Supplemental Materials and Methods (full description)

Preparations
Experiments were performed with brainstem-spinal cord preparations from 0-2 day old Wistar rats. The experimental protocols were approved by the Animal Research Committee of Showa University, which operates in accordance with Law No. 105 for the care and use of laboratory animals of the Japanese Government. Newborn rats were deeply anesthetized with ether in a 120-ml glass bottle until nociceptive reflexes induced by tail pinch were abolished. Respiratory movement halted temporarily at this level of anesthesia. The cerebrum was quickly removed by transection at the intercollicular level, and the brainstem and spinal cord were isolated according to methods described previously (Suzue, 1984; Onimaru and Homma, 1992). The preparation was superfused at a rate of 3.0 ml/min with the following artificial cerebrospinal fluid (ACSF) (Suzue, 1984) (in mM): 124 NaCl, 5.0 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgCl2, 26 NaHCO3 and 30 glucose, equilibrated with 95% O2 and 5% CO2, pH 7.4, at 26-27°C. Inspiratory activity corresponding to phrenic nerve activity was monitored from the fourth cervical ventral root (C4). Nerve activities were recorded through a glass suction electrode and high-pass filtered with a 0.3-s time constant. The μ-opiate receptor agonist [D-Ala2, N-Me-Phe4, Gly5-ol] enkephalin (DAMGO; Sigma-Aldrich Co.) was dissolved in the ACSF and applied by superfusion for 10-15 min.

Whole-cell patch-clamp recordings
Membrane potentials and input resistances of neurons in the parafacial region of the rostral medulla were recorded by a blind whole-cell patch-clamp method (Onimaru and Homma, 1992). The electrodes (inner tip diameter, 1.2-2.0 μm; resistance, 4-8 MΩ) were filled with the following pipette solution (in mM): 130 K-gluconate, 10 ethylene glycol bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES), 2 Na2-ATP, 1 CaCl2 and 1 MgCl2, with pH 7.2-7.3 adjusted with KOH. Membrane potentials were recorded with a single-electrode voltage-clamp amplifier (CEZ-3100; Nihon Kohden Corp., Tokyo, Japan) after compensation for series resistance (20-50 MΩ) and capacitance. We used three types of preparations for the whole-cell recordings for approaches from three directions: 1) An approach from the ventral surface for recording cells at various depths around the level of caudal end of the facial nucleus (n=21). For this purpose, conventional medulla-spinal cord preparations, which were rostrally and transversely
cut between the VIth cranial nerve roots and the lower border of the trapezoid body, were used with the ventral surface up. 2) An approach from the rostral cut surface for recording ventral superficial cells at the level of the rostral half of the facial nucleus \((n=10)\). For this, we used preparations with cutting levels similar to the above conventional preparations or cut slightly caudally, \(i.e. \ < 200 \mu m\) caudal to the anterior inferior cerebellar artery (AICA) and the preparation was positioned with the rostral cut surface up. 3) An approach from the caudal cut surface for recording ventral superficial cells at the level of the caudal half of the facial nucleus \((n=22)\). In this case, the brainstem was transversely cut at a level 100-200 \(\mu m\) caudal to the caudal end of the facial nucleus and the caudal half of the pons was retained in order to record from the facial nerve. The preparation was positioned caudal cut surface up. To obtain stable rhythmic facial nerve activity, 0.5 \(\mu M\) DAMGO was applied to the block preparation for 10–15 min and then washed out. It is known that the rhythmic facial nerve activity in such block preparations reflects burst activity of Pre-I neurons (Onimaru et al., 2006). Above transverse sectionings were performed with a vibrating-blade tissue slicer (laboratory-made) in the dissection chamber.

**Experimental protocol**

Responses to hypercapnia were assessed by changes in membrane potential and input resistance when CO2 concentration was changed from 2% to 8%, according to methods of previous studies (Okada et al., 1993; Kawai et al., 1996; Kawai et al., 2006). After establishment of the whole-cell recordings, the standard solution (5% CO2, pH 7.4) was replaced by an ACSF containing 0.5 \(\mu M\) tetrodotoxin (TTX, Sigma) equilibrated with 2% CO2 (pH 7.8). After a 15 min incubation with the 2% CO2 solution, the superfusate was replaced by a hypercapnic acidic ACSF equilibrated with 8% CO2 (pH 7.2) (Kawai et al., 2006). After a 5-6 min test of membrane potential responses in the 8% CO2 solution, the superfusate was returned to 2% CO2 solution. To detect input resistance change during hypercapnia, a 500 ms (10-40 pA) hyperpolarizing pulse was applied with 10-s interval. In some experiments, we applied biasing current to restore the resting membrane potential (in 2% CO2) when hypercapnia induced membrane depolarization. Since we confirmed that the difference of input resistance between with and without biasing current was not significant, application of biasing current during membrane depolarization was not routinely performed in the present study.

**Immunofluorescence**

For the preparation of the Phox2b antibody, a peptide corresponding to the C-terminus
of the mouse Phox2b protein (Pattyn et al., 1997) was synthesized and used for immunization of guinea pigs. We verified that this peptide dose-dependently suppressed Phox2b-like immunoreactivity in rat brain as detected in our experiments with our experimental protocols where complete elimination of immunoreactivity took place at 5 μg/μl of the peptide column. For histologic analysis of the recorded cells, the electrode tips were filled with 0.5% Lucifer Yellow (lithium salt; Sigma-Aldrich, Co.). After experiments, preparations were fixed for 2-3 hr at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS), immersed in 18% sucrose-PBS overnight, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA), then frozen on dry ice, and cut into 30 or 50 μm-thick transverse sections, followed by immunofluorescence. The following primary antibodies were used for immunofluorescence: rabbit anti-Lucifer Yellow (1:400 dilution, Molecular Probes/Invitrogen, Carlsbad, CA), guinea pig anti-Phox2b (1:1000 dilution), rabbit anti-tyrosine hydroxylase (TH) (1:500 dilution, Abcam, Cambridge, USA), rabbit anti-neurokinin-1 receptor (NK1R) (1:2000 dilution, BIOMOL International LP, PA). The secondary antibodies for fluorescence staining (1:1000 dilution) were Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 546 anti-rabbit IgG (Molecular Probes/Invitrogen), and Alexa Fluor 633 anti-guinea pig IgG or Alexa Fluor 546 anti-guinea pig IgG. 4,6-diamidino-2-phenylindole (DAPI, Sigma) was used for nuclear staining. To identify motor neuron nuclei in the medulla, the sections were routinely stained with NeuroTrace (435/455 blue fluorescence, Invitrogen) for Nissl stain. Images of immunofluorescent samples were obtained with 20× or 40× objectives on an Olympus FV1000 confocal microscope (Olympus Optical, Tokyo, Japan) or conventional fluorescence microscope (BX60, Olympus Optical).

**In situ hybridization**

Digoxigenin (DIG)-labeled riboprobe for in situ detection of vesicular glutamate transporter 2 (VGlut2) mRNA was prepared as reported (Yokota et al., 2007) using the plasmid containing VGlut2 cDNA as a template (kindly provided by Dr. Stornetta (Stornetta et al., 2002)). In situ hybridization was performed essentially as described previously on 30 μm thick cryosections (Yokota et al., 2007) with the following modifications: we did not treat sections with RNase A after hybridization and signals were detected with an anti-Dig antibody conjugated to alkaline phosphatase (Roche) and NBT/BCIP (Roche) for chromogen, followed by immunofluorescence using anti-Phox2b and anti-TH antibodies.
Lucifer Yellow-labeled neurons were reconstructed with the aid of a camera lucida attached to a fluorescence microscope (BX60; Olympus Optical Co., Ltd., Tokyo, Japan). Neuronal burst rates and nerve activity (bursts/min) were calculated from the mean burst activity for 3-5 min. Values are shown as mean ± SD. Statistical significance of differences ($P < 0.05$) were determined by Student’s $t$-test.

References


