Su, Chun-Kuei. Rhythmic sympathetic nerve discharges in an in vitro neonatal rat brain stem-spinal cord preparation. J. Appl. Physiol. 87(3): 1066–1074, 1999.—To understand the origination of sympathetic nerve discharge (SND), I developed an in vitro brain stem-spinal cord preparation from neonatal rats. Ascorbic acid (3 mM) was added into the bath solution to increase the viability of preparations. At 24°C, rhythmic SND (recorded from the splanchnic nerve) was consistently observed, but it became quiescent at <16°C. Respiratory-related SND (rSND) was discernible and was well correlated with C4 root activity. Power spectral analysis of SND revealed a dominant 2-Hz oscillation. In most preparations (86%), such oscillation was persistent, whereas it only slightly reduced its magnitude after isolation from the brain stem. The removal of neural structures rostral to the superior cerebellar artery (equivalent to the level of facial nuclei) reduced rSND, increased tonic SND, but did not affect the temporal coupling between SND and C4 root activity. Our data suggest a prominent contribution of SND from the neural mechanisms confined within the neonatal rat spinal cord. This ascorbic acid-enhanced in vitro preparation is a very useful model to study neural mechanisms underlying sympathoautonomic integration.

Rhythmic sympathetic nerve discharges in an in vitro neonatal rat brain stem-spinal cord preparation

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SYMPATHETIC NERVE DISCHARGE (SND) maintains the vasomotor tone for an appropriate blood perfusion to different organs. The origination of SND has been attributed mainly to the supraspinal neural structures, especially to those neurons located in the rostral ventrolateral medulla (5, 13, 31). Some rostral ventrolateral medulla neurons have pacemaker-like activity or receive tonic excitatory drives from other neurons and subsequently deliver these excitatory signals via their axonal projection to the sympathetic preganglionic neurons, intermediate lateral (IML) column cells, located in the thoracolumbar spinal cord (2, 14, 18, 21, 22, 35, 38). This laboratory has previously demonstrated that the neurons in dorsomedial medulla are also involved in the maintenance of vasomotor tone and have differential control over different sympathetic outflows (35, 37).

Spontaneous activities of IML cells can also contribute to the generation of SND. Substantial background SND is found in some cervical sympathetic preganglionic fibers or splanchnic nerves in a spinal preparation of cats (4, 23). Some intraspinal synaptic inputs to IML cells can maintain a low level of ongoing synaptic activities on a sympathetic preganglionic neuron (6, 10). In animals with a spinal preparation, rhythmic SND could still be elicited by asphyxia or through direct application of strychnine or kainic acid to the spinal cord (1, 12). These observations imply that the neural mechanisms, including IML cells in the spinal cord, may also contribute significantly to SND.

An in vitro brain stem-spinal cord preparation from neonates was recently developed to study the neural inputs from the brain stem to IML cells (7, 24). However, it is unclear whether such an in vitro preparation can indeed generate rhythmic SND endogenously. Comparable with the above-mentioned experimental model, there is also another in vitro brain stem-spinal cord preparation (extending from the lower brain stem to the cervical spinal cord) that can generate rhythmic respiratory activities (33, 40). Because the anatomic locations of cardiovascular and central respiratory neurons in the brain stem are within the proximity of each other (8, 17), it is very likely that this in vitro preparation could spontaneously generate SND as well.

In this study, we describe an in vitro brainstem-spinal cord preparation that can endogenously generate respiratory outflow at the C4 cervical spinal cord ventral root and SND at the splanchnic nerves. Sections were made at different levels of the brain stem and spinal cord to evaluate the contribution of different neural structures to SND. Our observations suggest that, in neonatal rats, a significant portion of SND is derived from the neural mechanisms confined in the spinal cord.

METHODS

Neonatal Sprague-Dawley rats (postnatal days 0–2) were used in this study. General procedures were modified from the methods previously described (36). The neural tissue that extends from the brain stem to the lumbar spinal cord, encased by the skull and vertebrae, was immersed in a 10°C bath solution for further dissection under microscope (Wild M32). Temperature of the bath solution was controlled by a circulation pump (B401-D, Firstek Scientific) and was monitored by a temperature amplifier (13–4615–474029 Gould) by using a thermoprobe (YSI probe 401, Yellow Springs Instruments). The pH of bath solution was also monitored (PHM83, Radiometer, Copenhagen, Denmark). Bath solution (in mM: 128 NaCl, 3 KCl, 1.5 CaCl2, 1.0 MgSO4, 24 NaHCO3, 0.5 NaH2PO4, and 30 D-glucose) was equilibrated with 95% O2-5% CO2. In some experiments, CO2 concentration ([CO2]) was adjusted by a gas proportioner (03218–50, Cole-Parmer) and monitored by a CO2 analyzer (Normocap CD-102–28–02, Datex). Complete exposure of the brain stem and cervical spinal cord was achieved by removing the surrounding bones. The cervical ventral roots were carefully preserved and were used later for monitoring the respiratory activities of the preparation. Dorsal parts of the vertebrae encasing the thoracic-lumbar spinal cord were also removed to allow for...
better perfusion, whereas the ventral parts of the vertebrae were left intact to preserve the sympathetic efferent pathways. Excess tissues attached to the ventral parts of the vertebrae were removed, retaining only minimal tissues in the bath. By tracing those nerves that exit from the sympathetic chain and innervate the celiac ganglion, located adjacent to the adrenal gland, we can easily identify the splanchnic nerves. The distal ends of the nerves were cut at the level before innervation of the celiac ganglion. The whole bundle of splanchnic nerves, containing both major and minor branches encapsulated by a nerve sheath, was used to record the preganglionic SND. This dissected preparation was fixed to the Sylgard-gel floor of the recording chamber (Fig. 1).

C4 ventral root and splanchnic nerve activities were recorded by suction electrodes. Neural signals were amplified, filtered (band-pass filter: 0.1–1 kHz; DAM50, WPI), and stored in a PCM-tape recorder (DR-890, Neuro-Corder). To acquire the envelope of C4 root activity (∫C4) or SND (∫SND), the signals were rectified and integrated by using a leaky integrator. The total SND was also measured by a time-based integrator (13–4615–70, Gould) with a resetting time of 5 s. Background noise level of SND recording was determined by integration of the signals at a bath temperature <15°C (when nerve activities were virtually quiescent) or after a transection at the level of T8 spinal cord. C4 root activity-triggered average of SND and power spectral analysis of the neurogram were conducted by using a Pclamp system (Axon Instruments) and were further analyzed by Axograph (version 3.0).

In constructing the C4 root activity-triggered average of SND, two analog-delay modules (NL740 Neurolog system, Digitimer) were used to acquire the pretriggering signals. Rhythmical oscillation of SND was examined by power spectral analysis of SND (low-pass filtered at 200 Hz), with each episode of the signal registering 8.192 s and sampling at 1 kHz. The averaged power spectrum was acquired from 32 episodes.

Student’s t-test was used to test whether neural signals were significantly altered after a treatment. The χ² test was used to evaluate the incidence ratio of certain observations. A P value of <0.05 was considered significant. All the values are presented as means ± SE.

RESULTS

Optimal conditions for recording endogenous SND in the in vitro preparation. After dissection was conducted in a 10°C bath solution, the preparation was promptly thawed back to room temperature while SND was monitored. As shown in Fig. 2, the recovery of rhythmical nerve activities is temperature dependent. At a bath temperature of <16°C, there was an absence of significant SND generated by the splanchnic nerves. SND became apparent as the bath temperature was raised to ∼20°C, reached a plateau at ∼24°C, and decayed progressively when bath temperature was increased (data not shown). When bath temperature was maintained at 27°C, supposedly an optimal temperature for recording respiratory activities in similar studies (3, 33, 40), SND decayed promptly. Therefore, the optimal temperature in this preparation to record SND was ∼24°C.

To acquire a sustainable preparation that can generate stable and long-lasting SND, the effects of ascorbic acid in preservation of the viability of brain slices were evaluated. The viability of the brain stem-splanchnic cord preparation in generating SND was compared between the experiments with or without incubation of 3 mM ascorbic acid. In the preparations incubated with 3 mM ascorbic acid, the rundown of SND was observed only in 2 of 17 (12%) experiments. (Rundown of SND: when bath temperature was maintained at 24 ± 1°C, the neural signals disappeared gradually within 2 h, leaving an integrated signal not different from the level when bath temperature was <15°C.) In contrast, in the absence of ascorbic acid, four of seven (57%) experiments showed an apparent rundown of SND within 2 h. The χ² test revealed that a significantly higher incidence of viable preparation occurred with incubation of 3 mM ascorbic acid (P < 0.05). These findings are consistent with the previous reports that ascorbic acid improves the conditions of neuronal growth and viability of brain slices (16, 19). In the presence of 3 mM ascorbic acid and with maintenance of the bath temperature at 24°C, SND could be sustained for more than 5 h without apparent deterioration. Figure 3 shows the acute effects of ascorbic acid. By adding 3 mM ascorbic acid to the bath solution, both SND and respiratory...
rhythmic discharge of C₄ root activity were increased significantly (SND: 25 ± 4%; P < 0.01; respiratory frequency: 38 ± 2%; P < 0.001; n = 4).

The effects on neural activities of changing [CO₂] in the bubbling air were also examined. The [CO₂] of the bubbling air was changed from 5 to 2 or 8%; this resulted in a change of the pH value in the bath solution from 7.39 to 7.14 or 7.64, respectively. As shown in Fig. 4, the change of SND only parallels the change of [CO₂] in an isolated spinal cord preparation, but not in an intact brain stem-spinal cord preparation. In isolated spinal cord preparations, SND consistently decreased when milieu [CO₂] was decreased from 5 to 2% (−31 ± 4%; n = 4; P < 0.01), but was only insignificantly increased when milieu [CO₂] was increased from 5 to 8% (13 ± 7%; n = 4). In intact preparations with the brain stem, the change of [CO₂] did not result in a consistent SND response. An increase of [CO₂] from 5 to 8%, did not change SND significantly (n = 3) or cause a biphasic SND response with either an initial decrement or increment followed by a delayed enhancement or depression (n = 3). A decrease of [CO₂] from 8 to 2%, might reduce (n = 3), enhance SND (n = 2), or cause an initial decrement followed by a delayed increment (n = 2). At equilibrium states (10 min after changing milieu [CO₂]), the overall effects induced by the change of [CO₂] on SND were pooled. In comparison with the level of SND at 5% [CO₂], SND was not significantly altered either by elevation (3 ± 15%; n = 6) or reduction (13 ± 16%; n = 6) of the milieu [CO₂]. However, the burst frequency of C₄ root activity consistently changed in parallel with [CO₂], i.e., the frequency of respiratory bursts was higher as [CO₂] increased and lower as [CO₂] decreased (n = 6). In comparison with the respiratory frequency at 5% [CO₂], an increase of [CO₂] to 8% accelerated the respiratory frequency significantly (34 ± 6%; P < 0.05), whereas a decrease of [CO₂] to 2% diminished the rate (−68 ± 17%; P < 0.05).

Brain stem- and spinal cord-derived components of SND. Surprisingly, not many preparations showed a significant amount of SND that depended on the synaptic inputs from the brain stem. Only in 2 of 14 (14%) preparations, was brain stem-derived SND component manifested by a significant diminution of SND after an acute section at the C₁ or C₈ level (Fig. 2). In most experiments (86%) after the section, SND was reduced only transiently and within 10 min could recover up to 90% of its activity before the section (Fig. 5). However, a further section at the T₇–T₉ spinal cord consistently
abolished SND (Fig. 5D). Because ascorbic acid may stimulate SND (Fig. 3, A and B), we further evaluated whether such prominent SND generated spontaneously from the isolated spinal cord is caused by a direct action of ascorbic acid to enhance SND at the level of spinal cord. In contrast to the observation in Fig. 3, A and B, the addition of 3 mM ascorbic acid to an isolated spinal cord did not alter SND (Fig. 3, C and D). This indicates that ascorbic acid mainly acts at the level of the brain stem, which also results in an increase of respiratory frequency (36 ± 3%, n = 4; P < 0.001). On the other hand, the transient and slight reduction of SND immediately after the brain stem section was also observed in experiments with no ascorbic acid added to the bath solution (n = 2). These results exclude the possibility of direct stimulant effects of ascorbic acid on SND preganglionic neurons. The prominent recovery of SND after isolation of the synaptic inputs from the brain stem suggests that a significant component of SND originates endogenously from the spinal cord.

In taking advantage of the landmark lying on the rostroventral surface of the medulla, we further deciphered whether the neural structure rostral to the superior cerebellar arteries (equivalent to the level at facial nuclei) is essential for the generation of SND. Figures 5 and 6 show a slight but persistent increase of SND after the removal of the neural structure rostral to the level of superior cerebellar arteries. Although the neural structure in the brain stem rostral to the facial nuclei is not essential for the generation of SND, we did notice that, in its absence, a dominant component of SND related to the respiratory activities (correlated with \( C_4 \) ventral root activities) was diminished (Fig. 6). To manifest the respiratory-related component of SND (rSND), a leaky integrator was used to reveal the envelope of SND fluctuation. After sectioning of the brain stem was performed at the level of superior cerebellar artery, respiratory frequency and amplitude increased, but rSND diminished (Fig. 6). Figure 7 shows the power spectral analysis of SND. The rhythmic oscillation of SND was dominant at a frequency of \( \approx 1-2 \) Hz. This basic rhythm was not altered after removal of the neural structure higher than the facial nuclei, despite the fact that a lower frequency component of SND (<1 Hz) was reduced after the section (Fig. 7B, n = 7). Also, the dominant rhythm of SND at 1-2 Hz persisted in isolated spinal cord preparations (Fig. 7). Figure 8 shows the average of neural activities...
triggered by C4 root activity. The peak activity of SND appeared consistently after the onset of inspiratory C4 activities with a delay of 103 ± 11 ms (range: 65–208 ms, n = 13). After sectioning was performed at the level of superior cerebellar arteries, there was a tonic elevation of SND, whereas the temporal relationship between peak C4 activities and SND remained unchanged (n = 7).

DISCUSSION

We have developed an in vitro neonatal rat brain stem-spinal cord preparation that can endogenously generate respiratory activities and SND. Because SND is discernible and well correlated with C4 respiratory-burst activities, this preparation could be a useful model to study the neural mechanisms that underlie the sympathosensory integration. Our results also clearly demonstrate that a significant portion of SND in neonatal rats is derived from the neural mechanisms of the spinal cord.

Optimal conditions for recording SND from the in vitro preparation. On the basis of our empirical observation, the optimal ambient bath temperature for this in vitro preparation is 24°C, which is a temperature lower than that for the preparation routinely used to study respiratory rhythmogenesis (27–28°C) (3, 29, 33). Oxygen consumption for neurons may be higher in a warmer environment due to the higher cellular metabolism. Thus, when less free oxygen is available for the neurons closer to the core of preparation, a microenvironment of hypoxic hypercapnia would result. In sinoaortic-denervated cats, hypoxia depresses inspiration-synchronous sympathetic activity while it increases the tonic component of the activity (32). Intravertebral injection of NaCN, to cause a local hypoxia in either the brain stem or an isolated spinal cord, stimulates sympathetic excitation (27). Asphyxia of sufficient duration in cats with a spinal preparation increased sympathetic nerve activities, characterized by a 2- to 3-Hz oscillation (12). Thus hypoxia or hypercapnia could have a direct stimulant effect on SND at the level of the spinal cord.
cord and might produce the spinal cord-derived component of SND. However, the lower optimal ambient temperature in our experimental conditions did not support such a view (that higher \([\text{CO}_2]\) in the core of preparation resulting from the higher ambient temperature is required to produce the endogenous SND). In fact, we observed that respiratory frequency is positively correlated with the change of \([\text{CO}_2]\) (Fig. 4). This observation indicates that the accumulated \(\text{CO}_2\) in the core of the preparation could be higher in a warmer environment to stimulate respiratory rhythmogenesis but not SND. Furthermore, there is no consistent effect of changing the \([\text{CO}_2]\) on SND in our intact in vitro experimental model. Only in the in vitro isolated spinal cord, but not in the intact brain stem-spinal cord preparation, did we observe a consistent SND response in parallel with change of \([\text{CO}_2]\) (Fig. 4). Such an observation indicates a complex action of \(\text{CO}_2\) on different orders of neurons responsible for eliciting SND, and, possibly, an inhibitory and excitatory action at the level of the brain stem and the spinal cord, respectively.

The mammalian brain contains a high level of ascorbic acid. The level is even higher in fetal and newborn rats than in adult rats (20). Ascorbic acid reduces lipid peroxidation and enhances viability of neurons (19, 26). All these findings are consistent with our observation that the viability of our in vitro preparation is significantly better in the presence of 3 mM ascorbic acid. In our study, ascorbic acid exerts no direct effect on the level of spinal cord, yet it enhances SND in intact brain stem-spinal cord preparations. This suggests a direct action of ascorbic acid on the brain stem neurons that generate SND (Fig. 3). Thus, in the presence of ascorbic acid, the overall SND could be biased to a stronger synaptic input from the brain stem. However, an acute isolation of the spinal cord from the influence of the brain stem only slightly reduced SND in most preparations. This observation also suggests that a major portion of SND is derived from the spontaneous neural activities confined within the neonatal rat spinal cord.

Characteristics of endogenous rhythmic SND in vitro. The most dominant rhythm of SND in our in vitro preparation is \(\sim 1-2\ \text{Hz}\). The power of rhythmic SND decayed promptly at the frequency \(>5\ \text{Hz}\). Such a pattern of activities was not altered after the section was made at the junction between the brain stem and the spinal cord. Intriguingly, other studies also suggest that the basic rhythmic generator of SND is located in the spinal cord. In spinal preparations of cats, splanchonic activities during asphyxia have a 2–3 Hz oscillations (12). In the isolated spinal cord of adult rats, a nonpatternized stimulation of neural circuits at the spinal cord (by exogenous application of kainic acid) is sufficient to elicit a patterned rhythmic 2–6 Hz SND (1). Furthermore, disruption of neural mechanisms in the brain stem by intracisternal injection of kynurenate to block glutamate synaptic transmission does not desynchronize the sympathetic activity (9). Thus, the neural circuits in the spinal cord in either neonatal or adult rats can be independent from the brain stem and can be sufficient to elicit a basic rhythmic SND.

By power spectral analysis of SND, we observed subtle changes of SND after sectioning was performed at different brain stem levels. The lower frequency component of SND (\(<1\ \text{Hz}\), Fig. 7), equivalent to rSND, was diminished yet persisted after sectioning of the brain stem at the level of superior cerebellar artery (Figs. 6–8). In contrast, the same sectioning increased tonic SND (Figs. 5–8). The appearance of rSND indicates a functioning neural circuit of our in vitro preparation that conveys the respiratory drives to the brain stem sympathetic premotoneurons and subsequently to.
Fig. 7. Power spectral analysis of SND envelope. A: power spectrum was acquired by an average of 32 episodes (8.192 s/episode, sampling at 1 kHz) collected under different bath temperatures (solid lines), revealing a rhythmic SND at ~2 Hz. Dashed lines, spectrum of SND envelope after serial sections made at level of superior cerebellar artery, C₈, and T₈, respectively. Arrow, reduction of lower frequency component (<1 Hz) after removal of neural structures rostral to superior cerebellar artery. Rhythmic SND at 1–2 Hz was sustained after section was performed at C₈, but power was virtually abolished after section was performed at T₈. Top right: each episode is 1 s, showing original envelopes of SND under different conditions. B: normalized change of power spectrum after section was performed at level of superior cerebellar artery. Data from 7 experiments were pooled. Gray lines, normalized change of individual experiments, acquired through dividing power spectrum after section was performed by that before the sectioning occurred. Black line, average of normalized change of power spectrum. Arrowhead (compare with arrow in A) indicates an averaged decrease of the power (<1 Hz) after section performed at level of superior cerebellar artery.

Fig. 8. Average of SND triggered by C₄ root activity. Each trace is average of 32 episodes. Leaky integration of neural activities, before and after section performed at superior cerebellar artery, is superimposed to reveal temporal coupling of \( \int \text{SND} \) and \( \int \text{C₄} \). Temporal coupling between activities was not altered, as shown by persistence of rSND after the section was performed. Compared with apparent rSND acquired from C₄-triggered average, random pulse-triggered average from same stretch of neural signals only revealed a general elevation of tonic SND after section was performed.
the splanchnic nerves. After sectioning was performed at the level of superior cerebellar artery, the latency between the peak activity of SND and the onset of C4 root inspiratory activity was not altered (Fig. 8). Studies of central circuits underlying the sympatho-respiratory coupling have recently elucidated some likely pathways that propagate respiratory messages toward sympathetic premotoneurons (13, 30, 39). To date, the neurons that can carry respiratory messages to sympathetic premotoneurons have been found in the Bötzinger complex and the caudal ventrolateral medulla (28, 39). Thus our findings further demonstrate that the neural structure rostral to the superior cerebellar artery (at the level of facial nucleus) is not essential to bridge these two systems.

Physiological significance of rhythmic SND in neonatal rats. At birth, the development of the rat sympathetic nervous system is incomplete (11, 25). Sympathetic ganglionic neurotransmission only functions after postnatal days 5–10 (15, 34). In the present study, we used rats with an age of 0–2 postnatal days and observed a significant spinal cord-derived component of SND. This observation may simply reflect a maturation process of the sympathetic nervous system and may not directly link to the vasomotor regulation at this developmental stage. The discrepancy between rats, in terms of maturation at birth, may in turn give rise to a variable dependency on the synaptic inputs from the brain stem. This possibility could be reflected by our observation that 14% of the preparations revealed a greater proportion of SND derived from the brain stem (Fig. 2). Undoubtedly, it would be of interest to examine the postnatal changes of SND genesis and to determine whether such a spinal cord-derived rhythmic SND is involved in the activity-dependent fine tuning of the spinal neural circuits in the neonates.

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