Novel Axonal Projection
from the Caudal End of the Ventrolateral Medulla
to the Intermediolateral Cell Column

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ABSTRACT

We used an optical imaging technique to investigate whether axons of neurons in the caudal end of the ventrolateral medulla (CeVLM), as well as axons of neurons in the rostral ventrolateral medulla (RVLM), project to neurons in the intermediolateral cell column (IML) of the spinal cord. Brainstem-spinal cord preparations from neonatal normotensive Wistar-Kyoto and spontaneously hypertensive rats were stained with a voltage-sensitive dye, and responses to electrical stimulation of the IML at the Th2 level were detected as changes in fluorescence intensity with optical imaging apparatus (MiCAM-01). The results were as follows: (1) depolarizing responses to IML stimulation during low-Ca high-Mg superfusion were detected on the ventral surface of the medulla at the level of the CeVLM, as well as at the level of the RVLM, and they had a latency of 24.0 ± 5.5 (SD) ms. (2) depolarizing responses were also detected on cross sections at the level of the CeVLM, and (3) antidromic action potentials in response to IML stimulation were demonstrated in the CeVLM neurons where optical images were detected, and (4) glutamate application to the CeVLM increased the frequency of excitatory postsynaptic potentials (EPSPs) and induced depolarization of the IML neurons. The optical imaging findings suggested a novel axonal and functional projection from neurons in the CeVLM to the IML. The increase in EPSPs of the IML neurons in response to glutamate application suggests that the CeVLM participates in the regulation of sympathetic nerve activity and blood pressure and may correspond to the caudal pressor area.

Key words: optical imaging, patch-clamp technique, monosynaptic bulbospinal projection, caudal pressor area, sympathetic nervous system
INTRODUCTION

Since neurons in the intermediolateral cell column (IML) of the spinal cord receive various inputs from higher neurons (the rostral ventrolateral medulla [RVLM], rostral ventromedial medulla [RVMM], paraventricular nucleus, raphe nucleus, etc.) (1, 4, 12, 21, 23, 26, 29), IML neurons play an important role in the regulation of sympathetic outflow. The precise location and function of the caudal pressor area (CPA) have recently been re-evaluated, and it is now known to be located more caudally than the caudal VLM (CVLM) (2, 8, 27, 28). Since microinjection of glutamate and homocysteine into the CPA in in vivo experiments induced sympathetic activation and an increase in blood pressure, and microinjection of glycine induced inhibition of sympathetic activity and blood pressure reduction, the neurons in the CPA have been considered to be sympathoexcitatory (16).

Much attention has been focused on afferent inputs into the CPA and efferent pathways out of the CPA. Natarajan and Morrison (16) demonstrated that the CPA neurons project to the sympathoexcitatory CVLM, whose neurons then project to the RVLM. Horiuchi et al. (8) reported that the CPA is modulated by prolactin releasing peptide, a hypothalamic hormone. Sun and Panneton (27,28) used anterograde and retrograde tracers to precisely localize the site of the CPA in the ventrolateral medulla. Li et al. (11) recently found a direct projection from the CPA to the IML in a retrograde tracer experiment. Studies to investigate whether the CPA neurons are anatomically and functionally bulbospinal neurons have just begun.

In the present study we used optical imaging, electrophysiological, and histological methods to determine whether an axonal projection exists from the medulla oblongata, including the CPA, to the IML. Since we did not measure blood pressure in this in vitro study, we will refer to the area as the caudal end of the VLM (CeVLM), instead of
as the CPA. Using optical imaging, we tried to detect depolarizing responses on the ventral surface of the medulla oblongata and cross sections of brain stem-spinal cord preparations to electrical stimulation of the axons or neural terminals of presympathetic neurons that projected to the IML region (IML stimulation). We also examined antidromic action potentials in response to the IML stimulation in the CeVLM area in which optical imaging revealed showed depolarizing responses. The preliminary results have been presented in the form of an abstract (9).

MATERIALS AND METHODS

Preparations and staining

Experiments were performed on brainstem-spinal cord preparations from 2- to 4-day-old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) (14,15,21). The experimental protocols were approved by the Animal Research Committee of Keio University School of Medicine in compliance with Japanese Law (No.105). Under deep ether anesthesia, the brainstem and spinal cord were isolated as previously described (14), and the spinal cord was sectioned at the second thoracic nerve root (Th2) level (Fig.1). Three different types of preparations were used: (1) a type in which the brainstem was sectioned at the distal end of the pons for observation of the ventral surface of the medulla oblongata, (2) a type in which the brainstem was sectioned between the posterior inferior cerebellar artery and the top of the hypoglossal nerve roots for observation of cross sections at the RVLM level, and (3) a type in which the brainstem was sectioned between the branch point of the basilar artery and the distal end of the exit points of the hypoglossal nerve roots for observation of cross sections at the level of the CeVLM.

The preparations were continuously superfused at 2-3 ml/min with a standard
solution, pH 7.4, consisting of (in mmol/L) 124 NaCl, 5.0 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, 1.2 KH₂PO₄, and 30 D-glucose, and equilibrated with 95% O₂ and 5% CO₂, at 26-27 °C. In most of the experiments the preparations were superfused with a low-calcium high-magnesium solution containing (in mmol/L) 0.2 CaCl₂ and 5.0 MgCl₂ so as to exclude ascending monosynaptic and polysynaptic projections and descending polysynaptic projections and detect descending monosynaptic projections alone.

Optical imaging

The brainstem-spinal cord preparations were incubated for 40-50 min in the standard solution containing the fluorescent voltage-sensitive dye Di-4-ANEPPS (0.1 mg/ml) or Di-2-ANEPEQ (50 µg/ml) (Molecular Probes, Eugene, OR) (19,20,30-32). The hydrophilic dye Di-2-ANEPEQ was used for observation at the brainstem surface because of its higher diffusion property into tissue than the lipophilic dye Di-4-ANEPPS. Di-4-ANEPPS was used for observation at the cut surface of the transverse sections, because it provides more stable staining with less noise than Di-2-ANEPEQ. Previous study reported that the depth for optical detection of spontaneous bursting activity was less than 500 µm and that the effective depth might be 200–300 µm for Di-2-ANEPEQ (20). The depth for optical detection of responses to electrical stimulation may be greater than for spontaneous burst activity.

The preparation was then placed in a perfusion chamber (volume 1 ml) mounted on a fluorescence microscope (BX50WIF-2; Olympus Optical, Tokyo), with the ventral surface or cross section of the medulla and spinal cord facing up.

Neuronal activity in the preparation was detected as a change in fluorescence of the voltage-sensitive dye with an optical imaging apparatus (MiCAM01; Brain Vision
Inc., Tsukuba, Japan) (19,20,31,32) and a tungsten-halogen lamp (150 W) through a 510-550 nm excitation filter, dichroic mirror, and 590 nm absorption filter (U-MWIG2 mirror unit; Olympus Optical). The head of CCD camera had an 8.4 x 6.5 mm² imaging area consisting of 180 x 120 pixels and a maximal time resolution of 3.5 msec. The power of the microscope was adjusted to 2x in most experiments so that the image sensor could cover an area measuring 4.2 x 3.25 mm². We examined optical images of the medulla oblongata for responses to electrical stimulation of the IML. Very thin stainless steel electrodes (tip diameter 20 \( \mu \)m, length 100 \( \mu \)m, impedance 700 kOhm) were used, and the position of the IML neurons was carefully identified through the CCD camera to enable stimulation (10-50 V, 100 \( \mu \)sec, single pulse) of only axons that projected to the IML neurons as much as possible by inserting the electrode perpendicularly into cross sections of the spinal cord (Fig.1). The IML was stimulated at the Th2 level, which is known to be the main target region of the cardiac sympathetic premotor neurons in the brainstem.

Most recordings were performed with an acquisition time of 10 msec. Fluorescence signals during a 3.4 sec period per trial were totaled and averaged for 10-20 electric stimulations of ipsilateral IML neurons. The fluorescence changes are expressed as ratios (fluorescence intensity divided by the fluorescence intensity of the reference image). The differential images were processed with a software-spatial filter for 2 x 2 pixels and presented as pseudocolor displays in which the “red” region corresponded to a decrease in fluorescence, meaning membrane depolarization. The intensity of the depolarizing response was strongest in the red region, and grew weaker in the yellow region, and still weaker in the green region. Decreases in fluorescence (depolarization) are presented as upward of the intensity of the depolarization. Since optical signals basically represent depolarization of the soma...
and not of the axons (30), we refer to the fluorescence change as “a depolarizing response”. Values are reported as means ± SD. Data were compared to assess statistical significance by the unpaired t test.

**Electrophysiological experiments**

We investigated whether the neurons in the CeVLM that exhibited depolarizing responses to IML stimulation detected by optical imaging projected to the IML. Neuronal activity was recorded extracellularly or intracellularly during low-Ca high-Mg superfusion (21), and the IML was stimulated to investigate the induction of antidromic action potentials. For the extracellular recordings, glass electrodes were filled with 2% pontamine sky blue in 0.5 mol/l sodium acetate (resistance 5-20 MΩ). For the intracellular recordings by the whole-cell patch-clamp technique, patch electrodes were filled with 0.5 % lucifer-yellow in a pipette solution of the following composition (mmol/l): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na₂-ATP, 1 CaCl₂, and 1 MgCl₂, with pH 7.2-7.3 adjusted with KOH. The IML was stimulated with a stainless steel electrode (5-15 V, 100 µsec, single pulse) to induce antidromic action potentials in the VLM.

In addition, to examine functional and orthodromic effects of CeVLM neurons on the IML, IML neurons were recorded by an intracellular technique (whole-cell patch-clamp) and an extracellular technique during superfusion with standard solution. Glutamate (1 mmol/L, 10-30 µl) was locally applied to the CeVLM in a preparation whose cross section was at the level of the CeVLM and from which more rostral regions, including the CVLM and RVLM, have been removed (type 3 preparation of METHODS section and Fig 1). To confirm and minimize glutamate diffusion in the cross section at the level of the CeVLM, the glutamate solution was mixed with Fast Blue.
Histological examination

After immersing the preparations in 10% formalin-phosphate buffer solution at 4°C for 48 hours, the medulla oblongata with spinal cord was cut into 100-µm sections, and the locations of neurons that had stained with lucifer-yellow or pontamine sky blue were identified in the medulla and spinal cord. All preparations were then stained with neutral red to compare the location of the CeVLM neurons with the location of surrounding nuclei.

At the conclusion of the optical imaging experiments, the IML that had been electrically stimulated was coagulated (20 V, 20 msec, single pulse) to confirm that the neurons that projected to the IML had been accurately stimulated.

To confirm that the neurons in the spinal cord that depolarized in response to glutamate application to the CeVLM were IML neurons, they were stained with lucifer-yellow at the conclusion of the intracellular recording.

RESULTS

Optical imaging on the ventral surface

Depolarizing responses were actually captured in the form of a motion picture. As shown in Fig. 2, during superfusion with standard solution, IML stimulation induced depolarizing responses on the continuous column of the surface of the rostrocaudal VLM, including the RVLM, the CVLM, and the CeVLM. In all preparations (n=10), the intensity of depolarization (fluorescence change) of the CeVLM (-0.098 ± 0.024%, mean ±SD) was significantly (p<0.01) stronger than that in the RVLM (-0.048 ± 0.024%) and CVLM. The latency between IML stimulation and the start and the peak of the depolarizing response at the CeVLM was 41 ± 15 ms and 82 ± 32 ms, respectively. No depolarizing responses were detected in other regions despite even
stronger stimulation of the IML.

As shown in Fig 3, the IML stimulation during low-Ca high-Mg superfusion also induced depolarizing responses on the surface of the rostrocaudal VLM, including the RVLM, the CVLM, and the CeVLM. The low-Ca high-Mg solution was used to so as exclude ascending monosynaptic and polysynaptic projections and descending polysynaptic projections and obtain only the depolarizing responses of descending monosynaptic projections. In all preparations (n=6), the depolarizing response of the CeVLM (-0.041 ± 0.009%) was significantly (p<0.05) stronger than that of the RVLM (-0.026 ± 0.010%). The latency between IML stimulation and the start and the peak of depolarization in the CeVLM was 28 ± 7 ms and 31 ± 11 ms, respectively. The intensity of the depolarizing response was weaker during low-Ca high-Mg superfusion than during perfusion with the standard solution.

**Optical imaging of the cross sections**

IML stimulation during low-Ca high-Mg superfusion induced depolarizing responses in restricted regions on cross sections at the level of the RVLM (n=6, Fig.4) and at the level of the CeVLM (n=8, Fig.5). The latency between IML stimulation and the start and the peak of the depolarizing response was 24 ± 5 ms and 36 ± 8 ms, respectively, at the level of the CeVLM. That was 27 ± 9 ms and 35 ± 10 ms, respectively, at the level of the RVLM.

To determine whether the depolarizing responses were derived from the somas and not from the axons, we examined the depolarizing responses after adding GABA to the superfusion solution. IML stimulation during superfusion with low-Ca high-Mg solution containing GABA (200 μmol/L, n=7) did not induce any depolarizing responses in the cross sections at the level of the CeVLM (Fig.6) or of the RVLM.
Electrophysiological experiments

Other brainstem-spinal cord preparations were fashioned for the electrophysiological experiments, and during low-Ca high-Mg superfusion antidromic action potentials in response to IML stimulation were clearly detected in nine CeVLM neurons by intracellular recordings (whole-cell patch-clamp technique, Fig.7) and in sixteen CeVLM neurons by extracellular recordings. The latency between IML stimulation and detection of the antidromic action potential was 21.6 ± 7.1 ms by extracellular recording and 27.2 ± 4.6 ms by the whole-cell patch clamp technique. These latencies are almost the same as the latency between IML stimulation and the depolarizing response in the CeVLM measured by optical imaging of cross sections (see Fig.5). The spontaneous action potentials of the CeVLM neurons (extracellular recording) collided with subsequent antidromically evoked action potentials.

Glutamate application to the CeVLM

During whole-cell patch-clamp recordings of neurons in the IML with standard solution (Fig.8a), glutamate was applied to the CeVLM locally after removing more rostral regions including the CVLM and RVLM (type 3 preparation, like in Figures 5 and 6). Local application of glutamate to the CeVLM increased the frequency of the excitatory postsynaptic potentials (EPSPs) and induced significant depolarization (5.6 ± 2.5 mV) of the IML neurons (n=8, Fig.8a). Each EPSP was clearly revealed by increasing the speed of the sweep (Fig.8c).

Histological examination

The locations of the neurons in the CeVLM in which depolarizing responses were
detected by optical imaging and that exhibited an antidromic action potential in response to IML stimulation were investigated histologically (Fig.9a). The neurons stained with lucifer-yellow or pontamine sky blue were located in the lateral side of the lateral reticular nucleus (LRT) or in the LRT, the caudal part of the nucleus ambiguus (NA), and the medial side of trigeminal spinal tract nucleus (Sp5) at the level of pyramidal decussation (Pyx) (Fig.9b, c). Thus, the anatomical location of the neurons in the CeVLM satisfied the criteria for the location of the CPA described by Sun and Panneton (27,28).

We tried to confirm that the location of the electrical stimulation in the spinal cord actually corresponded to the IML, and the results showed that location of the electrical stimulation and coagulation did correspond to the location of IML neurons (Fig.10a, b).

The location of neurons that exhibited depolarization and an increase in EPSP in response to local glutamate application to the CeVLM and stained with lucifer-yellow in the spinal cord also corresponded to the IML (Fig.10c.d).

**DISCUSSION**

*Characteristics of neurons in the caudal end of the VLM identified by optical imaging*

We used optical imaging and electrophysiological methods in brainstem-spinal cord preparations of neonatal SHRs to determine whether neurons in the caudal end of the VLM (CeVLM) axonally project to the IML. The optical imaging revealed strong depolarizing responses in the CeVLM to IML stimulation, and CeVLM neurons fired antidromic action potentials in response to the IML stimulation. Glutamate applied in the CeVLM induced depolarization in the IML neurons. These findings demonstrated a monosynaptic axonal and excitatory projection from the CeVLM to neurons in the IML.
A number of studies, including a retrograde tracer study (10), have reported a monosynaptic projection from the RVLM, the RVMM, and the raphe nucleus to the IML (1,4,23,26). Several studies have also demonstrated a direct projection from the CVLM to the IML in adult rats (6, 13), and the result of a recent tracer study using cholera toxin B subunit suggested a projection from the CPA to the IML of adult rats (11). A new region that differs from the CPA, the medullo-cervical pressor area (MCPA), has recently been reported to project to the IML in adult rats based on an *in vivo* retrograde tracer study (25). Consistent with these findings, the optical imaging in our study suggested that a continuous longitudinal rostrocaudal column in the ventrolateral medulla, including the RVLM, the CVLM, and the CeVLM gives rise to a monosynaptic projection to the IML. Since we detected the strongest depolarizing response in the CeVLM, we focused specifically on the CeVLM in the present study. To our knowledge, few studies have succeeded in optical visualization of the axonal projection from the CeVLM neurons that are involved in sympathetic nerve regulation to the IML. Since the existence of the projection from the CPA to the IML had been suggested only by a tracer study (11), the projection we have found must be discussed critically.

Earlier studies have shown that EPSPs or postsynaptic responses completely disappeared during superfusion with low-Ca high-Mg solution, suggesting a complete blockade of synaptic transmission (7,17,22). In our unpublished observation, many EPSPs were detected in the CeVLM neuron in response to IML stimulation during superfusion with standard solution, whereas during superfusion with low-Ca high-Mg solution, the EPSPs observed in the CeVLM neuron to IML stimulation completely disappeared. Thus, using a low-Ca high-Mg superfusion has the advantage of blocking all synapses and enabling detection of only descending monosynaptic projections alone, whereas during superfusion with standard solution, depolarizing
responses of descending polysynaptic projections and ascending projections are
detected as well as of descending monosynaptic projections. In the present study, the
regions that exhibited depolarizing responses during low-Ca high-Mg superfusion were
the same as those that displayed them during superfusion with the standard solution.
In contrast, the depolarizing responses were less intense during low-Ca high-Mg
superfusion, and the peak time of the depolarizing responses during low-Ca high-Mg
superfusion occurred earlier than with the standard solution. These results strongly
suggest that neurons in the CeVLM monosynaptically project to the IML neurons.

Previous studies found that low-Ca high-Mg solution did not affect neuronal
excitability (7, 18). If neuronal excitability decreases during superfusion with low-Ca
high-Mg solution, the intensity of the depolarizing responses detected in the present
study should be weaker than the actual depolarizing responses. Therefore, we did not
overestimate the depolarizing response by optical imaging.

Optical imaging findings on the ventral surface revealed that the depolarizing
response in the CeVLM was more intense than in the RVLM. This finding suggests that
in neonatal rats more neurons project from the CeVLM to the IML than from the RVLM
and CVLM to the IML, but does not imply that neuronal activity in the CeVLM is
stronger than in the RVLM. It may merely reflect the characteristics of neonatal rats.
However, we were unable to compare the depolarizing responses during the different
developmental stages of the rats in this study.

Since the depolarizing response is the sum of the signals in the somas (cell
bodies) and axon bundles, we investigated whether the depolarizing response
originated in the somas or in the axons. GABA should bind to receptors on the
surface of the soma, and as shown in Fig 6, the depolarizing response of the CeVLM
was completely abolished during superfusion with GABA. This finding suggests that the depolarizing responses detected by optical imaging reflected depolarization of the somas of CeVLM neurons, not depolarization of the axons. Since we have reported the existence of the CeVLM neurons for the first time, it is unknown whether GABA receptors are present on CeVLM neurons. However, the result that the IML stimulation during superfusion with low-Ca high-Mg solution containing GABA did not induce any depolarizing responses in the cross sections of the CeVLM suggests to us that the CeVLM neurons possess GABA receptors. An earlier study reported that CPA neurons possess GABA receptors (24), and if the CeVLM neurons correspond to CPA neurons, then CeVLM neurons probably possess GABA receptors.

We also obtained some optical imaging data in Wistar-Kyoto (WKY) rats as a control for the SHRs. The data showed similar depolarizing responses to IML stimulation in the CeVLM on the ventral surface (n=5) and cross sections (n=4) of WKY rats. Although careful experiments are required to make quantitative comparisons between the intensity of depolarizing responses in the CeVLM of WKY rats and SHRs, the results indicate to us that the projection from the CeVLM to the IML exists in both normotensive and hypertensive neonatal rats. It would be interesting to compare responses in the IML neurons of WKY rats and SHRs, when glutamate is locally applied to the CeVLM on the preparation in the absence of the CVLM and the RVLM regions.

Methodological limitations

We cannot completely rule out the possibility that we may have stimulated regions other than the axons and neural terminals of bulbospinal neurons in the IML region (e.g., ascending neurons). However, for the following reasons we think that the
extent of the electrical stimulation in this study was relatively restricted to IML. We
used very thin electrodes, and carefully identified and stimulated the position of the IML
neurons through the CCD camera so as to limit the stimulation point. Indeed, as
shown in Fig 10, we demonstrated that the stimulation point was accurately restricted
to within the IML region. The location of the depolarizing response in Fig. 4 therefore
includes RVLM neurons. The depolarizing response that was detected in the region of
the RVLM in response to IML stimulation served as a good positive control.

These results also suggested that the stimulation point accurately covered axons
and neural terminals of bulbospinal neurons in the IML. We were therefore able to
conclude that the depolarizing responses detected in the CeVLM on both ventral
surface and cross section were depolarizations of bulbospinal neurons. As shown in
Fig 8, we also demonstrated EPSPs on the IML neurons detected during chemical
stimulation of the CeVLM. The results confirmed the occurrence of orthodromic
responses, demonstrating the presence of a functional projection from the CeVLM to
the IML.

Developmental issues

Although a tracer study (10) found that neurons in the RVMM and in the raphe
nucleus project to the IML, we did not detect any depolarizing responses in the RVMM
or the raphe nucleus after IML stimulation. Since the intensity of the depolarizing
response depends on the number of neurons that respond, the number of neurons
projecting from these areas to the IML may be relatively small. For example, in the
raphe nucleus of rabbits between day 26 of gestation and 6 days of age, the dendrites
showed expansion, increased the number and length of neurons, and developed
abundant spines (5). During this period, the soma grew in size. After postnatal day 6 to adulthood, a mature pattern of dendritic branching was achieved. Therefore, the reason that we were unable to detect depolarizing responses in the midline raphe may be due to the immaturity of the neonatal rats.

Secondly, a previous study showed that gap junctions between neurons may be more abundant in newborn rats than in adult rats (3), and the depolarizing responses may have been exaggerated by the gap junctions because we used newborn rats that were only 2 to 4 days old. The decrease in gap junctions during development may weaken the depolarizing response.

**Physiological implications**

In this *in vitro* study we investigated whether the CeVLM corresponds to the CPA, an area that has been identified in *in vivo* studies (2,8,16,27,28). However, few *in vitro* studies have identified the anatomical location of the CPA. Our histological examination demonstrated that the location of the CeVLM neurons that exhibited an antidromic action potential and stained with lucifer-yellow or pontamine sky blue corresponded to the location that where depolarizing responses were observed by optical imaging. The histological examination also showed that the location of the depolarizing responses in the CeVLM neurons was almost identical to that of the CPA reported in earlier *in vivo* studies (11,27,28).

To further confirm that the CeVLM corresponds to the CPA, we investigated whether neurons in the CeVLM are involved in peripheral sympathetic nerve regulation. The IML neurons exhibited increased EPSPs and membrane depolarization in response to local application of glutamate to the CeVLM despite the absence of the RVLM and the CVLM. These results imply that neurons in the CeVLM send excitatory
input to the IML neurons and that CeVLM neurons are involved in sympathetic nerve regulation and blood pressure control. If these implications are true, we can conclude that at least some of the neurons in the CeVLM correspond to neurons in the CPA. Further study by spike-triggered averaging is needed to determine whether the spontaneously occurring action potentials of the CeVLM neurons induce EPSPs in the IML neurons.

Since earlier studies have demonstrated that the functions of the CPA are mediated via sympathoinhibitory (2) and sympathoexcitatory (16) neurons in the CVLM, the CPA neurons would be expected to project to the CVLM. However, if the CeVLM corresponds to the CPA, our optical imaging findings suggest that the CPA also projects to the IML.

Even if the CeVLM does not correspond to the CPA, the CeVLM may play a role in sympathetic nerve modulation. It is hard to imagine that the CeVLM affects sympathetic nerve activity in the same manner as the neurons in the RVLM do, and instead we postulate that the CeVLM neurons modulate sympathetic nerve activity determined by the RVLM. Horiuchi et al. (8) found that microinjection of prolactin-releasing peptide into the CPA increased blood pressure, but that microinjection of angiotensin II did not. Thus, some functions of the CeVLM, such as responses to stress, differ from those of the RVLM neurons.

In summary, the optical imaging findings in the brainstem-spinal cord preparations of neonatal SHRs suggest the existence of a continuous longitudinal column in the ventrolateral medulla, including the RVLM, the CVLM, and the CeVLM, that gives rise to a monosynaptic projection to the IML. The neurons in the CeVLM have a role in sympathetic nerve and blood pressure control. The projection pathway and function of the CeVLM neurons need to be investigated more precisely in a future study.
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FIGURE LEGENDS

**Fig. 1.** Brainstem-spinal cord preparation from a neonatal spontaneously hypertensive rat. Three different types of preparations were used for optical imaging and the whole-cell patch-clamp technique: (1) a type for observation on the ventral surface of the medulla oblongata, (2) a type for observation of cross sections at the level of the rostral ventrolateral medulla (RVLM), and (3) a type for observation of cross sections at the level of the caudal end of the VLM (CeVLM). Electrical stimulation was applied to the intermediolateral cell column (IML) at the Th2 level.

**Fig. 2.** Optical images of the ventral surface of the medulla oblongata during superfusion with standard solution. Depolarizing responses to IML stimulation were clearly detected on the ventral surface at the RVLM, the CVLM, and the caudal end of the VLM (CeVLM, n=10).

**Fig. 3.** Optical images of the ventral surface of the medulla oblongata during superfusion with low-Ca high-Mg solution. Depolarizing responses to IML stimulation were detected on the ventral surface at the RVLM, the CVLM, and the caudal end of the VLM (CeVLM, n=6).

**Fig. 4.** Optical images of a cross section at the level of the RVLM during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the RVLM (n=6). The latency between stimulation of the IML and the start and the peak of the depolarizing response at the RVLM was 27 ± 9 (mean ± SD) ms and 35 ± 10 ms, respectively.
Fig. 5. Optical images of a cross section at the level of the caudal end of the VLM (CeVLM) during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the CeVLM (n=8). The latency (mean ± SD) between stimulation of the IML and the start and the peak of depolarizing response at the CeVLM was 24 ± 5 ms and 36 ± 8 ms, respectively.

Fig. 6. Optical images of a cross section at the level of the caudal end of the VLM (CeVLM) before (upper two panels) and during GABA superfusion (lower two panels). IML stimulation during superfusion with low-Ca high-Mg solution containing GABA (200 μmol/L, n=7) did not induce any depolarizing responses at the CeVLM.

Fig. 7. Antidromic action potential of a neuron in the caudal end of the VLM (CeVLM) recorded by the whole-cell patch-clamp technique in response to electrical stimulation of the intermediolateral cell column [IML] at the Th2 level. The latency between stimulation and the start of the antidromic action potential was 27.2 ± 4.6 ms according to the whole-cell patch-clamp recordings (n=9).

Fig. 8. Intracellular recordings of a neuron in the IML at the Th2 level (n=8) during superfusion with standard solution, in the type (3) preparation, like Figures 5 and 6. (a) Local application of glutamate (1 mmol/L) to the CeVLM increased the number of excitatory postsynaptic potentials (EPSPs), induced depolarization (5.63 ± 2.50 mV), and increased the action potential spikes of the IML neurons. (b) Few EPSPs were seen before glutamate application. (c) Each EPSP and depolarization were clearly detected after local application of glutamate to the CeVLM.
Fig. 9.  (a) Location of neurons in the CeVLM that exhibited an antidromic action potential in response to electrical stimulation of the IML. This experiment was carried out during low-Ca, high-Mg superfusion. Closed circles: extracellular recordings, Open circles (O): intracellular recordings. (AP) area postrema, (12) hypoglossal nucleus, (Sp5C) spinal trigeminal nucleus, caudal part, (Pyx) pyramidal decussation, (LRT) lateral reticular nucleus, (RAmb) retroambigus nucleus, (IO) inferior olivary nucleus, (IOM) inferior olive, medial nucleus.  (b) Fluorescence image showing a neuron in the CeVLM that exhibited an antidromic action potential and stained with lucifer-yellow.  (c) The drawing shows the location of a neuron that exhibited an antidromic action potential and was stained with lucifer-yellow.

Fig. 10.  (a) Histological examination of the location of IML neurons at the Th2 level that was coagulated at the conclusion of electrical stimulation of the IML.  This panel shows a cross section stained with neutral red.  The top is dorsal, and the bottom is ventral.  (b) High-magnification photograph of (a) showing the location of electrical stimulation of the IML.  The location that was electrically stimulated and then coagulated (indicated by the arrow) actually corresponded to the IML neurons.  (c) A neuron in the IML that exhibited depolarization and an increase in EPSPs in response to glutamate application to the CeVLM.  This neuron stained with lucifer-yellow is the same neuron shown in Figure 8.  (d) Cross section at the Th2 level stained with neutral red.  The neuron that exhibited the increase in EPSPs, stained with lucifer-yellow and shown in panel (c), was actually located in the IML region (open circle).  The top is dorsal, and the bottom is ventral.
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Fig. 1. Brainstem-spinal cord preparation from a neonatal spontaneously hypertensive rat. Three different types of preparations were used for optical imaging and the whole-cell patch-clamp technique: (1) a type for observation on the ventral surface of the medulla oblongata, (2) a type for observation of cross sections at the level of the rostral ventrolateral medulla (RVLM), and (3) a type for observation of cross sections at the level of the caudal end of the VLM (CeVLM). Electrical stimulation was applied to the intermediolateral cell column (IML) at the Th2 level.
Fig. 2. Optical images of the ventral surface of the medulla oblongata during superfusion with standard solution. Depolarizing responses to IML stimulation were clearly detected on the ventral surface at the RVLM, the CVLM, and the caudal end of the VLM (CeVLM, n = 10).
Fig. 3. Optical images of the ventral surface of the medulla oblongata during superfusion with low-Ca high-Mg solution. Depolarizing responses to IML stimulation were detected on the ventral surface at the RVLM, the CVLM, and the caudal end of the VLM (CeVLM, $n = 6$).
Fig. 4. Optical images of a cross section at the level of the RVLM during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the RVLM (n = 6). The latency between stimulation of the IML and the start and the peak of the depolarizing response at the RVLM was 27 ± 9 (mean ± SD) ms and 35 ± 10 ms, respectively.
Fig. 5. Optical images of a cross section at the level of the caudal end of the VLM (CeVLM) during low-Ca high-Mg superfusion. A depolarizing response to IML stimulation was detected at the CeVLM (n = 8). The latency (mean ± SD) between stimulation of the IML and the start and the peak of depolarizing response at the CeVLM was 24 ± 5 ms and 36 ± 8 ms, respectively.
Fig. 6. Optical images of a cross section at the level of the caudal end of the VLM (CeVLM) before (upper two panels) and during GABA superfusion (lower two panels). IML stimulation during superfusion with low-Ca high-Mg solution containing GABA (200 µmol/L, n=7) did not induce any depolarizing responses at the CeVLM.
Fig. 7. Antidromic action potential of a neuron in the caudal end of the VLM (CeVLM) recorded by the whole-cell patch-clamp technique in response to electrical stimulation of the intermediolateral cell column [IML] at the Th2 level. The latency between stimulation and the start of the antidromic action potential was $27.2 \pm 4.6$ ms according to the whole-cell patch-clamp recordings ($n = 9$).
Fig. 8. Intracellular recordings of a neuron in the IML at the Th2 level (n = 8) during superfusion with standard solution, in the type (3) preparation, like Figures 5 and 6. (a) Local application of glutamate (1 mmol/L) to the CeVLM increased the number of excitatory postsynaptic potentials (EPSPs), induced depolarization (5.63 ± 2.50 mV), and increased the action potential spikes of the IML neurons. (b) Few EPSPs were seen before glutamate application. (c) Each EPSP and depolarization were clearly detected after local application of glutamate to the CeVLM.
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