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Research report

Spinal interneurons play a minor role in generating ongoing renal sympathetic nerve activity in spinally intact rats

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Abstract

The purpose of the present study was to determine whether spinal interneurons play a role in the regulation of sympathetic activity in spinally intact rats. In acutely spinally transected rats, we have described a population of spinal interneurons that, by virtue of correlations between their ongoing firing rates and the magnitude of ongoing renal sympathetic nerve activity (RSNA), are candidates for generators of sympathetic activity. Further evidence for a sympathetic role for these neurons comes from our observation that cervical spinal stimulation that reduces RSNA also reduces their discharge rates. In chloralose-anesthetized, spinally intact and spinally transected rats, we recorded ongoing RSNA and the ongoing activities of T_{10} dorsal horn and intermediate zone interneurons, and we determined the incidence of sympathetically related neurons in these rats by cross-correlating their activities with RSNA. The incidence of correlated neurons was much smaller in spinally intact than in spinally transected rats. We stimulated the dorsolateral, C_{2-3} spinal cord before and after acute C_1 spinal transection. Dorsolateral cervical stimulation in spinally transected rats reduced both RSNA and the activities of most T_{10} interneurons, but stimulation in spinally intact rats *increased* RSNA while still reducing the activities of most T_{10} interneurons. Both the low incidence of sympathetically correlated spinal neurons in intact rats and the dissociation between the effects of cervical stimulation on RSNA and the discharge rates of spinal interneurons argue against these neurons playing a major role in regulating sympathetic activity in intact rats.

Theme: Endocrine and autonomic regulation

Topic: Cardiovascular regulation

Keywords: Descending modulation; Spinal reflexes; Sympathetic nervous system

1. Introduction

A large body of evidence suggests that, in mammals with intact neuraxes, most sympathetic activity is generated by brainstem systems (see Ref. [3] for review). However, in anesthetized, acutely spinally transected rats, sympathetic outflow to abdominal organs (but not to skeletal muscle) is well maintained [20,21]. Although our laboratory has emphasized afferent sources of this ongoing activity [16], Taylor and Weaver have provided convincing evidence that spinal systems are capable of generating sympathetic activity to the abdominal viscera [20].

In vivo, sympathetic preganglionic neurons do not exhibit pacemaker potentials [6,9] or other evidence of spontaneous activity. Logically, therefore, spinal interneurons must be involved in the generation of sympathetic activity after spinal transection either by being excited by spinal afferents or by participating in endogenous spinal networks. In spinally transected rats, our laboratory has identified a population of spinal interneurons with activities that are correlated with both ongoing and stimulus-evoked renal sympathetic nerve activity (RSNA). RSNA frequently increases 20–140 ms after discharge of these interneurons. We have proposed that these spinal interneurons are members of spinal networks that play a role in generating sympathetic activity after spinal transection [5].

The present study was designed to determine whether, in spinally intact rats, a similarly correlated population of spinal interneurons existed. If so, these neurons would be candidates for generating ongoing sympathetic activity or

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for conveying brainstem-generated sympathetic drive to sympathetic preganglionic neurons. We were encouraged in this effort by Barman and Gebber's report of sympathetically correlated spinal neurons in spinally intact cats [2]. Two strategies were used. First, we cross-correlated the activity of spinal interneurons and RSNA to identify neurons whose action potentials preceded either increases or decreases in RSNA. Neurons that met several criteria with respect to these correlations (described in Section 2) were designated 'sympathetically correlated neurons.' If the incidence of correlated neurons in spinally intact rats was similar to or greater than that in spinally transected rats, we would conclude that spinal interneurons were likely to play an important role in exciting or inhibiting RSNA in intact rats.

Second, we took advantage of this laboratory's previous observation that low intensity electrical stimulation of the superficial, dorsaolateral, cervical spinal cord simultaneously *decreased* both the activities of spinal interneurons and RSNA in spinally transected rats [15], but that identical stimulation *increased* RSNA in intact rats [13]. Spinally elicited increases in RSNA in intact rats were most likely mediated by a spino–bulbo–spinal reflex, elicited by stimulation of excitatory, spinal inputs to the rostral ventrolateral medulla [19]. A role for spinal neurons in conveying brainstem drive to sympathetic preganglionic neurons in spinally intact rats would be supported if cervical stimulation increased the activities of spinal neurons in concert with stimulus-evoked increases in RSNA.

2. Materials and methods

All procedures used in these experiments were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Adult male Wistar rats (Taconic Farms) weighing 200–350 g were anesthetized with halothane followed by α-chloralose (100 mg/kg i.v.). The right femoral artery was cannulated for measurement of arterial pressure, and the trachea was cannulated for artificial respiration. Rats were mounted in a stereotaxic frame and paralyzed with gallamine triethiodide (Flaxedil, 40 mg/kg i.v.). Body temperature was monitored with a rectal probe and maintained at 35–37°C with a heating pad and a radiant heat lamp.

The rostral-most cervical spinal processes and dura were removed to expose the C_1–C_4 spinal segments for C_1-transection and for electrical stimulation between C_2 and C_4. A T_9 laminectomy was also performed to expose the T_{10} spinal segment for extracellular recording, and both regions of exposed spinal cord were bathed in warm mineral oil. A left pneumothorax was performed to reduce respiratory movements. The spinal cord was stabilized by clamping thoracic and sacral vertebrae. The left kidney was approached via a left flank laparotomy. After lateral retraction of the kidney, the renal nerve was dissected from surrounding tissue, placed on a bipolar hook recording electrode and covered with warm mineral oil.

Extracellular recordings were made with single-barrel, carbon-fiber microelectrodes connected to a high-impedance probe (Grass HIP5). The resulting signals were filtered (300–3000-Hz half power cutoff frequencies) and amplified (Grass P511 amplifier). The interneurons' action potentials were discriminated by a dual window discriminator (BAK Electronics). All neurons reported herein generated at least occasional pairs of action potentials with interspike intervals shorter than 50 ms, ensuring that they were spinal interneurons rather than sympathetic preganglionic neurons. Sympathetic preganglionic neurons rarely, if ever, discharge more rapidly than 20 Hz [7]. The loci of recorded neurons were marked by a small lesion, made by passing current through the recording electrode.

RSNA was recorded with bipolar electrodes, amplified by a factor of 200 000 (Grass P15 AC and Grass P511 AC amplifier) and filtered (300–1000-Hz half power cutoff frequencies). The amplified and filtered activity was full-wave rectified and smoothed at a time constant of 0.05 s. Zero nerve activity was determined at the end of experiments by crushing or cutting the renal nerve proximal to the recording electrode. Arterial pressure, RSNA, T_{10} interneuron activity, respiration, and stimulus markers were recorded on VHS tapes (A/D VCR Recorder Adapter, Medical Systems) for off-line analysis

Once the action potential for a thoracic interneuron was well isolated in either an intact or spinally transected rat, electrical stimulation (30 Hz, 10–80-μA pulses, 0.2-ms pulse duration, 1–5-s train duration) was delivered to the region between the second and third dorsal roots on the left dorsolateral surface of the spinal cord [15]. In many cases, the response to stimulation of RSNA and interneuron firing was very clear, and only two or three sets of stimuli were delivered. When no response was observed, as many as seven sets of stimuli were delivered at intensities that increased successively until RSNA either increased or decreased or the stimulation intensity reached 80 μA.

At the end of experiments, rats were perfused transcardially with buffered saline followed by 4% buffered formaldehyde. The relevant spinal cord segments were removed and stored in a solution of 30% sucrose in 4% phosphate-buffered formaldehyde (pH 7.4). Transverse 40-μm sections of the T_{10} segment were cut on a sliding microtome, mounted on gelatin-coated glass slides, and air dried. The sites of electrolytic lesions were identified microscopically. For neurons not identified by lesions, either deeper lesions in the same track were used as references or neurons' positions were estimated from the depth meter of the microdrive.

Final data acquisition and analysis were performed off-line. Action potential occurrences of T_{10} interneurons were converted to standardized pulses by a window discriminator and recorded with 0.8 ms resolution. Corre-

spondingly, RSNA was acquired at 1250 Hz. Cross-correlation functions were computed to assess correlations between the activities of T_{10} interneurons and RSNA. The pulses representing the original spike train of the interneuron were filtered in preparation for cross-correlation analysis by digital convolution with a sinc function, the width (number of points) of which was determined by the ratio of the sampling frequency (usually 1250 Hz) to the maximum firing frequency to be resolved (usually 125 Hz). This process tended to normalize the distribution of the neuronal time series, increasing the credibility of the values of the normalized cross-correlations.

Unlike spinally transected rats, some intact rats exhibited respiratory components in both their RSNA and interneuron firing patterns. Because we wanted to identify spinal interneurons that were candidates for relatively direct excitation or inhibition of RSNA, we attempted to eliminate interneurons that, logically, could not play that role. To this end, we established several criteria for 'sympathetically correlated neurons.' First, the T_{10} interneuron's cross-correlation function with RSNA had to exhibit a sharp increase for a positive correlation, or decrease for a negative correlation between 20 and 140 ms after the occurrence of the interneuron's action potentials (Fig. 1). This range of latencies was based on the window of occurrence of spike triggered averages from previous experiments in spinally transected rats [5] and from the logical consideration that effects on RSNA of spinal interneurons with either longer or shorter latencies would require unphysiologically slow or rapid conduction velocities, respectively. Second, the magnitudes of the positive or negative peaks in the correlogram had to exceed the envelope of at least 20 'dummy' cross-correlations (Fig. 1, upper left). Dummy correlations were calculated by repeatedly correlating the original RSNA signal with temporal randomizations of the interspike intervals of the interneuron's train of action potentials. This randomization preserved the average discharge rates of the original train. Correlations with insufficient magnitude (Fig. 1, upper right) or inappropriate latencies (Fig. 1, lower left) were frequently observed.

2.1. Statistical analysis

The significance of differences between incidences of variously classified neurons was determined with contin-

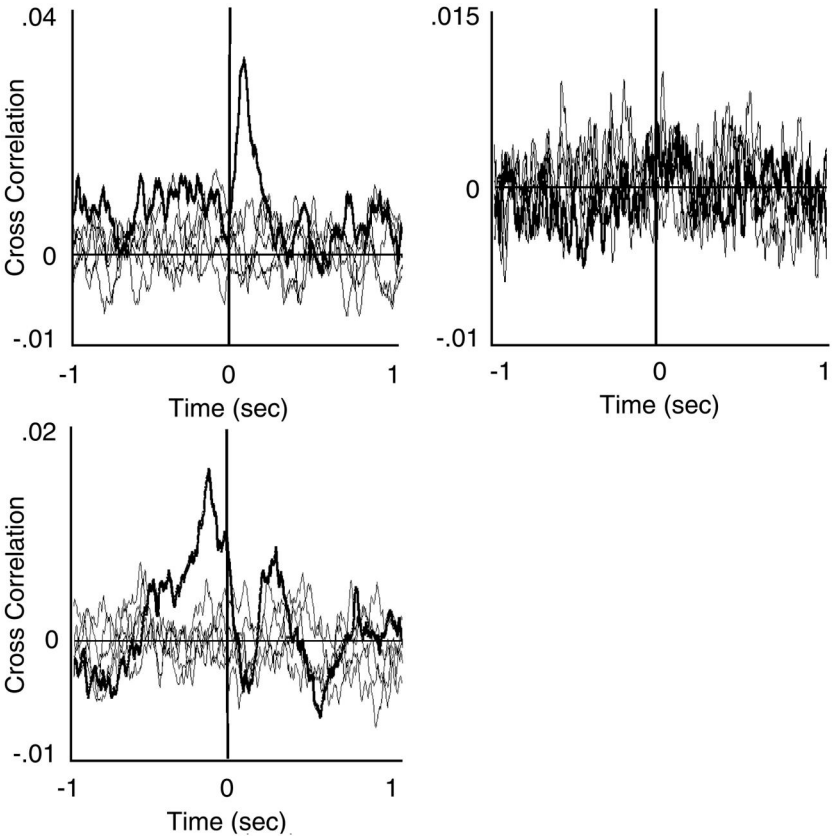

Fig. 1. Examples of cross-correlations (dark traces) and dummy cross-correlations (light traces) between RSNA and spinal neuronal activities in spinally intact rats. (Upper left) Correlogram exhibiting a sharp peak at an appropriate latency and larger than the envelope of dummy correlations. (Upper right) Correlogram of insufficient magnitude to exceed the dummy correlations. (Lower left) Correlogram with a peak that precedes the discharge of the interneuron. For clarity, only five dummy averages are shown for each correlation. Determination of the significance of correlations was based on comparisons of the actual correlation with the envelope of at least 20 dummy correlations.

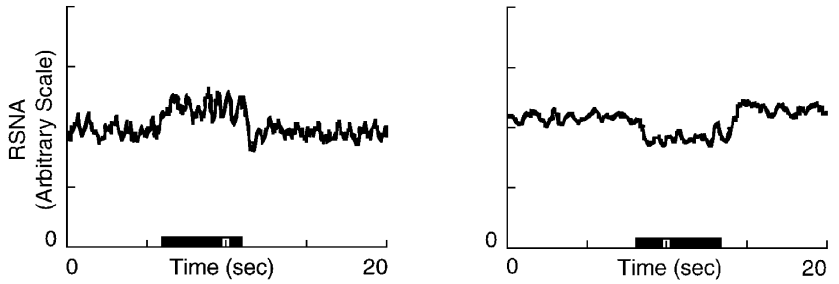

Fig. 2. Responses of renal sympathetic nerve activity to electrical stimulation of the cervical spinal cord in a spinally intact rat (left panel) and a spinally transected rat (right panel). Bars indicate stimulation periods.

gency tables, using the χ^2-test [18] Values of $P<0.05$ were considered significant. All values are expressed as means ± 1 S.E.M.

3. Results

The activities of 76 neurons were recorded in 20 spinally intact rats. The ongoing discharge rates of these neurons ranged from 0.3 to 59 Hz (mean 15 ± 1.6 Hz). Mediolaterally, the positions of the neurons ranged from the lateral spinal nucleus to the lateral portions of lamina X. Their depths were distributed as follows: 1–200 μ (6), 201–400 μ (15), 401–600 μ (23), 601–800 μ (13), 801–1000 μ (10), 1101–1200 μ (5), and 1201–1400 μ (4). In these spinally intact rats, the activities of few interneurons were positively correlated (9/76, 12%). The activities of two of these neurons and simultaneously recorded RSNA were strongly correlated with the period of artificial ventilation. Therefore, it was not possible to eliminate the possibility that the interneurons and the RSNA were being driven by a common oscillator. In the following analysis, therefore, we make the conservative assumption that all nine of these neurons were sympathetically correlated. The activities of even fewer interneurons were negatively (5/76, 7%) correlated with RSNA. Strong respiratory periodicity was observed in none of the neurons exhibiting negative correlations.

The incidence of positively correlated neurons was much larger after spinal transection (7/12, 58%, $P<$ 0.002). One negatively correlated neuron was observed after spinal transection. Strong respiratory periodicity was not observed in any neurons after spinal transection.

Confirming our previous observations [12,13,15], low intensity dorsolateral cervical stimulation decreased RSNA in all spinally transected rats (12/12, Fig. 2). RSNA was increased by cervical stimulation in most spinally intact rats (18/20, 90%). It was unaffected in two intact rats.

The effects of cervical stimulation on the activity of interneurons were more varied (Fig. 3). In spinally intact

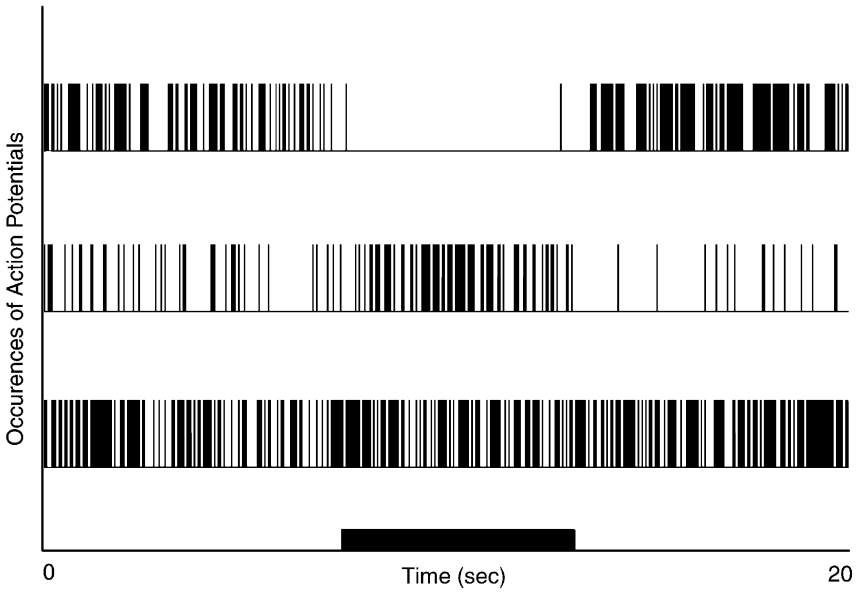

Fig. 3. Three effects of cervical stimulation on the discharges of T_{10} interneurons (shown converted to standardized pulses). (Upper trace) Neuron inhibited by stimulation. (Middle trace) Neuron excited by stimulation. (Lower trace) Neuron unaffected by stimulation. Bar indicates period of stimulation.

rats, cervical stimulation reduced the discharge rates of the majority of interneurons (48/76, 63%). Stimulation increased the discharge rates of relatively few interneurons (11/76, 15%). The discharge frequencies of some interneurons (17/76, 22%) were unaffected by stimulation. Equal numbers (four) of positively correlated neurons were excited and inhibited by cervical stimulation. One positively correlated neuron was unaffected by cervical stimulation. Four negatively correlated interneurons were inhibited by cervical stimulation. One negatively correlated neuron was unaffected by cervical stimulation. Although the responses of different interneurons to cervical stimulation varied greatly, the responses of the same interneuron to repeated stimulation varied little. Cervical stimulation did not activate previously silent neurons within our recording fields.

During 70 cervical stimuli that *increased* RSNA in spinally intact rats, the discharge rates of 62 neurons (89%) either *decreased* or remained unchanged. Finally, discharge rates were decreased during the six instances when cervical stimulation did not alter RSNA.

4. Discussion

The major observations of these experiments were (1) that fewer sympathetically correlated neurons were identified in spinally intact than in spinally transected rats and (2) that the effects of cervical stimulation on the activities of T_{10} interneurons and RSNA in spinally intact rats were incongruent. Both of these observations are consistent with the hypothesis that T_{10} interneurons play a minor role in regulating RSNA in spinally intact rats.

We chose to restrict our observations to the T_{10} segment of the spinal cord because the incidence of interneurons with activities correlated to RSNA is greatest in this segment [5]. The neurons from which we recorded were distributed between the most superficial lamina to the ventral portion of the intermediate zone of the spinal cord. Their modal location was in the nucleus proprius or central region of the dorsal horn. Because we did not attempt to obtain an anatomically unbiased sample of neurons [4], this profile is more closely related to the probability of isolating the first neuron in an electrode track than to the incidence of correlated or uncorrelated neurons at different depths.

The incidence of correlated neurons in spinally intact rats (12%) was not only smaller than their incidence in the 12 transected rats reported herein (58%), but it also was smaller than the incidence of correlated neurons recorded in the much larger samples of spinally transected rats previously reported in this laboratory [4,5]. Combining the results of those previous studies, the activities of 41 of 99 neurons (41%) were sympathetically correlated. The incidence of correlated neurons after spinally transecting the rats in the present study (66%) appears to be greater than that observed in the previous two studies. Although this difference is not statistically significant ($P>0.1$), we suspect that our adaptation of the cross-correlation method and the use of multiple dummy averages for determining the significance of the correlations may be more efficient in detecting sympathetically correlated neurons.

The incidence of neurons with activities correlated with decreases in RSNA was very low in both the spinally intact rats in the current study (5/76, 7%) and in previously reported [4] spinally transected rats (1/64, 1.5%). Although we can only speculate on the significance of this observation, it is consistent with the generally held opinion that most decreases in RSNA result from disfacilitation, rather than by direct inhibition, of renal sympathetic preganglionic neurons.

It is instructive to compare our observations with those of Barman and Gebber [2], who, based on their observation of sympathetically correlated spinal interneurons, suggested that spinal interneurons could play a role in sympathetic function in intact cats. They studied the relationship between the activities of rostral thoracic interneurons and the sympathetic activity in the inferior cardiac nerve. We studied the relationship between the activities of T_{10} interneurons and RSNA. Both Barman et al. [1] and Hayes and Weaver [8] have demonstrated substantial independence of brainstem regulation of different sympathetic nerves, and we have shown that spinal transection differentially affects sympathetic nerves to the abdominal viscera and muscle [21]. We might, therefore, expect segmental differences in the relative contributions to sympathetic activity of brainstem and spinal systems.

Despite this expectation, the incidence of sympathetically correlated neurons observed in this study was not markedly different from that reported by Barman and Gebber [2]. Adding the nine positively correlated neurons and the five negatively correlated neurons observed in our spinally intact rats, 14/76 (18%) were sympathetically correlated. Barman and Gebber reported that 18 of 59 interneurons (31%), located in the intermediate zone of the cat spinal cord, were sympathetically correlated. Thus, we would be safe in concluding, as did Barman and Gebber, that spinal interneurons might play some role in regulating sympathetic activity. However, the large discrepancy between the incidences of sympathetically correlated neurons in spinally intact versus spinally transected rats suggests that the role of spinal neurons in intact rats is minor when compared to direct descending projections to sympathetic preganglionic neurons.

We, like Barman and Gebber [2], compared the responses of spinal interneurons with the responses of sympathetic activity to electrical stimulation of the CNS. The rationale for these studies is that the responses of spinal interneurons should be closely related to the sympathetic responses to the same stimuli if, in fact, these interneurons are involved in generating or controlling sympathetic activity. In their experiments, Barman and

Gebber compared interneuronal and sympathetic responses to brainstem stimulation. Because we needed to use a system that would remain intact after spinal transection, we used our previous observations that low intensity electrical stimulation of the dorsolateral surface of the C_{2-3} spinal cord after C_1 spinal transection reduced ongoing RSNA by approximately 60% and that this stimulation simultaneously reduced the ongoing activities of most spinal interneurons [12,13,15].

Stimulation of cervical sites identical to those from which sympathoinhibition is elicited in spinally transected rats *excited* RSNA in most rats when the spinal cord was intact. However, cervical stimulation in spinally intact rats, while *increasing* RSNA, *decreased* the activity of most spinal interneurons. Indeed, even among the nine neurons whose activities were positively correlated with RSNA in intact rats, the activities of only four were increased by cervical stimulation. The activities of four others were decreased, and the activity of one was unchanged. Although we cannot prove that the small number of neurons that were excited by cervical stimulation were not involved in increasing RSNA during cervical stimulation, our observation that cervical stimulation either *reduced* or failed to affect the activities of most spinal interneurons in intact rats while increasing RSNA argues strongly against a major role for these neurons in conveying sympathetic drive to renal sympathetic preganglionic neurons.

Excitatory inputs to the spinal intermediate zone and sympathetic preganglionic neurons from the rostral ventrolateral medulla are well established [10,11,14,17]. The results of the present study suggest that these pathways probably represent the major source of sympathetic drive for RSNA in the spinally intact rat. Further, a paucity of negatively sympathetically correlated spinal neurons is consistent with the hypothesis that decreases in RSNA in both spinally intact and acutely spinally transected rats are mediated by disfacilitation rather than by direct inhibition of sympathetic preganglionic neurons.

Acknowledgements

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