness of this surgical sympathectomy approach (Kerman et al., 2003, Kerman et al., 2006a, Kerman et al., 2006b).

Examination of brains from Group 1 animals (Table 1; i.e. 96 h survival after adrenal and gastrocnemius injections – $n = 3$) revealed predominant infection with PRV-BaBlu, and to a lesser extent with PRV-152. Ventral gigantocellular nucleus (GiV) of the ventromedial medulla was the most prominently labeled brainstem region and contained PSPMNs in all of the animals. Additionally, the A7 (Köllicker-Fuse nucleus) and the C3 also contained PSPMNs in all three of the animals. In two of the animals PSPMNs were also detected within the LC, SubC, and the periaqueductal gray (PAG). In one animal PSPMNs were also observed in the hypothalamus, primarily within the lateral hypothalamus (LH) and paraventricular nucleus (PVN), and in the A5. Because of the predominance of the PRV-BaBlu infection in the Group 1 animals, in the other cases we injected gastrocnemius muscle 24 h before the adrenal injection to allow for additional transport time from the hindlimb. This resulted in more balanced infection, and in the Group 2 rats (Table 1; 96 h after adrenal injection and 120 h after gastrocnemius injection) PSPMNs were detected in the same brainstem regions observed in Group 1 as well as within the gigantocellular nucleus pars α (GiA), caudal raphe nuclei, and the rostral ventrolateral medulla. Hypothalamic labeling was more extensive in this group, and PSPMNs were detected in the PVN and LH in every case. Within Group 3 (Table 1; 108–112 h after adrenal injection and 132–136 h after gastrocnemius injection), PSPMNs became more prominent within the caudal ventrolateral medulla, NTS, PAG, Edinger-Westphal nucleus, posterior hypothalamus, and dorsomedial hypothalamus. Within Group 4 (Table 1; 111–119 h after adrenal injection and 143–144 h after gastrocnemius injection) there appeared additional labeling within the anterior hypothalamus, primarily the median pre-optic area; there was also extensive PRV-152 infection in the motor cortex with some PRV-BaBlu labeling and occasional co-localization of the two viruses there.

3.2. Distribution of TH-ir PSPMNs

Consistent with our previous observations, we detected PSPMNs (neurons infected with both PRV-152 and PRV-BaBlu) within multiple brainstem regions, including those that

contain catecholaminergic neurons: A1, C1, C2, C3, A5, LC, A7, and SubC. All of these areas contained TH-ir PSPMNs (Fig. 1).

Among these regions LC, SubC, and the A7 cell group appeared to be enriched in their content of TH-ir PSPMNs. Within the LC, TH-ir PSPMNs were detected at both the short and long survival times and their numbers increased with longer survival times (Fig. 2). At short survival times TH-ir PSPMNs were predominantly located at the ventrolateral edge of the LC within its caudal and middle subdivisions (Fig. 2 G, H). At longer survival times these neurons extended into the dorsolateral portions of the LC at the caudal and middle levels (Fig. 2 J, K). Very few of the TH-ir PSPMNs were detected within the rostral LC (Fig. 2 I, L).

Within the SubC, TH-ir PSPMNs were detected at the shortest survival times and their numbers increased with longer survival (Fig. 3). These neurons were distributed throughout the dorsoventral extent of this cell group, but were most numerous within the region immediately medial to the motor trigeminal nucleus (Fig. 3).

Within the A7 cell group, TH-ir PSPMNs were detected at both short and long survival times (Fig. 4). At short survival times TH-ir PSPMNs were primarily distributed within the lateral lemniscal fibers or within the region immediately medial to them (Fig. 4 G–I). At longer survival times the A7 TH-ir PSPMNs neurons were more numerous and occupied the same position in the immediate proximity to the lateral lemniscus. In addition, they also extended more medially to occupy an area dorsomedial to the rubrospinal tract (Fig. 4 J–L).

Within the C2 cell group no PRV-infected neurons were detected at the short survival times (data not shown). However, there we observed TH-ir PSPMNs throughout the C2 at the longer survival times (Fig. 5 A–E). Within the A2 cell group we detected PSPMNs, but virtually none of them expressed TH (Fig. 5 F–J). In addition, neurons infected with PRV-BaBlu, but not with PRV-152, co-localized with TH within the A2 cell group (Fig. 5 F–J).

3.3. Quantitative Analysis

Numbers of PRV-infected and TH-ir neurons were quantified within the following areas: A1, A2, C1, C2, C3, A5, LC, A7, and SubC. In our initial analyses we utilized one-way ANOVAs to determine whether there were differences in the numbers of PRV-infected cells among the four experimental groups (grouped by survival time after gastrocnemius muscle injection; Table 1). We used survival time as an independent factor and the total number of PRV-infected neurons as a dependent variable; this analysis was performed separately for each cell group. One-way ANOVAs were significant ($p < 0.05$) within: A1 ($F_{(3,8)} = 12.33$), A2 ($F_{(3,8)} = 8.27$), C1 ($F_{(3,8)} = 16.71$), C2 ($F_{(3,8)} = 4.86$), C3 ($F_{(3,8)} = 5.83$), and LC ($F_{(3,7)} = 7.86$), but not within A5 ($F_{(3,8)} = 1.73$, $p > 0.05$) or A7 ($F_{(3,8)} = 1.88$, $p > 0.05$). *Post-hoc* analyses indicated that there were no statistical differences for the number of PRV-infected neurons between Groups 1 and 2, or between Groups 3 and 4 for any of the areas examined.

Based on these results, we combined data from Groups 1 and 2 into a “Short Survival” analysis group, and data from animals in Groups 3 and 4 into a “Long Survival” analysis group (Table 1). Grouped data were then analyzed using two-way ANOVA, with analysis

group (i.e. “Short Survival” or “Long Survival”) and anatomical location as independent factors, and the total number of TH-ir neurons as a dependent variable. This analysis revealed a significant effect of anatomical location ($F_{(8,88)} = 368.3$, $p < 0.001$), but not of analysis group ($F_{(1,88)} = 0.186$, $p > 0.05$), indicating that the total numbers of TH-ir neurons did not change with survival times after PRV injections (Table 2, “Total TH-ir” column).

We then examined potential differences across the regions of interest in the numbers of PSPMNs and in their fraction of total TH-in neurons within the Long Survival analysis group. We focused on the Long Survival group because this is when we observed maximal labeling in the brainstem, and there were also too few neurons in the animals in the Short Survival Group for meaningful statistical analyses.

For the total number of PSPMNs, four regions contained the greatest numbers of these neurons, including: C1, LC, A7, and SubC. Statistical analysis using the Kruskal-Wallis test with anatomical location as an independent factor and the total number of PSPMNs as the dependent variable revealed a significant effect of anatomical location ($H_{(8)} = 26.715$; $p < 0.001$). *Post-hoc* analyses using the Mann-Whitney U test with a Bonferroni correction showed that the LC contained significantly more PSPMNs than A1, A2, and C2 cell groups, while the C1 and A7 cell groups contained significantly greater number of PSPMNs than the A1 and A2 cell groups (Table 2, “Total PSPMNs” column).

In the case of TH-ir PSPMNs three regions contained the greatest numbers of neurons, including: LC, A7, and SubC. Analysis using the Kruskal-Wallis test indicated a significant effect of anatomical location ($H_{(8)} = 39.991$, $p < 0.0001$). *Post-hoc* testing showed that the LC contained significantly more TH-ir PSPMNs than all the other cell groups with the exception of A7 and SubC (Table 2; “TH-ir PSPMNs” column). A7 contained significantly more TH-ir PSPMNs than five other cell groups (i.e. A1, A2, C2, C3, and A5), while SubC contained more neurons than three of the other cell groups (i.e. A1, A2, and C3; Table 2, “TH-ir PSPMNs” column).

We extended these analyses to determine the relative fraction of TH-ir neurons that are PSPMNs within each of these cell groups by dividing the number of TH-ir PSPMNs by the total number of TH-ir neurons in the long survival group. In this analysis, the A7 and SubC contained the greatest percentage of TH-ir PSPMNs. Kruskal-Wallis test revealed a significant effect of anatomical location ($H_{(8)} = 43.075$, $p < 0.0001$). *Post-hoc* testing revealed that the A7 and SubC both contained significantly higher percentage of TH-ir PSPMNs as compared to all of the other cell groups with the exception of A5 (Table 2, “% TH-ir PSPMNs” column).

4. Discussion

In our previous work we systematically mapped the organization of PSPMN circuits that likely mediate somatomotor-sympathetic integration, showing that distinct populations of neurons that make up this circuitry express orexins, melanin-concentrating hormone, oxytocin, arginine vasopressin, and serotonin within multiple brain regions (Kerman et al., 2006a, Kerman et al., 2006b, Kerman et al., 2007). We previously reported that within the

brainstem the greatest number of PSPMNs are distributed within the rostral ventromedial medullary complex and the caudal raphe nuclei, with the noradrenergic regions containing comparatively fewer such neurons (Kerman, 2008). Given the important role of central catecholamine circuits in regulating stress responsivity and the considerable number of PSPMNs within noradrenergic cell groups such as the LC, SubC and A5 (Kerman et al., 2003), the present study characterized the distribution of catecholaminergic PSPMN in the brainstem. We focused our analyses on the caudal midbrain, pons, and medulla and observed differences in the regional distribution of TH-positive PSPMN. The largest number of these cells was detected within the ventral LC and the A7, while virtually none of these neurons were observed within the A2. Our analyses revealed that the majority of TH-positive neurons within the A7 and SubC were PSPMN, suggesting that the major function of these cell groups may be dedicated to integrating somatomotor and sympathetic functions.

Our data suggest that the most direct descending noradrenergic presympathetic-premotor connections to the spinal originate within the A7, SubC, and LC. Our observations that these regions contain TH-ir PSPMN at the earliest survival times support this idea. Likewise, our quantitative data indicate that these regions contain the highest number of PSPMN at short and long survival times. Previous studies that have examined the connectivity of these regions are consistent with this notion. This includes demonstration of the presence of bulbospinal neurons within these noradrenergic cell populations (Olson and Fuxe, 1972, Tan and Holstege, 1986, Clark and Proudfit, 1991b, Bruinstroop et al., 2012).

Distribution of TH-ir PSPMN

It is likely that some of the cell groups identified here send direct bulbospinal projections, while others represent higher order neurons that project to synaptic relay sites. Classical anatomical investigations have identified a dense network of noradrenergic fibers within the IML in close proximity of the sympathetic preganglionic neurons (Carlsson et al., 1964) along with extensive innervation of the dorsal and ventral horns (Paxinos, 2004). Likewise, C1–C3 adrenergic groups also contain neurons with descending projections to the spinal cord (Minson et al., 1990, Card et al., 2006, Holloway et al., 2013). Our results are consistent with these previous data, because we identified TH-ir PSPMN at the shortest survival times within all of these groups with the exception of the C2.

In addition, we also detected TH-ir PSPMN within the SubC at the earliest survival times. In contrast to the LC, SubC is a group of heterogeneous cell populations that contains a relatively small number of noradrenergic neurons (Amaral and Sinnamon, 1977, Grzanna and Fritschy, 1991). These noradrenergic neurons send descending projections to the brainstem, including innervation of hypoglossal motoneurons, along with ascending projections to the hypothalamus (Olson and Fuxe, 1972, Aldes et al., 1992, Funk et al., 1994).

We observed differences in the regional distribution of TH-positive PSPMN. The largest number of these cells was detected within LC, while virtually none of these neurons were observed within the A2. When we analyzed cell counts as fractions of the total number of catecholaminergic neurons within each cell group, we observed that the A7 and SubC contained greater fraction of catecholaminergic PSPMN than the other areas examined.

Other catecholaminergic nuclei including the A1, A5, C1, C2, and C3 also contained TH-positive PSPMNs to some extent.

Within the LC, we observed topographical differences in the distribution of PSPMNs, with these cells distributed ventrally at the early survival times and extending more dorsally at longer survival times. These findings are consistent with reports of PRV-infected neurons being restricted to the ventral LC following injections into various sympathetically-innervated peripheral organs, including spleen, kidney, and brown fat (Cano et al., 2001, Cano et al., 2003, Cano et al., 2004). Our findings also agree with earlier studies utilizing monosynaptic tracers to demonstrate dorso-ventral topography. Specifically, neurons within the ventral portion of the LC primarily project to the spinal cord, while those located dorsally project to the cortex, septum, hippocampus, and hypothalamus (Mason and Fibiger, 1979, Loughlin et al., 1982, Waterhouse et al., 1983, Loughlin et al., 1986, Waterhouse et al., 1993). Therefore, it is likely that the ventrally distributed LC neurons that are infected at the short survival time send direct projections to the spinal cord. On the other hand, PSPMNs within the dorsal LC that are infected at longer survival times may instead project to synaptic relays within the forebrain.

In addition to neurons within the dorsal LC, noradrenergic cells within the A1 and the A2 send projections to the hypothalamus. Both the A1 and the A2 neurons innervate the forebrain through the ventral noradrenergic bundle, but the extent in the overlap in the innervation targets of these two groups has not been fully determined (Rinaman, 2011). The A1 and A2 neurons both project to hypothalamic areas, including various subdivisions of the paraventricular nucleus and the supraoptic nucleus (Sawchenko and Swanson, 1981, Raby and Renaud, 1989, Kvetnansky et al., 2009), the amygdala, particularly the central nucleus of the amygdala (Jia et al., 1992, Roder and Ciriello, 1993), and the subfornical organ (Tanaka et al., 2002). Although these two nuclei share projection target areas, it has been reported that A1 and A2 noradrenergic neurons innervate distinct subdivisions or type of neurons (Sawchenko and Swanson, 1981). It is also interesting to note that local connections from the A2 to the A1 area exist, although the majority of them seem to be non-catecholaminergic (Sawchenko and Swanson, 1981, Chan et al., 1995). We previously observed a large population of PSPMNs within the medial parvocellular ventral subdivision of the PVN (Kerman et al., 2006a), the region that receives the majority of its noradrenergic innervation from A1 and A2 with little input from the LC (Rinaman, 2011). Given that we observed a number of TH-ir PSPMNs within the A1 cell group, there may exist a population of PSPMNs in the PVN that is targeted by the ascending A1 noradrenergic neurons. However, because we used a trans-synaptic tract-tracing approach we cannot conclude this definitively. Future work utilizing monosynaptic tract-tracing will be required to properly address this issue. It is worth noting that a previous retrograde trans-synaptic tract-tracing study showed that the A2 noradrenergic neurons send descending projections to the brown adipose tissue (Cano et al., 2003). Our observation of the presence of TH-ir A2 neurons with trans-synaptic projections to the adrenal gland are consistent with this notion, because of cold-induced norepinephrine secretion from the adrenal medulla (Vollmer et al., 1992). These observations suggest that TH-ir A2 neurons may play a role in engaging multiple sympathetic efferents as part of the cold defense, but they likely do not have a prominent role in somatomotor-sympathetic activation.

Along with its noradrenergic innervation, the hypothalamus also receives adrenergic innervation from the C1, C2, and C3 cell groups (Minson et al., 1990, Card et al., 2006, Kvetnansky et al., 2009). Though we observed some PRV labeling within the C1 and C3, the most striking observation was made within the C2 cell group where TH-positive PSPMNs were detected only at the long survival times. Catecholaminergic neurons within the C1 project to the IML, LC, A1, A2, as well as the lateral hypothalamus, dorsomedial hypothalamus, and the paraventricular nucleus of the hypothalamus (Card et al., 2006, Holloway et al., 2013). In addition, C3 adrenergic neurons project primarily to lamina X of the spinal cord, the IML, ventrolateral periaqueductal grey, and the paraventricular nucleus of the hypothalamus (Sevigny et al., 2012). The C2 neurons primarily belong to the ascending portion of the brainstem catecholaminergic system and project to the hypothalamus and amygdala (Kvetnansky et al., 2009). The C2 also contains a population of spinally-projecting adrenergic neurons, albeit to a lesser extent than the C1 and C3 groups (Minson et al., 1990). Given that we did not detect any PSPMNs within the C2 at the short survival times, but there was extensive labeling there at the longer survival times, it is feasible that the TH-positive C2 PSPMNs project to the hypothalamus where they provide adrenergic input to spinally-projecting neurons.

TH-ir neurons within the medulla form two continuous rostro-caudal columns – one within its ventrolateral extent, which consists of the noradrenergic A1 neurons and the adrenergic C1 neurons, and the other within its dorsomedial extent, which consists of the noradrenergic A2 neurons and the adrenergic C2 neurons. Within both columns the noradrenergic neurons are located at the caudal poles and the adrenergic ones are located rostrally, with the middle parts of these columns containing intermingled populations of adrenergic and noradrenergic cells (Card et al., 2006). Hence, we carefully chose the sections to analyze these areas, so that the sections that contain the A1 and A2 cell groups (i.e. noradrenergic neurons) would be separated from the ones that contain the C1 and C2 cell groups (i.e. adrenergic neurons) by at least 720 μm to ensure that we were sampling from noradrenergic and adrenergic cell populations (Card et al., 2006, Paxinos and Watson, 2007).

One well-documented source of noradrenergic fibers in the spinal cord is the A7 cell group. Earlier findings have suggested that axons of the A7 neurons terminate mainly in the dorsal horn (Clark and Proudfit, 1991b), although the origin of these fibers was not clear due to the proximity of the A7 neurons to the Kölliker-Fuse nucleus, which also contains spinally-projecting neurons (Tan and Holstege, 1986). A recent report using conditional tract-tracing to produce GFP expression within TH-positive neurons showed that the A7 neurons project to the spinal cord with dense innervation of the ventral horn and the IML (Bruinstroop et al., 2012). These observations suggest that the catecholaminergic A7 neurons make synaptic contacts with spinal motoneurons and sympathetic preganglionic neurons. Our observation of TH-positive PSPMNs within the A7 at the earliest survival time is concordant with those results. Our findings also extend the earlier work, indicating that the major function of the A7 cells may be in the somatomotor-sympathetic integration since the majority of its TH-positive neurons are PSPMNs.

The A5 cell group has previously been shown to send heavy descending projections to the IML (Clark and Proudfit, 1993) and no ascending projections to the forebrain. These cells

were also suggested to be the major central source of sympathetic innervation to the adrenal gland and the pancreas (Strack et al., 1989, Jansen et al., 1997). A more recent study using a conditional tract-tracing approach confirmed this result, reporting that the A5 axons primarily terminate in the IML and in the dorsal commissural region where the majority of sympathetic preganglionic neurons are located; a smaller number of axons was identified in the dorsal and ventral horns (Bruinstroop et al., 2012). In accordance with these observations, we found that the A5 noradrenergic neurons send poly-synaptic projections to the adrenal gland, some of which also sent poly-synaptic projections to the gastrocnemius muscle.

Among the cell groups that we examined SubC contained an especially high percentage of TH-ir neurons that were PSPMN, suggesting that this cell group may play an especially important role in somatomotor-sympathetic integration. Noradrenergic neurons within the SubC have distinct developmental origins from those in the LC, and also project to discrete targets in the forebrain (Robertson et al., 2013). While the dorsal SubC neurons are thought to be part of the coeruleospinal inhibitory pathway, which is one of the main pain control systems (Tsuruoka et al., 2012), less is known about the more ventrally-located neurons. The nearby non-TH-ir glutamatergic neurons of the sublaterodorsal nucleus have been implicated in sleep regulation, however, the potential contribution of the noradrenergic SubC neurons to this function is not clear (Datta and Siwek, 2002, Lu et al., 2006, Simon et al., 2012). Our data extend these previous observations and suggest that the SubC contains a unique population of noradrenergic neurons that may play a role in modulating somatomotor-sympathetic integration.

Functional Considerations

As discussed above, it is likely that catecholaminergic PSPMN at multiple brainstem sites project directly to the spinal where they may synapse onto sympathetic preganglionic neurons and motoneurons. Physiological experiments have documented prominent modulatory effects of adrenergic receptor signaling on the activity of both of these classes of neurons. Recordings from sympathetic preganglionic neurons following norepinephrine administration have revealed dampening of the after-hyperpolarization and emergence of a dose-dependent calcium-dependent after-depolarization that can result in repetitive firing, rhythmic bursting, and rhythmic oscillations in membrane potential (Yoshimura et al., 1986, 1987a, b). Such effects are receptor dependent, so that norepinephrine application elicits excitatory and inhibitory effects via $\alpha 1$ -adrenoceptor-mediated depolarization and an $\alpha 2$ -adrenoceptor-mediated hyperpolarization (Fukuda et al., 1987, Parkis et al., 1995, Carette, 1999). Norepinephrine application also depolarizes motoneurons located throughout the spinal cord and brainstem in slice and *in vivo* recordings (Elliott and Wallis, 1992, Parkis et al., 1995, White et al., 1996). These effects enhance glutamate-evoked motoneuron firing over long periods of time and enhance motoneuron excitability (Funk et al., 1994, White et al., 1996). These actions of norepinephrine lead to an augmentation of motor reflex responses (Stafford and Jacobs, 1990a), which in awake behaving animals occurs following alerting stimuli (*e.g.*, loud clicks) as well as in response to stimuli that activate the vigilance system and the fight-or-flight response (*e.g.*, exposure to a predator or white noise) (Stafford and Jacobs, 1990b). Conversely, given the relative lack of norepinephrine release during

sleep, these mechanisms likely contribute to sleep state atonia and may facilitate the development of obstructive airway pathologies in the case of the hypoglossal motoneurons (Sauerland and Harper, 1976, Okabe et al., 1994, Parkis et al., 1995). Therefore, one function of the spinally-projecting catecholaminergic PSPMNs may be to coordinate parallel increases in the excitability of motoneurons and sympathetic preganglionic neurons during specific behavioral challenges. It is feasible that catecholaminergic PSPMNs within different brainstem regions are recruited by distinct stressors that require a coordinated increase in somatomotor and sympathetic nerve firing. It should be noted that these effects may not be necessarily mediated by catecholamines. For instance, while the C1 neurons express phenylethanolamine-N-methyltransferase and are capable of epinephrine synthesis, they do not appear to actually release epinephrine at the nerve terminals (Sved, 1989). Instead their function appears to be mediated by glutamate release (Holloway et al., 2013).

Limitations

When interpreting results from the present study it is important to keep in mind an important caveat in regards to the tract-tracing methodology that we used. We extended the survival time following PRV injections in short increments to get a sense of the hierarchical organization of the descending somatomotor-sympathetic circuits. There is a strong possibility that the neurons infected with PRV at longer survival times project to the peripheral targets via a synaptic relay located elsewhere in the brain, while the ones infected at shorter survival times send direct projections. However, we cannot definitively conclude this only from increasing the post-inoculation survival times as in our current study. Since the progression of infection depends on the density of synaptic connections within the projection field as well as on the number of intervening synapses (Aston-Jones and Card, 2000, Card, 2001), our results likely indicate that some of the noradrenergic cell groups contain a mixture of neurons with a different density of synaptic connections to the peripheral targets. Nevertheless, the neurons that are infected only at a specific survival time in a given cell group are likely to be connected to the target in the same manner, and they send distinct projections compared to other cells infected at a different survival time.

We report here the total number of TH-ir neurons in multiple adrenergic and noradrenergic cell groups in the brainstem, in which the LC contains the largest number of such neurons. While we examined optically-sectioned images from multiple focal planes, it is unlikely that detected all TH-ir cells within the LC due to their high packing density. Because we probably underestimated that the total number TH-ir LC neurons, it is likely that fraction of these neurons that are PSPMN is lower.

Similarly, because cell groups examined here are located within disparate parts of the brainstem, extend for different rostro-caudal distances, and are characterized by neurons of differing sizes and packing densities, we were not able to utilize systematic random sampling to determine true neuronal population numbers within the same brains. However, we believe that our semi-quantitative sampling is still valid in revealing significant differences both in the number and the percentage of TH-ir PSPMN across the different catecholaminergic cell groups in the brainstem.

Conclusions

Our data indicate that TH-ir PSPMNs are distributed predominantly across three brain regions: LC, SubC, and A7. When compared to other noradrenergic and adrenergic cell groups in the brainstem, SubC and A7 contain significantly greater fraction of TH-ir neurons that are PSPMNs. These findings suggest a prominent role for SubC and A7 in somatomotor-sympathetic integration. Future studies will be required to elucidate the functional role of these neurons.

Acknowledgments

This study was funded by NIMH grant MH081927 (IAK). PRV stocks were provided by Dr. Lynn Enquist from Princeton University from the National Center for Viral Vectors supported by NIH Virus Center grant number P40RR018604. Statistical analysis reported in this article was supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under award number UL1TR001417. We thank Ms. Nateka Jackson for her excellent technical assistance. We also thank Dr. Sarah M. Clinton for her comments on an earlier version of the manuscript.

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Figure 1.

Examples of catecholaminergic presympathetic premotor neurons. Low-magnification images are presented on the left for orientation (column i). Within each region multiple images from the same field of view were digitized at higher magnification (area indicated by white box in column i) to identify immunofluorescent labeling for: tyrosine hydroxylase (column ii), β -galactosidase (projection to adrenal gland; column iii), and GFP (projection to gastrocnemius muscle; column iv). Column v shows merging of the three signals. Each row represents images from a distinct catecholaminergic cell group, including: A1 (A), C1 (B), C3 (C), and A5 (D). All images were taken from animals in the Survival Time Group 2 (see Table 1); arrows indicate triple-labeled neurons. Abbreviations: 7n – facial nerve root; β Gal – β -galactosidase; GFP – green fluorescent protein; IO – inferior olive; IRt – intermediate reticular nucleus; LRt – lateral reticular nucleus; LSO – lateral superior olive; mlf – medial longitudinal fasciculus; rs – rubrospinal tract; s5 – sensory root of trigeminal nerve; sp5 – spinal trigeminal tract; TH – tyrosine hydroxylase. Scale bars: 500 μ m (column i); 50 μ m (columns ii–v).

Figure 2.

Distribution of PRV-infected neurons that co-localize with tyrosine hydroxylase in the locus coeruleus. Distribution of labeling is illustrated at three rostro-caudal levels presented at low-magnification in panels on the left: caudal (A), middle (B), and rostral (C). Higher magnification images taken from the middle portion of the locus coeruleus (indicated by a white box in B) illustrate labeling with PRV-BaBlu (adrenal projections; D), PRV-152 (gastrocnemius projections; E), and merging of the two signals together with cyan fluorescent labeling for tyrosine hydroxylase (F); arrows indicate triple-labeled neurons. Maps on the right illustrate location of PRV-infected neurons that express tyrosine hydroxylase from an animal in the Survival Time Group 2 (G-I) and from an animal in the Survival Time Group 4 (J-L). Maps illustrate labeling from caudal (G, J), middle (H, K), and rostral (I, L) levels of the locus coeruleus as illustrated in panels A–C. Each symbol

represents one neuron from one tissue section. Abbreviations: 4V–the fourth ventricle; 7n–facial nerve; Cb–cerebellum; CG–central gray; LC–locus coeruleus; Me5–mesencephalic trigeminal nucleus; scp–superior cerebellar peduncle. Scale bars: 200 μm (A–C), 50 μm (D–F).

Figure 3.

PRV-infected neurons and their relationship to the tyrosine hydroxylase-immunoreactive neurons within the nucleus subcoeruleus. Low-magnification examples of labeling are presented from caudal (A), middle (B), and rostral (C) anatomical levels. Higher magnification images were taken from the middle anatomical level (region indicated by a solid white box in panel B) and show: neurons infected with PRV-BaBlu (adrenal projections; D), neurons infected with PRV-152 (gastrocnemius projections; E), and merging of the three fluorescent signals to show relationship to tyrosine hydroxylase-immunoreactive

neurons (cyan; F). Arrows indicate triple-labeled neurons. Maps on the right illustrate labeling from animals within Survival Group 2 (G-I) and Survival Group 4 (J-L). Areas shown in each map are indicated by dashed white boxes in panels A-C and correspond to caudal (G, J), middle (H, K), and rostral (I, L) anatomical levels. Each symbol represents one neuron from one tissue section. Abbreviations: 4V–fourth ventricle; Bar–Barrington’s nucleus; CG–central gray; DMTg–dorsomedial tegmental area; mlf–medial longitudinal fasciculus; LPB–lateral parabrachial nucleus; m5–motor root of the trigeminal nerve; Me5–mesencephalic trigeminal nucleus; scp–superior cerebellar peduncle; Mo5–motor trigeminal nucleus; MPB–medial parabrachial nucleus; PnC–caudal pontine reticular nucleus. Scale bars: 200 μ m (A–C); 50 μ m (D–F).

Figure 5.

PRV-infected neurons and expression of tyrosine hydroxylase at different levels of the nucleus tractus solitarius. Images show immunofluorescent labeling within the C2 (A–E) and A2 (F–J) cell groups located at the rostral and caudal levels, respectively. Images at the top (A, F) are low-power photomicrographs of the panels shown below, which illustrate immunofluorescent labeling for tyrosine hydroxylase (B, G), β -galactosidase (C, H), GFP (D, I), and merged (E, J). Arrows indicate tyrosine hydroxylase-immunoreactive presympathetic-premotor neurons within the C2 cell group (B–E). While there is considerable number of presympathetic-premotor neurons within the A2 noradrenergic cell group (double arrows), they do not express tyrosine hydroxylase despite their close proximity to the catecholaminergic neurons. A small number of neurons singly-infected with PRV-BaBlu were co-localized with tyrosine hydroxylase within the A2 (arrowheads). Images were taken from an animal in Survival Group 4 (see Table 1). Abbreviations: 4V 4th ventricle; 10N–dorsal motor nucleus of vagus; 12N–hypoglossal nerve nucleus; cc–central canal; Cu–cuneate nucleus; Gr–gracile nucleus; NTS–nucleus tractus solitarius; sol–solitary tract. Scale bars: 200 μ m (A, F); 50 μ m (B–E, G–J).

Table 1

Experimental Groups.

Rat ID	Post-Injection Survival Times (hours)		Survival Time Group	Analysis Group
	Muscle	Adrenal		
1	96	96	1	Short
2	96	96		
3	96	96		
4	120	96	2	
5	121	96		
6	122	97		
7	132	108	3	Long
8	132	108		
9	136	112		
10	144	111	4	
11	144	112		
12	143	119		

Table 2

Quantification of different classes of neurons. Values show: 1) numbers of tyrosine hydroxylase-immunoreactive neurons, 2) numbers of presympathetic-premotor neurons, 3) numbers of tyrosine hydroxylase-expressing presympathetic-premotor neurons, and 4) percentage of tyrosine hydroxylase-immunoreactive neurons that are presympathetic-premotor neurons across the different cell groups. Cell counts were expressed as an average number of neurons per section and are presented as mean $\pm$ SEM for animals within the short and long survival analysis groups.

Region	Total TH-ir		Total PSPMNs		TH-ir PSPMNs		% TH-ir PSPMNs	
	Short	Long	Short	Long	Short	Long	Short	Long
A1	36.33 $\pm$ 2.37	39.64 $\pm$ 1.87	1.67 $\pm$ 1.21	12.25 $\pm$ 1.98 ^{&†#^}	0.92 $\pm$ 0.52	8.14 $\pm$ 1.24 ^{†#^}	20.01 $\pm$ 2.35 ^{#^}	
A2	51.17 $\pm$ 3.38	58.94 $\pm$ 5.76	1.75 $\pm$ 1.56	11.17 $\pm$ 2.81 ^{&†#^}	0.08 $\pm$ 0.08	2.00 $\pm$ 0.41 ^{†#^}	3.57 $\pm$ 0.83 ^{#^}	
C1	43.83 $\pm$ 2.82	49.93 $\pm$ 1.63	9.17 $\pm$ 5.06	31.19 $\pm$ 4.47	5.50 $\pm$ 2.86	12.94 $\pm$ 1.60 [†]	25.84 $\pm$ 3.09 ^{#^}	
C2	37.50 $\pm$ 4.70	43.50 $\pm$ 3.22	1.33 $\pm$ 1.15	14.92 $\pm$ 1.99 [†]	0.67 $\pm$ 0.67	9.83 $\pm$ 1.43 ^{†#}	22.23 $\pm$ 2.38 ^{#^}	
C3	24.17 $\pm$ 3.38	27.28 $\pm$ 2.23	3.33 $\pm$ 1.17	19.25 $\pm$ 4.69	2.00 $\pm$ 0.82	5.17 $\pm$ 1.34 ^{†#^}	17.93 $\pm$ 3.87 ^{#^}	
A5	29.92 $\pm$ 2.73	24.53 $\pm$ 2.06	7.92 $\pm$ 3.92	15.52 $\pm$ 4.26	4.83 $\pm$ 2.39	10.39 $\pm$ 2.75 ^{†#}	40.30 $\pm$ 8.04	
LC	339.20 $\pm$ 22.99	312.47 $\pm$ 15.19	12.40 $\pm$ 5.05	34.28 $\pm$ 6.26	12.40 $\pm$ 5.05	34.28 $\pm$ 6.26	10.67 $\pm$ 1.40 ^{#^}	
A7	47.83 $\pm$ 9.29	38.72 $\pm$ 4.49	17.50 $\pm$ 5.18	49.72 $\pm$ 9.67	16.00 $\pm$ 4.73	26.69 $\pm$ 3.82	68.46 $\pm$ 6.25	
SubC	23.80 $\pm$ 0.86	26.11 $\pm$ 1.68	10.40 $\pm$ 3.27	31.94 $\pm$ 7.28	7.60 $\pm$ 1.69	18.94 $\pm$ 2.63	70.94 $\pm$ 6.42	

Abbreviations: TH-ir—tyrosine hydroxylase-immunoreactive, PSPMN—presympathetic premotor neuron, LC—locus coeruleus, SubC—nucleus subcoeruleus. Symbols indicate *post-hoc* comparisons within the same survival group and for the same dependent variable:

- [&] – significantly fewer neurons than C1;
- [†] – significantly fewer neurons than LC;
- [#] – significantly fewer neurons than A7;
- [^] – significantly fewer neurons than SubC.

Critical level of significance set using Bonferroni correction.