Differential activation of adrenal, renal, and lumbar sympathetic nerves following stimulation of the rostral ventrolateral medulla of the rat

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ABSTRACT

Under acute and chronic conditions the sympathetic nervous system can be activated in a differential and even selective manner. Activation of the rostral ventrolateral medulla (RVLM) has been implicated in differential control of sympathetic output based on evidence primarily in the cat. Although several studies indicate that differential control of sympathetic outflow occurs in other species, only a few studies have addressed whether the RVLM is capable of producing varying patterns of sympathetic activation in the rat. Therefore, the purpose of the present study was to determine whether activation of the RVLM results in simultaneous and differential increases in pre-ganglionic adrenal (pre-ASNA), renal (RSNA) and lumbar (LSNA) sympathetic nerve activities. In urethane-chloralose anesthetized rats, pre-ASNA, RSNA and LSNA were recorded simultaneously in all animals. Microinjections of selected concentrations and volumes of glutamate increased pre-ASNA, RSNA, and LSNA concurrently and differentially. Pre-ASNA and RSNA (in most cases) exhibited greater increases compared to LSNA on a percent basis. By varying the volume or location of the glutamate microinjections, we also identified individual examples of differential and selective activation of these nerves. Decreases in arterial pressure or bilateral blockade of RVLM GABA_A receptors also revealed differential activation, with the latter having a 3-4 fold greater effect on sympathetic activity. Our data provide evidence that activation of the rat RVLM increases renal, lumbar and pre-ganglionic adrenal sympathetic nerve activities concurrently, differentially, and in some cases selectively.
INTRODUCTION

The sympathetic nervous system plays a critical role in resting and reflex control of arterial blood pressure (21). Overactivity of this system can contribute to cardiovascular diseases such as hypertension and heart failure (21; 79). Although the activity of sympathetic nerves can be highly coordinated, there is increasing evidence that suggests that sympathetic activity to distinct cardiovascular targets is affected differentially on both an acute and chronic basis (34; 37; 46). For example, sympathetic nerves are affected differentially during acute changes in blood volume (5; 19; 53; 76). Under more chronic, pathophysiological conditions such as hypertension and heart failure, the activity and responsiveness of a variety of sympathetic nerves may be increased, decreased, or unchanged (34; 49; 78). Determining the mechanisms and central nervous system (CNS) substrates for differential control of sympathetic nerves is important since it will better define the contribution of different sympathetic nerves and CNS sites to a given physiological or pathophysiological process (37; 49).

Several brain regions have been implicated in the generation of differential sympathetic outflow to distinct cardiovascular targets (8; 40; 46) including the periaqueductal gray (6; 30), nucleus tractus solitarius (65), paraventricular nucleus of the hypothalamus (10; 69) and the rostral ventrolateral medulla (RVLM) (17; 21; 41). The RVLM is of important consideration since it is one of the most critical brain regions involved in resting and reflex control of sympathetic activity and blood pressure (13; 21; 51). In addition, the RVLM has been implicated in disease processes associated with sympathetic overactivity including hypertension and congestive heart failure (15; 28; 70; 71; 74; 77). Although sympathoexcitation attributed to the RVLM is typically associated
with vasoconstriction, the RVLM also regulates cardiovascular function via activation of a variety of functional diverse sympathetic outflows (21). For example, activation of pre-ganglionic sympathetic nerves to the adrenal gland elicits release of epinephrine which in addition to vasoconstriction, can produce skeletal muscle vasodilation and increases in cardiac rate and contractility (17; 32; 38). On the other hand, increases in renal sympathetic nerve activity are known to influence Na⁺ reabsorption and renin release as well as mediate renal vasoconstriction (18).

The purpose of the present study was to determine whether activation of the RVLM under a variety of conditions results in simultaneous and differential activation of sympathetic nerve activity to the adrenal medulla, kidney, and hindquarter vasculature. Based on previous data primarily in the cat (17; 33; 39; 40), we hypothesized that activation of the RVLM in the rat would reveal concurrent and differential increases in pre-ganglionic adrenal, renal, and lumbar sympathetic nerve activities.

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals endorsed by the American Physiological Society and published by the National Institutes of Health.

All procedures were described in detail previously with only slight modification (47; 60; 61; 66). Briefly, male Sprague-Dawley rats (300-350g; Harlan, Indianapolis, IN; n = 15) were anesthetized with a mixture of α-chloralose (80 mg/kg) and urethane (500
mg/kg, ip). Animals were connected to a small-animal respirator (SAR-830, CWE, Ardmore, PA), and artificially ventilated via tracheostomy with a 40% oxygen-60% nitrogen mixture. Adequacy of ventilation was determined at the end of the experiment in a subset of animals via analysis of blood gases (Radiometer, ABL500, OSM3; Copenhagen, Denmark). Average values measured were \( P_{O_2} = 140.7 \pm 15.1 \) Torr, \( P_{CO_2} = 38.3 \pm 2.9 \) Torr, and \( pH = 7.36 \pm 0.03 \) (\( n = 7 \)). Arterial pressure was monitored with a catheter in the left femoral artery. Two or three catheters were placed in the left femoral vein for infusion of anesthesia (12–21 mg/kg/h of \( \alpha \)-choloralose and 76–133 mg/kg/h of urethane dissolved in 2.4–4.2 ml/kg saline), and injection of drugs. The level of anesthesia was adjusted for each animal to completely inhibit corneal reflexes and the withdrawal response to firm toe pinch. Rectal temperature was maintained between 37 and 38°C by a water heating pad (Baxter, Pharmceal Division, model K20; Valencia, CA). In each experiment, simultaneous recordings from pre-ASNA, RSNA, and LSNA were performed. The renal and adrenal nerves were exposed retroperitoneally, the lumbar nerve was exposed through a midline abdominal incision, and neural recordings were accomplished as described previously (60; 61; 66). Each nerve was amplified (2,000 –20,000) with bandwidth set at 100-1,000 Hz depending on the level of raw activity recorded. Raw nerve activities were monitored on an oscilloscope. Raw blood pressure and nerve activity signals were digitized and recorded using a Hemodynamic and Neural Data Analyzer (Biotech Products, Greenwood, IN). Nerve activity was rectified and averaged over 1-s intervals and stored on hard disk for subsequent analysis. Background noise was determined 30–60 min after the animal was euthanized. Noise was subtracted from the recordings prior to performing any
calculations of evoked changes in SNA. A direct physiological comparison of the absolute level of nerve activity across nerves is not possible because of non-physiological factors (e.g. nerve electrode contact, size of nerve bundle) and the ambiguity in interpreting how a given increase in voltage in one nerve relates to an increase in voltage in another nerve. Thus all nerve activities were defined to be at their baseline physiological state just prior to their activation. These activities were normalized to 100% and percent change was used to compare the magnitude of increase across nerves from these physiological baselines.

An intravenous bolus injection of a small dose of the short-lasting (1–2 min) ganglionic blocker Arfonad (trimethaphan, 2 mg/kg; Hoffmann- La Roche; Basel, Switzerland) was used initially to estimate the amount of preganglionic activity in ASNA recordings. In all recordings included in this study ASNA increased in response to Arfonad. This was reevaluated at the end of each experiment with hexamethonium (20-30 mg/kg iv) and the data using hexamethonium were used to quantify the ratio of pre-versus post-ganglionic nerve activity in each nerve. According to the criteria established in our laboratory’s previous studies (60; 66; 67), ASNA was considered predominantly preganglionic (pre-ASNA) if the activity remaining after ganglionic blockade at the end of each experiment was ~75%. Average pre-ASNA measured after ganglionic blockade was 104.8 ± 5.8% (n = 15). The increase in pre-ASNA over 100% was likely due to an arterial baroreflex response caused by the decrease in MAP after ganglionic blockade. RSNA was almost completely postganglionic and LSNA was mostly postganglionic since 3.6 ± 1.7% and 30.9 ± 6.2% of the respective activities persisted after ganglionic blockade.
RVLM Microinjections

Rats were placed in a Kopf stereotaxic apparatus, a midline incision was performed at the back of the head, and muscle overlying the base of the skull was dissected. A partial occipital craniotomy was performed and an incision was made through the atlanto-occipital membrane to expose the brainstem. RVLM microinjections were performed similar to previous studies by our laboratory (47) and others (45). Triple-barrel glass micropipettes (outside tip diameter 30-60 μm) were inserted into the brainstem at a 90° angle relative to the brainstem with the aid of a surgical dissecting microscope. Initial stereotaxic coordinates used to locate the RVLM were 1.0 mm rostral and 1.8 mm lateral to calamus scriptorius, and 3.2 mm ventral to the dorsal surface of the medulla. Microinjections were achieved with the aid of a commercial pressure microinjection system (Pneumatic Picopump PV820; WPI; New Haven, CT). We measured microinjection volumes (15-90 nl) directly by monitoring the fluid meniscus in each micropipette barrel using a 150X compound microscope fitted with a calibrated reticule. In all studies, the RVLM was identified functionally by observing a pressor (>15 mmHg) and sympathoexcitatory response to glutamate microinjections (10 mM, 30 nl).

Experimental Protocols

**Direct Glutamatergic Excitation of RVLM**

Concentration-related responses to glutamate-mediated excitation of the RVLM. Glutamate was microinjected into the RVLM unilaterally in a fixed volume (30 nl) at different concentrations (1, 10 and 100 mM or 30, 300, 3000 pmol). Each
concentration of glutamate was microinjected in a random order from separate barrels of the same pipette and a minimum of five minutes of recovery was allowed between responses.

**Volume related responses to glutamate-mediated excitation of RVLM.**

Glutamate was microinjected into the RVLM unilaterally at a fixed concentration (10 mM) at different volumes (15, 30, 60 and 90 nl). Each volume of glutamate was microinjected in a random order from the same pipette barrel and a minimum of five minutes of recovery was allowed between responses.

**Reduction in GABAergic inhibition (Disinhibition) of RVLM**

*Effect of baroreceptor unloading by decreasing MAP.* Animals received ramp infusions of sodium nitroprusside (SNP, 200ug/ml) to decrease MAP until a peak SNA response was achieved in all nerves. Graded constant infusions were performed to produce stepwise (5–8 steps), sustained changes (1–2 min) in MAP to allow adequate time for full expression of steady state sympathetic response. The doses of SNP ranged from 2.5 to 80 μg/kg/min. Total volume administered during decreases in MAP was 300 to 450 μl. At least 30 minutes was allowed for recovery and stabilization of all variables to pre-infusion levels before subsequent injections of bicuculline were performed (see below).

*Effect of bilateral GABA_A receptor blockade in RVLM.* GABA_A receptors were blocked bilaterally with the GABA_A receptor antagonist, bicuculline (5 mM, 90nl or 450 pmol). Bilateral injections occurred within one minute of each other. The concentration of bicuculline used was based on previous studies by us (47) and others (24; 44; 45) in which bicuculline was shown to inhibit activation of endogenous GABA_A
receptors in the RVLM after activation of the NTS or CVLM (45; 47) or by blocking changes in sympathetic nerve activity in response to manipulations in arterial pressure (4; 14; 22; 47; 72). As mentioned above, all animals receiving bicuculline were also tested for baroreflex mediated sympathoexcitation in order to facilitate comparison between these two conditions.

To reduce the overall number of rats, we performed more than one protocol in most of the animals. Due to the short lasting and reversible effects of glutamate, glutamate concentration or volume response curves were generated in six of the eight animals that also received sodium nitroprusside injections and bicuculline microinjections. The remaining two animals received only sodium nitroprusside injections and bicuculline microinjections after identification of the RVLM with a single dose and concentration of glutamate (10 mM, 30 nl). Glutamate concentration and volume response curves were generated in two animals that did not receive sodium nitroprusside injections or bicuculline microinjections. Due to its duration of action and significant pressor and sympathoexcitatory response, bilateral microinjections of bicuculline were always performed as the last protocol.

**Histology**

The RVLM was functionally identified with glutamate microinjections in every animal. In addition, microinjection sites in the RVLM were successfully marked with 2% Chicago sky blue dye (30 nl) in 14 of 17 animals). At the end of the experiments, animals were overdosed with Fatal-Plus euthanasia solution (0.2 ml, Vortech, Dearborn, MI) and brains were removed and placed in 4% phosphate buffered formalin solution.
Following post fixation, brains were transferred to 30% sucrose for a minimum 48-hour infiltration. The hindbrain was frozen and cut into 50 μm sections on a cryostat. Alternating coronal sections were mounted on gel-coated slides. One set of slides was stained with neutral red to reveal surrounding structures. A bright-field microscope (Olympus BH-2, Center Valley, PA) was used to determine the center of the dye spot from the unstained sections, and the caudal pole of the facial nucleus was determined from the alternate sections stained with neutral red. Dye spot locations were reconstructed graphically by placing them on a series of sections modified from a rat brain atlas (50).

**Drugs**

α-chloralose, urethane, L-glutamate, bicuculline methiodide, and sodium nitroprusside were obtained from Sigma Chemical (St. Louis, MO). All drugs for microinjection were dissolved directly in filtered artificial cerebrospinal fluid (aCSF). aCSF was pH adjusted to 7.3-7.5 using sodium hydroxide or hydrochloric acid. Glutamate was short acting with recovery typically within 5 minutes or less.

**Data analysis**

Mean arterial pressure (MAP) and heart rate (HR) changes across concentrations or volumes of glutamate were analyzed by one-way analysis of variance (ANOVA) with repeated measures. Responses of the three different nerves relative to the concentrations or volumes of glutamate were analyzed by two-way analysis of variance (ANOVA) with repeated measures. When ANOVA indicated a significant interaction, differences between individual means were assessed by post hoc Holm-Sidak test according to a commercially available software package (SigmaStat 3.0, SPSS Inc.,
Chicago, IL). Responses of the three nerves to baroreceptor unloading with nitroprusside were initially compared to RVLM microinjections of bicuculline using two-way analysis of variance (ANOVA) with repeated measures. Since variances observed in nitroprusside and bicuculline protocols were highly different and obscured differences observed across nerves previously observed with nitroprusside, these data were analyzed separately using one way ANOVA with repeated measures. A probability of \( p < 0.05 \) was considered statistically significant and data are expressed as mean ± s.e.m.

RESULTS

**Direct Glutamatergic Excitation of RVLM**

*Concentration-Related Responses to Glutamate-Mediated Excitation of RVLM.* Varying concentrations of glutamate (1, 10 and 100 mM or 30, 300, 3000 pmol) in a fixed volume (30 nl) were microinjected into the RVLM to determine the effect of increasing concentrations on MAP, HR, pre-ASNA, RSNA, and LSNA. Examples of these injections in a single animal are shown in Figure 1 and the average responses across all animals are shown in Figure 2. Glutamate increased MAP and activated all three nerves. There was a significant main effect of dose, nerve recorded, and interaction between dose and nerve (\( p < 0.05 \) for each). Only pre-ASNA had a significant increase between Glu 10 mM and Glu 100 mM; whereas significant increases between Glu 1 mM and Glu 10 mM occurred for both RSNA and pre-ASNA. Finally, when comparing responses to Glu 1 mM and Glu 100 mM, all three nerves responded to a significantly greater extent at the higher dose. When comparing between nerves at each concentration, increases in pre-ASNA were significantly greater at the highest
concentration of glutamate (100 mM) compared to RSNA and LSNA, and increases in RSNA were significantly greater than increases in LSNA.

**Volume-related Responses to Glutamate-Mediated Excitation of RVLM.**

Varying volumes of glutamate (15, 30, 60, 90 nl or 150, 300, 600, 900 pmol) in a fixed concentration (10 mM) were microinjected into the RVLM to determine the effect of volume of injectate on MAP, HR, pre-ASNA, RSNA, and LSNA. Individual examples of these injections in a single rat are shown in Figure 3 and the average values across animals are shown in Figure 4. Increasing volumes of glutamate into the RVLM produced a general pattern of increasing SNA in each nerve. There was a significant overall effect of volume and nerve recorded as well as a volume/nerve interaction (all p<0.05). Only increases in pre-ASNA were significantly augmented when the volume of glutamate was increased from 15 nl to 30 nl and 30 nl to 90 nl. Increases in both RSNA and pre-ASNA were significantly augmented when comparing 15nl to 60nl or 15 nl to 90nl. Finally, increasing the volume of glutamate microinjection had no significant effect on the increases in LSNA at any volume tested.

When comparing between nerves at each volume, increases in pre-ASNA and RSNA were greater than increases in LSNA at 30, 60 and 90 nl of glutamate. Injections of increasing volumes of artificial cerebrospinal fluid (15, 30, 60, 90 nl) into the RVLM of a subgroup of animals (n=4) produced very little or no change in MAP, pre-ASNA, RSNA, and LSNA (data not shown). These results are similar to our previous study using 90 nl vehicle injections in the RVLM (47).

**Responses to infusion of SNP versus microinjection of bicuculline.**

Animals received ramp infusions of sodium nitroprusside to decrease MAP until peak
SNA responses were observed. An example of a ramp infusion of sodium nitroprusside in an individual animal is shown in Figure 5 and average values are shown in Figure 7. As arterial pressure was lowered, there was a reflex activation of all three nerves, however, increases in pre-ASNA were significantly greater than increases in LSNA. Differences in the increases in RSNA compared to increases in pre-ASNA or LSNA did not reach significance.

In the same animals that had received infusions of nitroprusside, GABA\textsubscript{A} receptors were blocked bilaterally in the RVLM with bicuculline (5 mM, 90nl or 450 pmol each side). An example of bilateral injections of bicuculline in the RVLM of one animal is shown in Figure 6 and the average data in all rats is shown in Figure 7. As expected, MAP increased along with substantial activation of all three nerves. Increases in pre-ASNA were significantly greater than LSNA following either bicuculline or SNP and increases in RSNA were significantly greater than increases in LSNA after bicuculline only. In addition, there was a significantly greater increase (i.e. 3-4 fold) in activation of the three nerves after bicuculline compared to nitroprusside (Figure 7).

**Verification of injection sites.** In addition to functionally identifying the RVLM with microinjections of both glutamate and bicuculline, we verified our microinjection sites histologically by analyzing dye spots from dye injections performed after the final protocol. Dye spots were successfully recovered in 14 of 17 animals and Figure 8 represents a composite of the injection sites relative to other structures in the rat brainstem. Histological analysis of microinjection sites verified that the pipettes were located within 500 \( \mu \)m of the caudal pole of the facial nucleus; that is, the previously defined cardiovascular region of the RVLM (21; 58).
DISCUSSION

The primary purpose of this study was to determine whether activation of the RVLM under a variety of conditions in the rat results in simultaneous and differential activation of SNA to the adrenal medulla, kidney, and hindquarter vasculature. By recording from all three nerves simultaneously we were able to examine activation of these nerves in the same animal under identical experimental conditions. Our primary new findings are: 1) pre-ASNA is simultaneously increased with RSNA and LSNA when the RVLM is activated by several different mechanisms. 2) pre-ASNA is activated to a greater extent than both RSNA and LSNA when the RVLM is activated by increasing concentrations of a fixed volume of glutamate. 3) Both pre-ASNA and RSNA are differentially increased compared to LSNA when the RVLM was activated by increasing volumes of a fixed concentration of glutamate. 4) Disinhibition of the RVLM by blocking tonic GABAergic inhibition resulted in several fold higher levels of sympathoexcitation in all three nerves compared with disinhibition produced by baroreceptor unloading. The functional consequences of activation of pre-ASNA along with RSNA and LSNA are likely to be important primarily in terms of the actions of epinephrine released from the adrenal gland and the actions of norepinephrine released from post-ganglionic nerve terminals.

Direct Activation of the RVLM simultaneously increases pre-ASNA, RSNA, and LSNA. Activation of pre-ASNA has been demonstrated under a variety of conditions including: acute decreases in blood pressure (60); activation of 5-HT sensitive cardiopulmonary receptors (19); CNS osmotic stimulation (2); and hemorrhage (5; 64; 76). In addition, microinjections into a number of brain regions result in
activation of sympathetic nerve activity to the adrenal gland (10; 26; 65). These previous studies and the current study suggest that a number of brain regions could provide central anatomical substrates for increasing pre-ASNA alone or via connections to the RVLM. Consistent with this idea, direct activation of the RVLM has been shown to increase plasma epinephrine levels (38; 48; 56) and elicit hindlimb vasodilation (32). In addition, in our previous studies similar increases in pre-ASNA produced by stimulation of adenosine receptor subtypes in the NTS are associated with hindlimb vasodilation that is abolished by β-adrenergic blockade or adrenalectomy (27; 42; 43; 66; 67). Collectively, these studies not only highlight the functional importance of sympathetic activation of the adrenal gland, but also emphasize the need to understand the extent to which release of epinephrine may act to contribute to a given blood pressure response via its actions on the vasculature and the heart.

**Reduction in GABAergic Inhibition (Disinhibition) of RVLM.** The tonic activity of RVLM neurons is critical to resting sympathetic outflow and arterial blood pressure and it is well known that these neurons are also under strong tonic GABAergic inhibition (21). The majority of this inhibition comes from inputs from the caudal ventrolateral medulla (CVLM) (35; 59), although there is evidence of non-CVLM mediated inhibition as well (7; 31; 45; 68). The tonic inhibition from the CVLM occurs via both baroreceptor and non-baroreceptor mediated mechanism (11; 12; 35). In the present study, we were able to compare the level of baroreceptor mediated inhibition of the RVLM versus the total amount of GABAergic inhibition mediated via GABA_α receptors. Disinhibition of the RVLM by blocking GABA_α receptors with bicuculline resulted in several fold higher levels of sympathoexcitation compared with disinhibition
produced decreases in arterial pressure (i.e. baroreceptor unloading). These data suggest that in our chloralose/urethane anesthetized preparations, the majority of GABAergic inhibition of the RVLM comes from non-baroreceptor dependent sources. The concentration and volume of bicuculline injections used in our study were sufficient to block the majority (if not all) of the GABAergic inhibition coming from the CVLM since similar bilateral injections of bicuculline inhibit endogenous activation of GABA<sub>A</sub> receptors in the RVLM through direct stimulation of the CVLM (45), the NTS (47) or by manipulations in arterial pressure (4; 14; 22; 47; 72). Interestingly, Natajaran and Morrison demonstrated that inhibition of the CVLM produced a selective rise in norepinephrine; whereas injections of bicuculline into the RVLM produced increases in both norepinephrine and epinephrine (48). Based on these data we suggest that the additional activation of the pre-ASNA with bicuculline compared to baroreceptor unloading in our study produces release of epinephrine via mechanisms that may not involve the arterial baroreflex or the CVLM.

Further evidence supporting the release of epinephrine during disinhibition of the RVLM has been provided recently by Verberne and Sartor (75). In these studies, increases in plasma glucose levels produced by bilateral injections of bicuculline into the RVLM were abolished by adrenalectomy, even though the pressor response was essentially unchanged. These data provide a functional role for epinephrine in regulating glucose homeostasis, but call into question the significance of epinephrine in contributing to the cardiovascular response to disinhibition of the RVLM. As mentioned, our previous studies have demonstrated hindquarter vasodilation following selective increases in pre-ASNA that were similar to the increases in pre-ASNA observed in the
current study. Rather than discount a cardiovascular influence of epinephrine, we suggest that the substantial activation of sympathetic vasoconstrictor nerves under these conditions may obscure the vasodilatory actions of epinephrine on the hindlimb vasculature. Furthermore these vasodilatory actions that would tend to reduce blood pressure may be offset by the actions of epinephrine on the heart to increase contractility and cardiac output. Certainly these hypotheses need to be tested more rigorously to determine the ultimate functional effects of RVLM-mediated increases in epinephrine on the cardiovascular system.

**Technical considerations.** There are a few technical aspects of our study that are important to consider. First, changes in all three nerves were quantified using percent change from baseline levels that were normalized to 100%. This quantification is based on our previous studies examining responses across functionally diverse nerves (60-64; 67). We have not reported any direct comparisons of absolute voltages (μV) across nerves for several reasons. Although arguments for making comparisons between the same nerve across various conditions have been made (20), it is our contention that it is simply not physiologically relevant to compare the absolute voltages across nerves since there are several factors independent of the nerve activity traveling through the nerves that contribute to the voltage in a given recording. According to Ohm’s law (Voltage = Current X Resistance), these factors will directly and proportionally influence the amount of voltage recorded in any preparation. Because these factors vary considerably across the nerves recorded in this study the amount of actual “nerve activity” contained within the voltage signal is unknown. In addition, the adrenal, renal, and lumbar nerve bundles are each of mixed composition in their fibers
and subserve various functions in the target organ in which they innervate. When differences in absolute voltages are observed in different sympathetic nerves, even when measured in the same animal, they do not necessarily indicate that the functional state of these outputs is different. Without the ability to estimate or correct for these factors, we instead favor defining all nerves to be at their baseline physiological state just prior to a manipulation; normalizing the values to 100%; and then calculating a percent change as an estimate of the amount of activation across nerves from their respective physiological baselines. We firmly believe that this is the best approximation of changes in relative function and facilitates comparisons of the responsiveness and differential control of these nerves across several conditions. Independent of these considerations, we were able to firmly conclude that pre-ASNA is increased under various conditions of direct and indirect activation of the RVLM, and along with RSNA and LSNA is likely to influence the blood pressure responses observed under these same conditions.

In our experiments we used glutamate to activate neurons in the RVLM since ionotrophic glutamate receptors in the RVLM are required for several sympathoexcitatory reflexes (21). We used increasing volumes of the same concentration of glutamate to increase the spread of the injection and activate additional populations of neurons in the RVLM. In addition, we used increasing concentrations of glutamate at similar volumes to uncover potential differences in the sensitivity of sympathetic premotor neurons located in the same area but projecting to different sympathetic outputs. In doing so, it is likely that we also increased the spread of glutamate because the diffusion gradient was almost certainly greater with the higher concentrations. Although we used fairly
small injections in some experiments (15 and 30 nl), we also used up to 90 nl injections for other injections. Several previous studies have used similar volumes (90-100 nl) to affect neurotransmitter receptors in the RVLM (1; 25; 29; 45). Although we concede that our glutamate microinjections do not activate RVLM neurons in the exact same manner as endogenous glutamate, our injections were located in the well defined pressor region of the RVLM which contains neurons which we were able to activate and influence sympathetic output in a graded fashion.

Lastly, in order to record from the three nerves simultaneously and perform microinjections into the RVLM, we felt the use of anesthesia was required. Although RVLM microinjections have been performed in conscious rats (36), including some with RSNA recordings (57), microinjections in conscious animals do not allow one to alter the position of the injector, which we were able to do very precisely in the present study to not only find the pressor region of the RVLM but also place additional, discrete injections within different portions of this region (see below).

PERSPECTIVES

Although the idea of a viscerotopic organization of sympathetic outflow within the RVLM has been fairly well established in the cat (16; 17; 32; 33; 41), a limited number of studies performed in the rat have suggested only a preferential control of regional SNA by the RVLM (3; 23). An advantage of our study was our ability to compare responses across the three nerves to individual injections. In doing so we found several instances where nerves responded differentially in magnitude and in some cases direction. This was particularly true when changing the volume of injectate or
repositioning the pipette. Figure 9 contains individual examples from animals in which increasing the volume of glutamate injection produced differential enhancement of one or more nerves. In Figure 9B, for example, a 15 nl injection of glutamate increased only LSNA; whereas, a 30 nl injection activated all three nerves. Further injection of 60 nl of glutamate enhanced the increase in RSNA and LSNA but did not appear to affect the increase in pre-ASNA. Figure 10 contains examples of injections made in the more traditional area in the RVLM (i.e. pressor response ≥ 15 mmHg) compared with moving the pipette caudally. Microinjection in more caudal spots resulted in preservation of the pre-ASNA excitatory response but diminished both the RSNA and LSNA responses. In Figure 10B the pressor response was converted to a depressor response consistent with peripheral vasodilation instead of vasoconstriction.

We believe that these directionally different changes in nerve activity may provide some of the first evidence of specific control of regional SNA in the rat RVLM. These types of differential responses along with the differential sensitivity of these nerves to both concentration and volume are indicative of separate populations of neurons that have the capacity to drive selective sympathetic outflows. The lack of evidence for differential control in the RVLM of the rat may be due to its smaller size compared to the cat, which could limit the number and size of injections necessary to demonstrate a viscerotopic organization (46). Alternatively, since the time of the earlier studies of Weaver and colleagues (3; 23), the RVLM of the rat has been defined more discretely in terms of localization of barosensitive neurons that project to the spinal cord. These neurons are typically found within 500 μm of the caudal pole of the facial nucleus (21; 58). Given that the experiments in the rat by Weaver and colleagues preceded this
stricter definition and utilized rostral-caudal injections that were spaced 500 μm apart, it is understandable why evidence for a viscerotopic organization may not have been observed (3; 23). In the current study we observed instances of differential control when the pipette was moved only 100-200 μm or when the volume of glutamate injected was increased by as little as 15 nl. By using these strategies, our contention is that different subpopulations of neurons were activated when moving the pipette location and additional subpopulations of neurons were recruited by remaining in the same location and using larger volumes of injectate. Further activation of some but not other regional sympathetic outputs could be due to a functional segregation of subgroup of neurons or what has been termed previously as “anatomical parcellation of function” by Sved and colleagues (73). Alternatively or in addition, there could be a difference in the sensitivity of specific pre-sympathetic neurons to glutamate (or generalized stimulation) due to variation in glutamate receptor or other ion channel neurotransmission. These possibilities warrant more extensive studies in the future including discrete mapping studies to determine whether a true viscerotopic organization does indeed exist in the rat and can be demonstrated as clearly as it has in the cat (9; 16; 17; 32; 33; 41).

Our finding of more selective activation of pre-ASNA in more caudal areas of the RVLM is consistent with previous functional studies. Ritter and colleagues reported greater Fos activation in the caudal but not the rostral aspects of the RVLM in response to 2-deoxy-D-glucose (55), a stimulus that promotes the release of epinephrine via activation of C1 cells in the RVLM (54). Interestingly, these data are in contrast to anatomical data from Pyner and Coote (52) who used anterograde and retrograde tracing techniques to establish the extent to which neurons projecting from regions of
the RVLM innervate adrenal preganglionic neurons. In these studies neurons projecting to the adrenal preganglionic neurons were found to originate more heavily from rostral rather than the caudal areas of the RVLM (52). The disparities between the functional and anatomical data provide a further rationale for the additional mapping studies already mentioned above.

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FIGURE LEGENDS

**Figure 1.** Individual examples of glutamate microinjections into the RVLM while simultaneously recording pre-ASNA, RSNA, and LSNA. Injecting increasing concentrations of glutamate (1, 10, 100 mM) in a fixed volume (30 nl) produced increases in MAP, HR and all three nerves. MAP = mean arterial pressure; HR = heart rate; pre-ASNA = preganglionic adrenal sympathetic nerve activity; RSNA = renal sympathetic nerve activity; LSNA = lumbar sympathetic nerve activity; RVLM = rostral ventrolateral medulla.

**Figure 2.** Peak changes in MAP, HR and SNA in response to microinjections of increasing concentrations of glutamate (1-100 mM, 30 nl each) into the RVLM (n=7). Glutamate produced significant concentration-dependent increases in MAP (†, p<0.05), but not HR. Glutamate also produced greater concentration-dependent increases in pre-ASNA (―, ●) as indicated by significant differences between all concentrations of glutamate (#, p<0.05 for pre-ASNA). Increases in RSNA (– –, ▼) were significantly different between 1 and 10 mM and 1 and 100 mM (*, p<0.05 for RSNA). Increases in LSNA (---, ■) were only significantly different between 1 and 100 mM glutamate ($, p<0.05 for LSNA). At 100 mM glutamate, pre-ASNA had significantly greater increases compared to increases in RSNA and LSNA, and RSNA had significantly greater increases than increases in LSNA (see brackets, p<0.05 for all).

**Figure 3.** Individual examples of glutamate microinjections into the RVLM at a fixed concentration (10 mM) using increasing volumes (15, 30, 60, 90 nl) in the same animal. Glutamate increased MAP, HR, pre-ASNA, RSNA, and LSNA in a volume related manner. Abbreviations as defined in Figure 1 legend.
**Figure 4.** Peak changes in MAP, HR and SNA in response to microinjections of increasing volumes of glutamate (10 mM, 15-90 nl) into the RVLM (n=7). Glutamate produced significant volume-dependent increases in MAP (†, p<0.05), but not HR. Glutamate also produced greater volume-dependent increases in both pre-ASNA (—, ●) and RSNA ( – –, ▼) as indicated by significant differences between all volumes of glutamate (# and *, p<0.05, for pre-ASNA and RSNA, respectively); whereas, increases in LSNA were not significantly between any volume tested (p>0.05). At volumes of 30 nl of glutamate and higher, increases in pre-ASNA and RSNA were significantly greater than increases in LSNA (# and *, p<0.05 for pre-ASNA and RSNA, respectively). Abbreviations as defined in Figures 1 legend.

**Figure 5.** Individual example of an infusion of sodium nitroprusside while recording pre-ASNA, RSNA, and LSNA in the same animal. Decreasing MAP increased HR, pre-ASNA, RSNA, and LSNA. Black arrow denotes beginning of sodium nitroprusside infusion. Abbreviations as defined in Figure 1 legend.

**Figure 6.** Individual example of bilateral injections of bicuculline (5 mM, 90 nl each side of the RVLM) while recording MAP, HR, pre-ASNA, RSNA, and LSNA in the same animal. Bicuculline injections (double arrows) increased MAP, pre-ASNA, RSNA, and LSNA; however, the increases in pre-ASNA and RSNA were larger than the increase in LSNA. Abbreviations as defined in Figure 1 legend.

**Figure 7.** Peak changes in pre-ASNA (solid bars), RSNA (hatched bars), and LSNA (double hatched bars) in animals receiving sodium nitroprusside (SNP) infusions and microinjections of bicuculline (Bic, 5 mM, 90 nl) into the RVLM (n=8). Increases in all three nerves were significantly greater after Bic compared to SNP (‡, p<0.05 for main
effect). Increases in pre-ASNA were significantly greater than LSNA following SNP or Bic (#, p<0.05). Increases in RSNA were significantly greater than increases in LSNA after bicuculline (*, p<0.05). Abbreviations as defined in Figure 1 legend.

**Figure 8.** Modified graphical representation of the RVLM and surrounding structures based on a rat atlas (50). Microinjection sites were successfully marked with pontamine sky blue (2%, 30 nl) in the RVLM of 14 animals. Only animals in which the center of the dye spot was located within 500 μm of the facial nucleus were included in this study.

**Figure 9.** Individual examples of differential control of regional SNA by varying the volume of injection of glutamate into the pressor region of the RVLM (10 mM, 15-60 nl). (A) In one animal, increasing the volume of injection from 15 to 30 nl increased pre-ASNA while have little effect on peak RSNA and only slightly increasing LSNA. (B) In another animal a 15 nl injection increased LSNA only; whereas the 30 nl injection activated all three nerves. Injection of 60 nl of glutamate enhanced the RSNA and LSNA responses but did not appear to change the pre-ASNA response. Black arrows denote time of injection of glutamate. Gray arrows denote directional change as in Figure 3. Abbreviations as defined in Figure 1 legend.

**Figure 10.** Individual examples of differential control of pre-ASNA, RSNA, and LSNA produced by glutamate microinjections into the RVLM. Glutamate (30 nl, 10mM) increased MAP, pre-ASNA, RSNA, and LSNA (A and B, left panels). Moving the pipette 100 μm caudal (A, right panel) maintained the increase in pre-ASNA but significantly diminished the increases in RSNA and LSNA. In another experiment, moving 200 μm caudal and 200 μm medial (B, right panel) enhanced the increase in pre-ASNA response but appeared to abolish the increases in RSNA and LSNA. Importantly, the
MAP response converted from a pressor to a depressor response in this case. Black arrow = glutamate injection. Gray arrows = directional change in individual nerves across conditions to facilitate comparison. Abbreviations as defined in Figure 1 legend.

References


6. Carrive P, Bandler R and Dampney RA. Somatic and autonomic integration in the midbrain of the unanesthetized decerebrate cat: a distinctive pattern evoked


FIGURE 1

Glutamate (30 nL)

MAP (mmHg)

HR (bpm)

RSNA (%)

pre-ASNA (%)

LSNA (%)

TIME (s)

0        30        0        30        0        30       60

0        30        0        30        0        30       60

0        30        0        30        0        30       60

0        30        0        30        0        30       60

1 mM  10 mM  100 mM
FIGURE 3
FIGURE 4

- \( \Delta \text{MAP (mmHg)} \)
- \( \Delta \text{HR (bpm)} \)
- \( \Delta \text{SNA (\%)} \)

Glutamate (nl)
FIGURE 5
FIGURE 6
FIGURE 7
FIGURE 9