Synaptic Transmission from the Supratrigeminal Region to Jaw-closing and
Jaw-opening Motoneurons in Developing Rats

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Abstract

The supratrigeminal region (SupV) receives abundant orofacial sensory inputs and descending inputs from the cortical masticatory area and contains premotor neurons that target the trigeminal motor nucleus (MoV). Thus, it is possible that the SupV is involved in controlling jaw muscle activity via sensory inputs during mastication. We used voltage-sensitive dye, laser photostimulation, patch-clamp recordings and intracellular biocytin labeling to investigate synaptic transmission from the SupV to jaw-closing and jaw-opening motoneurons in the MoV in brainstem slice preparations from developing rats. Electrical stimulation of the SupV evoked optical responses in the MoV. An antidromic optical response was evoked in the SupV by MoV stimulation, whereas synaptic transmission was suppressed by substitution of external Ca\(^{2+}\) with Mn\(^{2+}\). Photostimulation of the SupV with caged glutamate evoked rapid inward currents in the trigeminal motoneurons. Gramicidin-perforated and whole-cell patch-clamp recordings from masseter motoneurons (MMNs) and digastric motoneurons (DMNs) revealed that glycinergic and GABAergic postsynaptic responses evoked in MMNs and DMNs by SupV stimulation were excitatory in P1-4 neonatal rats and inhibitory in P9-12 juvenile rats, whereas glutamatergic postsynaptic responses evoked by SupV stimulation were excitatory in both neonates and juveniles. Furthermore, the axons of biocytin-labeled SupV neurons that were antidromically activated by MoV stimulation terminated in the MoV. Our results suggest that inputs from the SupV excite MMNs and DMNs through activation of glutamate, glycine, and GABA\(_A\) receptors in neonates, whereas glycinergic and GABAergic inputs from the SupV inhibit MMNs and DMNs in juveniles.
INTRODUCTION

The supratrigeminal region (SupV) is located dorsal to the trigeminal motor nucleus (MoV) and medial to the trigeminal main sensory nucleus (Lorente de Nó 1933; Mizuno and Sauerland 1970). The SupV receives abundant orofacial sensory inputs, including periodontal and spindle afferents (Dessem and Taylor 1989; Jerge 1963; Mizuno 1970; Nishimori et al. 1986; Nomura and Mizuno 1985; Shigenaga et al. 1989), and descending inputs from the cortical masticatory area (Hatanaka et al. 2005; Yasui et al. 1985). Retrograde axonal tracing studies have shown that premotor neurons targeting the MoV are present in the SupV (Landgren et al. 1986; Mizuno et al. 1983; Travers and Norgren 1983; Turman and Chandler 1994a; Turman and Chandler 1994b). Premotor neurons identified by antidromic activation of the MoV are also present in the SupV (Donga and Lund 1991; Inoue et al. 1992; Westberg et al. 1995). Therefore, it is possible that the SupV is involved in controlling jaw-closing and jaw-opening muscle activity via sensory and descending inputs during mastication.

It is assumed that glycinergic SupV neurons inhibit jaw-closing motoneurons during the jaw-opening reflex (JOR) because the number of spike discharges evoked from SupV neurons by peripheral stimulation is proportional to the amplitude in the early phase of the inhibitory post-synaptic potentials (IPSPs) of jaw-closing motoneurons and the early phase of the IPSPs were decreased by strychnine (Kidokoro et al. 1968; Nakamura et al. 1973); SupV stimulation induced monosynaptic IPSPs in those motoneurons (Goldberg and Nakamura 1968; Kidokoro et al. 1968; Kolta 1997). In contrast, we showed previously that stimulus pulses to the SupV nearly always elicited masseter electromyography responses at short latencies (Takamatsu et al. 2005). When the latency from the motoneuron to the muscle is considered, excitatory premotor neurons that target jaw-closing motoneurons are likely present in the SupV. However, the detailed mechanism of the synaptic transmission from SupV neurons to
jaw-closing and jaw-opening motoneurons, particularly the neurotransmitters involved, are not clear. Furthermore, because feeding behavior changes dramatically from suckling to mastication during the early postnatal period, synaptic transmission might also change during postnatal development.

The aim of the present study was to characterize synaptic transmission from the SupV to jaw-closing and jaw-opening motoneurons and to examine postnatal changes in synaptic transmission in brainstem slice preparations from neonatal and juvenile rats. We found that inputs from the SupV excite jaw-closing and jaw-opening motoneurons through activation of glutamate, glycine and GABA<sub>A</sub> receptors in neonatal rats, whereas glycinergic and, most likely, GABAergic inputs to these motoneurons are inhibitory in juveniles.

**METHODS**

All experiments were approved by the International Animal Research Committee of Showa University in accordance with Japanese Government Law No. #105 for the care and use of laboratory animals.

*Slice preparation*

Experiments were performed with brainstem slices from postnatal day (P) 1-12 Wistar rats (<i>n</i> = 107). The day of birth was defined as P0. Animals were anesthetized deeply with ether and decapitated. Each brain was removed rapidly and placed in cold oxygenated artificial cerebrospinal fluid (ACSF). The brainstem was cut into 400 or 500 µm transverse sections with a microslicer (Pro 7, Dosaka EM, Kyoto, Japan). For cutting P7-12 rat brainstems, we used sucrose-based modified ACSF because it has been shown that the use of this ACSF during the slicing procedure is essential to maintain viable motoneurons in adult rats.
Modified ACSF contained (in mM) 260 sucrose, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 glucose. Viable motoneurons were obtained from P1-6 rats by using normal ACSF during slice preparation, as previously shown in spinal (Takahashi and Berger 1990), hypoglossal (Berger et al. 1992) and trigeminal (Del Negro and Chandler 1998) motoneurons of neonatal rats. Normal ACSF contained (in mM) 130 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 glucose. To compare membrane properties of motoneurons in slices cut in normal and modified ACSF, some slices from P1-6 rats were prepared by using modified ACSF. There was no significant difference in the resting membrane potential between motoneurons obtained from the two kinds of slices in P1-6 rats (normal ACSF, -68.2 ± 1.0 mV, n = 36; modified ACSF, -70.9 ± 0.9 mV, n = 8; P > 0.05). Furthermore, gramicidin-perforated patch-clamp recordings (see below) from motoneurons (n = 8) in the slices prepared with modified ACSF in P1-6 rats showed that all the eight motoneurons depolarized by pressure application of 1 mM glycine or bath application of 200 µM GABA similarly in the motoneurons prepared with normal ACSF.

Normal ACSF and modified ACSF were aerated continuously with a 95% O₂-5% CO₂ gas mixture. Slices from P1-6 rats were incubated at 35°C for 1 h in normal ACSF. Slices from P7-12 rats were incubated at 35°C in modified ASCF for 20 min followed by a 20-min incubation with a 50:50 mixture of modified and normal ACSF and then incubation in normal ACSF for an additional 20 min. All slices were maintained at room temperature (25-27°C) in normal ACSF. We also used normal ACSF for recordings.

Optical recordings

Fifty-four slices from 41 P1-6 animals were stained in normal ACSF containing 100 µg ml⁻¹ of fluorescent voltage-sensitive dye (Di-4-ANEPPS; Molecular Probes, Eugene, OR) as
described by Tominaga et al. (2000) for 1 h under 0.4 kgf cm⁻² of 95% O₂-5% CO₂ gas. After staining, unbound dye was removed by rinsing the sections in ACSF for 15 min. Slices were gently transferred into a recording chamber that was mounted on an upright fluorescence microscope (BX51WI; Olympus, Tokyo, Japan). The slices were superfused continuously with normal ACSF at a rate of 2.0 ml min⁻¹ at room temperature with a peristaltic pump (EYELA MP-1000; Tokyo Rikakikai, Tokyo, Japan).

Stimulus-evoked responses in the slice preparations were measured as the fractional change in fluorescence of the voltage-sensitive dye with an optical imaging system (MiCAM01 or MiCAM Ultima, Brain Vision, Tsukuba, Japan) equipped with a 510-550 nm excitation filter, a dichroic mirror and a 590 nm absorption filter (U-MWIG2 mirror unit, Olympus) and 150-watt tungsten-halogen lamp (MHF-G150LR, Moritex, Tokyo, Japan). The CCD-based camera head had a 3.0 x 2.0 mm² imaging area (96 x 64 pixels, MiCAM01) and the CMOS camera head had a 10.0 x 10.0 mm² imaging area (100 x 100 pixels, MiCAM Ultima), and the microscope magnification was adjusted to a 4x objective lens (0.28 NA, XLFluor4x/340; Olympus) so that an area of 0.75 x 0.5 mm² (MiCAM01) or 2.5 x 2.5 mm² (MiCAM Ultima) was covered by the image sensor. Each optical recording was acquired at a rate of 3.0 ms/frame for 512 frames. Fluorescence signals for each 1.5 sec per trial, including 300 ms before stimulation, were recorded at 3-sec intervals and averaged over 16-128 trials. A Teflon-insulated tungsten electrode (impedance 1 MΩ at 500 Hz, TOG204-045, Unique Medical, Tokyo, Japan) was used for single-pulse slice stimulation (0.33 Hz, 20-30 µA, 0.2 ms pulse duration). Fluorescence changes were expressed as a ratio (percent fractional change) of the change in the intensity of fluorescence relative to that of the reference image. The differential image, processed with a 2 x 2 (pixels in the image plane) and 2 x (time frames) software filter, was represented by a pseudo-color display in which red corresponded to
fluorescence decrease and membrane depolarization. Optical data were collected and stored on a personal computer controlled by MiCAM01 or MiCAM01 Ultima-associated software (Brain Vision). To represent the time course of fluorescence change, the optical signals were inverted with an upward deflection corresponding to depolarization. The MoV was visualized in the slices as an opaque bright oval region through the optical imaging system. The optical responses in the MoV evoked by stimulation were measured in the central region (3 x 3 pixels) of the MoV.

At the end of each experiment, brainstem stimulation sites were marked by passing a 10 sec, 20 µA negative current through the electrodes in select slices. The slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for at least 1 day at -4°C. Slices were then rinsed with 0.1 M phosphate buffered-saline (PBS) (pH 7.4) and incubated overnight in 5-25% sucrose/PBS at -4°C. Transverse 20-µm thick frozen sections were then cut with a cryostat and stained with cresyl violet. The location of stimulation sites was confirmed by microscopic examination.

**Photostimulation**

We used laser photolysis of caged glutamate to determine whether SupV neurons send excitatory inputs to the MoV. Eight slices from five animals at P1-5 were imaged with a monochrome CCD camera (C9100, Hamamatsu Photonics, Hamamatsu, Japan). A pulsed nitrogen laser (365 nm wavelength, 600 ps pulse duration, Micropoint, Photonic Instruments, Kawasaki, Japan) was directed into the epifluorescence attachment of the microscope, onto a dichroic mirror, and into the back aperture of the 4x objective. The location of the photostimulation was visualized directly through the objective, and the laser beam was focused on an approximately 10-µm diameter area of tissue. At the beginning of the experiment,
4-methoxy-7-nitroindolinyl-caged L-glutamate (Tocris Cookson, Ellisville, MO) was added to 25 ml ACSF to yield a concentration of 300 µM, which was circulated. All photostimulation experiments were started at least 15 min after the addition of caged glutamate. Single pulses of photostimulation were delivered to the MoV or SupV at 10 sec intervals to trigger focal photolysis of caged glutamate. We varied the strength of photostimulation by neutral density filters of varying optical densities.

_Retrograde labeling of jaw-closing and jaw-opening motoneurons_

In experiments that examined the nature and development of synaptic transmission between the SupV and MoV, we used a fluorescence labeling technique to record from jaw-closing and jaw-opening motoneurons. One to 2 days before slices were prepared, animals were anesthetized with ether, and 2-5 µl of 10% 3,000 or 10,000 MW dextran-tetramethylrhodamine-lysine (DRL, Molecular Probes) in distilled water was injected bilaterally in the masseter or digastric muscles with a microsyringe (1010RN, Kloehn, Las Vegas, NV). After the animals recovered from anesthesia, they were returned to their mothers while the DRL diffused. Forty-one masseter motoneurons (MMNs) from 23 rats and 31 digastric motoneurons (DMNs) from 18 rats were retrogradely labeled by 3,000 or 10,000 MW DRL solution. MMNs or DMNs could be labeled 1 day after injection of 3,000 MW DRL solution. When 10,000 MW DRL was used, we had waited for 2 days after DRL injection to prepare slices. Thus, to prepare slices at P1, 3,000 MW DRL solution was injected into the muscles 1 day before slice preparation (i.e. at the day of birth, P0).

_Electrophysiological recordings_

Whole-cell and gramicidin-perforated patch-clamp recordings were performed with
infrared videomicroscopy (BX51WI, Olympus) and a 40x water immersion objective with differential interference contrast and epifluorescence optics. In preparations from 41 DRL-injected animals at P1-12, the epifluorescence DRL-labeled MMNs and DMNs were quickly identified with the CCD camera. 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 Instruments, Novato, CA). Voltage-clamp experiments during whole-cell recordings were done with an internal solution of (in mM) 103 K-gluconate, 27 KCl, 1 CaCl₂, 10 HEPES, 11 EGTA, 2 MgCl₂, 0.3 NaGTP and 2 NaATP (pH 7.3, 285-300 mOsm). For current-clamp experiments in the whole-cell configuration, electrodes were filled with a solution of (in mM) 115 K-gluconate, 25 KCl, 9 NaCl, 16 HEPES, 0.2 EGTA, 1 MgCl₂, 3 K₂ATP and 1 NaATP. Biocytin (Sigma-Aldrich, St. Louis, MO) at a concentration of 5 mg ml⁻¹ was added to the internal solution for recording from SupV neurons. For gramicidin-perforated patch-clamp recordings, an internal solution containing (in mM) 150 KCl, 10 HEPES and 5 lidocaine N-ethylbromide (QX-314) was used. QX-314 is a sodium channel blocker; it is a large molecule and can enter the cell only when the membrane is ruptured. Voltage-activated sodium current was checked at the beginning and at the end of each cell recording. Gramicidin was dissolved in 10 mg ml⁻¹ dimethyl sulfoxide and diluted in the pipette-filling solution to a final concentration of 20 µg ml⁻¹ just before the experiment. Pipette resistance ranged from 2.5 to 5.0 MΩ when the electrodes were filled.

Stimulus-evoked postsynaptic currents (PSCs), postsynaptic potentials (PSPs) evoked by electrical stimulation of the SupV and membrane potentials were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). A tungsten electrode (impedance 400 kΩ; Unique Medical) was used for single-pulse stimulation (5-20 µA, 0.2 ms pulse duration). Series resistance compensation was set to 50-60% for gramicidin-perforated patch-clamp
recordings and to 70-80% for whole-cell recordings. Data were acquired at 2-20 kHz, filtered at 2-20 kHz, digitized with a Digidata 1332A interface (Molecular Devices) and stored and analyzed on a personal computer with pCLAMP 8.2 software (Molecular Devices). PSC data from five to eight trials were averaged and used for analyses, except for PSCs evoked by photostimulation, which were analyzed on a trial-by-trial basis. Liquid-junction potentials of 11 mV for voltage-clamp experiments and 12 mV for current-clamp experiments were subtracted from all membrane potentials in the whole-cell patch configuration. No correction of liquid-junction potential for gramicidin-perforated patch-clamp recordings was made. All experiments were performed at room temperature.

**Analyses of the morphology of SupV neurons projecting to the MoV**

Whole-cell recordings were obtained from SupV neurons of P1-5 rats (n = 20) to examine their characteristics. To determine which SupV neurons projected to the ipsilateral MoV, the MoV was stimulated with a tungsten electrode (impedance 400 kΩ; Unique Medical). The morphology of SupV neurons that met the following criteria was studied further: (i) the neuron responded to MoV stimulation less than 25 µA at a short and constant latency, and (ii) the neuron responded to 100 Hz triple shocks to the MoV when synaptic transmission was suppressed by substitution of external Ca\(^{2+}\) with Mn\(^{2+}\).

Neurons were filled by passive diffusion of biocytin from the patch pipette during and after the recording period (15-20 min) without application of current. After recording, patch pipettes were carefully detached from cells, and the slices remained in the recording chamber for an additional 30 min to allow for biocytin transport into dendrites and axons. The slices were then fixed in 4% paraformaldehyde (Sigma) in 0.1 M PB at -4°C for at least 24 h and then left overnight in 30% sucrose in 0.1 M PB at -4°C. Transverse, 100-µm thick serial sections
were cut on a microslicer. Sections were incubated overnight at 4°C in streptavidin conjugated
with horseradish peroxidase (1:800; Dako, Kyoto, Japan) in 0.05 M Tris-buffered saline (TBS)
containing 1% Triton X-100. After several rinses with TBS, sections were reacted with 0.04%
3,3’-diaminobenzidine tetrahydrochloride, 0.002% H$_2$O$_2$ and 0.07% nickel ammonium sulfate
in 0.05 M TBS and mounted on ovalbumin-coated slides. Stained cells were reconstructed
from multiple sections with a camera lucida.

**Drug applications**

For optical imaging experiments, the following drugs were applied with pressure to the
MoV at 1.5 ml min$^{-1}$ through a microperfusion system (BPS-8 PR-10, ALA Scientific
Instruments, Westbury, NY): 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM, Sigma),
DL-2-amino-5-phosphonopentanoic acid (APV, 20 µM, Sigma), strychnine (5-10 µM, Sigma)
and bicuculline (20 µM, Sigma). For patch-clamp experiments, CNQX, APV, strychnine,
SR95531 (5-10 µM, Sigma), bicuculline and tetrodotoxin (TTX, 0.5 µM, Sigma) were applied
to the bath. Glycine (1 mM, Sigma) was applied with pressure through a patch pipette to the
soma of the recorded neurons with a Picospritzer III (General Valve, Fairfield, NJ).

**Statistics**

Values are presented as mean ± S.E.M. Data obtained before, during and after drug
application within groups were subjected to one-way repeated measures analysis of variance
(ANOVA). Differences in data between groups were analyzed by the Student's $t$ test and
two-way ANOVA. ANOVA was followed by Tukey's post-hoc multiple comparison test
when appropriate. Probability values of less than 0.05 were considered significant.
Statistical analyses were conducted with SPSS 13.0J and Microsoft Excel 2003.
RESULTS

Premotor neurons in the SupV

To examine which region, when stimulated, elicits excitatory optical responses in the MoV, single-pulse electrical stimulation was applied systematically to the reticular formation around the MoV every 250 µm in the mediolateral and dorsoventral directions in 40 brainstem slices from 32 rats (P1-6) stained with Di-4-ANEPPS. Optical responses in the MoV were evoked by stimulation of the reticular formation dorsal to the MoV in 33 of 40 slices examined. Stimulus delivery is seen in the upper two frames (arrowheads) of Fig. 1A, and the optical response in the MoV is shown in the third frame. Histological analysis revealed that the stimulation sites were in the lateral SupV. Stimulation applied to sites outside of the SupV did not evoke optical responses in the MoV (data not shown). However, this does not indicate that excitatory premotor neurons are located only in the SupV in the slice preparations we used. Previous immunohistochemical studies revealed that glutamatergic premotor neurons for the trigeminal motor nucleus are also located in the principal sensory trigeminal nucleus and the intertrigeminal region (Kolta et al. 2000; Turman and Chandler 1994b). It is possible that activation of premotor neurons in these areas did not cause excitation of trigeminal motoneurons large enough to be detected by our optical recording system.

We then examined whether the SupV contains neurons that project to the MoV in five rats (P1-6). We stimulated the MoV at an intensity of 20 µA while synaptic transmission was suppressed by substituting Ca^{2+} (2 mM) in the perfusate with the inorganic calcium channel blocker Mn^{2+} (2 mM). Under these conditions, an optical response was observed in the SupV immediately after the stimulus was delivered in 5 of 17 slices tested (Fig. 1B), suggesting that some SupV neurons were likely to be activated antidromically by MoV stimulation in the five
slices and that excitatory optical signals were evoked in their somas.

We then used focal laser photolysis of bath-applied caged glutamate to examine whether SupV neurons send excitatory inputs to the MoV. As mentioned above, electrical stimulation of the SupV evoked optical responses in the MoV. However, electrical stimulation may have activated axons of passage in the SupV and therefore, optical responses in the MoV may have been due to activation of neurons located in areas other than the SupV. To exclude this possibility, we examined whether selective activation of SupV neurons with caged glutamate photolysis induced excitatory postsynaptic currents (EPSCs) in trigeminal motoneurons of P1-5 rats (n = 5). Whole-cell patch-clamp recordings were obtained from trigeminal motoneurons from 8 slices (n = 9; Fig. 2A), and the lens was then switched to the 4x objective. When photostimulation was applied around the tip of the patch electrode, membrane depolarization was elicited at latencies of 0.7 ± 0.2 ms (n = 9; Fig. 2B). To minimize damage to the motoneurons or SupV neurons by the laser beam, the intensity of the laser was adjusted to be slightly stronger than the threshold for generating an action potential. One or two action potentials were consistently evoked in the motoneurons at latencies of 20.3 ± 3.5 ms (7.0-28.5 ms; n = 9) by each photostimulation under these conditions (Fig. 2B). Such long latency was probably due to the small amount of glutamate uncaged by the laser around the motoneurons.

Next, the laser beam was focused on the SupV. In the presence of 5-10 µM strychnine, a glycine receptor antagonist, and 4-10 µM SR95531, a GABA<sub>A</sub> receptor antagonist, photostimulation evoked rapidly rising inward currents in all motoneurons except one at latencies of 20.4 ± 3.8 ms (7.8-42.6 ms; n = 8) in voltage-clamp mode (Fig. 2C). The focus of the laser was set on the spot in the SupV where the rapidly rising inward currents were most frequently evoked. Some trigeminal motoneuron dendrites reportedly extend into the SupV in cats (Shigenaga et al. 1988), thus, uncaged glutamate in the SupV might have stimulated
motoneuron dendrites. To exclude this possibility, we examined the effects of photostimulation of the SupV after addition of 0.5 µM TTX. TTX abolished the rapidly rising inward currents in all seven neurons tested (Fig. 2D). Thus, the rapidly rising inward currents were most likely glutamatergic EPSCs. However, small, slowly-rising inward currents were evoked immediately after photostimulation in three of the seven neurons (Fig. 2D, arrow). It is likely that they may have resulted from direct stimulation of the dendrites of recorded cells by uncaged glutamate.

**Postnatal changes in MoV optical responses mediated by CNQX-, APV- and strychnine-sensitive receptors**

To characterize the nature of the transmitters involved in synaptic transmission between the SupV and the MoV in developing rats, we tested the effects of several receptor antagonists of fast synaptic transmission on SupV stimulation-induced optical responses in the MoV. Seventeen slices from 15 neonatal animals (P1-6) and 14 slices from 9 juvenile animals (P7-12) were used in this experimental series. In P1-6 rats, exposure of the MoV to a combination of the non-N-methyl-D-aspartate (non-NMDA) receptor antagonist CNQX (20 µM) and the NMDA receptor antagonist APV (20 µM) for 20 min significantly reduced the peak value of optical responses in the MoV by $42.8 \pm 20.7\%$ ($n = 17, P < 0.01$) compared to control responses (Fig. 3A, G). The optical responses recovered to $89.8 \pm 20.7\%$ of the control level after the tissue was washed for 20 min with the normal ACSF. Interestingly, application of strychnine (10 µM) to the MoV significantly suppressed optical responses in the MoV by $55.4 \pm 19.8\%$ ($n = 16, P < 0.01$) (Fig. 3B, G). The responses recovered to $82.8 \pm 11.8\%$ of control values after the tissue was rinsed for 20 min with the normal ACSF.

In P7-12 rats, SupV stimulation also elicited an excitatory optical response in the MoV.
Focal application of CNQX (20 µM) and APV (20 µM) significantly reduced the peak responses by 56.7 ± 13.1% (n = 10, P < 0.01) compared to control responses (Fig. 3C, E). The optical responses recovered to 81.1 ± 14.8% of the control level 20 min after washout of CNQX and APV. The rate of decrease in the optical responses at P7-12 did not differ significantly from that of P1-6 (P > 0.2, Fig. 3G). However, the degree to which strychnine (5-20 µM) inhibited optical responses decreased after P5 (Fig. 3F), and strychnine significantly enhanced the optical responses by 19.0 ± 23.1% in the slices from P7-12 rats (n = 14, P < 0.05, Fig. 3D). The rate of strychnine-induced changes in optical responses at P7-12 differed significantly from the rate of strychnine-induced changes at P1-6 (P < 0.01, Fig. 3G). These results suggest that glycineric inputs from the SupV to the MoV changed from excitatory to inhibitory during postnatal development.

SupV stimulation-evoked postsynaptic potentials and currents in developing jaw-closing and jaw-opening motoneurons

Immature neurons have relatively high intracellular Cl− concentrations ([Cl−]i) (DeFazio et al. 2000; Plotkin et al. 1997; Shimizu-Okabe et al. 2002). Thus, glycineric excitatory synaptic transmission from the SupV to the MoV in P1-6 rats might be due to high [Cl−]i in trigeminal motoneurons. To confirm that glycineric or GABAergic PSCs evoked by SupV stimulation were excitatory, we performed gramicidin-perforated patch-clamp recordings of MMNs (jaw-closing motoneurons) or DMNs (jaw-opening motoneurons) that left [Cl−]i undisturbed (Kyrozis and Reichling 1995). We also examined the contribution of glutamatergic receptors to SupV stimulation-evoked PSCs in MMNs and DMNs.

For recording from MMNs at P1-4 or P7-12, 20 slices from 15 neonatal animals and 11 slices from eight juvenile animals were prepared, respectively. For recording from DMNs at
P1-4 or P7-12, 18 slices from 10 neonatal animals and 10 slices from eight juvenile animals were prepared, respectively. The resting membrane potentials of MMNs were \(-69.6 \pm 1.0 \text{ mV}\) for P1-4 \((n=24)\) and \(-68.1 \pm 3.0 \text{ mV}\) for P9-12 \((n=10)\), and the resting membrane potentials of DMNs were \(-67.0 \pm 1.6 \text{ mV}\) for P1-4 \((n=20)\) and \(-70.2 \pm 3.4 \text{ mV}\) for P9-12 \((n=10)\). There were no significant between-group differences in resting potential \((P > 0.05)\).

At P1-4, electrical stimulation of the SupV evoked short latency PSCs at 2.8 \pm 0.4 ms in all MMNs \((n=6)\) and at 3.6 \pm 0.3 ms in all DMNs \((n=5)\) tested (Fig. 4A). Because all PSCs were observed as transient inward currents at a holding potential of -60 mV, SupV stimulation elicited EPSCs at this age. Combined bath application of CNQX (20 \(\mu\text{M}\)) and APV (20 \(\mu\text{M}\)) significantly reduced the amplitude of EPSCs in MMNs by 66.2 \pm 8.0\% \((n=6, P<0.01)\) and in DMNs by 69.4 \pm 2.6\% \((n=5, P<0.05)\). The remaining PSCs were inward. Subsequent addition of strychnine (10 \(\mu\text{M}\)) almost completely abolished the remaining PSCs in MMNs and DMNs (Fig. 4A). Partial recovery of EPSCs was observed in MMNs and DMNs after the antagonists were washed out for 20 min with normal ACSF. In current-clamp mode, SupV stimulation evoked depolarizing PSPs in MMNs \((n=6)\) and DMNs \((n=4)\) at membrane potentials of -60 mV in the presence of 20 \(\mu\text{M}\) CNQX and 20 \(\mu\text{M}\) APV (Fig. 4B). Under these conditions, an action potential (arrowhead) could be triggered by an EPSP evoked by SupV stimulation alone (Fig. 4B) or SupV stimulation with simultaneous injection of a depolarizing subthreshold intracellular current pulse (see Gulledge and Stuart 2003). Addition of 10 \(\mu\text{M}\) strychnine completely abolished the remaining PSPs. Pressure application of 1 mM glycine to MMNs \((n=6)\) and DMNs \((n=5)\) induced membrane depolarization and a remarkable decrease in membrane resistance in all neurons tested at P1-4 in the presence of 20 \(\mu\text{M}\) CNQX and 20 \(\mu\text{M}\) APV (Fig. 4C). Bath application of 200 \(\mu\text{M}\) GABA to MMNs \((n=4)\) and DMNs \((n=5)\) induced membrane depolarization and a remarkable decrease in membrane resistance in all
neurons tested (data not shown).

It is possible that 10 µM strychnine reduced GABAergic and glycinergic currents in MMNs and DMNs. Therefore, we used whole-cell patch-clamp recordings to examine the effects of GABA_A receptor antagonists on MMNs and DMNs in P1-5 rats. In five of eight MMNs, SupV stimulation-evoked PSCs were significantly reduced by the GABA_A receptor antagonists bicuculline (20 µM) or SR95531 (10 µM) by 52.4 ± 5.9% (n = 5, P < 0.01) in the presence of both 20 µM CNQX and 20 µM APV (Fig. 4D, left). In the remaining three neurons, SupV stimulation-evoked PSCs were not altered by 20 µM bicuculline or 10 µM SR95531 (Fig. 4D, right), and addition of 10 µM strychnine abolished the PSCs. In five of seven DMNs, SR95531 (10 µM) reduced SupV stimulation-evoked PSCs by 33.9 ± 8.5% (P < 0.05) in the presence of 20 µM CNQX and 20 µM APV (Fig. 4E, left). SR95531 did not affect PSCs in the remaining two DMNs (Fig. 4E, right). Addition of 10 µM strychnine abolished remaining PSCs in all eight DMNs. These results suggest that some MMNs and DMNs received GABAergic and glycinergic inputs from the SupV.

In P9-12 rats, gramicidin-perforated patch-clamp recordings revealed that SupV stimulation evoked EPSCs at a holding potential of -60 mV with a latency of 2.7 ± 0.3 ms in all MMNs (n = 7) and 2.8 ± 0.3 ms in all DMNs (n = 6) examined (Fig. 5A). However, combined application of CNQX (20 µM) and APV (20 µM) revealed outward PSCs in all neurons tested (MMNs, n = 6; DMNs, n = 6). The remaining PSCs in MMNs and DMNs were almost completely abolished by subsequent addition of strychnine. We then performed whole-cell recordings to examine effects of SR95531 on MMNs and DMNS in P9-12 rats. In four of five MMNs, SupV stimulation-evoked PSCs were significantly decreased by 10 µM SR95531 application by 36.5 ± 6.3% (n = 4, P < 0.05). In four of five DMNs, SR95531 also significantly reduced SupV stimulation-evoked PSCs by 56.1± 11.3% (n = 4, P < 0.01). In the
remaining one MMN and one DMN, SupV stimulation-evoked PSCs were not altered by SR95531. Addition of 10 µM strychnine abolished remaining PSCs in all five MMNs and five DMNs. These results suggest that some MMNs and DMNs of P9-12 rats also received GABAergic and glycinergic inputs from the SupV similarly in P1-4 rats. To determine the reversal potentials of the PSCs in the presence of CNQX and APV, we evoked PSCs by SupV stimulation at various holding potentials (Fig. 5B). In slices from P1-4 rats, reversal potentials of strychnine-sensitive PSCs in MMNs and DMNs were -36.1 ± 5.2 mV (n = 6) and -27.4 ± 8.7 mV (n = 5), which was remarkably more depolarized than the resting potentials for both (Fig. 5C, D). In contrast, the reversal potentials of the strychnine-sensitive PSCs for MMNs and DMNs at P9-12 were -74.1 ± 3.9 mV (n = 6) and -75.6 ± 3.2 mV (n = 5), which were significantly more hyperpolarized than their resting potentials (P < 0.05) and the reversal potentials at P1-4 (P < 0.01, Fig. 5C, D). These results suggest that glutamatergic, glycinergic and GABAergic PSCs in MMNs and DMNs evoked by SupV stimulation were excitatory at P1-4, and glycinergic and most likely GABAergic PSCs, changed from excitatory to inhibitory as the rats developed.

Properties of SupV neurons projecting to the MoV

We examined the physiological and morphological properties of SupV neurons projecting to the MoV. Whole-cell patch recordings were made from 143 SupV neurons in 23 slices from 20 P1-5 animals. Twelve (8.4%) neurons were antidromically activated by MoV stimulation at intensities of less than 25 µA and at constant latencies of 3.0 ± 0.1 ms (Fig. 6Aa). Subtracting the 0.2 ms utilization time for initiation of the spike potential from and adding a 0.5 ms synaptic delay to the mean antidromic latency of 3.0 ms yields a 3.3 ms latency that approximated the 2.8 ms mean latency for EPSCs in MMNs and 3.6 ms in DMNs that were
evoked by electrical stimulation of the SupV. Ten of the 12 antidromically activated SupV neurons tested showed tonic firing after we injected a 1-sec depolarizing current pulse at the resting membrane potential (Fig. 6Ab). The remaining two neurons showed a rapid adaptation of firing in response to the current pulse injection. The responses of SupV neurons to serial hyperpolarizing and depolarizing current pulses are shown in Fig. 6Ac. Nine neurons had no depolarizing sag and showed a relatively linear I-V relation over hyperpolarized potentials. Three neurons had small depolarizing sags during step hyperpolarization.

During and after experiments, we injected biocytin into the recorded neurons through patch pipettes, and the morphology of four neurons could be analyzed after the immunohistochemical procedure. All four neurons showed tonic firing in response to depolarizing current pulse injections (Fig. 6A). Cell bodies of all biocytin-labeled neurons were oval (Fig. 6D) and located in the lateral part of the SupV (Fig. 6C). The dendrites extended in the various directions in the reticular formation dorsal to the MoV. The axon of one neuron projected in the ventral direction to the MoV (Fig. 6B, C). Under high magnification, we identified many swollen boutons and end terminals in the MoV, indicating that SupV neurons make synaptic contacts with trigeminal motoneurons (Fig. 6E). It has been shown that jaw-closing and jaw-opening motoneurons are distributed in the dorsolateral and ventromedial divisions, respectively, of the MoV in mammals (Mizuno et al. 1975; Voogd et al. 1998). In rats, the dorsolateral division exists at the whole rostrocaudal level of the MoV, whereas the ventromedial division is found only at the caudal half of the MoV (Jacquin et al. 1983; Sasamoto 1979; Uemura et al. 2007). The neuron shown in Fig. 6 was recorded in the slice from the rostral part of the motor nucleus where only the dorsolateral division exists. Therefore, this neuron likely projected to the jaw-closing motoneuron pool. The axon of one other neuron that traveled to the MoV in the lateral-ventral direction mainly reached the
dorsolateral division. Axons of the remaining two neurons projected to the MoV in the medial-ventral directions and ramified broadly within the MoV, however, it was difficult to judge from our histological results whether their axons entered the ventromedial division as well as the dorsolateral division of the MoV. Thus, all four of the biocytin-labeled neurons likely projected to the jaw-closing motoneuron pool; however, further studies are necessary to confirm whether the SupV premotor neurons project to either jaw-closing or jaw-opening motoneurons, or both.

**DISCUSSION**

In the present study, we showed for the first time the existence of excitatory glutamatergic SupV neurons projecting to MMNs and DMNs in developing rats. We also showed that MMNs and DMNs receive excitatory glycinergic and GABAergic inputs from the SupV in neonatal rats. In juvenile rats, glycinergic and likely GABAergic inputs are inhibitory, whereas the glutamatergic inputs remain excitatory. The differential effects of the synaptic transmission from the SupV to the MoV during postnatal development may reflect the transition from suckling to mastication.

*Glutamatergic inputs from the SupV to the MoV*

Electrical stimulation of the SupV evoked optical responses and PSCs in the MoV. The stimulus intensities which were delivered to the SupV were 20-30 µA for optical recording, and the area which was directly activated by electrical stimulation was confined within the SupV (see Fig. 1). For patch-clamp recordings the SupV was stimulated at weaker intensities (5-20 µA) than those used for optical recordings. Therefore, the stimulus field produced by the stimuli was likely to be confined within the SupV. Antidromic optical responses could be
evoked in the SupV after MoV stimulation when synaptic transmission was suppressed by substitution of Mn$^{2+}$ for external Ca$^{2+}$. Twelve SupV neurons were antidromically activated by MoV stimulation, and 4 of the 12 neurons made synaptic contacts with the motoneurons. These results suggest that a certain number of premotor neurons in the SupV target the MoV.

Turman and Chandler (1994b) showed histochemically that the SupV contains glutamatergic premotor neurons that target the MoV. In the present study, application of CNQX and APV attenuated optical responses in the MoV and EPSCs in MMNs and DMNs that were induced by electrical stimulation of the SupV. Furthermore, in the presence of strychnine and SR95531, activation of SupV neurons by focal laser photolysis of bath-applied caged glutamate evoked the rapidly rising inward currents in P1-5 trigeminal motoneurons. The focus of the laser beam was set on the spot in the SupV where the rapid inward currents were most frequently evoked. Photostimulation became less effective to evoke the rapid inward currents, when the focus of the laser was moved away from the spot (data not shown). These results suggest that glutamatergic excitatory premotor neurons projecting to MMNs and DMNs are likely located in the SupV.

Bourque and Kolta (2001), however, suggested interconnection between the SupV, the intertrigeminal region and the medial peritrigeminal area in which premotor neurons targeting the MoV are located. Thus, photostimulation of the SupV might have evoked EPSCs polysynaptically in trigeminal motoneurons, and the shortest latency of EPSCs in trigeminal motoneurons evoked by SupV photostimulation (7.8 ms) was longer than the mean latencies for EPSCs in MMNs (2.8 ms) and DMNs (3.6 ms) evoked by electrical stimulation of the SupV. However, this discrepancy in the latencies was likely due to a delay in spike generation in SupV neurons after photostimulation, because it took at least 7.0 ms to generate an action potential in trigeminal motoneurons by direct photostimulation at the intensities used in the present study.
Therefore, the EPSCs in the motoneurons were likely evoked monosynaptically by the photostimulation, although we can not rule out the possibility for involvement of polysynaptic pathways especially in cases of long latencies of the EPSCs.

**Glycinergic and GABAergic inputs from the SupV to the MoV**

The SupV has been shown histochemically to contain glycinergic and GABAergic premotor neurons that target the MoV (Li et al. 1996; Turman and Chandler 1994a). In the present study, application of strychnine, bicuculline and SR95531 altered optical responses in the MoV and EPSCs in MMNs and DMNs that were induced by electrical stimulation of the SupV.

The PSCs that remained after CNQX and APV application were almost completely abolished by strychnine. Bicuculline and SR95531 reduced the remaining PSCs by 52% in five of eight MMNs and by 34% in five of seven DMNs from neonates and by 37% in four of five MMNs and by 56% in four of five DMNs in juveniles. Therefore, 10 µM strychnine reduced GABAergic and glycinergic PSCs. However, strychnine abolished remaining PSCs after the addition of bicuculline and SR95531 to CNQX and APV; apparently, all MMNs and DMNs receive glycinergic inputs from the SupV. In contrast, bicuculline and SR95531 did not abolish the remaining PSCs after application of CNQX and APV, and they had little effect on the remaining PSCs in three MMNs and two DMNs in the neonates and in one MMN and one DMN in the juveniles. Apparently, MMNs and DMNs receive GABAergic inputs that are weaker than glycinergic inputs from the SupV. The number of glycine-immunoreactive boutons synapsing on MMNs and DMNs in cats is close to twice the number of GABA-immunoreactive boutons (Bae et al. 1999; Shigenaga et al. 2005). The SupV may be the origin of glycinergic and GABAergic axon terminals at MMNs and DMNs. Our
observation of stronger glycinergic than GABAergic inputs from the SupV to the MoV in the present study is consistent with the abundance of glycine-immunoreactive boutons at MMNs and DMNs reported in the studies cited above.

Glycine and GABA evoke membrane depolarizations during fetal and early postnatal life in many areas of the nervous system (Ben-Ari et al. 1989; Luhmann and Prince 1991; Owens et al. 1996), including the spinal cord (Reichling et al. 1994; Wu et al. 1992) and brainstem (Kandler and Friauf 1995; Singer et al. 1998), as well as adult spinal laina I neurons following peripheral nerve injury (Coull et al. 2003). In the present study, strychnine reduced optical responses evoked in the MoV by SupV stimulation, and the CNQX/APV-insensitive inward currents of MMNs and DMNs in perforated patch-clamp recordings were almost abolished by strychnine in the brainstem of P1-6 neonatal rats. The mean reversal potentials of strychnine-sensitive PSCs in neonate MMNs and DMNs were -36.1 mV and -27.4 mV, respectively, which were much more depolarized than their resting membrane potentials. Application of glycine and GABA depolarized neonate MMNs and DMNs. However, strychnine enhanced the optical responses in the MoV, and strychnine-sensitive PSCs in MMNs and DMNs became outward in the brainstems of P7-12 juvenile rats. These results indicate that glycinergic and GABAergic inputs from the SupV depolarize MMNs and DMNs in neonates, whereas SupV glycinergic and likely GABAergic inputs hyperpolarize MMNs and DMNs in juvenile rats. Such differences in the synaptic transmission between neonates and juveniles were not due to the different use of the ACSFs during slice preparation because application of glycine or GABA also evoked depolarization in all motoneurons tested obtained from the slices prepared using modified ACSF, similarly in the motoneurons prepared with normal ACSF.

GABA- and glycine-induced membrane depolarization has been shown to be caused by
Cl⁻ efflux due to high [Cl⁻]ᵢ maintained in immature cells (Ben-Ari 2002; Owens et al. 1996). Increased expression of K-Cl cotransporter isoform 2 (KCC2) is believed to underlie the functional conversion of GABA from excitatory to inhibitory (DeFazio et al. 2000; Rivera et al. 1999). Because KCC2 mRNA is expressed in the MoV of adult rats (Toyoda et al. 2005), developmental upregulation of KCC2 may occur in MMNs and DMNs and lead to a transition from membrane depolarization to membrane hyperpolarization in response to glycine and/or GABAᵦ receptor activation.

**Roles of premotor neurons in the SupV**

The present study showed that a wide variety of inputs, i.e., glutamatergic, glycinergic and GABAergic inputs, is sent to MMNs and DMNs from the SupV. Intracellular staining of four SupV premotor neurons also shows that there were, at least, three patterns of axonal projection to the MoV. Recently, Hsiao et al. (2007) reported a morphological and electrophysiological diversity in SupV neurons, although the SupV neurons recorded in their study were not identified as premotor neurons for targeting the MoV. Such diversities of SupV premotor neurons may indicate that those neurons play a wide variety of roles in orofacial motor function.

Periodontal and spindle afferents (Dessem and Taylor 1989; Jerge 1963; Mizuno 1970; Nishimori et al. 1986; Nomura and Mizuno 1985; Shigenaga et al. 1989) and orofacial afferents (Goldberg 1972; Sessle 1977) facilitate jaw-closing muscle activity, and the SupV is a recipient of such afferents. Therefore, it is possible that excitatory glutamatergic premotor neurons in the SupV are involved in increasing jaw-closing muscle activity via sensory inputs during mastication.

Glycinergic interneurons that inhibit jaw-closing motoneurons during the JOR are
assumed to exist in the SupV (Kidokoro et al. 1968; Nakamura et al. 1973). This assumption is strongly supported by our present finding that SupV stimulation elicits IPSCs in MMNs of juvenile rats. The JOR contributes to protecting the mouth by inhibiting jaw-closing muscle activity when a hard piece of food or a stone is bitten or when sharp material, such as a fish bone, pierces the oral mucosae during mastication. However, inhibitory glycinergic and GABAergic effects from the SupV emerged after P7 in the present study, and this emergence preceded the initiation of immature mastication at around P12 (Westneat and Hall 1992). At around P7, the teeth begin to erupt (Asahito et al. 1999; Nakakura-Ohshima et al. 1993), and the tongue or buccal mucosa may then be bitten by the erupting teeth. Thus, inhibition of jaw-closing muscle activities by elicitation of the JOR is likely needed to prevent the oral mucosa from bites after tooth eruption. In fact, latency of the JOR decreases drastically after P6 (Iriki et al. 1983), suggesting that the JOR comes into function upon tooth eruption. In the present study, glycinergic and GABAergic inputs from the SupV to the MoV were excitatory in neonatal rats. Because neonates feed by suckling and ingest only milk, inhibition of the jaw-closing muscles may not be necessary. Instead, such excitatory glycinergic and/or GABAergic synaptic transmission to the MoV might promote the maturation of local neural circuits by Ca\(^{2+}\) influx in immature neurons through voltage-gated Ca\(^{2+}\) channels (see Flint et al. 1998; Hsiao et al. 2005; Lo et al. 1998). Thus, the developmental switch of glycinergic and GABAergic effects from the SupV to MMNs and DMNs could play an important role in the smooth transition from suckling to mastication by function development of the JOR prior to the initiation of mastication.

SupV stimulation evoked glutamatergic EPSCs and EPSPs in DMNs of neonates and juveniles in the present study. SupV neurons, however, do not appear to be responsible for excitation of DMNs during the JOR because lesions of the oral and interpolar parts of the spinal
trigeminal nucleus that spared the SupV abolished the JOR induced by stimulation of the inferior alveolar nerve (Sumino 1971) and because application of lidocaine to the caudal part of the spinal trigeminal nucleus significantly suppressed temporomandibular joint-evoked reflex of the digastric muscles (Cairns et al. 2001).

Stimulation of the SupV also evoked glycinergic and GABAergic IPSCs in DMNs of juvenile rats. When there is a low level of continuous background activity in the jaw-opening muscles in cats and humans, the jaw-jerk elicited by tapping on the chin occasionally inhibits the jaw-opening muscles (Matthews 1975). Because the SupV receives spindle afferents from the jaw-closing muscles, SupV neurons might inhibit DMNs in a way similar to the Ia inhibitory interneurons for limb muscles. However, stimulation of the mesencephalic trigeminal nucleus, which contains the spindle cell bodies, does not evoke short-latency PSCs in DMNs of anesthetized cats (Kidokoro et al. 1968). The inhibition of DMNs during the jaw-jerk reflex might be easily masked in anesthetized animals.

Kogo et al. (1996) induced rhythmic activity in the motor branches of the trigeminal nerves in isolated brainstem preparations from neonatal rats by application of N-methyl-D,L-aspartate (NMA). They suggested, by transection of the preparations, the existence of rhythmogenetic circuits between the trigeminal and facial motor nuclei, which include the SupV. Enomoto et al. (2002) developed slice preparations that consist of islands attached to a 300-µm area surrounding the MoV and including the SupV. They recorded rhythmic motoneuronal activity during NMDA application and proposed that premotor neurons that drive NMDA-induced rhythmic activity are located in the 300-µm area surrounding the MoV. In anesthetized adult rats, the rhythmic neuronal activity corresponding to the masticatory rhythm during cortically-induced fictive mastication can be recorded from the SupV (Inoue et al. 1992). Furthermore, Hsiao et al. (2007) showed that some of these neurons
have intrinsic burst generating capabilities. Thus, it is possible that SupV neurons are involved in generation of suckling and/or masticatory rhythm. It is also possible that SupV neurons transmit trigeminal rhythm inputs from the rhythm generator possibly located in the SupV or another area. The SupV may be involved in facilitating or inhibiting activity of the jaw-closing and jaw-opening muscles to ensure appropriate jaw movement during feeding.

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FIGURE LEGENDS

FIG. 1. Examples of optical responses evoked by electrical stimulation in a transverse brainstem slice preparation. A: consecutive optical images (3.0 msec/frame) showing excitation of the MoV induced by stimulation of the lateral SupV. B: consecutive images showing antidromic excitation of the SupV by MoV stimulation while synaptic transmission was suppressed by substitution of Ca\(^{2+}\) (2 mM) with Mn\(^{2+}\) (2 mM) in the perfusate. Stimuli were delivered at the second frames (arrowheads). MoV, trigeminal motor nucleus; SupV, supratrigeminal region; Dor, dorsal; Lat, lateral. Scale bar = 500 µm.

FIG. 2. Laser photolysis of caged glutamate in the SupV. A: video image of a transverse slice preparation from a P2 rat showing the location of the recorded neuron and photostimulation (asterisk). The dashed line surrounds the MoV. B: current-clamp recording showing membrane depolarization elicited by direct photostimulation of the recorded motoneuron. C: voltage-clamp recordings showing excitatory inputs from the SupV to the MoV. Photostimulation of the SupV evoked rapidly rising inward currents in the motoneuron in the presence of strychnine and SR95531 (upper trace). D: addition of TTX abolished the rapid inward currents. E: after the slice was washed in the presence of STR and SR95531, the rapid inward currents recovered. The dotted line indicates the onset of photostimulation. Arrows indicate small, slowly rising inward currents. Scale bar = 100 µm.

FIG. 3. Effects of receptor antagonists on MoV optical responses evoked by SupV stimulation. A and B: time course of fluorescence change in MoV optical signals before (open circles), during (filled squares), and after (open squares) application of CNQX + APV (A) or strychnine (B) in P3 neonatal rats. C, time course of change in MoV optical signals before (open circles),
during (filled triangles) and after (open squares) application of CNQX and APV in a slice from a P8 juvenile rat.  

D, time course of change in MoV optical signals before (open circles) and during (filled squares) application of strychnine and addition of CNQX and APV to strychnine (filled circles) at P11.  Note that application of strychnine enhanced the optical response.  
Fluorescence decrease (i.e., depolarization) is upward.  Values are mean ± SEM of 16-128 trials.  The sampling interval is 3 sec.  

E, F, plots of rate of change in optical responses during application of CNQX + APV (E) or strychnine (F) versus rat age.  

G, summary of age-related changes in the effects of CNQX + APV (filled squares) and strychnine (filled circles) on optical signals.  The rate of change by strychnine in P1-6 neonates differed significantly from the rate in P7-12 juveniles.  * $P < 0.01$ (ANOVA).

**FIG. 4.** Gramicidin-perforated (A-C) and whole-cell (D, E) patch-clamp recordings of jaw-closing (MMNs) and jaw-opening (DMNs) motoneurons in P1-4 neonatal rats.  

**A:** examples of EPSCs evoked by SupV stimulation in a P1 MMN (left) and a P3 DMN (right).  
Inward currents were elicited by SupV stimulation at a holding potential of -60 mV, and EPSCs that remained after application of CNQX + APV continued to be inward.  Addition of strychnine completely abolished the EPSCs in both motoneurons.  

**B:** depolarizing PSP evoked by SupV stimulation.  An action potential (arrowhead) was triggered by the EPSP evoked by SupV stimulation.  The action potential is truncated in this figure.  

**C:** membrane depolarization and decrease in membrane resistance of a P4 MMN induced by pressure application of 1 mM glycine.  

**D and E:** Effects of GABA$_A$ receptor antagonist on EPSCs of jaw-closing and jaw-opening motoneurons elicited by SupV stimulation at P1-5.  

**D:** In five of eight MMNs, EPSCs were significantly suppressed by SR95531 (left).  In the remaining three neurons, EPSCs were not altered by SR95531 (right).  

**E:** in five of seven DMNs, EPSCs were
depressed by SR95531 (left). In the remaining two neurons, SR95531 did not abolish the EPSCs (right).

**FIG. 5.** Postnatal development of strychnine-sensitive PSCs evoked by SupV stimulation.  
A: SupV stimulation-evoked IPSCs and EPSCs in P9-12 MMNs and DMNs. Effects of CNQX + APV or strychnine on PSCs in a MMN (left) and DMN (right) obtained from gramicidin-perforated patch-clamp recordings. Application of CNQX + APV revealed outward PSCs in both motoneurons.  
B: examples of the strychnine-sensitive PSCs at various holding potentials in P2 MMN and P9 MMN.  
C: examples of current/voltage relation of SupV stimulation-evoked PSCs in P2 and P9 MMNs, and P4 and P11 DMNs.  
D: mean ± SEM of the reversal potentials of SupV stimulation-evoked PSCs of neonates and juveniles. The reversal potentials in MMNs and DMNs became more hyperpolarized with age. All PSCs were obtained from gramicidin-perforated patch-clamp recordings in the presence of 20 µM CNQX and 20 µM APV. **P < 0.01.

**FIG. 6.** Physiological and morphological properties of SupV neurons.  
A: SupV neuron antidromically activated by MoV stimulation (a) showed tonic firing (b) by injection of a depolarizing current pulse from the resting membrane potential and a linear I-V relation over hyperpolarized potentials (c).  
B and C: camera lucida reconstruction of a biocytin-filled SupV neuron showing soma (arrow), axon (arrowhead) and dendrites. Location of the SupV neuron in relation to the MoV and the trigeminal main sensory nucleus is shown in C.  
D and E: photographs of a soma (D) and axonal terminals in the MoV (E) as indicated by the area outlined in B. The SupV neuron sent its axon in the medial-ventral direction to the MoV (B, C). Under high magnification the ovoid body of the SupV neuron is visible (D) and its many
swollen boutons and end terminals could be clearly identified in the MoV (E). Scale bars = 200 µm (B, C) and 20 µm (E). Dor, dorsal; Med, medial; Vp, trigeminal main sensory nucleus; MoV, trigeminal motor nucleus; SupV, supratrigeminal region.
A

SupV

MoV

100 μm

B

Direct stimulation

20 mV

20 ms

C

STR 10 μM + SR95531 10 μM

D

TTX 0.5 μM + STR + SR95531

E

Wash (STR + SR95531)