Patterning of somatosympathetic reflexes reveals nonuniform organization of presynaptic drive from C1 and non-C1 RVLM neurons

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Burke PGR, Neale J, Korim WS, McMullan S, Goodchild AK. Patterning of somatosympathetic reflexes reveals nonuniform organization of presynaptic drive from C1 and non-C1 RVLM neurons. Am J Physiol Regul Integr Comp Physiol 301: R1112–R1122, 2011. First published July 27, 2011; doi:10.1152/ajpregu.00131.2011.—To determine the organization of presynaptic vasomotor drive by phenotypic populations of rostral ventrolateral medulla (RVLM) neurons, we examined the somatosympathetic reflex (SSR) evoked in four sympathetic nerves together with selective lesions of RVLM sympathetic neurons. Urethane-anesthetized (1.5 g/kg ip), paralyzed, vagotomized and artificially ventilated Sprague-Dawley rats (n = 41) were used. First, we determined the afferent inputs activated by sciatic nerve (SN) stimulation at graded stimulus intensities (50 sweeps at 0.5–1 Hz, 1–80 V). Second, we recorded sympathetic nerve responses (cervical, renal, splanchnic, and lumbar) to intensities of SN stimulation that activated A-fiber afferents (low) or both A- and C-fiber afferents (high). Third, with low-intensity SN stimulation, we examined the cervical SSR following RVLM microinjection of somatostatin, and we determined the splanchnic SSR in rats in which presympathetic C1 neurons were lesioned following intraspinal injections of anti-dopamine-β-hydroxylase-saporin (anti-DBH-saporin). Low-intensity SN stimulation activated A-fiber afferents and evoked biphasic responses in the renal, splanchnic, and lumbar nerves and a single peak in the cervical nerve. Depletion of presympathetic C1 neurons (59 ± 4% tyrosine hydroxylase immunoreactivity profiles lesioned) eliminated peak 2 of the splanchnic SSR and attenuated peak 1, suggesting that only RVLM neurons with fast axonal conduction were spared. RVLM injections of somatostatin abolished the single early peak of cervical SSR confirming that RVLM neurons with fast axonal conduction were inhibited by somatostatin. It is concluded that unmethylated RVLM presynaptic neurons, presumed to be all C1, innervate splanchnic, renal, and lumbar but not cervical sympathetic outflows, whereas methylated C1 and non-C1 RVLM neurons innervate all sympathetic outflows examined. These findings suggest that multiple levels of neural control of vasomotor tone exist; methylated populations may set baseline tone, while unmethylated neurons may be recruited to provide actions at specific vascular beds in response to distinct stressors.

catecholamines; somatostatin; anti-DBH-saporin

BULBOSPINAL BAROSENSITIVE sympathoexcitatory neurons in the rostral ventrolateral medulla (RVLM) provide synaptic drive to sympathetic preganglionic neurons (SPN) that control vascular smooth muscle to maintain blood pressure (9, 15, 41). The RVLM also integrates feed-forward and reflex adjustments to vasomotor outflow as central and peripheral inputs converge onto sympathoexcitatory RVLM neurons (3, 38, 44).

The somatosympathetic reflex (SSR) increases sympathetic nerve activity (SNA) in response to a variety of challenges, including exercise and noception (7, 49). Sympathetic activation generated by SSR originates from both spinal and supraspinal levels (4, 45). In anesthetized animals, the short-latency spinal SSR is rarely observed due to tonic suppression by descending supraspinal inputs from several sources, including the RVLM and A5 and A6 regions (10, 11, 19, 55). With an intact neuraxis, the SSR is generated wholly by supraspinal inputs dependent on RVLM circuitry (38). Inhibition of RVLM presynaptic neurons by chemical or electrical lesion (31, 38, 61) or by activation of arterial baroreceptors (22, 24, 26, 33) abolishes the supraspinal component of the SSR. Excitatory sensory inputs from somatic afferents converge on bulbospinal vasomotor RVLM neurons (i.e., those inhibited by baroreceptor inputs) (12, 33, 38, 54). In anesthetized rat, single stimuli applied to somatic afferents activates > 90% of all bulbospinal barosensitive RVLM neurons (38, 54).

Although sympathetic nerves are activated by somatic stimuli, the temporal pattern and the magnitude of sympathetic response differ between sympathetic nerves of the same species (22, 33) or between the same sympathetic nerves across mammalian species (e.g., renal; 18, 39). These observations led to our hypothesis that subgroups of RVLM neurons provide activation of select sympathetic outflows that ultimately innervate the vasculature when the SSR is evoked.

RVLM presynaptic subgroups can be distinguished by their neurochemical content, receptors expression, and/or functional attributes. Approximately 70% of presynaptic neurons are adrenaline-synthesizing (C1), glutamatergic neurons (37, 40, 52). The remaining presynaptic neurons have a non-C1 phenotype with many, but not all, expressing preproenkephalin (6, 51). Furthermore, ∼35% of presynaptic neurons express inhibitory somatostatin 2A receptor, including the RVLM and A5 and A6 regions (10, 11, 19, 55). The proportion of presynaptic RVLM neurons with a vasomotor function is unknown. For instance, a subset of presynaptic C1 cells is barosensitive, with slowly conducting axons and control sympathetic output to epinephrine-secreting adrenal chromaffin cells (29, 36, 57).

The objective of this study, therefore, is to describe the SSR response in different sympathetic outflows to identify distinct projections of RVLM bulbospinal barosensitive neurons by function (fast or slow axonal conduction) and phenotype (C1 or somatostatin-sensitive). This study has four aims: (1) to deter-
mune the effects of somatic afferent A- or A/C-fiber activation on splanchnic SNA with single-pulse sciatic stimuli at graded intensities to describe the subgroups of neurons activated in the RVLM and their time course of activation; 2) to determine the temporal patterns in multiple, simultaneously recorded sympathetic nerves following stimulation of somatic afferents at low (A-fiber) and high (A- and C-fiber) intensities to determine the spinal projection patterns of subgroups of neurons activated in the RVLM; 3) to determine the temporal pattern of the SSR evoked in the splanchnic sympathetic nerve following deple-
tion of one subgroup of RVLM neurons, the bulbospinal C1 cells, using intraspinal injections of antidopamine β-hydroxy-
lyase conjugated to saporin; 4) to determine the temporal pattern of the SSR evoked in the cervical sympathetic nerve after RVLM microinjection of somatostatin, which selectively inhib-
its a subgroup of bulbospinal RVLM neurons.

MATERIALS AND METHODS

Animals

Experiments were carried out on male Sprague-Dawley rats (350–
500 g, n = 41) with the approval of the Royal North Shore Hospital/
University of Technology, Sydney and Macquarie University Animal Care and Ethics Committees and were conducted in accordance with Australian guidelines. Rats were housed at 23°C in open-top cages, initially in groups with a 12:12-h light-dark cycle and with food and water available ad libitum. After microinjection of saporin conjugates into the thoracic spinal cord, animals were housed singularly for a further 2–4 wk.

Electrophysiological Experiments

General preparation. Anesthesia was induced with halothane (5% in O3) followed by urethane (10% in saline, 1.3 g/kg ip). Anesthetic was maintained at a level at which noxious pinch of the tail or hindpaw resulted in < 5 mmHg changes in blood pressure (BP). Supplemental urethane (0.2 ml iv) was administered as required. Body temperature was maintained at 36 to 37.5°C using a thermoregulated heating blanket (Harvard Apparatus, Holliston, MA).

The trachea was cannulated to permit artificial ventilation. The right jugular vein and carotid artery were cannulated to administer drugs and record BP, respectively. Rats were mounted in a stereotaxic apparatus (Kopf Instruments), artificially ventilated with O2-enriched air, immobilized with pancuronium bromide (2 mg/ml, induction: 0.4 mg/ml; maintenance: 0.2 ml/h iv; Astra Pharmaceuticals, Australia) and vagotomized. End-tidal CO2 was maintained at 4–5%. The dorsal medullary surface was exposed by occipital craniotomy for experi-
ments requiring chemical microinjection or electrical stimulation of the RVLM. In saporin-lesioned rats, the right tibial nerve (TN) was isolated, tied, cut, and mounted on bipolar silver stimulating elec-
trodes. In all other experiments, the right or left sciatic nerve (SN) was stimulated instead.

Nerve recordings and electrical stimulation. Whole nerve recordings were made from postganglionic renal and lumbar sympathetic nerves, preganglionic cervical and greater splanchnic sympathetic nerves, and from the L4 and L5 dorsal roots. The left cervical nerve was dissected proximal to the carotid bifurcation, tied with 6-0 silk thread and cut. The left splanchnic, renal, and lumbar sympathetic nerves were approached via a retrosurgical incision, as described previously (5, 6). Dorsal roots were exposed by an L1–L4 laminectomy and removal of the dura.

Nerves were mounted on bipolar, silver-hook electrodes and bathed in paraffin oil. Sympathetic neurograms were amplified, band-pass filtered (0.1–3 kHz), sampled at 2 kHz (1401plus, CED, Cambridge, UK) and recorded using Spike2 software (CED). Dorsal root poten-
tials were sampled at 15 kHz.

Waveform averages of SNA or dorsal root potentials (DRPs) to 50 cycles of SN or TN stimulation (0.2 ms duration, 1–80 V, 0.5, or 1 Hz) were generated. Voltage intensity was increased incrementally from 1 V (subthreshold) to 80 V (supramaximal) at intervals: 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 40, 50, 60, 80 V, and the stimulus was repeated twice at each voltage. The threshold voltage for SSR re-
sponses was ~3–4 V. Stimulus intensities between 4 and 12 V activated A-fiber afferents and were classed as low intensity; > 30 V activated A- and C-fiber afferents and was classed as high intensity (see Fig. 1). In three experiments, stimuli using graded constant current (100–500 μA) were used to determine lumbar sympathetic responses to both RVLM and SN stimulation, consistent with previous studies (20).

RVLM microinjections and electrical stimulation. Single or multi-
barreled glass micropipettes were used for RVLM drug injection.

The RVLM was identified by characteristic > 30 mmHg increases in blood pressure in response to glutamate microinjection. Drugs were then injected bilaterally when required within 1 min of each other. Injection sites were marked by a 50-nl injection of colloidal gold or pontamine sky blue. The lumbar sympathetic response to single-pulse electrical stimulation of the RVLM (n = 3) was determined using a fine-tipped monopolar electrode with negative pulses of 0.6 ms width (100–500 μA) delivered at 0.5 Hz for 100 sweeps. The spinal SSR (n = 6) was determined by performing a medullocervical C1 spinal cord transection. Rats were euthanized with a bolus injection of 3 M KCl iv and the brainstem was removed and placed in fixative (4% formaldehyde in saline) overnight.

Coronal sections (80 μm) were cut using a vibratome, and colloidal gold injection sites were processed using a silver enhancement kit (model SE-100; Sigma-Aldrich, Australia). Sections were mounted sequentially on gelatinized glass slides, dehydrated, counterstained using cresyl violet, and cover-slipped.

Data Analysis

Neurograms were rectified and normalized between the maximum activity of SNA achieved following KCl administration (100%) and the postmortem levels (0%). Waveforms averages of rectified neurograms were constructed from 50 consecutive sweeps and pooled using Microsoft Excel and Graphpad Prism 4.0, as previously described (33). All values are expressed as means ± SE. Student’s paired t-test was used to compare the area under the curve (AUC) of the evoked peaks of SNA elicited by SN stimulation. AUC was quantified as arbitrary units (au). P < 0.05 indicated statistical significance.

Neurotoxin-Treated Rats

Microinjections of saporin conjugates into the thoracic spinal cord. Rats (n = 19) were anesthetized with halothane (5% in O2) followed by injection of pentobarbital sodium (Nembutal; 60 mg/kg ip). Addi-
tional doses of pentobarbital sodium (6 mg/kg ip) were administered if required.

The spinal cord was exposed between levels T1 and T2, and antidopamine-β-hydroxylase-saporin (anti-DβH-SAP) (n = 10; Ad-
vanced Targeting Systems, San Diego, CA) or the control conjugate IgG-saporin (n = 9; Advanced Targeting Systems) was bilaterally injected (12 ng/100 nl, 200 nl per side), targeting the intermediolateral column [lateral, 0.6 mm; ventral, 1 mm; 2 injections per side (100 nl each) separated by >1 mm rostrocaudally]. Following injections, wounds were sutured, and animals were given 5% glucose (1 ml ip) and monitored closely.

Rats were allowed to recover for 18–30 days. Resting systolic blood pressure measurements were made by tail cuff plethysmogra-
phy under light halothane anesthesia before and then 2–3 times per week after the neurotoxin or control injections. Two to four weeks after treatment, baroreflex and SSR responses to TN stimulation were examined under urethane anesthesia, as described above. Sympathetic baroreflex responses to ramp increases in BP were induced by bolus administration of phenylephrine (PE; 10 μg/kg iv). BP and rectified normalized splanchnic SNA (sSNA) were smoothed using 1-s time constants, and baroreflex curves (sSNA vs. BP) were plotted using linear regression (y = mx + b) where the slope value (m) is taken as the sensitivity of the baroreflex. Only data sets with an $r^2 \geq 0.9$ were included.

**Immunohistochemical verification of anti-DβH-SAP lesion.** At the end of recordings, rats were deeply anesthetized with a supplemental dose of urethane (0.5 ml of 10% in saline iv) and were transcardially perfused with 500 ml of DMEM nutrient mixture (cat. no. D8900; Sigma, Australia) followed by 500 ml of 4% formaldehyde in 0.1 M sodium phosphate buffer (NaPB; pH 7.4). The brainstem and spinal cord (C3- T5) were removed and placed overnight in the same fixative and then sectioned coronally (50 μm) on a vibrating microtome. Free-floating brainstem sections were processed for DβH using standard techniques [primary antibody: mouse anti-DβH (1:1,000, Chemicon); secondary: biotinylated donkey anti-mouse IgG (1:500, Rockland)]. Neurons expressing DβH were visualized by treating sections with ExtrAvidin-peroxidase (1:1,000; Sigma, Australia) and a nickel DAB reaction (35).

**Imaging and Quantification**

DβH-immunoreactive (ir) neurons were counted extending caudally from the facial nucleus (−11.3 to −11.9 mm caudal to Bregma) from sections at 200-μm intervals. Images were acquired and processed using a Spot 2 digital camera and software. Counts are expressed as means ± SE.

**RESULTS**

**Afferent Fiber Activation and Sympathetic Response to SN Stimulation**

Figure 1 shows a representative example of simultaneous recordings of sSNA responses (Fig. 1, Ai, Bi, and Ci) and DRPs (Fig. 1, Aii, Bii, and Cii) to SN stimulation at graded intensities. The threshold intensity for the SSR was 3 V (not shown). Low-intensity SN stimulation (4, 8, or 10 V) evoked a reflex increase in sSNA consisting of two distinct peaks (Fig. 1 Ai) with latencies of 82 ± 2 ms and 183 ± 3 ms (n = 3). DRP exhibited a biphasic response (Fig. 1Aii, Table 1), corresponding to the activation of $\alpha/\beta$- and $\delta$-fiber afferents, as described previously (38). The increase in stimulus intensity from 4 to 10 V increased the peak amplitude of both DRP and sSNA. For each intensity (4, 8, 10V), the amplitude of the second splanchnic peak was 42 ± 5% of peak 1.

High-intensity SN stimulation (40, 60, 80 V; Fig. 1Bi) evoked a triphasic splanchnic SSR (Fig. 1Bii) with a latency to
peak at 83 ± 2 ms, 181 ± 4 ms, and 293 ± 4 ms. The amplitude of peak 2 roughly doubled (240 ± 75%) that of peak 1; peak 3 was half that of peak 1 (60 ± 22%). The DRP for the same intensities yielded the biphasic response present at lower intensities and a late response (Fig. 1Bi, Table 1) corresponding to activation of C-fiber afferents, as described previously (38). The threshold for this late response was 30–40 V (n = 2).

**Sympathetic Responses to Electrical Stimulation of RVLM vs. SN**

Single-pulse stimulation (200 μA, 0.6 ms duration, 0.5 Hz, 100 stimuli; n = 3) of the RVLM (Fig. 2A) evoked a biphasic increase in lumbar SNA (Fig. 2B). The latencies to peak lumbar SNA were 94 ± ms and 216 ± ms, consistent with previous reports (16). Low-intensity single-pulse SN stimuli (500 μA, 0.2 ms duration, 0.5 Hz, 50 stimuli; n = 3) also evoked a biphasic increase in lumbar SNA with latencies to peak activity of 117 and 238 ms. The interval to peak lumbar SNA after RVLM stimulation preceded the lumbar SSR by ~25 ms (Fig. 2, grey shading) for both early and late peaks.

**SSRs in Multiple Sympathetic Outputs**

Responses to SN stimulation were recorded in combinations of sympathetic nerves (Fig. 3): cervical (cSNA), sSNA, renal (rSNA), and lumbar (lSNA).

Figure 3 (left) shows at low-intensity (6–10 V) that SN stimulation evoked a single peak in cervical SNA with a latency of 93 ± 2 ms (n = 5). SN stimulation evoked a biphasic response in splanchnic, renal, and lumbar SNA with peak latencies of 83 ± 2 ms and 184 ± 4 ms (splanchnic, n = 5); 115 ± 1 ms and 214 ± 6 ms (renal, n = 4); 118 ± 2 ms and 240 ± 4 ms (lumbar, n = 6). The amplitude of splanchnic peak 2 was 42 ± 5% of peak 1 (n = 5); AUC of splanchnic peak 2 was 60% of peak 1 (0.3 ± 0.1 vs. 0.2 ± 0.1 au², n = 5; Fig. 4A). By contrast, the amplitude of peak 2 for renal and lumbar SSRs was greater than peak 1: 123 ± 12% (n = 4) and 144 ± 48% (n = 6), respectively. Similarly, the AUC of peak 2 for renal and lumbar SSRs were 178% (0.4 ± 0.2 vs. 0.7 ± 0.2 au², n = 4) and 143% (0.3 ± 0.1 vs. 0.4 ± 0.1 au², n = 6) greater than peak 1, respectively (Fig. 4A). After spinal C1 transection, the splanchnic SSR yielded a single volley of SNA at a latency of 32 ± 1 ms, consistent with a spinal SSR mediated A-fiber afferent activation.

Figure 3 (right) shows high intensities (40–60 V), the cervical SSR yielded a biphasic activation of SNA with latencies to peak of 94 ± 3 ms and 186 ± 6 ms (n = 5), whereas the splanchnic, renal, and lumbar SSR became triphasic. Peak latencies were: splanchnic 79 ± 2 ms, 175 ± 4 ms and 298 ± 3 ms (n = 5); renal 113 ± 2 ms, 214 ± 6 ms, and 323 ± 4 ms (n = 4); lumbar 115 ± 1 ms, 238 ± 4 ms and 330 ± 6 ms (n = 6). After spinal C1 transection, the splanchnic SSR yielded a biphasic activation of SNA at a latencies of 33 ± 3 ms and 125 ± 4 ms (n = 5) consistent with the coactivation of A- and C-fiber afferents, respectively. Figure 4B summarizes the AUC of the sympathoexcitatory peaks evoked at high-intensity SN stimulation.

**DeSTRUCTION OF THE BULBOSPINAL C1 CELL POPULATION WITH ANTI-DBH-SAP**

DBH-ir neurons extended caudally from the caudal pole of the facial nucleus (~11.3 mm caudal to Bregma) to 1 mm caudal to the obex (~15.3 mm Bregma). Intraspinal treatment with 24 ng/side of anti-DBH-SAP depleted 59 ± 4% (range 44 to 66%) of the DBH-ir neurons in the rostral RVLM (~11.3 to ~11.9 mm Bregma) compared with animals treated with IgG-saporin, similar to previous reports (47) (Fig. 5A). The DBH-ir cell population extending caudally was not significantly altered. However, a marked depletion of catecholaminergic neurons in the pontine A5 region (Fig. 5B) and the locus coeruleus was also evident; the extent of this cell loss was not quantified.

Tail cuff recordings of systolic pressure between treatment groups during the 2–4 wk recovery period were not significantly different. Under urethane anesthesia, resting MAP for both C1-depleted and control rats during the 2–4 wk recovery period were not significantly different. Under urethane anesthesia, resting MAP for both C1-depleted and control rats were not significantly different. Under urethane anesthesia, resting MAP for both C1-depleted and control rats (90.6 ± 2.0 mmHg, n = 5) were also not significantly different. The gain of the sympathetic baroreflex in IgG-saporin treated rats was 4.13 ± 0.27%/mmHg and in anti-DBH-SAP-treated rats was 1.83 ± 0.59%/mmHg, a 55% reduction (P < 0.05).

**Effects of Bulbospinal C1 Lesion on the Splanchnic SSR**

Low-intensity TN stimulation (6–8 V) evoked a biphasic volley of splanchnic SNA in IgG-SAP-treated rats (Fig. 5C). Anti-DBH-SAP pretreatment eliminated peak 2 of SSNA responses to TN stimulation (6–8 V) and reduced the amplitude and duration of peak 1, although the onset latencies of peak 1 response were unchanged. Furthermore, anti-DBH-SAP pretreatment, but not IgG-SAP, partially unmasked the spinal SSR (grey shading).

**FIG. 2.** Averaged lumbar SNA (B) evoked following single-pulse electrical stimuli applied to the rostral ventrolateral medulla (RVLM) (200 μA, 0.2 ms, 0.5 Hz; A) or to the SN (500 μA, 0.2 ms, 0.5 Hz) in 3 rats. The grey shaded box indicates a 25-ms interval. The delay between RVLM stimulus-induced peaks and SN-induced peaks remains constant at ~25 ms.
Low-intensity SN Stimulation after Chemical Inhibition of the RVLM

Somatostatin (100 nl, 1.5 mM, \( n = 4 \)) injected bilaterally at pressor sites in the RVLM (Fig. 6A) abolished peak 1 of the cervical sympathetic nerve evoked by low-intensity stimulation of the SN (Fig. 6B).

DISCUSSION

The major findings of this study are as follows. SN stimulation at intensities sufficient to evoke short latency responses in the dorsal root, corresponding to the activation of Aα/β- and Aδ-fiber afferents, evokes qualitatively different SSR responses consisting of monophasic or biphasic bursts of SNA that are unique to the output measured. High-intensity SN stimulation activates an additional group of C-fiber afferents and evokes biphasic or triphasic bursts of SNA depending on the output measured. From these data it was hypothesized that biphasic SNA responses to low-intensity SN stimulation are generated by A-fiber inputs driving two classes of bulbospinal sympathoexcitatory RVLM neurons with fast or slow axonal conduction. Bimodal axonal conduction of bulbospinal sympathoexcitatory neurons was supported by observing identical latencies to peak lumbar SNA evoked by either electrical stimulation of the SN or the RVLM. Lesioning a significant population of bulbospinal C1 neurons with intraspinal anti-DβH-SAP abolished peak 2 of the splanchnic SSR and reduced the amplitude and duration of peak 1. RVLM injections of somatostatin abolished peak 1 of the cervical SSR and consistent with our previous observations (6) that somatostatin abolishes the first, but not the second, peak of the splanchnic SSR.

From these data it is concluded that the fast descending volley is generated by activity of myelinated C1 and non-C1 bulbospinal RVLM neurons that are sensitive to somatostatin, and the slow response is composed of unmyelinated C1 neurons. These data also show that unmyelinated...
RVLM presympathetic neurons, presumed to be all C1, innervate splanchnic, renal, and lumbar but not cervical sympathetic outflows, whereas myelinated C1 and non-C1 neurons innervate all sympathetic outflows examined. These findings indicate that myelinated RVLM presympathetic neurons have an output distribution that would make them ideal candidates for generating basal sympathetic vasomotor tone.

Sciatic Afferent Stimulation and Corresponding SSR

Our data show that low-intensity, single-pulse SN stimuli activate Aαβ- and Aδ-fiber afferents and evoke two peaks in splanchnic SNA. High-intensity, single-pulse SN stimuli activates both A- and C-fiber afferents resulting in a triphasic response in splanchnic SNA. We observed a 10-fold difference in stimulus thresholds and similar conduction velocities for each afferent fiber type recorded within the dorsal rootlet, as originally described (38). We also confirm that biphasic activation of SNA correlated with the stimulation of A-fibers. We report for the first time that a third sympathetic peak that emerged with suprathreshold stimulation of unmyelinated afferent C-fibers. Some methodological differences, including wider band-pass filtering (0.1–3 KHz vs. 0–50 Hz) and longer analysis of poststimulus periods (~100 to 800 ms) may indicate why this was not reported by Morrison and Reis (38). Nonetheless, there is evidence from other studies of a third 260- to 300-ms peak in rat SSR following high-intensity somatic nerve stimulation, including cardiac (23, 26, 27), splanchnic, and lumbar sympathetic nerves (34). Several observations provide evidence that a triphasic SSR response is generated by bimodally distributed RVLM presympathetic drive activated by both A- and C-fiber afferents.

First, RVLM neurons receive excitatory inputs from both A- and C-fiber afferents. The temporal response pattern of bulbospinal barosensitive units has been consistently reported to exhibit mean latencies to activation of ~20 ms (A-fiber) and ~110 ms (C-fiber); the latter period of activation occurs within a poststimulus range of 90 to 150 ms (12, 33, 38). For instance, Morrison and Reis (38) showed that 95% of barosensitive bulbospinal RVLM neurons (n = 37/39) were activated by the A-fiber input with a mean response latency of 17 ms. Of those 37 cells, 81% (n = 30) exhibited a latter period of excitation with a mean latency of 115 ms.

Second, bulbospinal sympathoexcitatory RVLM neurons exhibit bimodal axonal conduction. In the present study, single-pulse stimulation of the RVLM evoked a biphasic increase in lumbar SNA with latencies to peak activity of 94 ms and 216 ms, similar to that described previously (16, 20). Biphasic SNA responses are attributable to the activation of myelinated and unmyelinated bulbospinal RVLM neurons with mean axonal conduction velocity of ~3.5 m/s and ~0.8 m/s, respectively (20, 37). The lumbar SNA response to RVLM stimulation was compared with the lumbar SNA response to low-intensity SN stimulation, which also evoked a biphasic increase with latencies to peak activity of 117 and 238 ms. The interval to peak lumbar SNA after RVLM stimulation preceded the lumbar SNA after SN stimulation by ~23 ms for both early and late peaks. This 23-ms interval is equal to the latency to early excitation of RVLM neurons following SN stimulation (12, 33, 38, 54). Our data also suggests that the transit time from lamina I cells in the dorsal horn to the RVLM was ~15 ms and would necessitate a monosynaptic projection with fast axonal conduction at ~10 ms, as previously suggested by Zagon and Hughes (60).

Thus our model shows (Fig. 7), in keeping with the original scheme proposed by Morrison and Reis (38), that stimulation of A-fiber afferents in the SN simultaneously excite fast- and slow-conducting bulbospinal, sympathoexcitatory RVLM neurons (Fig. 7Ai) resulting in a biphasic increase in splanchnic SNA with a latency to peak of ~90 ms (peak 1) and ~190 ms (peak 2) (Fig. 7Ai). Stimulation of C-fiber afferents excites a subset of fast- and slow-conducting bulbospinal, sympathoexcitatory RVLM neurons, ~100 ms after the A-fiber volley (Fig. 7Bi). C-fiber stimulation activates fast-conducting RVLM neurons, whose descending excitation is summed with that of slow-conducting RVLM neurons activated by A-fiber afferents, significantly increasing the burst amplitude of peak 2 (~190 ms). C-fiber stimulation also activates slow-conducting RVLM neurons that contribute peak 3 with a latency of ~290 ms (Fig. 7Bii).
Unique Patterns of the SSR Recorded in Sympathetic Nerves

The temporal patterns evoked in multiple, simultaneously recorded sympathetic nerves at low (A-fiber) and high (A- and C-fiber) intensities were determined. In agreement with previous studies, we observed a single early peak response in the cervical SNA (33) and biphasic SSR responses in the splanchnic (38), renal (39), and lumbar SNA (25, 34) with low-intensity SN stimulation. The relative amplitude of the peaks making up the biphasic responses was dependent upon the sympathetic nerve type.

We describe for the first time in rat the expression of C-fiber-driven SSR in all nerves examined. As discussed above, A- and C-fiber inputs converge on the same population of RVLM neurons (33, 38). Hence, the SNA responses observed at high-intensity SN stimulation was a duplicate of the low-intensity response, with the additional C-fiber-generated volley offset by ~100 ms. For example, the cervical SSR exhibited a monophasic volley of SNA (93 ms latency) with low-intensity SN stimulation and became biphasic (94 ms and 186 ms) with high-intensity SN stimulation. Similarly, biphasic renal and lumbar responses became triphasic at high intensities. Thus, the unique patterns of SSR reflect nonuniformity of RVLM premotor drive to SPN. Furthermore the SSR also accurately represent the afferent input(s) to RVLM presympathetic neurons. RVLM single unit recordings show that C-fiber inputs were half as strong (200% vs. 400% increase in basal activity) but longer in duration than A-fiber inputs (12, 33, 38). This pattern of activation is mirrored in the cervical SSR, where C-fiber-evoked sympathoexcitation was half as intense and relatively longer in duration than the A-fiber-evoked sympathoexcitation.

Projection Patterns of Bulbospinal Sympathoexcitatory RVLM Neurons

The major objective of this study was to establish whether single-pulse SN stimuli evoked different patterns of SNA that originate from the activity of distinct RVLM sympathoexcitatory populations projecting to SPN. To test this idea, we compared the sympathetic responses to low-intensity SN stimulation in several nerves as well as across two treatment groups: intraspinal anti-D\textsubscript{1}H-SAP and RVLM injections of somatostatin. The interpretation of our data can be restricted to the projection patterns of bulbospinal barosensitive RVLM neurons for several reasons: 1) A-fiber afferent stimulation activates most (>90%) of bulbospinal barosensitive neurons (38); 2) baroreceptor activation abolishes supraspinally generated SSR (22, 24, 26, 33), indicating somatic afferents selectively target bulbospinal barosensitive RVLM neurons; 3) inhibition of the RVLM with bilateral injections of muscimol abolishes the supraspinally generated SSR (6, 38); and 4) bulbospinal neurons in other brainstem regions, including the midline raphé (32, 42), A5, locus ceruleus and lateral parabrachial nucleus (38, 50), and higher brain structures (38) make no significant contribution to SSR.

Fig. 5. Following intraspinal IgG-SAP or anti-D\textsubscript{1}H-SAP responses, splanchnic sympathetic responses were evoked following stimulation of tibial nerve (TN). Dopamine \textsubscript{\beta}-hydroxylase immunoreactivity is shown in the RVLM (A) and A5 region (B) following intraspinal IgG-SAP or anti-D\textsubscript{1}H-SAP. C, somatosympathetic reflex (SSR) evoked with low-intensity single-pulse TN stimulation (≈8 V, 0.2 ms, 0.5 Hz, 50 stimuli). IgG-SAP (n = 4) had no effect on the early or late supraspinal volleys observed in splanchnic nerve; anti-D\textsubscript{1}H-SAP (n = 7) eliminated the late supraspinal peak as well as much of the early peak response, but spared supraspinal inputs with the shortest latency (fastest axonal conduction). Anti-D\textsubscript{1}H-SAP also unmasked the spinally generated sympathoexcitatory response to TN stimulation (shaded line). The effects of SN nerve stimulation on splanchnic nerve in rats spinally transected at the C1 spinal level are shown for comparison. Data are mean (black line) ± SE (grey line).
Our SSR data indicate that bulbospinal barosensitive RVLM neurons with fast axonal conduction provide drive to all sympathetic outflows examined. By contrast, premotor drive arising from unmyelinated bulbospinal barosensitive RVLM neurons was pronounced in renal and lumbar sympathetic outflow, was relatively weak in splanchnic sympathetic and absent from cervical sympathetic outflow. A monophasic early peak response is also characteristic of the cardiac SSR (27), suggesting that unmyelinated RVLM projections to upper thoracic SPN are sparse.

Our study is the first to examine the SSR following ablation of bulbospinal C1 RVLM cell population with anti-DβH-SAP (30, 43, 47, 48, 59). A depletion of ~60% of C1 neurons within 800 μm caudal to the facial nucleus was produced, which contains the bulk of bulbospinal C1 neurons (40, 47). The lesions in individual animals were within the range of individual C1 depletions reported in other studies (44–89%) using intraspinal injection of the toxin, albeit lower than average depletions (61–74%) previously reported (47, 48, 59). Whether our use of DβH-ir to reveal unlesioned cells, as opposed to phenylethanolamine N-methyl transferase immunoreactivity, alters the visible depletion at the time intervals following injection of the toxin is difficult to know. We suggest that the depletion was, in fact, underestimated as significant functional loss was evident in the study that was, if anything, slightly greater than the deficits in baroreceptor gain seen in previous studies (47), and the variability in functional responses between animals was small. Furthermore, this depletion of C1 neurons eliminated peak 2 of the splanchnic SNA and reduced peak 1 again demonstrating significant functional effectiveness of such lesions. Only the shortest latency activity was present in the first supraspinal peak, suggesting that RVLM neurons with the fastest axonal conduction velocities were spared by the toxin. This finding is supported by previous studies that showed that bulbospinal RVLM neurons with slow (unmyelinated) axonal conduction are mostly, if not exclusively, catecholaminergic, whereas <50% of bulbospinal RVLM neurons with fast (myelinated) axonal conduction are catecholaminergic (44, 46, 51, 58).

In addition, our data show that RVLM injections of somatostatin eliminated the cervical SSR. This is consistent with our previously reported findings that somatostatin selectively abolished peak 1 of the splanchnic SSR, but did not attenuate peak 2 (6) and provides further evidence that somatostatin selectively inhibits bulbospinal RVLM neurons with fast axonal conduction.

**Tonic Suppression of the Spinal SSR**

A very short-latency spinal SSR was partially unmasked after lesion of bulbospinal catecholaminergic neurons. Similar responses have been described by us and others after inhibition of the RVLM (6, 10, 23, 38). The mechanism by which RVLM neurons gate the spinal SSR remains unknown. Other sources of descending tonic suppression of spinal SSR may also arise from A5 or A6 neurons as many catecholamine neurons in these regions were also destroyed by spinal treatment with anti-DβH-SAP, as described previously (47). Both A5 and A6 neurons project to the dorsal horn of the spinal cord (21, 55) and inhibit dorsal horn neurons through their actions on postsynaptic α2-adrenergic receptors (53). For example, low-intensity SN stimulation strongly activates contralateral A6 neurons with a brief latency of 14–18 ms (17) and increases norepinephrine release at the level of the dorsal horn, emanating from A6 neurons (19).

**Conclusions**

Thus we have demonstrated that stimulation of somatic A-fibers activates both myelinated and unmyelinated bulbospinal barosensitive sympathoexcitatory RVLM neurons and drives mono- or biphasic bursts in SNA, depending on the output measured. When C-fiber afferents are additionally recruited, activation of both RVLM populations with a consistent delay of ~100 ms results in the generation of bi- or triphasic bursts of SNA. Using this information, combined with chemical or neurotoxic lesions, we have demonstrated that unmyelinated RVLM (C1 neurons) have limited spinal SPN projections, with predominant innervation of lower thoracic outflows. In contrast, RVLM neurons with fast axonal conduction and express somatostatin receptors drive sympathetic vasomotor outflow at all levels of the spinal cord.

**Perspectives and Significance**

Sympathetic vasomotor activity is defined by its ongoing activity or tone and cardiac rhythmicity (2), and it is reflected in recordings from all segments of thoracolumbar spinal cord. Our observations advance the idea that basal sympathetic vasomotor tone is derived from a subpopulation of RVLM presympathetic neurons with myelinated axons (15, 56). It is known that rapidly conducting RVLM neurons generate four
times as much activity at rest compared with slowly conducting neurons \([19 \pm 3 \text{ vs. } 4 \pm 1 \text{ Hz}; \ P < 0.05, \text{ (see Refs. } 1, 46)\] making them ideal candidates to maintain basal levels of vasomotor tone. Our data shows that these neurons innervate all levels of the sympathetic outflow. Finally, we have demonstrated that somatostatin microinjection in the RVLM selectively inhibits this fast-conducting RVLM population as demonstrated in the present study and eliminates vasomotor tone (6). The relative contributions/roles of myelinated C1 and non-C1 phenotypes to the genesis of basal tone remain to be determined. What then is the primary role of slowly-conducting barosensitive, bulbospinal RVLM neurons, for which our data demonstrates preferential innervation of lower thoracic outflows? These unmyelinated cells are exclusively C1 and represent the majority of bulbospinal, barosensitive C1 cells (44, 46). As elimination of the C1 population has little bearing on vasomotor tone (30, 48), but causes significant deficits in sympathetic reflexes (29, 47), we suggest that unmyelinated RVLM neurons are recruited to provide sympahtoactivation in response to distinct stressors. In keeping with this idea, hypotension (hydralazine or sodium nitroprusside) produces fos labeling only in SPN of lower thoracic spinal cord (13). Furthermore, under similar stimulus conditions, 85% of Fos neurons in the RVLM region were C1 (8), although this percentage is controversial (52). We suggest that the detection of Fos in C1 cells, and their target SPN, is largely due to the low/latent activity of these unmyelinated C1 cells at rest. This is in contrast to the above-average spontaneous activity of myelinated RVLM cells that most likely provides basal vasomotor tone.

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