Electrophysiological and morphological properties of rat supratrigeminal premotor neurons targeting the trigeminal motor nucleus

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Nakamura S, Nakayama K, Mochizuki A, Sato F, Haque T, Yoshida A, Inoue T. Electrophysiological and morphological properties of rat supratrigeminal premotor neurons targeting the trigeminal motor nucleus. J Neurophysiol 111: 1770–1782, 2014. First published February 5, 2014; doi:10.1152/jn.00276.2013.—The electrophysiological and morphological characteristics of premotor neurons in the supratrigeminal region (SupV) targeting the trigeminal motor nucleus (MoV) were examined in neonatal rat brain stem slice preparations. With Ca2+ imaging, whole cell recordings, and intracellular biocytin labeling. First, we screened SupV neurons that showed a rapid rise in intracellular free Ca2+ concentration ([Ca2+]i) after single-pulse electrical stimulation of the ipsilateral MoV. Subsequent whole cell recordings were generated from the screened SupV neurons, and their antidromic responses to MoV stimulation were confirmed. We divided the antidromically activated premotor neurons into two groups according to their discharge patterns during the steady state in response to I-s depolarizing current pulses: those firing at a frequency higher (HF neurons, n = 19) or lower (LF neurons, n = 17) than 33 Hz. In addition, HF neurons had a narrower action potential and a larger afterhyperpolarization than LF neurons. Intracellular labeling revealed that the axons of all HF neurons (6/6) and half of the LF neurons (4/9) entered the MoV from its dorsomedial aspect, whereas the axons of the remaining LF neurons (5/9) entered the MoV from its dorsolateral aspect. Furthermore, the dendrites of three HF neurons penetrated into the principal sensory trigeminal nucleus (Vp), whereas the dendrites of all LF neurons were confined within the SupV. These results suggest that the types of SupV premotor neurons targeting the MoV with different firing properties have different dendritic and axonal morphologies, and these SupV neuron classes may play unique roles in diverse oral motor behaviors, such as suckling and mastication.

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**EXPERIMENTAL PROCEDURES**

Animal care and experimental procedures were approved by the Institutional Animal Research Committee of Showa University in accordance with Japanese Government Law No. 105. All efforts were made to minimize the number of animals used and their suffering.

**Slice preparation.** Transverse brain stem slices (400 μm thick) including the MoV and the SupV were prepared from 46 Wistar rats (P1–6) as described previously (Nakamura et al. 2008). Briefly, the animals were anesthetized deeply with ether and then decapitated after deep anesthesia was ensured with toe and tail pinches. Each brain was removed rapidly and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 130 NaCl, 3 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose. ACSF was continuously bubbled with a 95% O2–5% CO2 gas mixture (carbogen) to establish a pH of 7.4. Transverse sections were cut on a vibrating microslicer (Pro 7, Dosaka EM, Kyoto, Japan) with a Leica Microsystems, Tokyo, Japan), and the slices were allowed to recover in a holding chamber containing ACSF at 34°C for 1 h and then maintained at room temperature (25–27°C) in ACSF prior to bulk loading with Ca2+ indicator fura-2 AM or fluo-8 AM (see below).

**Ca2+ imaging of SupV neurons.** Intracellular Ca2+ levels in neurons located in the SupV were measured by Ca2+ imaging with fura-2 AM (Dojindo, Kumamoto, Japan), a membrane-permeant Ca2+ indicator dye. The stock solution of fura-2 AM (1 mM) was dissolved in 10% Cremophor EL (Sigma-Aldrich, St. Louis, MO) and dimethyl sulfoxide (Sigma-Aldrich) and stored in frozen aliquots. Fura-2 AM was added to ACSF in a loading chamber to achieve a final concentration of 10 μM fura-2 AM. In some experiments, the slice preparation was loaded with 20 μM fluo-8 AM (AAT Bioquest, Sunnyvale, CA) as a Ca2+ indicator dye. The fluo-8 AM stock solution was dissolved in 20% Pluronic F-127 (Life Technologies, Carlsbad, CA) and dimethyl sulfoxide. Fluo-8 AM was added to ACSF in the loading chamber to achieve a final concentration of 20 μM fluo-8 AM. Forty-six slices corresponding to 46 animals were used in this study. Each slice was incubated at 34°C for 30 min for fura-2 AM and 1 h for fluo-8 AM in the carbogenated dye solution. After a 15-min recovery and dye washout period, the slices were transferred to a recording chamber, where they were continuously superfused with carbogenated ACSF at a rate of 2.0 ml/min at room temperature (25–27°C) in ACSF prior to bulk loading with Ca2+ indicator fura-2 AM or fluo-8 AM (see below).

**Electrophysiological recordings.** Whole cell patch-clamp recordings in current-clamp configuration were performed with infrared videomicroscopy (BX51WI, Olympus) and a ×40 water immersion objective with differential interference contrast. Patch electrodes were constructed from single-filament 1.5-mm-diameter borosilicate capillary tubing (GD-1.5, Narishige, Tokyo, Japan) with a microelectrode puller (P-97, Sutter Instrument, Novato, CA). Electrodes were filled with an internal solution of (in mM) 130 K-glucopon, 10 KCl, 10 HEPES, 0.4 EGTA, 2 MgCl2, 2 Mg-ATP, and 0.3 Na2-GTP (pH 7.3, 285–305 mosM). Biocytin (Sigma-Aldrich) at a concentration of 5 mg/ml was added to the internal solution for morphological analysis. Pipette resistance ranged from 2.5 to 5.0 MQ when the electrodes were filled. Action potentials and passive membrane properties were recorded with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and a Digidata 1332A interface (Molecular Devices). The data were filtered at 10 kHz, digitized at 20 kHz, and stored and analyzed on a personal computer with pCLAMP 9.2 (Molecular Devices) and Microsoft Excel software. The measured liquid junction potential of 12 mV between the pipette filling and bath solutions was subtracted from all membrane potentials, and series resistance compensation was set to 70–80% in the whole cell patch configuration. To assess antidromic responses of the recorded neurons to MoV stimulation, we applied a 100-Hz triple stimulation (200-μs pulse duration) of the MoV at intensities ≤25 μA through the stimulating electrode at 30 ms prior to the fourth frame by the imaging computer. The magnitude of the stimulus was set at ≤25 μA, which would result in the activation of neurons within the MoV and possibly axon terminals of the premotor neurons innervating to these motoneurons, based on the estimation of 10 μA as an effective spread of the stimulus current through a monopolar electrode, as proposed by Abzug et al. (1974). The imaging data were averaged over four trials. The averaged Ca2+ levels over the respective cell areas were sequentially plotted against time as the ratio F340/F380. When the ratio of fluorescence obtained from neurons immediately after MoV stimulation increased by ≥0.01 (0.02 ± 0.001, n = 37), we judged that these neurons displayed Ca2+ responses due to the MoV stimulation. If we did not find any neurons that displayed a Ca2+ response in the first imaging trial on a slice preparation, we repeated the trials in a slightly different imaging field and/or different focal plane up to four times on the same slice.

In some experiments, Ca2+ imaging was performed with a confocal microscope system (A1R, Nikon Instruments, Tokyo, Japan) equipped with a water immersion ×40 (0.8 NA) objective (Nikon Instruments). Fluo-8-loaded neurons were excited with a 488-nm laser. The changes in fluorescence were captured with an imaging scanner and imaged at a flame rate of 4 Hz with an image acquisition and analysis system (NIS-Elements, Nikon Instruments).

Averages were calculated using the SigmaPlot 13.0 software (Systat Software, San Jose, CA). All data were normalized to the average value of the first imaging trial on a slice. Statistical analysis was performed using two-way or one-way ANOVA, followed by Tukey’s test. Statistical significance was set at P ≤ 0.05.

**Slice stimulation.** The activity of each neuron was assessed by applying a 100-Hz triple stimulation (200-μs pulse duration) of the MoV at intensities ≤25 μA through the stimulating electrode at 30 ms prior to the fourth frame by the imaging computer. The magnitude of the stimulus was set at ≤25 μA, which would result in the activation of neurons within the MoV and possibly axon terminals of the premotor neurons innervating to these motoneurons, based on the estimation of 10 μA as an effective spread of the stimulus current through a monopolar electrode, as proposed by Abzug et al. (1974). The imaging data were averaged over four trials. The averaged Ca2+ levels over the respective cell areas were sequentially plotted against time as the ratio F340/F380. When the ratio of fluorescence obtained from neurons immediately after MoV stimulation increased by ≥0.01 (0.02 ± 0.001, n = 37), we judged that these neurons displayed Ca2+ responses due to the MoV stimulation. If we did not find any neurons that displayed a Ca2+ response in the first imaging trial on a slice preparation, we repeated the trials in a slightly different imaging field and/or different focal plane up to four times on the same slice.

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**Histology.** The neurons were filled by passive diffusion of biocytin from the patch pipette during the whole cell recording period (15–20 min) without the application of current. After recording the patch pipettes were carefully detached from the cells, and the slices remained in the recording chamber for an additional 30 min to allow biocytin transport into dendrites and axons. The slices were then fixed in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (PB) at 4°C for at least 24 h and left overnight in 30% sucrose in 0.1 M PB at 4°C. Some slices were cut into 80- to 100-μm-thick sections with a freezing microtome. The slices were incubated with avidin-biotin-horseradish peroxidase (HRP) complex (ABC kit; Vector, Burlingame, CA) in 0.05 M Tris-buffered saline (TBS) containing 1% Triton X-100 for 3 h at room temperature. After several rinses with TBS, the slices were reacted with 0.04% 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.002% H2O2, and 0.07% nickel ammonium sulfate in 0.05 M TBS and mounted on ovalbumin-coated slides. The slices were finally counterstained with cresyl violet or Neutral Red. The stained cells were reconstructed with a camera lucida.

**Statistics.** Statistical analyses were conducted with SPSS 13.0J (SPSS Japan, Tokyo, Japan) and Excel (Microsoft Japan, Tokyo, Japan). Values are presented as means ± SE. Differences between groups were analyzed with Student’s t-test, two-way ANOVA, and Fisher’s exact test. ANOVA was followed by Scheffé’s post hoc multiple-comparison test when appropriate. Probability values of <0.05 were considered significant.

**RESULTS**

Identification of SupV premotor neurons with Ca2+ imaging and whole cell recordings. To efficiently find SupV premotor neurons that targeted V motoneurons, we utilized a Ca2+ imaging method and searched for SupV neurons that showed a rapid rise in somatic [Ca2+]i, in response to single-pulse electrical stimulation applied to the ipsilateral MoV. We analyzed 40 brain stem slices stained with fura-2 AM and 6 slices stained with fluo-8 AM, which were obtained from 40 rats (P1–6) and 6 rats (P2–6), respectively. Fluorescence was detected in many neurons stained with fura-2 AM in the SupV (Fig. 1B), and we selected 20–89 (45.6 ± 3.8) SupV neurons with fluorescence per slice as ROIs. The data obtained from fluo-8-stained slices were not included in this analysis, because we could not count most fluo-8-labeled neurons because of the weak fluorescence of stained neurons before MoV stimulation. However, when we stimulated the MoV, we found neurons that clearly increased their fluorescence signal in real time (see below). The stimulating electrode was placed in the central region of the MoV (at a distance of ≥300 μm from the recording site), where the SupV neurons were situated (Fig. 1A), similar to that described previously (Nakamura et al. 2008), and a single pulse was delivered at an intensity ≤25 μA for Ca2+ imaging. In 34 of 40 preparations, 137 of 1,201 neurons (11.4%, 4.0 ± 0.9 neurons per slice) were located in the SupV showed an obvious increase in [Ca2+]i, in response to MoV stimulation (Fig. 1D), which was most likely produced by action potentials in the somas of these neurons. These action potentials may have been antidromically conducted from the axons of the SupV neurons distributed within the MoV, or they may have been orthodromically generated with interneurons directly or indirectly activated by MoV stimulation. In contrast, the remaining 1,064 neurons (88.6%) did not exhibit significant changes in fluorescence following stimulation (Fig. 1E). In 6 of the 40 slices examined, we could not find any neurons that responded with an increase in [Ca2+]i, after MoV stimulation.

To confirm that the SupV neurons with the stimulus-evoked [Ca2+]i increase directly sent their axons to the MoV, we used whole cell patch-clamp recording to examine whether these neurons were antidromically activated by MoV stimulation (Fig. 1C). The neurons selected for electrophysiological analysis were those demonstrating stable resting membrane potentials more negative than −50 mV and exhibiting overshooting action potentials larger than 80 mV (measured from the resting
membrane potential to the spike peak) in response to a brief (3 ms) injection of depolarizing current. We successfully collected whole cell recordings from 37 of the SupV neurons stained with Fluoro-3 AM and 6 of the SupV neurons stained with fluo-8 AM that had shown a significant increase in [Ca$_{2+}$]$_i$ by MoV stimulation in 40 slices from 40 animals. Thirty-seven (86.0%) of the 43 neurons followed a 100-Hz triple stimulation of the MoV at intensities of 19.9 ± 1.8 μA (n = 37) without action potential failure and underlying excitatory postsynaptic potentials (EPSPs; Fig. 1F). The mean latency to the action potentials initiated by each stimulus was fairly constant (1st: 2.67 ± 0.13 ms, 2nd: 2.78 ± 0.13 ms, 3rd: 2.82 ± 0.15 ms). In some experiments, we examined the effects of blockade of excitatory synaptic transmission on the responses of SupV neurons to triple stimulation of the MoV. We previously demonstrated that glycineric and GABAergic responses as well as glutamatergic responses are excitative in V motorneurons of P1–4 neonatal rats and that simultaneous application of the non-N-methyl-D-aspartate (NMDA) receptor antagonist CNQX (20 μM) and the NMDA receptor antagonist APV (20 μM) and also the GABA$_A$ receptor antagonist SR95531 (10 μM) and the glycine receptor antagonist strychnine (10 μM) abolishes postsynaptic responses to SupV stimulation (Nakamura et al. 2008). Therefore, we examined the effects of the simultaneous application of these four antagonists on the responses of SupV neurons. We performed whole cell recordings in four SupV neurons that had responded with an increase in [Ca$_{2+}$]$_i$ after MoV stimulation. All four SupV neurons followed a 100-Hz triple stimulation of the MoV before and during the simultaneous application of 20 μM CNQX, 20 μM APV, 10 μM SR95531, and 10 μM strychnine (data not shown). These results suggest that the responses of SupV neurons that followed a 100-Hz triple stimulation of the MoV were most likely to be antidromic. Therefore, it is most likely that the 37 neurons that followed the triple stimulation of the MoV sent their axons to the MoV and that they were premotor neurons targeting the MoV. The mean antidromic latencies (2.77 ± 0.13 ms, n = 37) in the present study and our previous study (3.0 ± 0.1 ms, n = 12; Nakamura et al. 2008) were longer than the latencies of trigeminal premotor neurons located around the MoV in anesthetized adult rats (0.4–1.4 ms; Inoue et al. 1992) and in brain stem slice preparations, which were maintained at a temperature of 29–31°C and obtained from 1- to 4-wk-old rats (0.6 ms; Bourque and Kolta 2001). Because the conduction velocities of axons are slow in neonatal animals (Nakamura et al. 1987; Walton and Navarrete 1991) and at low temperature and they decrease with cooling (Chatfield et al. 1948; Gasser 1931), the long antidromic latencies in this study were likely due to the immature state of the neurons and/or low temperature in the perfusion solution.

The remaining 6 of the 43 neurons did not follow the 100-Hz triple shock, even at intensities >25 μA. All six neurons responded with one or two action potentials by MoV stimulation, suggesting that the responses of these six neurons were most likely mediated by synaptic inputs from interneurons that were located in MoV or other areas adjacent to the MoV rather than antidromic activation. We also performed whole cell recordings in SupV neurons that had not responded with an increase in [Ca$_{2+}$]$_i$ after MoV stimulation (n = 6); however, none of these neurons antidromically responded to 100-Hz triple stimulation of the MoV (Fig. 1G).

**Firing characteristics of antidromically activated SupV premotor neurons.** We next examined the firing properties of SupV neurons antidromically activated by MoV stimulation in response to current injection of a 1-s constant depolarizing pulse from the resting membrane potential. We found two types of antidromically activated SupV neurons based on their steady-state firing (average firing frequency of overshooting action potentials over the last half of an injected 1-s constant depolarizing current pulse from the resting membrane potential). Figure 2A shows an example of the first type of neurons that were characterized as neurons with high-frequency tonic spiking ability throughout the duration of the depolarizing pulse at high current intensities (HF neurons). At an intensity of 0.05 nA, near the rheobase, this neuron type showed a sustained repetitive firing discharge throughout the current pulse with virtually no spike frequency adaptation. Moreover, the firing frequency progressively increased with increases in the current intensity, and the firing frequency of the steady-state firing was beyond 38 Hz even at 0.15 nA (Fig. 2A and B). Another type of neurons was characterized by low-frequency firing properties in the steady state (LF neurons). In response to current pulses near the rheobase (0.05 nA), the second neuron type also showed sustained tonic firing with slight spike frequency adaptation, for which the firing frequency was similar to that of HF neurons at 0.05 nA (Fig. 2C). However, further depolarization progressively caused a decrease in the action potential amplitude and an increase in the action potential duration. Because such changes in the spike form resulted in an increase in nonovershooting action potentials, the maximum frequency of the steady-state firing stayed at 24 Hz in this example (Fig. 2D). One SupV premotor neuron fired at higher than 33 Hz during the steady state only when the neuron was depolarized by >50 mV. Thus we excluded this neuron from both HF and LF neurons.

When we constructed a histogram of the maximum firing frequency of the steady-state firing in the SupV neuron, the histogram clearly showed a bimodal distribution with two peaks, which were separated by ~33 Hz (Fig. 2E). On the basis of this observation, we defined HF (n = 19) and LF (n = 17) neurons as those firing in the steady state at an average firing frequency higher and lower than 33 Hz, respectively. The majority of HF neurons (13/19) fired at average frequencies higher than 50 Hz, and the average firing frequencies were significantly higher in HF neurons than the firing frequencies in LF neurons at current injection of 0.1–0.4 nA (Fig. 2F). In contrast to steady-state firing, the mean first interspike interval (ISI) of both HF and LF neurons rose as the current intensity increased and attained ~100 Hz at 0.4 nA [HF: 112 ± 3.1 Hz (n = 19); LF: 93.5 ± 8.8 Hz (n = 17); Fig. 2G].

Hsiao et al. (2007) reported that 37% of SupV neurons, which were not identified as premotor neurons for the MoV, displayed a delayed-firing pattern in response to a 1-s depolarizing current pulse from a holding membrane potential of ~80 mV. When the membrane potentials of the neurons were clamped around ~50 mV or 50 μM, 4-aminopyridine (4-AP), a blocker for A-type voltage-gated K$^+$ current, was applied, a depolarizing current pulse evoked sustained firing, and the first spike of the spike train occurred almost immediately after the current injection. Therefore, we also examined whether the firing characteristics of HF (n = 4) or LF (n = 4) neurons that were classified on the basis of firing characteristics at the
resting membrane potential were altered in response to a depolarizing current pulse from −80 mV. However, none of the HF or LF neurons changed their firing characteristics to the alternate type of discharge pattern. Only one neuron demonstrated single-spike characteristics, with a solitary action potential at the onset of a depolarizing current pulse at both resting and hyperpolarized (−80 mV) membrane potentials.

Hsiao et al. (2007) also reported that membrane depolarization produced a rhythmic burst discharge in a subpopulation of SupV interneurons in P8–12 rats. Therefore, we next examined whether SupV premotor neurons generated rhythmic burst firing in response to prolonged depolarizing current injection in eight HF neurons and seven LF neurons. In six of the eight HF and two of the seven LF neurons, prolonged membrane depolarization induced an obvious tonic burst discharge, and the firing frequency increased as the current intensity increased (Fig. 3A). In five of the seven LF neurons, prolonged membrane depolarization did not produce a burst discharge but instead produced an irregular spike discharge (Fig. 3B). Only two HF neurons among the eight HF and seven LF neurons tested showed a rhythmic burst discharge when depolarized by prolonged current injection (Fig. 3C). The ability to generate rhythmic burst discharge was retained even when the cells were depolarized to −42 mV. This result suggests that a small population of SupV premotor neurons have the ability to produce burst discharges, even those from P1–5 neonatal rats, which is similar to the result described by Hsiao et al.

Passive and active membrane properties of antidromically activated SupV neurons. We next compared passive and active membrane properties between HF and LF neurons. Resting membrane potentials, input resistances, membrane time constants, and cell capacitances were not significantly different between HF and LF neurons (Table 1). In response to serial hyperpolarizing current pulses from −200 to 0 pA in 50-pA increments, the majority of HF and LF neurons demonstrated no depolarizing sags and showed a relatively linear current-voltage (I–V) relationship in the range from the resting membrane potential to approximately −100 mV (Fig. 4A and B). In addition, some inward rectification was observed at more hyperpolarized potentials. Only one HF and one LF neuron displayed small depolarizing sags at the termination of hyperpolarizations to currents more negative than approximately

Fig. 2. Two types of discharge characteristics of SupV premotor neurons. A: sample responses of a high-frequency-firing (HF) neuron following injection of different depolarizing current pulses (left, 0.05 nA; center, 0.15 nA; right, 0.25 nA) from the resting membrane potential. B: the instantaneous frequency for the 1st interspike interval (ISI) and steady-state discharge frequency (left) and a frequency-time plot for 3 different current intensities for the HF neuron shown in A (right). C: sample responses of a low-frequency-firing (LF) neuron following injection of different depolarizing current pulses (left, 0.05 nA; center, 0.15 nA; right, 0.25 nA) from the resting membrane potential. D: the 1st ISI and steady-state discharge frequency (left) and frequency-time plot for 3 different current intensities for the LF neuron shown in C (right). The steady-state discharge was calculated according to the mean number of spikes over the last 500 ms of a 1-s current pulse. Note that the HF neuron showed a sustained tonic firing discharge with little adaptation, whereas the LF neuron exhibited a rapid spike frequency adaptation during the spike train at higher current intensities. E: distribution of maximum firing frequencies in the steady state. Gray and open bars indicate the cell numbers of HF and LF neurons, respectively. The lines indicate the fitting of Gaussian distributions of HF and LF neurons. F: average steady-state discharge frequency. The steady-state frequency was significantly higher in HF neurons than in LF neurons, whereas there was no significant difference in the 1st ISI between HF and LF neurons. G: summary plot for the mean 1st ISI. SE bars are indicated in each plot. Significant differences: *P < 0.05, **P < 0.01.
-90 mV. Postinhibitory rebound spikes were observed at the offset of hyperpolarizing current pulses in 32% (6 of 19) of HF neurons and 6% (1 of 17) of LF neurons. There were no significant differences in the presence of the depolarizing sag and rebound spike between HF and LF neurons (Fisher’s exact test, \( P = 0.69 \) and 0.09, respectively).

Single action potentials were elicited by injections of 3-ms depolarizing current pulses from the resting membrane potential (Fig. 4, C–E). The amplitudes of the action potentials were not significantly different between HF and LF neurons, whereas both the half-duration and the 90–10% decay time of the action potential were significantly shorter in HF neurons than in LF neurons (\( P < 0.05 \); Table 1). Moreover, both HF and LF neurons exhibited only monophasic afterhyperpolarization (AHP); however, the AHP amplitude was significantly larger in HF neurons than in LF neurons (\( P < 0.05 \); Table 1), whereas the AHP half-width was not significantly different between HF and LF neurons (\( P = 0.61 \); Table 1).

Morphological properties of antidromically activated SupV premotor neurons. Of the 37 antidromically activated SupV neurons that were electrophysiologically characterized with whole cell recordings, 6 HF and 9 LF neurons were successfully stained by intracellular injection of biocytin through patch pipettes during whole cell recordings. All of these neurons sent their axons to the MoV.

The cell bodies of the HF neurons were scattered from the middle to the lateral SupV (Fig. 5). HF neuron morphology was characterized by axonal trajectories to the MoV, i.e., the axons of all six HF neurons entered the MoV from the dorsomedial aspect (class I morphology; Table 2). Figure 6 shows an example of reconstructions of a class I HF neuron. The primary dendrites extended in various directions (Fig. 6, A, C, and D). This neuron could fire at >33 Hz in the steady state (Fig. 6B). A stem axon originated from a primary dendrite (Fig. 6, A and D, arrowheads), ran dorsally to a certain extent, and then produced two branches. One branch ran medially toward the midline and further issued two collaterals that ran ventrally to the MoV. The two collaterals entered the MoV from the dorsomedial aspect and ramified broadly and terminated within the MoV. In 50% of HF neurons (3/6), the axon collaterals could be identified in both the dorsal and ventral halves of the MoV. The axons of the remaining HF neurons (3/6) were not fully stained by intracellular injection of biocytin through patch pipettes during whole cell recordings. All of these neurons sent their axons to the MoV.

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Table 1. Passive and active membrane properties of antidromically activated SupV premotor neurons

<table>
<thead>
<tr>
<th></th>
<th>HF Neurons</th>
<th>LF Neurons</th>
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<tr>
<td>Total cells</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>-62.2 ± 1.6</td>
<td>-64.3 ± 0.8</td>
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<tr>
<td>Input resistance, MΩ</td>
<td>524.0 ± 47.1</td>
<td>474.4 ± 58.2</td>
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<tr>
<td>Membrane time constant, ms</td>
<td>33.2 ± 3.8</td>
<td>29.1 ± 4.8</td>
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<tr>
<td>Cell capacitance, pF</td>
<td>63.1 ± 4.1</td>
<td>62.6 ± 3.4</td>
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<tr>
<td>Action potential</td>
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<td></td>
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<tr>
<td>Amplitude, mV</td>
<td>95.0 ± 2.0</td>
<td>92.7 ± 2.5</td>
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<tr>
<td>Half-width, ms</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1*</td>
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<tr>
<td>Rise time, ms</td>
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<td>3.7 ± 0.2</td>
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<tr>
<td>Decay time, ms</td>
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<td>3.1 ± 0.5*</td>
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<tr>
<td>Firing threshold, mV</td>
<td>-54.0 ± 0.8</td>
<td>-51.0 ± 1.4</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>8.4 ± 1.3</td>
<td>3.4 ± 0.5*</td>
</tr>
<tr>
<td>AHP half-width, ms</td>
<td>86.1 ± 6.1</td>
<td>79.7 ± 6.8</td>
</tr>
</tbody>
</table>

Data are means ± SE. SupV, supratrigeminal region; HF, high frequency firing; LF, low frequency firing; AHP, afterhyperpolarization. *P < 0.05, t-test.
confined within the dorsal half of the MoV. Under high magnification, the terminal arbors with boutons terminaux and en passant could be found within the MoV in the six HF neurons (Fig. 6E). The other branch ran laterally and entered the dorsal part of the Vp, and the axon collaterals formed terminal arbors in the Vp (Fig. 6A and C). Interestingly, three of the six HF neurons had dendrites that extended in the lateral direction and penetrated into the dorsal part of the Vp (Fig. 6C). In the remaining three HF neurons, the dendrites were confined within the SupV (Table 3).

The cell bodies of the LF neurons were scattered more widely than in HF neurons from the middle to the lateral SupV (Fig. 5). In five of the nine LF neurons, their stem axons entered the MoV at the dorsolateral aspect (class II morphology), although axons of the latter half of the LF neurons entered the MoV from its dorsomedial aspect (class I). The proportion of LF neurons with class I morphology was significantly lower than that of HF neurons with class I morphology (Fisher’s exact test, \( P < 0.05 \); Table 2). Figure 7 shows an example of an LF neuron with class II morphology. In this example, the slices were cut to a 100-\( \mu \)m thickness to detect synaptic contacts of SupV premotor neurons on the V motoneurons. The primary dendrites extended in various directions, and one branch entered the MoV to a small extent (Fig. 8A and C). A stem axon originated from a primary dendrite (Fig. 8D), coursed laterally for some distance, and then turned ventrally to the MoV as in the example shown in Fig. 7; however, the stem axon entered the MoV from its dorsomedial aspect. The boutons terminaux and en passant were distributed in the dorsal and ventral part of the MoV, and a significant number of them were found to contact the somata and/or juxtasomatic regions of the V motoneurons, i.e., this neuron was a premotor neuron for V motoneurons. In other LF neurons with class I morphology, a stem axon coursed medially and then ran ventrally and entered the MoV from its dorsomedial aspect, similar to the class I HF neurons. However, all class I LF neurons had dendrites that were confined within the SupV, in contrast to the class I HF neurons.

The axon collaterals of six of the nine LF neurons (67\%), which were three class I and three class II LF neurons, were distributed in both the dorsal and ventral halves of the MoV. In the remaining three LF neurons (1 class I LF neuron and 2 class
II LF neurons), the axons were confined within the dorsal half of the MoV. None of the dendrites of the nine LF neurons extended to the Vp but were confined within the SupV (Table 3). The occurrence of penetration of dendrites into the Vp was significantly higher in HF neurons than in LF neurons (Fisher’s exact test, $P < 0.05$). These results suggest that HF and LF SupV premotor neurons possess different morphological features.

**DISCUSSION**

In the present study, we first used Ca$^{2+}$ imaging to screen SupV premotor neurons with functional axonal connection with the MoV in slices containing the MoV. Subsequent whole cell recordings were made from the screened SupV neurons, from which antidromic responses to MoV stimulation were further confirmed. We then examined the electrophysiological properties of the antidromically activated SupV premotor neurons, and our data demonstrated the presence of two types of premotor neurons on the basis of their firing properties during the steady state: HF and LF neurons. Intracellular biocytin labeling revealed that HF and LF neurons differed in their morphological characteristics, specifically, axonal and dendritic trajectories. All HF neurons and half of the LF neurons possessed axons that entered the MoV at the dorsomedial aspect of the MoV (class I), whereas the other half of the LF neurons issued axons that entered the MoV at the dorsomedial aspect of the MoV (class II). Furthermore, compared with LF neurons, more HF neurons had dendrites that entered the dorsal part of the Vp. These results suggest that the SupV premotor neurons with distinct physiological properties also had different morphological features based on dendritic and axonal morphologies.

**Identification of V premotor neurons in the SupV.** In the present study, to detect SupV premotor neurons targeting the MoV, we first screened SupV neurons demonstrating a rapid increase in [Ca$^{2+}$], in response to single-pulse electrical stimulation of the ipsilateral MoV and identified 137 candidates for premotor neurons out of 1,201 neurons from 40 preparations stained with fura-2 AM (~4 neurons per slice). We subsequently performed whole cell recordings for 37 neurons of the 139 candidates stained with fura-2 AM and examined whether these neurons antidromically responded to MoV stimulation. We detected as many as 31 (84%) of the 37 SupV neurons that followed a 100-Hz triple stimulation of the MoV without action potential failure. When we performed whole cell recordings for randomly selected SupV neurons in a previous study, only 12 (8.4%) of the 143 SupV neurons tested responded antidromically to MoV stimulation (Nakamura et al. 2008). The detection rate (8.4%) of antidromically activated SupV neurons in that study is similar to the rate (9.6%) of antidromically activated SupV neurons among total SupV neurons demonstrating fluorescence in the present study, as estimated by extending the whole cell recording data to the entire neuronal population of the slices (determined by multiplying 137 by 0.84 and dividing by 1,201). Subsequent intracellular labeling experiments further demonstrated that all neurons successfully stained with biocytin were premotor neurons targeting the MoV. Thus our screening method utilizing Ca$^{2+}$ imaging yielded a 10-fold higher efficiency in identifying premotor neurons targeting the MoV than random sampling.

**Firing characteristics of SupV premotor neurons.** In the SupV, we detected HF and LF premotor neurons showing sustained repetitive firing frequencies that exceeded 33 Hz and were restricted to $<33$ Hz, respectively. Similar firing properties have been reported for interneurons located in the SupV and the reticular formation surrounding the MoV, although these interneurons have not been identified as premotor neurons for the MoV (Bourque and Kolta 2001; Brocard et al. 2006; Hsiao et al. 2007; McDavid et al. 2008; Min et al. 2003; Minkels et al. 1995). We previously reported that laser photolysis of caged glutamate in the lateral SupV of P1–5 rats evoked high-frequency (>50 Hz) burst PSCs more frequently in masseter motoneurons than in digastric motoneurons, even in the presence of the glycine receptor antagonist strychnine and the GABA$_A$ receptor antagonist SR955531 (Nonaka et al. 2012). Because PSCs evoked by electrical stimulation of the SupV in P1–12 rats are completely abolished by the combined application of the non-NMDA receptor antagonist CNQX and the NMDA receptor antagonist APV in addition to strychnine and SR955531 (Nakamura et al. 2008), the PSC components that are insensitive to both strychnine and SR95531 are most likely produced through activation of ionotropic glutamate receptors. Thus it is possible that certain HF neurons in the SupV send excitatory burst inputs to jaw-closing motoneurons and may contribute to increasing jaw-closing muscle activity in response to periodontal and spindle afferent inputs after the weaning period.

**Table 2. Distribution of SupV premotor neurons according to discharge classification and axonal trajectory**

<table>
<thead>
<tr>
<th>Class</th>
<th>HF Neurons</th>
<th>LF Neurons</th>
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<tbody>
<tr>
<td>I</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>5</td>
</tr>
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In the present study, two HF premotor neurons exhibited a rhythmical burst discharge during prolonged current injections. Similar intrinsic bursting activity has been found in SupV interneurons (Hsiao et al. 2007) and Vp neurons located adjacent to the SupV (Brocard et al. 2006). Because these SupV and Vp neurons may be responsible for the generation of suckling and/or masticatory rhythms (Morquette et al. 2012), it is possible that some HF neurons are involved in the suckling and/or masticatory rhythm generator and could therefore directly send rhythmical inputs to V motoneurons.

In addition, HF premotor neurons in the SupV may send inhibitory inputs to V motoneurons. It has been demonstrated that high-frequency burst discharges are evoked in SupV neurons by single electrical pulses of the inferior alveolar nerve or the lingual nerve in the cat (Goldberg and Nakamura 1968; Kidokoro et al. 1968). These SupV neurons are assumed to be inhibitory premotor neurons for jaw-closing motoneurons in the jaw-opening reflex because the amplitude of the monosynaptic inhibitory postsynaptic potentials (IPSPs) of the jaw-closing motoneurons evoked by the stimulation applied to the inferior dental nerve or the lingual nerve varied in parallel with the spike number of the SupV neurons evoked by the stimulation (Kidokoro et al. 1968; Nakamura et al. 1973). These SupV neurons have also been shown to be monosynaptically activated by stimulation of the inferior alveolar nerve and the lingual nerve (Goldberg and Nakamura 1968; Kidokoro et al. 1968). However, no HRP-labeled terminals were observed in the SupV of the cat after injection of HRP into primary afferents from the oral facial areas (Shigenaga et al. 1986; Table 3. Distribution of SupV premotor neurons according to discharge classification and dendritic characteristics

<table>
<thead>
<tr>
<th>Dendrites in Vp</th>
<th>HF Neurons</th>
<th>LF Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>

Vp, principal sensory trigeminal nucleus.
Tsuru et al. 1989). In the present study, the dendrites of three of six HF neurons penetrated into the Vp, and such HF neurons may directly receive primary afferent inputs in the Vp, which may account for the previously reported monosynaptic activation of SupV neurons by afferent stimulation in the cat (Goldberg and Nakamura 1968; Kidokoro et al. 1968). On the other hand, neither the remaining three HF neurons nor the LF neurons ($n = 9$) extended their dendrites into the Vp. SupV neurons in anesthetized rats were reported to respond to stimulation of the inferior alveolar nerve at significantly longer latencies than the interneurons located in the reticular formation medial to the oral spinal trigeminal nucleus (5.8 ± 2.3 ms vs. 3.1 ± 1.2 ms) (Inoue et al. 1992). Therefore, certain populations of SupV neurons are likely to receive afferent inputs di- or polysynaptically.

In contrast to HF neurons, current injections of $>0.15$ nA to LF neurons led to an increase in nonovershooting action potentials, resulting in a drop of the firing frequency during the steady state. Hsiao et al. (2007) reported that a subset of SupV neurons in rats display an initial burst of action potentials and spike cessation during injection of a 1-s depolarizing current pulse and have smaller AHPs compared with SupV neurons with tonic firing properties. These findings correspond to the properties of LF neurons described in the present study, although the SupV neurons were not identified as premotor neurons for V motoneurons in the previous study. These previous authors proposed that a slowly inactivating or noninactivating low-threshold $K^+$ current may contribute to the observed firing property, because the SupV neurons were transformed into toniclike neurons following the application of 4-AP, which blocks A-type voltage-gated $K^+$ currents. However, these $K^+$ currents are less likely to be responsible for the firing patterns of LF premotor neurons, because the LF firing pattern never shifted to the HF firing pattern when the membrane potential was held at $-80$ mV. Enomoto et al. (2007) demonstrated that, even in the presence of 4-AP, the onset of reduction in the number of spikes and complete spike inactivation in mesencephalic trigeminal neurons with increasing...
stimulus intensity occurred at lower stimulus intensities in Na1.6-null mice, which have smaller persistent and resurgent Na⁺ currents compared with wild-type mice. Therefore, smaller persistent and/or resurgent Na⁺ currents compared with HF neurons may underlie the firing pattern of LF neurons.

Relationships between functional properties and axonal trajectories of SupV premotor neurons. In the present study, SupV premotor neurons were classified as class I or class II neurons on the basis of their axonal trajectories. The proportion of HF neurons with class I morphology (6/6) was significantly higher than that of LF neurons with class I morphology (4/9), indicating that SupV premotor neurons with different firing properties also had different axonal trajectories. Studies using intra-axonal HRP staining of premotor neurons in the Vp (Yoshida et al. 1998) and the oral spinal trigeminal nucleus (Vo) (Yoshida et al. 1994) of the cat demonstrated axonal trajectories of the premotor neurons to the MoV. These authors showed that there were two types of premotor neurons in the Vo: 1) DL neurons projecting to the dorsolateral subdivision of the MoV, where jaw-closing motoneurons are located, and 2) VM neurons projecting to the ventromedial subdivision of the MoV, where jaw-opening motoneurons are located. DL neurons possess receptive fields in more posterior oral structures in the maxillary region than VM neurons, and their axon collaterals enter the MoV from its lateral aspect. In contrast, the receptive fields of VM neurons are located in the mandibular region, and their axon collaterals enter the MoV from its more caudomedial aspect. These results from studies of Vo premotor neurons in the cat suggest that different axonal trajectories of premotor neurons to the MoV may imply differential acceptance of peripheral sensory inputs and synaptic connections of the premotor neurons to the jaw-closing or jaw-opening motoneurons. However, all premotor neurons in the Vp just lateral to the SupV project to the dorsolateral subdivision of the MoV, although their axon collaterals enter the MoV from its dorsomedial and/or dorsolateral aspects.
ors such as suckling and mastication. Different physiological and morphological properties may play...and the lateral hypothalamus, masticatory rhythm inputs from the masticatory area and the primary...mentioned above, the SupV receives diverse inputs including different dendritic and axonal morphologies. Moreover, as...the MoV had distinct physiological characteristics as well as different dendritic and axonal morphologies. Moreover, as...inputs to individual SupV premotor neurons and the target motoneurones to which the SupV premotor neurons project.

We demonstrated that the SupV premotor neurons targeting the MoV had distinct physiological characteristics as well as different dendritic and axonal morphologies. Moreover, as mentioned above, the SupV receives diverse inputs including descending inputs from the masticatory area and the primary orofacial motor area of the cerebral cortex (Hatanaka et al. 2005; Yasaki et al. 1985; Yoshida et al. 2009), the amygdala, and the lateral hypothalamus, masticatory rhythm inputs from the CPG for mastication, and abundant afferent inputs from the oral facial structures. Thus SupV premotor neurons with different physiological and morphological properties may play distinct roles related to complicated and diverse motor behaviors such as suckling and mastication.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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Ohta M, Moriyama Y. Supratrigeminal neurones mediate the shortest, disynaptic pathway from the central amygdaloid nucleus to the contralateral


