Abstract
To determine the organization of presympathetic vasomotor drive by phenotypic populations of RVLM neurons, we examined the somatosympathetic reflex (SSR) evoked in four sympathetic nerves together with selective lesions of RVLM presympathetic neurons. Urethane-anesthetized (1.3g/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 0.5-1Hz, 1-80V). 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-/C-fiber afferents (high). Third, with low intensity SN stimulation, we examined the cervical SSR following RVLM microinjection of somatostatin (SST), and we determined the splanchnic SSR in rats in which presympathetic C1 neurons were lesioned following intraspinal injections of anti-dopamine-beta-hydroxylase-saporin (anti-DβH-SAP). 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% TH-ir profiles lesioned) eliminated the 2nd peak of the splanchnic SSR and attenuated the 1st peak suggesting that only RVLM neurons with fast axonal conduction were spared. RVLM injections of SST abolished the single early peak of cervical SSR confirming that RVLM neurons with fast axonal conduction were inhibited by SST. It is concluded 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 RVLM neurons innervate all sympathetic outflows examined. These findings suggest that multiple levels of neural control of vasomotor tone exist: myelinated populations may set baseline tone whilst unmyelinated neurons may be recruited to provide actions at specific vascular beds in response to distinct stressors.
Introduction

Bulbospinal barosensitive sympathoexcitatory neurons in the rostral ventrolateral medulla (RVLM) provide synaptic drive to sympathetic preganglionic neurons (SPN) that control vascular smooth muscle in order 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 in response to a variety of challenges, including exercise and nociception (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, 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 presympathetic 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 presympathetic subgroups can be distinguished by their neurochemical content, receptors expression and/or functional attributes. Approximately 70% of presympathetic neurons are adrenaline-synthesizing (C1) glutamatergic neurons (37, 40, 52). The remaining presympathetic neurons have a non-C1 phenotype with many, but not all, expressing preproenkephalin (PPE) (6, 51). Further, approximately ~35% of presympathetic neurons express inhibitory somatostatin 2A receptor (sst2A), including ~54% of bulbospinal C1 and ~30% of bulbospinal PPE expressing neurons (6). Functionally, vasomotor presympathetic RVLM neurons exhibit bimodally distributed axonal conduction: lightly myelinated (1-8
m/s) or unmyelinated (<1 m/s) (37). Those with unmyelinated spinal axons appear to be exclusively C1 (44, 46). Conversely, barosensitive cells with rapidly conducting spinal axons are mainly non-C1 (28, 44, 46, 47, 51). The proportion of presympathetic RVLM neurons with a vasomotor function is unknown. For instance, a subset of presympathetic C1 cells is baroinsensitive, 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 in order to identify distinct projections of RVLM bulbospinal barosensitive neurons by function (fast or slow axonal conduction) and phenotype (C1 or SST-sensitive). This study has four aims:

1. To determine the effects of somatic afferent A- or A/C-fiber activation on splanchnic sympathetic nerve activity with single pulse sciatic stimuli at graded intensities in order 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 in order 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 depletion of one subgroup of RVLM neurons, the bulbospinal C1 cells, using intra-spinal injections of anti-dopamine β hydroxylase conjugated to saporin.

4. To determine the temporal pattern of the SSR evoked in the cervical sympathetic nerve after RVLM microinjection of SST which selectively inhibits a subgroup of bulbospinal RVLM neurons.
Materials and Methods

Animals
Experiments were carried out on male Sprague-Dawley (SD) 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 hour 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 weeks.

Electrophysiological experiments

General preparation
Anesthesia was induced with halothane (5% in O2) 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 (AP). Supplemental urethane (0.2 ml, iv) was administered as required. Body temperature was maintained at 36 - 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 AP, respectively. Rats were mounted in a stereotaxic apparatus (Kopf Instruments, USA), artificially ventilated with O2-enriched air, immobilized with pancuronium bromide (2 mg/ml, induction: 0.4 ml iv; maintenance: 0.2 ml/hr iv, Astra Pharmaceuticals, Australia) and vagotomized. End tidal CO2 was maintained at 4-5%. The dorsal medullary surface was exposed by occipital craniotomy for experiments 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 electrodes. 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 & 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 retroperitoneal 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 Ltd., UK) and recorded using Spike2 software (CED Ltd. Cambridge, UK). Dorsal root potentials were sampled at 15 kHz.

Waveform averages of sympathetic nerve activity (SNA) or dorsal root potentials (DRP) to 50 cycles of SN or TN stimulation (0.2 ms duration, 1 – 80V, 0.5 or 1 Hz) were generated. Voltage intensity was increased incrementally from 1V (sub-threshold) to 80V (supra-maximal) 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 responses was approximately 3-4V. Stimulus intensities between 4 and 12 V activated A-fiber afferents and were classed as ‘low intensity’; >30V activated A- and C-fiber afferents and was classed as ‘high intensity’ (see Figure 1). In three experiments, stimuli using graded constant current (100 – 500 μA) were used to determine lumbar sympathetic responses to both RVLM and sciatic nerve stimulation, consistent with previous studies (20).

**RVLM microinjections and electrical stimulation**

Single or multi-barreled glass micropipettes were used for RVLM drug injection. L-glutamic acid (100 mM, Sigma-Aldrich), somatostatin (SST; 1.5 mM, Auspep, Australia), muscimol (10 mM, Sigma-Aldrich) and colloidal gold (25% v/v; Sigma-Aldrich) were dissolved in phosphate buffered saline (0.1 M PBS, pH 7.4) and pressure injected in 50-100 nl volumes (14). PBS was microinjected as a volume and vehicle control.

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 one minute of each other. Injection sites were marked by 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 0.6ms width (100 - 500 μA) delivered at 0.5 Hz for 100 sweeps. The spinal SSR (n=6) was determined by performing a medullo-cervical C1 spinal cord transection. Rats were euthanized with a bolus injection of 3M KCl iv, the brainstem was removed and placed in fixative (4% formaldehyde in saline) overnight.
Coronal sections (80 µm) were cut using a vibrating microtome and colloidal gold injection sites were processed using a silver enhancement kit (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 post-mortem 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 ± SEM. Students paired t-test was used to compare the area under the curve (AUC) of the evoked peaks of sympathetic nerve activity 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 sodium pentobarbitone (Nembutal, 60mg/kg ip). Additional doses of sodium pentobarbitone (6 mg/kg ip) were administered if required.

The spinal cord was exposed between T1 and T2 and anti-DβH-saporin (n = 10, Chemicon International, USA) or the control conjugate IgG-saporin (n = 9, Chemicon International, USA) was bilaterally injected (12 ng/100 nl, 200 nl per side), targeting the intermediolateral cell column (IML) (lateral 0.6mm, ventral 1mm, 2 injections per side (100nl 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 plethysmography 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 AP were induced by bolus administration of phenylephrine (PE, 10 µg/kg iv). AP and rectified normalized sSNA were smoothed using 1s time constants and baroreflex curves (sSNA versus AP) 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 Dulbecco’s modified Eagle’s medium nutrient mixture (DMEM D8900, Sigma, Australia) followed by 500 ml of 4% formaldehyde in 0.1M 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 (1:1000, Chemicon); secondary: biotinylated donkey anti-mouse IgG (1:500, Rockland, USA). Neurons expressing DβH were visualized by treating sections with ExtrAvidin-peroxidase (1:1000, 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 mean ± SEM.

**Results**

**Afferent fiber activation and sympathetic response to SN stimulation**

Figure 1 shows a representative example of simultaneous recordings of splanchnic SNA (sSNA) responses (Fig 1Ai, Bi and Ci) and dorsal root potentials (DRP) (Fig 1Aii, Bii and Cii) to SN stimulation at graded intensities. The threshold intensity for the SSR was 3V (not shown). Low intensity SN stimulation (4, 8 or 10V) evoked a reflex increase in sSNA consisting of two distinct peaks (Figure 1Ai) with latencies of 82 ± 2 ms and 183 ± 3 ms (n=3). DRP exhibited a biphasic response (Figure 1Aii, Table 1), corresponding to the activation of Aα/β- and Aδ-fiber afferents, as described previously (38). The increase in stimulus intensity from 4 to 10V increased the peak amplitude of both DRP and sSNA. For each intensity (4, 8, 10V), the amplitude of the 2nd splanchnic peak was 42 ± 5% of the 1st peak.
High intensity SN stimulation (40, 60, 80 V; Figure 1Bi) evoked a triphasic splanchnic SSR (Figure 1Bii) with a latency to peak at 83 ± 2 ms, 181 ± 4 ms and 293 ± 4 ms. The amplitude of 2nd peak roughly doubled (240 ± 75%) that of the 1st peak; the 3rd peak was half that of the 1st (60 ± 22 %). The DRP for the same intensities yielded the biphasic response present at lower intensities and a late response (Figure 1Bii, Table 1) corresponding to activation of C-fiber afferents, as described previously (38). The threshold for this late response was 30-40V (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 (Figure 2A) evoked a biphasic increase in lumbar SNA (Figure 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 (Figure 2: grey shading) for both early and late peaks.

**Somatosympathetic reflexes in multiple sympathetic outputs**

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

Figure 3 (left panel) shows at low intensity (6-10V) 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 the 2nd splanchnic peak was 42 ± 5% of the 1st peak (n=5); AUC of the 2nd splanchnic peak was 60% of the 1st peak (0.3 ± 0.1 vs. 0.2 ± 0.1 au², n=5; Figure 4A). By contrast, the amplitude of the 2nd peak for renal and lumbar SSRs was greater than the 1st: 123 ± 12% (n=4) and 144 ± 48% (n=6), respectively. Similarly, the AUC of the 2nd peak 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 the 1st peak, respectively (Figure 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 panel) shows at high intensities (40-60V), 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 co-activation 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-DβH-saporin.

DβH -ir neurons extended caudally from the caudal pole of the facial nucleus (-11.3 mm caudal of Bregma) to 1 mm caudal to the obex (-15.3 mm Bregma). Intraspinal treatment with 24 ng/side of anti-DβH-SAP depleted 59 ± 4 % (range 44 – 66 %) of the DβH-ir neurons in the rostral RVLM (-11.3 to -11.9 mm Bregma) compared to animals treated with IgG-saporin, similar to previous reports (47) (Figure 5A). The DβH -ir cell population extending caudally was not significantly altered. However, a marked depletion of catecholaminergic neurons in the pontine A5 region (Figure 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 week recovery period were not significantly different. Under urethane anesthesia, resting MAP for anti-DβH-SAP treated rats (89.5 ± 3.5 mmHg, n=8) and IgG-SAP treated 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-DβH-SAP treated 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 (6-8V) evoked a biphasic volley of splanchnic SNA in IgG-SAP pretreated rats (Figure 5C). Anti-DβH-SAP pretreatment eliminated the 2nd peak of sSNA responses to TN stimulation (6-8V) and reduced the amplitude and duration of the 1st peak, although the onset latencies of the 1st peak response were unchanged. Furthermore, anti-DβH-SAP pretreatment, but not IgG-SAP, partially unmasked the spinal SSR (grey shading).

Low intensity SN stimulation after chemical inhibition of the RVLM
SST (100 nl, 1.5 mM, n=4) injected bilaterally at pressor sites in the RVLM (Figure 6A) abolished the 1st peak of the cervical sympathetic nerve evoked by low intensity stimulation of the SN (Figure 6B).

**Discussion**

The major findings of this study are as follows. Sciatic nerve (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 sympathetic nerve activity (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 intra-spinal anti-DβH-SAP abolished the 2nd peak of the splanchnic SSR and reduced the amplitude and duration of the 1st peak. RVLM injections of SST abolished the 1st peak of the cervical SSR and consistent with our previous observations (6) that SST abolishes the 1st, but not the 2nd, peak of the splanchnic SSR.

From these data it is concluded that the fast descending volley is generated by the activity of myelinated C1 and non-C1 bulbospinal RVLM neurons that are sensitive to SST, 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 & corresponding SSR*

Our data show that low intensity single pulse SN stimuli activate Aα/β- and Aδ-fibre 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 supratheshold stimulation of unmyelinated afferent C-fibers. Some methodological differences including wider band pass filtering (0.1 – 3 KHz vs. 0-50Hz) and longer analysis of poststimulus periods (-100 to 800 ms) may indicate why this was not reported by Morrison & Reis (38). Nonetheless, there is evidence from other studies of a 3rd 260-300 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.

Firstly, 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 ~20ms (A-fiber) and ~110 ms (C-fiber); the latter period of activation occurs within a post-stimulus range of 90-150 ms (12, 33, 38). For instance, Morrison & 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.

Secondly, bulbospinal sympathoexcitatory RVLM neurons exhibit bimodal axonal conduction. In the current 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 to 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 m/s, as previously suggested by Zagon & Hughes (60).

Thus our model shows (Figure 7), in keeping with the original scheme proposed by Morrison & Reis (38), that stimulation of A-fiber afferents in the sciatic nerve simultaneously excite fast and slow conducting bulbospinal, sympathoexcitatory RVLM neurons (Figure 7Ai) resulting in a biphasic increase in splanchnic SNA with a latency to peak of ~90 ms (1st peak) and ~190 ms (2nd peak) (Fig 7Aii). Stimulation of C-fiber afferents excites a subset of fast and slow conducting bulbospinal, sympathoexcitatory RVLM neurons, ~100 ms after the A-fiber volley (Figure 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 the 2nd peak (~190ms). C-fiber stimulation also activates slow conducting RVLM neurons which contribute the 3rd peak with a latency of ~290 ms (Figure 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 off-set 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 non-uniformity 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: Intra-spinal anti-DβH-SAP and RVLM injections of SST. 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). 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.

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 800μm 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 PNMT-ir, 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 present 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 the 2nd peak of the splanchnic SNA and reduced the 1st peak again demonstrating significant functional effectiveness of such lesions. Only the shortest latency activity was present in the 1st 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 SST eliminated the cervical SSR. This is consistent with our previously reported findings that SST selectively abolished the 1st peak of the splanchnic SSR, but did not attenuate the 2nd peak (6) and provides further evidence that SST 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 post-synaptic 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 bi-phasic bursts in sympathetic nerve activity depending on the output measured. When C fiber afferents are additionally recruited, activation of both RVLM populations with
a consistent delay of ~100ms results in the generation of bi- or tri-phasic bursts of sympathetic nerve activity. 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 on-going activity, or ‘tone’, and cardiac rhythmicity (2), and it is reflected in recordings from all segments of thoraco-lumbar 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 to slowly-conducting neurons (19 ± 3 Vs 4 ± 1 Hz; P < 0.05, (see 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 SST 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 sympathoactivation in response to distinct stressors. In keeping with this idea hypotension (hydralazine or sodium nitroprusside) produces fos labelling only in sympathetic preganglionic neurons 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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**Figure Legend**

**Figure 1** Averaged evoked activity from simultaneously recorded splanchnic SNA and L3 dorsal root potentials following 50 SN stimuli (0.2 ms duration, 0.5 Hz) at intensities ranging from 4 - 80V. **Ai.** Low intensity SN stimulation (4, 8, 10 V) evoked a biphasic increase in SNA. **Ai.** Corresponding dorsal root potentials exhibited two early peaks (I and II) indicative of Aα/β- and Aδ-fibre afferent activity. **Bi.** High intensity SN stimulation (40, 60, 80 V) evoked a triphasic increase in SNA. **Bi.** Dorsal root potentials exhibited two early peaks (I and II) and a third peak (III), indicative of C-fiber afferent activation. **Ci-ii.** Averaged evoked SNA and dorsal root potentials shown at all intensities. Note the increase in amplitude of the 190 and 290 ms volleys of SNA with C-fiber activation.

**Table 1** Characteristics of dorsal root potentials following SN stimulation applied 70 mm distal to the dorsal root recorded. Values are mean ± SEM; n = 3.

**Figure 2** Averaged lumbar SNA (B) evoked following single pulse electrical stimuli applied to the RVLM (200 μA, 0.2 ms, 0.5 Hz (A)) or to the sciatic nerve (SN; 500 μA, 0.2 ms, 0.5 Hz) in three rats. The grey shaded box indicates a 25 ms interval. The delay between RVLM stimulus induced peaks and sciatic nerve induced peaks remains constant at about 25 ms.

**Figure 3** The sympathetic response in multiple sympathetic outputs evoked by stimulation of the sciatic nerve (SN). Responses from cervical (n=5), splanchnic (n=5), renal (n=4) and lumbar (n=6) sympathetic nerves following single pulse SN stimulation (0.2 ms, 0.5-1 Hz, 50 stimuli). **Left Panel:** Low intensity (4-8V) SN stimulation evokes a monophasic response characterized by a single early peak in the cervical sympathetic nerve and biphasic responses characterized by two peaks in the splanchnic, renal and lumbar sympathetic nerves. C1 spinal transection (C1 SpX) eliminated both supraspinally generated peaks and unmasked the spinal sympathetic response characterized by a single very early peak (32 ± 1 ms latency, n=5) and is indicative of A-fiber afferent activation. **Right Panel:** High intensity SN stimulation evokes a biphasic response characterized by two peaks in the cervical sympathetic nerve and triphasic responses characterized by three peaks in the splanchnic, renal and lumbar sympathetic nerves. C1 spinal transection (C1 SpX) eliminated supraspinal inputs; the spinal response exhibited two peaks in the splanchnic nerve (n=5), indicative of A & C fiber afferent activation. Data are mean (black line) ± SEM (grey line).

**Figure 4** Grouped data showing the area under the curve (AUC) of the sympathetic peaks elicited by low (A) or high (B) intensity SN stimulation as shown in Figure 3. **A.** There is no 2nd peak generated in cervical sympathetic nerve whereas in splanchnic nerve the 2nd peak is smaller than the first whereas in renal and lumbar nerve the 2nd peak is larger than the 1st when A fibres are stimulated alone. **B.** Recruiting C-fibres in addition to the A-fibre activation seen in A shows the appearance of an additional peak in all outflows measured. Data are mean ± SEM. Significance is indicated if P < 0.05

**Figure 5** Following intraspinal IgG-SAP or anti-DβH-SAP responses splanchnic sympathetic responses were evoked following stimulation of tibial nerve (TN). Dopamine β-hydroxylase immunoreactivity is shown in the RVLM (A) and A5 region (B) following intraspinal IgG-SAP or anti-DβH-SAP. **C.** Shows the SSR evoked with low intensity single pulse TN stimulation (TN; ~8V, 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-DβH-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\(\beta\)H-SAP also unmasked the spinally generated sympathetic 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) ± SEM (grey line).

**Figure 6** RVLM microinjection of somatostatin (SST) abolishes the response in the cervical sympathetic nerve following stimulation of the sciatic nerve (SN). A. shows the SST (1.5 mM) injection sites B. Responses from the cervical sympathetic nerve following low intensity single pulse SN stimuli before (Bi) and after bilateral injection of somatostatin into the RVLM (Bii; SST, 1.5 mM, 100 nl, n=4). Data are mean (black line) ± SEM (grey line).

**Figure 7** Schematic model illustrating the proposed mechanism by which stimulation of myelinated and unmyelinated somatic afferents generate patterns of splanchnic SNA. Ai. A-fiber inputs simultaneously activate fast (myelinated, thick hatched lines) and slow (unmyelinated, thin lines) conducting bulbospinal, sympathoexcitatory RVLM neurons and evoke a biphasic response in splanchnic SNA (Aii). Unmyelinated bulbospinal RVLM neurons and half of the myelinated bulbospinal neurons are C1 cells and are destroyed by intraspinal anti-DBH-SAP following which activation of SSR evokes only the early part of P1. Bulbospinal sympathoexcitatory neurons expressing the sst2A receptor and exhibiting fast axonal conduction are inhibited by somatostatin following which SSR activation evokes only P2. Bi. C-fiber inputs (grey arrowed dotted lines) activate a subset of fast and slow conducting bulbospinal, sympathoexcitatory RVLM neurons ~100 ms after the A fiber activation (black arrowed lines). Hence, a second biphasic volley activates splanchnic SNA (grey inputs to Peaks 2 & 3). The second sympathetic peak (P2, 190 ms) is a summation of both slow conducting bulbospinal RVLM neurons activated by A-fiber afferents, as well as fast conducting bulbospinal RVLM neurons activated by C-fiber afferents. The third sympathetic peak (290 ms) is generated by slow conducting bulbospinal C1 neurons activated by C-fiber afferents. Bii. Splanchnic SNA triphasic response generated following high intensity somatic afferent stimulation.
References


Figure 1.
<table>
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Table 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.

A. RVLM injection sites

RVLM injection sites

-200 μm Vlm

-400 μm Vlm

1.5 mM SST

B. Cervical

Bi

SN ↓

Bii

SN ↓

SST

control

Time (ms)
Figure 7.