Substance P Modulation of Hypoglossal Motoneuron Excitability During Development: Changing Balance Between Conductances

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Adachi T, Huxtable AG, Fang X, Funk GD. Substance P Modulation of Hypoglossal Motoneuron Excitability During Development: Changing Balance Between Conductances. J Neurophysiol 104: 854–872, 2010. First published June 10, 2010; doi:10.1152/jn.00016.2010. Although Substance P (SP) acts primarily through neurokinin 1 (NK1) receptors to increase the excitability of virtually all motoneurons (MNs) tested, the ontogeny of this transmitter system is not known for any MN pool. Hypoglossal (XII) MNs innervate tongue protruder muscles and participate in several behaviors that must be functional from birth including swallowing, sucking and breathing. We used immunohistochemistry, Western immunoblotting, and whole cell recording of XII MNs in brain stem slices from rats ranging in age from postnatal day zero (P0) to P23 to explore developmental changes in: NK1 receptor expression; currents evoked by SPNK1 (an NK1-selective SP receptor agonist) and; the efficacy of transduction pathways transforming ligand binding into channel modulation. Despite developmental reductions in XII MN NK1 receptor expression, SPNK1 current density remained constant at 6.1 ± 1.0 (SE) pA/pF. SPNK1 activated at least two conductances. Activation of a pH-insensitive Na+ conductance dominated in neonates (P0–P5), but its contribution fell from ~80 to ~55% in juveniles (P14–P23). SPNK1 also inhibited a pH-sensitive, two-lore domain K+ (TASK)-like K+ current. Its contribution increased developmentally. First, the density of this pH-sensitive K+ current doubled between P0 and P23. Second, SPNK1 did not affect this current in neonates, but reduced it by 20% at P7–P10 and 80% in juveniles. In addition, potentiation of repetitive firing was greatest this current in neonates, but reduced it by 20% at P7–P10 and 80% in juveniles. In addition, potentiation of repetitive firing was greatest this current in neonates, but reduced it by 20% at P7–P10 and 80% in juveniles. In addition, potentiation of repetitive firing was greatest this current in neonates, but reduced it by 20% at P7–P10 and 80% in juveniles.

Parallel maturation of neurotransmitter systems that modulate MN excitability (either by altering membrane and synaptic properties or MN responses to synaptic inputs), however, is hypothesized as critical for maintaining MN excitability during development and, as behavioral repertoire increases, for mediating changes in excitability that are specific to a given arousal state or behavior (Rekling et al. 2000).

Modulatory inputs from neurons in the caudal raphe to cranial and spinal MNs (Manaker and Tischler 1993; Manaker et al. 1992) feature prominently in this regard. They exert a powerful, diverse, and primarily excitatory influence on MN excitability through the state-dependent release (Jacobs and Fornal 1991, 1993), or corelease, of serotonin (5HT) and thyrotropin-releasing hormone (TRH) or 5HT and Substance P (SP) (Deun et al. 1993; Henry and Manaker 1998; Kachidian et al. 1991; Manaker and Zucchi 1998). Postsynaptically, these modulators act on MNs (including XII MNs) in large part through convergent pathways to block two-lore domain K+ (TASK) channels (Berg et al. 2004; Talley and Bayliss 2002; Talley et al. 2000) and activate a mixed cationic current (Bayliss et al. 1992; Fisher and Nistri 1993; Parkis et al. 1995). The excitatory actions of 5HT and TRH increase with postnatal development in most MN pools (Rekling et al. 2000). SP excites MNs primarily through NK1 receptors (Rekling et al. 2000), but little is known of how its actions on MN excitability change postnatally.

Labeling studies are inconclusive. In general, NK1 receptor binding and SP immunolabeling within the brain stem decrease postnatally (Moss and Laferriere 2002; Quirion and Dam 1986; Rodier et al. 2001; Sakanaka et al. 1982). However, NK1 receptor levels appear to be differentially regulated between MN pools (Charlton and Helke 1986; Lewis and Travagli 2001; Manaker and Zucchi 1998; Nakamura et al. 2006; Nakaya et al. 1994; Tanaka-Gomi et al. 2007; Yashpal et al. 1990).

Electrophysiological studies are lacking. Neonatal and juvenile MNs (Ptak et al. 2009; Talley et al. 2000; Yasuda et al. 2001) respond to SP, but these studies were not developmental in nature. In spinal MNs, including phrenic MNs, SP effects are characterized by slow inward currents or depolarizations, increases in input resistance, and variable potentiation of repetitive firing behavior (Fisher et al. 1994; Lehenkuehler et al. 1993; Matsuto et al. 1984; Ptak et al. 2000, 2009; Svensson et al. 2002). In the brain stem, SP excites MNs in the ambiguous (NA) (Massari et al. 1994), dorsal motor vagal (Lewis and Travagli 2001) and XII nuclei. XII MNs respond to SP through...
mechanisms similar to those described for 5HT and TRH (Rekling et al. 2000).

In this study, we focus on the developmental modulation of XII MNs by SP for several reasons. XII MNs innervate the intrinsic muscles of the tongue and participate in multiple reflex and rhythmic motor behaviors that must be functional at birth, including chewing, swallowing, and suckling (Bartlett et al. 1990; Miller 2002). XII MNs are also important in maintaining airway patency during breathing (Remmers et al. 1978). In fact, changes in the activity of modulatory neurons that innervate these MNs, including SP-containing raphe neurons, are hypothesized to contribute to the decrease in genioglossus activity during sleep that contributes to obstructive apnea (Remmers et al. 1978). In addition, neonate and adult XII nuclei contain NK1 receptor binding sites (Manaker and Rizio 1989; Rodier et al. 2001), and SP immunoreactive fibers (Gatti et al. 1999; Hinrichsen and Weston 1999; Tallaksen-Greene et al. 1993; Yasuda et al. 2001). In adults, SP immunoreactive fibers make synaptic contact with XII MNs (Connaughton et al. 1986; Gatti et al. 1996; Richardson and Gatti 2004). Finally, while we have only a limited understanding of the endogenous conditions or factors that activate this signaling system, the observations that inspiratory XII MNs depolarize in response to SP-mediated, raphe obscurus input (Ptak et al. 2009) and hyperpolarize in vitro in response to NK1 antagonists (Yasuda et al. 2001) suggest that SP is important in modulating inspiratory motor output.

The objectives of this study were therefore to combine immunohistochemistry, Western immunoblotting, and whole cell recording in transverse medullary slices from Wistar rats ranging in age from P0–P21 to test the hypotheses that during postnatal development 1) NK1 receptor expression in the XII nucleus decreases; 2) the effects of NK1 receptor activation on XII MNs are mediated by at least two conductances; and 3) that these effects decrease, and the consequences of NK1 receptor-mediated actions on XII MN excitability and action potential firing behavior diminish.

METHODS

Animals

Experiments were performed on Wistar rats ranging in age from P0 to P23. For immunohistochemical and the majority of electrophysiological studies, animals were placed in two age groups, P0–P5 and P14–P23. Note that for immunohistochemistry and Western immunoblotting, all rats of the oldest age group were P20 to P21. A third intermediate group ranging from P7–P10 was included in only two sets of electrophysiological experiments designed to characterize the developmental changes in the magnitude of the pH-sensitive K+ current and the relative contribution of this current to the total SP current. All procedures were approved by University of Auckland or University of Alberta Animal Ethics Committees.

Immunohistochemistry

Rats were anesthetized deeply with sodium pentobarbital (0.3 ml, 65 mg/kg ip) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Following perfusions, brain stems were removed and postfixed for 1–2 days in 4% paraformaldehyde 4°C. For long term storage, tissue was placed in phosphate buffered saline (PBS) with 1% sodium azide (PB-Azide, Fisher). Subsequently, brainstems were sliced into 50 μm transverse sections on a Leica VT 1000S vibratome and stored in PB-Azide until processing for immunofluorescence.

Tissue from the different age groups was processed simultaneously to facilitate comparison of NK1 receptor expression in P0–P5 and juvenile animals. NK1 receptor immunofluorescence was performed on free-floating sections using two polyclonal antibodies. Both were targeted to the 15 amino acid sequence 393–407 of the C-terminus of the NK1 receptor. The Chemicon NK1 receptor antisera was developed in Guinea Pig (1:1000, Chemicon). The Advanced Targeting Systems (ATS) NK-1 receptor histochemical antisera was developed in rabbit using the same peptide sequence but in this case the synthetic peptide was conjugated to bovine thyroglobulin via paraformaldehyde (1:1,000). The specificity of these antibodies has been previously characterized (Manthey et al. 1997; Vigna et al. 1994).

All sections were initially washed three times with PBS for 15 min. All washes mentioned in the following text similarly comprised three washes of 15 min each. All incubations were performed on an oscillating shaker at room temperature. Processing for the guinea pig and rabbit primary antibodies was identical except that sections processed with the guinea pig primary antibody were incubated for 30 min in 1% sodium borohydride (dissolved in distilled water) and then washed. All sections were then incubated in 1% hydrogen peroxide (in PBS) for 15 min, washed, and then placed in 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) and 0.3% Triton X-100 (in PBS) for 1 h. Sections were placed overnight (14–16 h) in the primary antibody solution consisting of an antibody, 0.1% BSA, and 0.3% Triton X-100 (in PBS).

Following incubation in the primary antibody, sections were washed and then incubated for 2 h in a solution containing secondary antibodies conjugated to secondary probes, Cy2-conjugated donkey anti-rabbit IgG (Cy2-DAR, Jackson ImmunoResearch Laboratories, West Grove, PA) or Cy3-conjugated donkey anti-guinea pig IgG (Cy3-DAGP, Jackson ImmunoResearch), and 0.1% BSA in PBS. Sections were washed and mounted on Fisher Tissue Path Superfrost Plus Gold Slides and coverslipped with Fluorosave Reagent (Calbiochem, San Diego, CA). Control sections were processed in an identical manner with the exception that the primary antibody was omitted from the incubation step.

Localization of NK1 receptor immunolabeling to MNs or glia was examined in P21 animals using primary antibodies against choline acetyl transferase (ChAT; 1:300; Chemicon; generated in goat) and glial fibrillary associated protein (GFAP; 1:500; Chemicon; monoclonal), respectively. In these experiments, sections were processed for NK1 receptor immunofluorescence as described in the preceding text for the antibody generated in guinea pig (1:1,000, Chemicon) except that the NK1 primary and the ChAT or GFAP primary antibodies were added together. Sections were washed and placed overnight (room temperature) in the primary antibody solution consisting of the antibodies (NK1 and ChAT or NK1 and GFAP), 0.1% BSA for ChAT but 0.1% BSA for GFAP, and 0.3% Triton X-100 (in PBS). After incubation, sections were washed and then incubated for 2 h in a solution containing secondary antibodies conjugated to fluorescent probes for both primary antibodies (NK1 and ChAT, or NK1 and GFAP) (NK1, 1:1,000, Cy3-conjugated donkey anti-guinea pig IgG, Jackson ImmunoResearch; ChAT, 1:1,000, Cy5-conjugated donkey-antigoat IgG, Jackson ImmunoResearch; GFAP, 1:1,000, Cy5 donkey anti-mouse IgG, Jackson ImmunoResearch). Sections were washed and mounted as described in the preceding text.

Confocal images were acquired using a Zeiss 510 Confocal Laser Scanning Microscope system with an Axiovert 100M microscope. Images (1,024 x 1,024 pixels) were acquired with ×10 (Fluar, NA 0.5), ×20 (Fluar, NA 0.75) or ×40 objectives (Plan-Neofluar NA 1.3). Consecutive z-axis images were acquired under ×20 and ×40 magnification. Cy2-DAR was visualized using an Argon laser set to 488 nm and an LP505 emission filter. Cy3-DAGP was visualized using a HeNe laser set to 543 nm and an LP560 emission filter. Optical section thicknesses for Cy2-DAR images were 0.7, 0.8, and
Western immunoblotting

P3 and P21 rats were anesthetized with isoflurane or sodium pentobarbital. Brain stems were rapidly removed and then cut to produce a block extending from the midpons rostrally to the spinomedullary junction caudally. Tissue blocks were glued rostral-end down on a metal platform in front of an agar block and transferred to the bath of a vibratome containing ice-cold, artificial cerebrospinal fluid (ACSF; bubbled with 95% O2-5% CO2) and serial transverse 200 μm sections were cut in the caudal to rostral direction with a Leica (VT 1000S) vibratome until the XII nucleus was clearly visible in the 200 μm section. Two 300-μm sections were then taken from each P3 brain stem while two 400-μm sections were taken from P21 preparations. This placed the rostral margin of the first slice at or just rostral to obex for both P3 and P21 animals (see Fig. 3). The second slice was then taken (i.e., the sampled tissue bracketed obex caudally and rostrally). Given the larger size of the P21 animals, thicker sections were taken from this age group to sample a similar relative portion of the entire nucleus. Sections were pinned on a silicone-elastomer (Sylgard) coated bath, and the XII nuclei were removed bilaterally using 23 and 18 gauge tissue punches for P3 and P21 animals, respectively. Punch size was selected to obtain as large a sample as possible without sampling the midline where NK1 receptor-expressing glia are concentrated (Horie et al. 2000).

Tissue sections were homogenized in lysis buffer [50 mM Tris-HCl, 3 mM sucrose, 0.1% Triton X-100, and 1 mM protease inhibitor cocktail (Cat #P8340, Sigma), and centrifuged at 10,000g for 10 min at 4°C. The supernatant was removed, and protein content was estimated using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

The presence and relative abundance of NK-1 receptor and Glp protein were determined using Western immunoblotting. Aliquots from the homogenates of XII tissue samples were diluted in reducing sample buffer (Tris-Cl 0.5 M, B-mercaptoethanol, 87% glycerol, 10% SDS, 1% bromophenol blue). Protein (20 μg/well; see following text for determination of loading concentration) was loaded into 10% polyacrylamide gels. Proteins were separated by electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) then blocked for nonspecific binding in a 7% skimmed milk solution at room temperature for 1 h prior to recording. Tissue blocks were transferred to the bath of a vibratome containing ice-cold, sucrose-substituted ACSF (Aghajanian and Rasmussen 1989; Bellingham and Berger 1996) (bubbled with 95% O2-5% CO2). Juveniles were decapitated at the midcervical level, a craniotomy performed, and the brain, from just caudal to the olfactory bulbs to the midcervical cord, was rapidly removed and placed in ice-cold ACSF. A tissue block extending from the mid-cervical spinal cord to the rostral midbrain from which the cerebellum had been removed was isolated and glued rostral end down on a metal platform in front of an agar block. Tissue blocks were transferred to the bath of a vibratome containing ice-cold, bicarbonate-based ACSF bubbled with 95% O2–5% CO2, and transverse sections (300 μm thick) were cut in the dorsal to ventral direction with a vibratome (Leica VT 1000S) and sapphire blade (Delaware Diamond Knives). In P0–P5 rats, two to three slices containing the XII nucleus were collected. In juveniles, the XII nucleus was larger allowing collection of three to four slices per animal. Slices were transferred to a recovery chamber containing standard ACSF bubbled with 95% O2–5% CO2 and left for at least 40 min at 34°C to increase viability of surface MNs (Miles et al. 2004; Talley et al. 2000). They were then transferred to a holding chamber containing standard ACSF at room temperature for at least 1 h prior to recording.

MN identification

Neurons were assumed to be XII MNs based on their location within the XII nucleus and characteristic morphology, both of which are easily established under visualization with infrared differential interference contrast (IR-DIC) microscopy. The XII nucleus is a large nucleus containing a dense cluster of neurons immediately ventral and lateral to the central canal or fourth ventricle. It is relatively homogeneous; <5% are interneurons (Viana et al. 1990). Morphological criteria included a large cell soma that was >15 μm along the shortest axis, 20–30 μm in the longest axis and a ratio of small axis/long axis of between 0.60 and 0.7 (Nunez-Abades and Cameron 1995; Nunez-Abades et al. 1994). Cell soma dimensions change little during postnatal development so these criteria were applied for all ages (Nunez-Abades and Cameron 1995; Nunez-Abades et al. 1994). Electrophysiologically, cells were assumed to be XII MNs if input resistance values fell between 50 and 150 MΩ (Berger et al. 1996; Nunez-Abades and Cameron 1995; Nunez-Abades et al. 1994). MNs were included in the analysis if they had a resting membrane potential of ~55 mV or more hyperpolarized, demonstrated repetitive firing under current-clamp conditions in response to current pulses at 3×
threshold, and generated action potentials (APs) >70 mV in amplitude.

Whole cell recording

Slices were submerged in a recording chamber (500 µl) mounted on the fixed stage of an upright microscope (Zeiss Axioskop II FS) and perfused at 3–4 ml/min. Whole cell patch-clamp recordings were made under direct visualization using a ×40 water immersion objective (Zeiss 0.9 NA), IR-DIC optics and an infrared-sensitive CCD camera (Ikegami) and monitor (National Electronics) (Stuart et al. 1993). Patch electrodes (resistance: 3.0–4.0 MΩ) were pulled on a Sutter P-97 puller from 1.2 mm OD filamented borosilicate glass (Clark Electromedical). Whole cell current signals recorded under voltage clamp conditions were amplified and filtered (low-pass 2 kHz Bessel filter) with an Axopatch 200B or Multiclamp 700A amplifier (Axon Instruments, Union City, CA), and acquired (8 kHz) using a Digidata 1200B or Digidata 1322 A/D board and pClamp 6.0 or 9.0 software and Axoscope (version 9.0, Axon Instruments). Current-clamp recordings of membrane potential, repetitive firing and action potential waveform were performed with the multi-clamp 700A amplifier (Axon Instruments). Signals were also recorded continuously via pulse code modulation for off-line analysis (Model 402, A.R.Veter) or via an additional Digidata 1322 A/D board controlled by Axoscope software. Off-line acquisition of pulse-code-modulated data were performed using AxoScope 1.1 software (Axon Instruments) and signals were analyzed using Clampfit 9.0 software. Baseline and peak currents or depolarizations elicited by SPNK1 were measured using a 1 s averaging window within Clampfit 9.0 software.

To investigate the current/voltage (I/V) relationships of SPNK1-mediated currents and the effects of NK1 receptor activation on input resistance (Ri) under voltage-clamp mode, ramps, (from −90 to −50 mV; 600 ms; 0.1 V/s) were delivered immediately prior to drug application, at the peak of drug response, and following drug washout. Ramps were used over pulses simply because they facilitated faster application, at the peak of drug response, and following drug washout. Drug application, at the peak of drug response, and following drug washout.

MNs Rik was measured as the inverse of the slope of the I/V relationship. The equilibrium potential for K+ was calculated according to the Nernst equation (−97 mV). Series resistance averaged 9.5 ± 0.1 and 10.8 ± 0.5 MΩ in P0–P5 and P14–P23 MNs, respectively. It was compensated ≥80% and monitored throughout the experiment. Data were discarded if series resistance changed by >10% between control and test conditions. As described elsewhere, currents were reported relative to membrane capacitance (measured from the compensation circuitry of the amplifier) as current densities (pA/pF) to facilitate comparison between different age groups. All recordings were performed at room temperature (22–24°C) to facilitate comparison with existing data (Ptak et al. 2009; Talley et al. 2000; Yasuda et al. 2001) Results are expressed as means ± SE. Comparisons between groups were made using Student’s t-test (paired or unpaired as dictated by the data) or ANOVA in conjunction with Bonferroni correction for multiple comparisons when appropriate (GraphPad Prism 4.2). Changes were considered significant if P < 0.05. Precise P values are provided in all cases when P is near 0.10 (i.e., <0.20). Precise P values are not provided for larger P values because such P values “are consistent with a true zero effect” (Curran-Evenett and Benos 2004).

Details of specific voltage- and current-clamp protocols are provided within RESULTS.

Solutions and drugs

Standard bicarbonate ACSF contained (in mM) 120 NaCl, 3 KCl, 1 CaCl2, 2 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, and 20 n-glucose (equilibrated with 95% O2-5% CO2, at room temperature, pH = 7.55). The sucrose-substituted bicarbonate ACSF in which brain tissue was sliced was the same as the standard ACSF with the exception that NaCl (120 mM) was replaced with 216 mM sucrose to maintain true iso-osmolarity (Bellingham and Berger 1996).

The contribution of Na+ to SPNK1-mediated currents was examined using standard bicarbonate ACSF in which choline chloride (120 mM) was substituted for NaCl (120 mM). Most recordings were performed with standard bicarbonate ACSF. However, the effect of extracellular pH on membrane current was examined in standard bicarbonate ACSF as well as HEPES-based ACSF. The effect of pH on the magnitude of SPNK1-mediated currents was examined in HEPES-based ACSF. For these experiments, pH of bicarbonate and HEPES-based ACSF was adjusted using 1.0 N HCl or 1.0 N NaOH. HEPES-based ACSF contained (in mM) 140 NaCl, 3 KCl, 1 CaCl2, 2 MgSO4, 10 HEPES, 1.25 NaH2PO4, and 20 n-glucose (bubbled with 100% O2). Note that the K+ concentration in HEPES and standard ACSF, and therefore the calculated Erev, for K+, were the same in both solutions (−97 mV).

The intracellular pipette solution contained in (mM) 122.5 potassium-glucuronate, 17.5 KCl, 9 NaCl, 1 MgCl2, 10 HEPES, 0.2 EGTA, 3 ATP-Mg2+, salt, and 0.3 GTP-Tris salt (pH 7.2–7.3 adjusted with KOH). To facilitate developmental comparison of SPNK1 effects, all XII MNs were examined under the same conditions using the same intracellular and extracellular solutions.

The NK1 receptor agonist, [Sar9,Met(O2)11]-SP (SPNK1) (Tocris Cookson) (Tousignant et al. 1990), was used because NK1 is the main subtype mediating the actions of SP on MN excitability (Rekling et al. 2000). SPNK1 was prepared as a stock solution in distilled water, aliquoted, and stored at −4°C. Immediately prior to use, aliquots were thawed and diluted in extracellular recording solution to final concentrations ranging from 1 to 10 µM. Aliquots were not re frozen. The pH of extracellular and SPNK1-containing extracellular solutions was the same. Tetrodotoxin (TTX, 0.5 µM, Alomone Labs) was added to the bath in some experiments to block synaptic transmission.

Drug application

SPNK1 was applied into the bath (1.0 µM), or pressure injected (1.0–10 µM) via triple-barreled pipettes (~6 µm per barrel, outside-tip diameter) that were pulled from borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, UK). These concentrations were selected based on observations in spinal (Lepre et al. 1993, 1996) and XII MNs (Talley et al. 2000; Yasuda et al. 2001) of rodents. The EC50 values for the excitatory action of SP on spinal MNs (means ± SE) obtained in rat [0.4 ± 0.2 µM (Lepre et al. 1996); 0.95 ± 1.0 µM (Lepre et al. 1993)] and gerbil [0.47 ± 0.26 µM (Lepre et al. 1993)] preparations were comparable. In XII MNs of P7-14 rat, 1.0 µM SPNK1 completely blocked the pH-sensitive TASK1 current (Talley et al. 2000). In XII MNs of mouse, local application of SPNK1 at concentrations ranging from 0.001 to 10 µM established that 0.001 is without effect on XII activity; 1 µM evoked the maximum potentiation of XII nerve inspiratory activity; 10 µM reduced inspiratory burst amplitude due to a disproportionate increase in tonic discharge that obscured inspiratory activity (Yasuda et al. 2001). Thus bath application of 1 µM and local application of 10 µM are near saturating levels. Higher concentrations were not used due to the excessive time required for MNs to recover.

Triple-barreled drug pipettes were placed superficial to the surface of the slice ~25–50 µm upstream (as determined by monitoring flow of fluorescent dye from drug pipette) of the MN soma as this configuration virtually eliminates pressure effects. Pipettes typically contained NK1 agonist in two barrels. The third barrel contained...
vehicle solution to control for pressure artifacts. Pressure effects were rarely observed but were easily distinguished from drug actions (whether evoked by vehicle or agonist) in that they were associated with a rapid, almost step change (typically a reduction) in holding current and an increase in series resistance, neither of which recovered during drug washout. Drugs were delivered at 10 psi, and injections were controlled via a programmable stimulator (Master-8, AMPI, Jerusalem, Israel) linked to a solenoid. Leakage of drug from the pipette was tested in preliminary experiments by including Lucifer yellow (di-potassium salt) in the agonist or SPNK1-containing barrels and visualizing the electrode tip with epifluorescence. Leakage was never observed with outside tip diameters in the range of 6–7 μm per barrel. Many protocols required repeated applications of SPNK1 to an individual MN. Consecutive applications were separated by an interval of ≥15 min, which, as shown in previous experiments (Funk, unpublished observations) and similar preparations that the concentration of drug decreases exponentially with distance from the pipette tip (Nicholson 1985), which accounts for observations in this (Funk, unpublished observations) and similar preparations that the concentration of drug in the pipette must be ~10-fold greater than the bath-applied concentration to produce similar effects (Liu et al. 1990).

RESULTS

NK1 receptor immunoreactivity in XII MNs decreases developmentally

Based on SP binding assays in the XII nucleus of piglets (Rodier et al. 2001), we hypothesized that NK1 receptor immunoreactivity in the XII nucleus would follow the pattern observed in other areas of the brain stem (Pagliardini et al. 2003; Quirion and Dam 1986; Sakanaka et al. 1982) and decrease during postnatal development. Transverse sections (50 μm) from P0 to P5 and P20 medulla were processed simultaneously and under identical conditions for NK1 receptor immunolabeling using the anti-rabbit antibody (ATS). Labeling was visualized with FITC (or rhodamine) fluorescence. The experiment was repeated seven times with each replicate using tissue from different P0–P5 and P20 rats. The staining pattern was similar in all trials and is shown for one replicate in Fig. 1. In the first series of panels (Fig. 1, A and B), confocal settings were selected to optimize imaging in the P0 tissue. Identical conditions were then used to image the P20 sections to facilitate developmental comparison. As shown for the individual animals in Fig. 1, NK1 receptor immunolabeling within the XII nucleus was stronger in P0–P5 compared with P20 animals. At birth, NK1 receptor immunoreactivity was strongest in midline structures separating left and right XII nuclei, consistent with the well-documented high level of NK1 receptor expression in midline glial cells at this age (Horie et al. 2000). Aside from midline structures, labeling in the XII nuclei was higher than in surrounding tissue. Hence, under low magnification, the nuclei were easily distinguished (Fig. 1A, left). Within the nuclei, a dense plexus of immunoreactive structures could be distinguished. At higher magnification (Fig. 1, B and C, left), NK1 immunolabeling appears to outline the cell bodies and extend along proximal dendrites in a pattern described previously in brain stem neurons (Gray et al. 1999; Guyenet et al. 2002; Pagliardini et al. 2003, 2005).
margins are even more apparent in Supplementary Fig. S1, which includes expanded versions of Fig. 1C in which brightness was also increased 20%. Outlined MNs are indicated with arrows. The dimension and morphology of these “outlined” cells is consistent only with MN labeling, but this does not exclude the possibility that glial labeling contributes to the signal.

With development, midline labeling decreased in intensity and was absent by P20 (Fig. 1A, right) as previously documented (Horie et al. 2000). Labeling also decreased within the XII nucleus, such that at P20 labeling was only barely visible when visualized using imaging parameters that were sufficient to reveal extensive immunolabeling in the P0–P5 rats, as shown for individual rats in Fig. 1, A and B. Detector gain was therefore increased and offset adjusted in Fig. 1C (right) to more clearly illustrate NK1 receptor expression in the XII nucleus of a P20 rat. NK1 receptor immunolabeling is apparent surrounding individual MN somata and some proximal dendrites (Fig. S1). Note that despite the increase in detector gain, NK1 receptor immunofluorescence still appears lower at P20 compared with neonatal sections. There was no obvious developmental redistribution of immunolabeling from dendrites to soma or vice versa, but this was not examined in detail.

In addition, although the P0 (near obex) and P20 sections (rostral to obex) are from slightly different rostrocaudal levels, NK1 labeling intensity was lower throughout the XII nucleus of juveniles.

To establish that the greater NK1 receptor immunolabeling in P0–P5 compared with P20 was not an artifact associated with, for example, greater diffusion of antibody into P0–P5 tissue, we examined the compact division of nucleus ambiguus (cNA), which has moderate to strong NK1 receptor immunofluorescence still appears lower at P20 compared with neonatal sections. There was no obvious developmental redistribution of immunolabeling from dendrites to soma or vice versa, but this was not examined in detail. In addition, although the P0 (near obex) and P20 sections (rostral to obex) are from slightly different rostrocaudal levels, NK1 labeling intensity was lower throughout the XII nucleus of juveniles.

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The NK1 receptor immunolabeling experiments were repeated with a different NK1 receptor antibody generated in guinea pig (Chemicon). Although the intensity of NK1 receptor immunofluorescence was somewhat lower using the guinea pig antibody, developmental changes in immunofluorescence within the XII were similar to those seen with the rabbit antibody (Supplementary Fig. S2). In addition, the stronger cytoplasmic signal associated with this antibody revealed cell morphology consistent with MN labeling. Nevertheless, to confirm that MNs contributed to the NK1 immunolabeling in the XII nucleus, we performed double immunolabeling experiments in two P21 rats using antibodies against the MN marker ChAT and the NK1 receptor (guinea pig; Chemicon). Figure 2 clearly shows double-labeled MNs. Note that this does not exclude the possibility that glia contribute to the NK1 signal. In fact, double-immunolabeling in tissue from P21 rats with antibodies against the glial marker GFAP, and the NK1 receptor (guinea pig) suggest that glia contribute to the signal in the XII nucleus (Supplementary Fig. S3). The most important point, however, is that during postnatal development NK1 immunolabeling appears to decrease throughout the XII nucleus (Fig. 1 and Supplementary Figs. S1 and S2); i.e., in both MNs and glia.

Western immunoblotting

To further explore the possibility that NK1 receptor expression in the XII nucleus decreases postnatally, we performed western immunoblot analysis of NK1 receptor protein extracted from XII tissue punches of P3 and P21 rats (Fig. 3, A and B). Visual inspection of the Western blots from two P3 (1 of which represents tissue pooled from 6 animals and 1 represents tissue pooled from 2 animals) and three P21 tissue samples shows that the NK1 receptor signal is greater at P3 than at P21 while the Gβ protein signal is similar between age groups. When NK1 receptor levels are expressed as the ratio of NK1 receptor OD to Gβ protein OD, NK1 receptor levels at P3 were 0.14 ± 0.013 compared with 0.019 ± 0.013 at P21 (Fig. 3C).

The online version of this article contains supplemental data.
SPNK1 current density does not change postnataally

We next tested the hypothesis that postnatal changes in SPNK1 current density parallel changes in NK1 receptor immunolabeling and decrease developmentally. Resting potential averaged $-62.3 \pm 1.7$ (n = 20) and $-62.4 \pm 1.5$ mV (n = 20) in P0–P5 and P14–P23 age groups. Membrane capacitance was also similar across age groups (39 ± 3 pF; P0–P5; 33 ± 3 pF; P14–P23), while $R_N$ decreased from 109 ± 13.2 MΩ in P0–P5 to 61 ± 12.3 MΩ in P14–P23 (P = 0.0018).

SPNK1 evoked slowly developing, reversible inward currents in all cells studied, whether it was applied in the bath (1 μM, 30 s) or applied locally (1–10 μM, 15 s; Fig. 4A). SPNK1 current density was assessed by adding the agonist (1.0 μM) directly to the bath. This was essential for developmental comparison because with local application, differences in drug diffusion/tissue permeability between age groups could result in different agonist concentrations at the MN (Lehmenkühler et al. 1993). SPNK1 current density did not change developmentally, averaging 6.1 ± 1.0 pA/pF (n = 20) in P0–P5 and 6.2 ± 1.0 pA/pF (n = 20) in P14–P23 rats (Fig. 4, A and D).

Bath application has the advantage over local drug application in that agonist concentration is more precisely known and therefore helps to ensure that P0–P5 and P14–P23 MNs are exposed to similar concentrations of SPNK1. It has the disadvantage, however, that responses are slow in onset and recovery is prolonged, making repeated applications difficult. Experiments designed to explore mechanism of action required repeated SPNK1 applications and therefore utilized local application techniques.

Current/voltage relationships, obtained using voltage ramps from $-90$ to $-30$ mV applied at the peak of the SPNK1 current, revealed that SPNK1 currents were associated with a 36 ± 6 or 44 ± 7% increase in $R_N$ from 109 ± 13 to 148 ± 19 MΩ in P0–P5 (n = 20, P < 10$^{-4}$) compared with 61 ± 12 to 88 ± 19 MΩ in P14–P23 MNs (n = 20; Fig. 4C, P < 10$^{-4}$). Extrapolation of these I/V relationships suggested that SPNK1 currents reversed near $-128 ± 5$ mV in P0–P5 and $-96 ± 4$ mV in P14–P23 (Fig. 4B).

Postsynaptic location of theNK1 receptors was established by the observation that 0.5 μM TTX in the bath eliminated most excitatory postsynaptic currents (EPSCs) but did not change the SPNK1-mediated inward current in any age group (Fig. 4A, right). In P0–P5 rats, the current evoked by local application of SPNK1 (10 μM) in TTX was 119 ± 32% of control (n = 4), whereas in P14–P23, the current in TTX was 83 ± 7% of control (n = 5). The SPNK1-mediated increase in $R_N$ was also similar in control and TTX. In P0–P5 rats, it increased 149 ± 4% in control and 136 ± 4% in TTX (n = 4).

In the P14–P23, $R_N$ increased 143 ± 15% in control and 131 ± 14% in TTX (n = 4).

In summary, SPNK1 currents are mediated by postsynaptic receptors and are associated with a significant increase in $R_N$. In P0–P5 neonates, extrapolation of the I/V relationships suggest that the SPNK1 currents reverse at a potential hyperpolarized to the equilibrium potential for K$^+$ ($E_{K^+}$). Develop-
mentally, the extrapolated reversal potential shifts in the depolarizing direction and approaches $E_{K^+}$. Most importantly, the SPNK1 currents do not decrease in parallel with NK1 receptor signal levels obtained with both immunohistochemistry and western blots, but remain constant over the first 3 wk of postnatal development.

### Ionic basis of SPNK1 actions

Given immunohistochemical and Western blot data suggesting that NK1 receptor immunolabeling within the XII nucleus decreases markedly during development, but electrophysiological data that NK1 receptor-mediated current density remains constant, we next addressed the hypothesis that SPNK1 current density is maintained through developmental increases in the efficacy of effector systems (ion channels) mediating the actions of SPNK1.

$I_h$ increases developmentally but is not modulated by SPNK1

The neuromodulators norepinephrine (NE), TRH, and SPNK1 occlude each other’s action in XII MNs (Parkis et al. 1995; Yasuda et al. 2001), suggesting common signaling cascades. This, combined with the observations that NE and 5HT enhance the hyperpolarization activated inward current, $I_{h}$, in XII (Parkis et al. 1995), spinal (Larkman and Kelly 1997), or trigeminal (Hsiao et al. 1997) MNs and that $I_h$ increases developmentally in XII MNs (Bayliss et al. 1994a), led us to test two hypotheses: that SPNK1 modulates $I_h$ and that increased modulation of $I_h$ by SPNK1 with development contributes to the fact that SPNK1 current density does not decrease in parallel with NK1 receptor density.

To determine whether SPNK1 affected $I_h$, membrane potential was stepped from a holding potential of $-60$ to $-130$ mV in a series of 5 steps (1st step was $-10$ mV; remaining steps were $-15$ mV; 800 ms step duration, 20 s between pulses) to activate $I_h$ before, during, and after local application of SPNK1. Currents activated by this series of voltage steps are shown in Fig. 5A. The instantaneous current jump ($I_{inst}$) was measured immediately following the capacitive transient (Fig. 5A, $\phi$).

The steady-state current ($I_{steady}$) measured at the end of the 800 ms hyperpolarizing pulse (Fig. 5A, %) revealed an inwardly rectifying conductance (Fig. 5B). The difference between $I_{steady}$ and $I_{inst}$ was defined as the $I_h$ current based on its voltage-dependence (Fig. 5B), and its sensitivity to the organic blocker ZD7288 (locally applied 10–100 μM; tested in P0–P5 only, data not shown). The $I_h$ current first became apparent with steps to $-85$ mV, and it increased in magnitude with further hyperpolarization (Fig. 5, $A$ and $B$). The difference between $I_{steady}$ and $I_{inst}$ was defined as the $I_h$ current based on its voltage-dependence (Fig. 5B), and its sensitivity to the organic blocker ZD7288 (locally applied 10–100 μM; tested in P0–P5 only, data not shown). The $I_h$ current first became apparent with steps to $-85$ mV, and it increased in magnitude with further hyperpolarization (Fig. 5, $A$ and $B$). The difference between $I_{steady}$ and $I_{inst}$ was defined as the $I_h$ current based on its voltage-dependence (Fig. 5B), and its sensitivity to the organic blocker ZD7288 (locally applied 10–100 μM; tested in P0–P5 only, data not shown). The $I_h$ current first became apparent with steps to $-85$ mV, and it increased in magnitude with further hyperpolarization (Fig. 5, $A$ and $B$). The difference between $I_{steady}$ and $I_{inst}$ was defined as the $I_h$ current based on its voltage-dependence (Fig. 5B), and its sensitivity to the organic blocker ZD7288 (locally applied 10–100 μM; tested in P0–P5 only, data not shown). The $I_h$ current first became apparent with steps to $-85$ mV, and it increased in magnitude with further hyperpolarization (Fig. 5, $A$ and $B$).
slices (−908 ± 113 pA, n = 10, P = 0.0016). SPNK1, however, did not significantly alter $I_h$ current magnitude in either age group. Following SPNK1, $I_h$ activated with steps from −60 to −130 mV measured −224 ± 47 and −842 ± 101 pA in P0–P5 and P14–P23 MNs, respectively, which corresponded to 89 ± 21 and 95 ± 4% of control (Fig. 5C).

**pH-sensitive, resting $K^+$ conductance increases postnatally**

In XII MNs from P7 to P14 rats, one of the main mechanisms mediating the actions of SP is its inhibition of a pH-sensitive, background $K^+$ leak conductance to which the TASK1 channel contributes significantly (Talley et al. 2000). With acidic shifts in extracellular pH, this channel closes, leading to membrane depolarization. With alkaline shifts in extracellular pH, the channel opens leading to membrane hyperpolarization. Whether the expression of this pH-sensitive $K^+$ current changes developmentally is not known. We tested the hypothesis that its expression increases developmentally in XII MNs by measuring pH-mediated changes in membrane current. This group was included to facilitate comparison with the original work (Talley et al. 2000) describing the properties and density of pH-sensitive $K^+$ currents in XII MNs of rat that examined animals between P7 and 14. Passive properties including resting potential (−61.8 ± 1.4 mV, n = 19), membrane capacitance (38 ± 2 pF) and $R_N$ (80 ± 4.5 MΩ) of this intermediate age group were consistent with those seen in younger and older groups (see preceding text).

The time course of changes in membrane holding current that accompanied changes in extracellular pH is shown for a single P5 and P19 XII MN in Fig. 6A. The average differences in holding current (Fig. 6B) and neuronal $R_N$ (Fig. 5C), measured in each age group between pH values of 6.5 and 7.3 or 6.5 and 8.4 are also shown. There were three main observations. First, in almost every case (3 age groups; 2 pH ranges), an increase in pH caused a significant outward current that was associated with a significant decrease in $R_N$. The only exception was in the youngest group where the significant outward current associated with the smaller pH shift from 6.5 to 7.3 was not associated with a significant decrease in $R_N$.

Second, for neonates and juveniles, the magnitude of the current increased with greater changes in extracellular pH. The outward current evoked by a pH change from 6.5 to −8.4 was greater than that evoked by a change from 6.5 to 7.3 in neonates ($P = 0.0035$) and juveniles ($P < 10^{-3}$). Third, the magnitude of the pH-induced outward current increased developmentally. The density of the outward current associated with pH shifts between 6.5 to 7.3 averaged 1.6 ± 0.4 pA/pF ($n = 12$), 2.5 ± 0.5 pA/pF ($n = 6$), and 2.9 ± 0.4 pA/pF ($n = 11$) in P0–P5, P7–P10 and P14–P23, respectively; the current in P14-23 was greater than that at P0-5 ($P = 0.009$). Shifts in pH between 6.5 and 8.4 saw even larger current densities that increased from 3.0 ± 0.4 (n = 12) to 3.9 ± 0.7 ($n = 5$) and 6.3 ± 0.8 pA/pF ($n = 11$) with progressing age; the current evoked at P14-23 by this larger pH step was greater than that at P0-5 ($P = 0.009$).
These pH-induced outward currents were associated with significant reductions in $R_N$, the magnitude of which increased with the size of the pH shift and with development (Fig. 6C). In P0–P5 neonates, $R_N$ remained at 100 ± 2% of control with the pH shift from 6.5–7.3, but $R_N$ decreased significantly to 95 ± 3% of control with the larger pH shift from 6.5–8.4 ($P = 0.035$). At P7–P10, $R_N$ decreased to 94 ± 3 and 90 ± 2% of control with pH shifts from 6.5 to 7.3 and 6.5 to 8.4, respectively. These changes were not significantly different from the $R_N$ changes observed in neonates. In juveniles, as seen in P0–P5 neonates, the $R_N$ change evoked by the large pH step averaged 79 ± 3% and was greater than that associated with the smaller pH step where $R_N$ fell to only 87 ± 3% of control ($P = 0.035$). The I/V relationship of the pH-mediated current was calculated by subtracting the whole cell I/V curve obtained in pH 8.4 from that obtained at a pH of 6.5 (Fig. 6D). Taking into consideration the −6.7 mV difference in liquid junction potential for the different pH solutions, the acidification-induced inward current reversed near $E_{K^+}$ at −93 ± 5 and −84 ± 4 mV in P0–P5 ($n = 5$) and P14–P23 MNs ($n = 6$), respectively.

As the pH-sensitive currents measured in bicarbonate ACSF in Wistar rats were smaller than previously recorded in juvenile Sprague-Dawley rats using HEPES solution (Talley et al. 2000), we repeated the preceding experiments in HEPES-based ACSF. Results were not significantly different from those obtained in bicarbonate ACSF. The currents induced by pH-shifts from 6.5 to 8.4 averaged 3.9 ± 0.6 pA/pF in P0–P5 ($n = 13$), 4.2 ± 0.8 pA/pF in P7–P10 ($n = 12$) and 7.2 ± 1.8 pA/pF in P14–P23 MNs ($n = 9$).

$pH$-sensitive current does not involve TREK-1 or TRAAK

While a number of K$^+$ channels are pH-sensitive, only TASK1 and TRAAK show the open rectification that, while not shown here, characterizes the pH-sensitive K$^+$ conductance in XII MNs (Fink et al. 1998; Talley et al. 2000). TRAAK channels however, are not pH-sensitive. TREK-1 channels, on the other hand, are outwardly rectifying but pH-sensitive in the relevant range (Patel et al. 1999). To exclude involvement of these two channels, which together might contribute to either the pH-mediated conductance or the $SP_N_{K,1}$-mediated effects, we tested the effects of lyso phosphatidylcholine (LPC) on membrane current in juvenile XII and VII MNs. LPC is a specific activator of TRAAK and TREK-1 but not TASK1 (Maingret et al. 2000). Juvenile animals were used because this age group has the largest pH-sensitive K$^+$ current. VII MNs were included as a positive control for LPC because immunolabeling indicates a high level of TREK-1 expression in this MN pool (Hervieu et al. 2001). As noted previously for arachidonic acid (Talley et al. 2000), local application of 1 mM LPC to XII MNs...
induced an inward shift in the holding current of $-49 \pm 24$ pA ($n = 3$) rather than the outward shift expected with activation of a $K^+$ current (data not shown). In addition, XII MN $R_h$ did not change noticeably. In VII MNs, however, LPC produced a $70 \pm 8$ pA ($n = 3$) outward current that reversed at $-73 \pm 2$ mV and was associated with a reduction in $R_h$ to $88 \pm 3\%$ of control ($P = 0.036$, data not shown). These data suggest that XII MNs do not express functional TRAAK or TREK-1 channels.

$SP_{NK1}$ currents have a pH-sensitive component that increases developmentally

If this pH-sensitive $K^+$ current is modulated by $SP_{NK1}$, its increased postnatal expression may contribute to our finding that $SP_{NK1}$ current density remains constant in the face of reduced NK1 receptor expression. To explore this possibility, we tested whether the $SP_{NK1}$ response is pH-sensitive and whether the pH sensitivity of the $SP_{NK1}$ current increases developmentally.

As in the preceding text, the magnitude of the pH-sensitive current was estimated by the decrease in holding current associated with transition from pH 6.5, where TASK is maximally inhibited (for these purposes, we will assume that TASK1 is completely closed) to pH 8.4 where TASK1 is maximally activated (Talley et al. 2000).

These experiments were performed in HEPES-based ACSF. Drugs for these experiments were therefore dissolved in HEPES-based ACSF as well. Whole cell recordings were established at pH 7.3. pH was then changed to 6.5, and $SP_{NK1}$ was applied (5 $\mu$M, 15 s). Following recovery, which took between 10 and 15 min, pH was changed from pH 6.5 to 8.4 and $SP_{NK1}$ was reapplied. Following recovery, $SP_{NK1}$ was applied once more at pH 6.5 to compare with the initial response. In some cells, a final application was made at pH 7.3.

The time course of changes in membrane current associated with changing pH and local application of $SP_{NK1}$ is shown for a single juvenile MN in Fig. 7A. As seen for this MN, $SP_{NK1}$ produced a significant inward current at pH 8.4 ($I_{SP8.4}$). An inward holding current then developed with transition to pH 6.5 where the SP current ($I_{SP6.5}$) was significantly attenuated relative to that produced at pH 8.4. With transition to pH 7.3, both membrane holding current and the $SP_{NK1}$ current returned to levels intermediate between those seen at pH 8.4 and 6.5. Extrapolation of I/V relationships of $I_{SP6.5}$ for a P1 and P17 MN indicated that the relationship in P14–P23 crossed the x axis close to $E_{K^+} = (-101 \pm 4$ mV, $n = 6$) and that it crossed at potentials that were numerically but not significantly, more hyperpolarized in P0–P5 MNs ($-122 \pm 10$ mV, $n = 6$, Fig. 7B).

Potentiation of $SP_{NK1}$ currents by increased pH [calculated as $(I_{SP8.4}/I_{SP6.5}) \times 100$] increased significantly during development. In the P0–P5 MNs, the $SP_{NK1}$ current was not affected by pH (Fig. 7, B and C). $I_{SP8.4}$ ($-139 \pm 19$ pA, $n = 6$) was 97 $\pm$ 18% of $I_{SP6.5}$, and its I/V relationship virtually overlapped with that produced at pH 6.5 (Fig. 7B). In P7-10 MNs,
the increase in pH from 6.5 to 8.4 increased $I_{SP}$ by 24 ± 6% from 131 ± 20 to 166 ± 30, respectively (Fig. 7C), but this was not significant. In P14–P23 MNs, $I_{SP8.4}$ (−197 ± 28 pA) was potentiated by 160 ± 41% relative to $I_{SP6.5}$ (−88 ± 18 pA, $n = 11$, Fig. 7C, $P = 0.0075$). While the extrapolated I/V relationships of $I_{SP8.4}$ and $I_{SP6.5}$ crossed the x axis at approximately the same potential, the I/V relationship for $I_{SP8.4}$ was much steeper than for $I_{SP6.5}$ (Fig. 7B, $P = 0.0008$) and was similar to the I/V relationship of the current activated with a shift in extracellular pH from 8.5 to 6.5 (Fig. 6D).

We then estimated the portion of the SPNK1 current that was mediated by the pH-sensitive K$^+$ current. $I_{SP6.5}$ represents the pH-insensitive component of the SPNK1 current. The current evoked at pH 8.4 represents the pH-insensitive plus the pH-sensitive component. Thus the ratio of the difference between $I_{SP8.4}$ and $I_{SP6.5}$ and $I_{SP8.4}$ represents the proportion of $I_{SP}$ that is mediated through inhibition of a pH-sensitive conductance [i.e., $(I_{SP8.4} - I_{SP6.5})/I_{SP8.4}$]. In P0-5 MNs, SPNK1 currents at pH 8.4 and 6.5 were not different. ($I_{SP8.4} - I_{SP6.5})/I_{SP8.4}$ was −17 ± 17% (Fig. 7D). In P7–P10 MNs, the fraction of $I_{SP}$ mediated by inhibition of a pH-sensitive current was 17 ± 5% ($n = 12$) and in P14–P23, it increased significantly to 53 ± 6% ($n = 10$, $P < 10^{-3}$). The proportion of the SPNK1 current mediated through inhibition of a pH-sensitive K$^+$ current therefore increases significantly during development.

**Modulation of a pH-sensitive K$^+$ current by SPNK1 increases in efficacy with postnatal development**

Modulation of the pH-sensitive K$^+$ current by SPNK1 requires not only the expression of the NK1 receptor and the ion channel in the postsynaptic membrane, it also requires activation of an as yet unidentified second messenger cascade (for discussion, see Talley et al. 2000) that may also change postnatally. In other words, while SPNK1 receptors and pH-sensitive K$^+$ channels are present, the ability of SP to modulate these channels may change developmentally. We tested this hypothesis indirectly by determining, at the different ages, the magnitude of the current evoked by increasing pH from 6.5 to 8.4 and then measuring the ability of SPNK1 to inhibit this pH-sensitive current (Parkis et al. 1995), it is also clear that they activate a Ba$^{2+}$-insensitive cationic conductance that is primarily carried by Na$^+$ (Parkis et al. 1995; Rekling et al. 2000). To assess the proportion of the SPNK1 current mediated by activating Na$^+$ versus closing K$^+$ conductances and how this proportion changes developmentally, we measured the current evoked by locally applying SPNK1 (5 μM, 15 s) over XII MNs in control solution ($I_{SP}$), when 120 mM NaCl was replaced with 120 mM choline chloride ($I_{SPCH}$), and after washout of choline chloride (Fig. 8). Note that these experiments were performed in bicarbonate buffer. Thus extracellular Na$^+$ was reduced to 26 mM (~18% of control) but not removed.

We first established that the effects on membrane properties of replacing NaCl with choline-chloride increased developmentally. In P0–P5 MNs, effects were inconsistent. In 7 of 15 neurons, holding current did not change in response to choline substitution. In the remaining 8/15 MNs, choline substitution was associated with an average outward current of 2.1 ± 0.3 pA/pF ($n = 8$) and an insignificant increase in $R_N$ from 154 ± 22 to 165 ± 24 MΩ. In contrast, choline substitution was associated with an outward current in all P14–P23 MNs ($n = 15$) that was larger (3.8 ± 0.6 pA/pF, $n = 8$) than in the P0–P5 age group (0.836 ± 0.422 pA/pF, $n = 15$, $P = 0.002$). $R_N$ did not change significantly (control, 56 ± 8 MΩ; choline chloride, 61 ± 7 MΩ). These data are consistent with postnatal development of a Na$^+$-dependent leak conductance that may or may not be modulated by SPNK1.

As our main interest was in the SPNK1-sensitive Na$^+$ current and how its contribution to the total SPNK1 current changes developmentally, we next compared SPNK1 currents evoked in control ACSF with those evoked in choline chloride-substituted ACSF. The SPNK1 current was significantly reduced in choline chloride solution compared with control. In choline chloride solution, it was 22 ± 2% ($n = 15$, $P < 10^{-3}$) and 42 ± 4% ($n = 12$, $P < 10^{-4}$) of the current evoked in normal

![FIG. 8. Relative contribution of a Na$^+$-dependent current to the total SPNK1 current decreases developmentally. A: whole cell voltage-clamp recordings showing, for an individual P0–P5 and P14–P23 XII MN, currents evoked by locally applied SPNK1 (5 μM) under control conditions, after substitution of Na$^+$ in the bicarbonate-based ACSF with choline chloride and after washout of choline chloride. B: histogram plotting the magnitude of the SPNK1 current evoked in choline chloride relative to that evoked in standard ACSF ($I_{SPCC}/I_{SP}$) × 100). (*) significant reduction in the SPNK1 current in choline chloride ACSF relative to control; φ, significant difference between P0–P5 and P14–P23 in the contribution of the Na$^+$ conductance to the SPNK1 current).
To explore the mechanisms that might underlie the greater potentiation of firing frequency, we examined the effects of SPNK1 on the medium afterhyperpolarization (mAHP) by eliciting single action potentials with brief (5 ms) suprathreshold current pulses before, during, and after application of SPNK1. A mAHP was apparent in both groups, but SPNK1 was not differentially affected by SPNK1, thus data are not shown. As documented previously (Nunez-Abades et al. 1993), the mAHP in P0–P5 MNs (68 ± 10 ms, n = 7) was longer in duration than in P14–P23 MNs (41 ± 1.4 ms, n = 12, P = 0.003). From the time of the fast AHP, it took 56 ± 12 ms to reach the maximum hyperpolarization (nadir) of the mAHP in P0–P5, whereas in P14–P23 MNs, it took 30 ± 1 ms. In spite of these differences in onset time, SPNK1 increased the time to the nadir by the same relative amount in P0–P5 (30 ± 9%; 74.8 ± 18.1 ms, P = 0.039) and juvenile MNs (17.0 ± 6.4%; 34.3 ± 1.7 ms, P = 0.022). The amplitude of the mAHP (measured from membrane potential prior to the action potential; approximately −60 mV) was similar in P0–P5 (2.6 ± 0.4 mV, n = 7) and P14–P23 MNs (3.1 ± 0.4 mV, n = 12) and unaffected by SPNK1, where it measured 2.7 ± 0.41 and 3.1 ± 0.4 mV in P0–P5 and P14–P23 MNs, respectively. Small SPNK1-mediated increases in the amplitude of the afterdepolarization (ADP) in P0–P5 (115 ± 9% of control, n = 5) and P14–P23 MNs (109 ± 3%, n = 6; data not shown), were not significant.

**DISCUSSION**

NK1 receptor expression decreases developmentally

NK1 receptor expression and SP levels in the brain stem are generally highest in early life and decrease thereafter. However, there is considerable regional variability in the ontogeny of SP and NK1 receptor expression (Quirion and Dam 1986; Sakanaka et al. 1982; Walker et al. 1991) even between MN pools. In adult, NK1 receptor labeling is high in spinal, particularly phrenic and pudendal, MN pools, moderate in other pools such as compact nucleus ambiguus (cNA) and XII, and low in facial and trigeminal MN pools (Charlton and Helke 1986; Holtman 1988; Manaker and Zucchi 1998; Nakaya et al. 1994; Tallaksen-Greene et al. 1993; Yashpal et al. 1990). Developmentally, the number of SP immunoreactive fibers surrounding phrenic MNs reaches adult levels by P8 (Charlton and Helke 1986), whereas around other spinal MNs it increases between P0 and P28 and then decreases by 50% into adulthood (Ozaki et al. 1992). In the trigeminal motor nucleus, the number of receptors and SP-immunoreactive fibers increases between E19 and P7 and then decreases into adulthood (Nakamura et al. 2006; Tanaka-Gomi et al. 2007). Moderate levels
of SP immunoreactivity or NK1 receptor labeling within the XII nucleus have been observed in neonatal human (Jordan et al. 1995, 1997; Rikard-Bell et al. 1990), adult rabbit (Gin-gras et al. 1988), cat (Gatti et al. 1996, 1999; Richardson and Gatti 2004), and rat (Hinrichsen and Weston 1999) and neonatal mouse (Yasuda et al. 2001). Developmentally, our immunohistochemical and Western blot analyses indicate that NK1 receptor expression in the XII nucleus decreases postnatally. In addition, the double-immunolabeling of XII cells for ChAT and NK1 and XII structures for GFAP and NK1 receptors suggest that MNs and glia, respectively, both express NK1 receptors. This, combined with the observation in NK1 labeling appears to fall similarly throughout the XII nucleus, is the basis of our conclusion that NK1 receptor expression on XII MNs decreases postnatally.

Although our immunohistochemical data do not allow us to quantify how protein expression changes developmentally, they provide valuable qualitative information because tissue from all age groups was processed simultaneously under identical conditions. We also demonstrate that NK1 receptor im-

**FIG. 9.** Potentiation of repetitive firing by SP\(_{\text{NK1}}\) increases postnatally. A: repetitive firing responses evoked by square-wave pulses of current (600 ms) under control conditions and in the presence of SP. Pulses that produced similar firing frequencies in P0–P5 and P14–P23 MNs under control conditions were selected for visual comparison. B: plots of average f/I relationships in control and during local application of SP\(_{\text{NK1}}\) for a single P0–P5 (left) and P14–P23 MN (right). C: cartoon showing method used to assess the magnitude of SP\(_{\text{NK1}}\)-mediated changes in the slope and leftward shift (\(b_2 - b_1\)) in the f/I relationship. D: histograms summarizing the effects of SP\(_{\text{NK1}}\) on the slope and leftward shift of f/I relationship in P0–P5 (\(n = 10\)) and P14–P23 MNs (\(n = 10\)). (*, parameter in SP\(_{\text{NK1}}\) is significantly different from in control; \(\phi\), significant difference from neonate).

**FIG. 10.** f/I relationships, based on the first ISI of the spike train, in control and after local application of SP\(_{\text{NK1}}\) for a single P0–P5 and P14–P23 MN.
munolabeling of the cNA increased over the same time frame (P0–P21) that XII labeling decreased. This strongly suggests that the developmental reduction in NK1 receptor immunolabeling in the XII nucleus does not reflect reduced penetration of antiserum into the juvenile tissue (Lehmenkuhler et al. 1993). In summary, our data, combined with the developmental reduction in $^{125}$I SP labeling in the XII nucleus of swine (Rodier et al. 2001), and reduced brain stem labeling for NK1 receptor or SP (Moss and Laferriere 2002; Quirion and Dam 1986; Rodier et al. 2001; Sakanaka et al. 1982), all suggest that in mammals NK1 receptor density in the XII nucleus decreases postnatally.

**$SP_{NK1}$ current density remains constant during development**

$SP_{NK1}$ current density in XII MNs remains constant postnatally, suggesting that a developmental increase in components of the NK1 receptor signaling cascade compensate for the developmental reduction in NK1 receptor expression. We first consider the mechanisms by which $SP_{NK1}$ modulates the excitability of juvenile XII MNs and then discuss developmental changes in the actions of $SP_{NK1}$ on XII MN excitability.

**$SP_{NK1}$ MODULATION OF JUVENILE XII MNS; MULTIPLE EFFECTS.** The first indication that SP has multiple actions on XII MNs in both neonatal and juvenile rats is that the $SP_{NK1}$ current reverses at potentials hyperpolarized to $E_{K+}$. If only a K$^+$ channel was blocked, the current would reverse at $E_{K+}$. Activation of a Na$^+$ (or Ca$^{2+}$)-dependent current and closure of a K$^+$ channel would shift the reversal potential in the hyperpolarizing direction relative to $E_{K+}$. Many modulator-induced currents reverse at potentials hyperpolarized to $E_{K+}$. For example, in XII MNs of rat the extrapolated reversal potential for $SP_{NK1}$ currents is $-96 \pm 4$ and $-128 \pm 5$ mV at P14–P23 and P0–P5, respectively. In XII MNs of neonatal mouse, $SP_{NK1}$ currents reverse at $-114 \pm 2$ mV (Yasuda et al. 2001). Other modulators produce currents with similarly hyperpolarized reversal potentials. For example, in juvenile rats TRH currents reverse at $-101$ mV ($E_{K+}$ at $-91$ mV) (Bayliss et al. 1992), and NE currents reverse at $-104 \pm 10$ mV (Parkis et al. 1995). These data, plus the observations in XII MNs that NE and TRH (Parkis et al. 1995), $\sim 40%$ of the 5HT current (Talley et al. 2000), most of the TRH-induced depolarization (Bayliss et al. 1992) in juvenile SD rats and $\sim 60%$ of the $SP_{NK1}$ current in juvenile Wistar rats. The identity of this channel remains to be established. The fact that it mediates a significant current without affecting $R_N$ suggests that it may have a dendritic location and help regulate the flow of distal synaptic inputs to the soma. A primarily Na$^+$-dependent persistent inward current (PIC) is located in XII MN dendrites (Powers and Binder 2003), but this may not correspond to the $SP_{NK1}$-sensitive, Na$^+$-dependent, voltage-independent, TTX-insensitive conductance studied here because the PIC is TTX sensitive and voltage dependent (Powers and Binder 2003).

**PH-SENSITIVE CURRENT HAS PROPERTIES CONSISTENT WITH TASK CHANNELS.** As reviewed previously (Duprat et al. 1997; Talley and Bayliss 2002; Talley et al. 2000), the distribution pattern (Talley et al. 2001) and pharmacological and voltage-dependent properties of TASK1 channels indicate that they contribute to the pH-sensitive, $SP_{NK1}$-modulated K$^+$ conductance in XII MNs. TASK3 subunits may also contribute as they are strongly expressed in somatic MNs (Talley et al. 2001), and heteromeric TASK1/3 channels share many properties with TASK1 homomeric channels (Berg et al. 2004; Talley and Bayliss 2002).

Several other K$^+$ channels are also sensitive to extracellular pH. TRAAK channels show the open rectification characteristic of the pH-sensitive K$^+$ conductance in XII MNs but are pH-insensitive (Fink et al. 1998; Talley et al. 2000). TRED-1 channels are pH-sensitive in the correct range but outwardly rectifying (Patel et al. 1999). These two channels together could underlie the pH-mediated conductance, but TRED-1 immunoreactivity in the XII nucleus is low (Hervieu et al. 2001). In addition, we demonstrated that LPC, which evokes outward currents through specific activation of TREK-1 and TRAAK channels (Maingret et al. 2000), evokes only small inward currents in XII MNs. Other pH-sensitive K$^+$ channels including TASK2 (Talley et al. 2001), Kir 1.1 (Wu et al. 2004), Kir 2.2–2.4 (Karschin and Karschin 1997; Karschin et al. 1996; Topert et al. 1998; Wu et al. 2004), 4.1, and 5.1 (Xu et al. 2000; Yang et al. 2000) can also be excluded based on their low expression in XII, a low pH-sensitivity or a pH that does not match the pH-sensitive, $SP_{NK1}$-modulated conductance, or that their I/V relationship does not show the open rectification typical of this current in XII MNs (Talley et al. 2001). Thus the most likely
candidate for the pH-sensitive K⁺ current remains TASK1 or TASK1/3 heteromeric channels (Berg et al. 2004; Talley et al. 2000).

RELATIVE CONTRIBUTION OF THE SPₕNK₁-MODULATED CONDUCTANCES CHANGES DEVELOPMENTALLY. The NE- and TRH-mediated enhancement of XII MN excitability (Bayliss et al. 1992, 1994b) and potentiation of inspiratory activity (Funk et al. 1994) increase postnatally (Bayliss et al. 1994b) and are largely attributed to increased receptor expression (Bayliss et al. 1994b). In the case of SPₕNK₁, the overall current density remains constant but the relative contribution of the Na⁺-dependent component falls developmentally as indicated by the reduced sensitivity of the SPₕNK₁ current to Na⁺ substitution and the shift in the extrapolated reversal potential toward $E_{K⁺}$ from hyperpolarized potentials.

The large contribution of the Na⁺-dependent conductance to the SPₕNK₁ current is difficult to reconcile with SPₕNK₁-evoked increase in $R_N$. Nevertheless, these observations are consistent with previous analyses of modulatory actions in MNs. As indicated in the preceding text, the Ba²⁺-insensitive Na⁺-dependent component of the modulator-induced current mediates between 40% and almost 100% of the current (or depolarization) evoked in XII MNs by SPₕNK₁, NE, TRH, and 5HT but has minimal or no effect on $R_N$ (Bayliss et al. 1992; Parkis et al. 1995). $R_N$ is only affected if the Ba²⁺-sensitive K⁺ current is inhibited in which case $R_N$ increases. This may simply reflect the greater driving force for Na⁺ currents at rest compared with K⁺ currents; i.e., for a Na⁺ conductance and a K⁺ conductance to generate a similar current at resting potential (~70 mV), the K⁺ conductance must undergo a much greater change because the driving force for this current is minimal ($E_{K⁺} - E_{RMP} = -20$ mV) compared with that of a Na⁺ current ($E_{Na⁺} - E_{RMP} = -122$ mV). It may also reflect a predominantly dendritic location of the Na⁺ current where its influence on somal dendritic resistance is difficult to measure (Parkis et al. 1995).

In contrast to the Na⁺-dependent component, the contribution of the pH-sensitive K⁺ current to the total SPₕNK₁ current increased from minimal levels in the P0–P5 MNs to ~45% in P14-23 MNs. This increase reflects at least two factors. First, the pH-sensitive K⁺ current increased twofold between P0–P5 and P14–P23; this is consistent with postnatal increases in TASK1 protein in brain stem neurons (Kanjhan et al. 2004) and anecdotal reports that TASK1 mRNA in XII MNs is lower in P7 compared with adult rats (Talley et al. 2000).

Second, the ability of SPₕNK₁ to inhibit the pH-sensitive K⁺ conductance increases during postnatal development. In P0–P5 MNs, a pH-sensitive K⁺ current is present but not inhibited by SPₕNK₁. By P14–P23 SPₕNK₁ inhibits ~80% of this current. We propose that this reflects postnatal maturation of the transduction mechanisms through which SPₕNK₁ inhibits the pH-sensitive K⁺ current. We cannot exclude the possibility that the pH-sensitive K⁺ current in P0–P5 rat MNs is not mediated by TASK but by other SPₕNK₁-insensitive, pH-sensitive K⁺ channels, in which case, the increased efficacy of SP would simply reflect a developmental increase in the expression of TASK channels. However, the presence in neonatal XII MNs of TASK1 transcript and immunoreactivity, combined with the linear I/V relationship of the pH-sensitive K⁺ current in P0–P5 MNs suggest that TASK channels are present in neonatal XII MNs.

Additional confounding factors include possible developmental increases in NK1 receptor number or NK1 receptor affinity or redistribution of NK1 receptors or effector channels. An increase is NK1 receptor number is unlikely as our immunohistochemical and Western blot data in the XII nucleus, like the bulk of previous work, suggest that NK1 receptor expression in the brain stem and brain stem motor nuclei falls developmentally (Moss and Laferriere 2002; Nakamura et al. 2006; Quirion and Dam 1986; Rodier et al. 2001; Sakanaka et al. 1982; Tanaka-Gomi et al. 2007). Whether increases in the affinity of NK1 receptor for SP contribute is not known. Different agonist affinities of central and peripheral NK1 receptors that have identical sequences (Lepre et al. 1996) raises the possibility that posttranslational modifications produce NK1 receptors with properties that are specific to specific classes of neuron, tissue, or developmental stage. A developmental redistribution of NK1 receptors or TASK channels from distal to proximal dendrites and soma could cause us to underestimate the ability of SPₕNK₁ to inhibit the pH sensitive K⁺ channels in neonates. An unidentified K⁺ leak conductance does redistribute from distal processes to a uniform distribution over the first three postnatal weeks (Cameron et al. 2000). Redistribution, however, is unlikely to be the only factor because the actions of locally applied drugs are not limited to the soma, and both the transcript (Talley et al. 2000, 2001) and channel for TASK1 (Kanjhan et al. 2004) are abundant in the somata of neonatal XII MNs.

The greater quality of voltage/space clamp typically achieved in neonatal MNs due to their smaller size and higher $R_N$ (Nunez-Abades and Cameron 1995; Nunez-Abades et al. 1994) can also confound interpretation. However, in this case, space clamp errors would have caused a relatively greater underestimation of the SPₕNK₁ current in P14–P23 compared with P0–P5 MNs. We are therefore confident that the SPₕNK₁ current density does not decrease developmentally.

EFFECTS OF SPₕNK₁ ON REPETITIVE FIRING INCREASE POSTNATALLY. Potentiation of repetitive firing behavior by SPₕNK₁ increased postnatally despite constant SPₕNK₁ current density. Developmental modulation by SPₕNK₁ of currents underlying the mAHP is unlikely to have contributed. SPₕNK₁ inhibits the AHP in some central neurons (Gilbert et al. 1998) and the slow AHP in spinal MNs (Lepre et al. 1996) but has only modest effects on the AHP in phrenic MNs (Ptk et al. 2000; Rekling et al. 2000). In XII MNs, SPₕNK₁ did not affect the amplitude of the mAHP at any age, and small changes in mAHP kinetics were consistent between age groups.

Two factors may have contributed to the greater potentiation of firing in juveniles (Nunez-Abades and Cameron 1995; Nunez-Abades et al. 1994). First, SPₕNK₁ fostered generation of spike doublets at the onset of discharge in a majority of P14–P23 but not P0–P5 MNs, suggesting potentiation of the ADP. However, the ADPs in the two age groups were similar in magnitude and were not significantly affected by SPₕNK₁. Thus the underlying mechanism is unclear. Second, the pH-sensitive K⁺ channel is present at higher levels in P14–P23 MNs and is more strongly inhibited by SPₕNK₁ in P14–P23 than P0–P5 MNs. Its inhibition by SPₕNK₁ and the associated in-
crease in $R_N$ are likely to contribute to the greater influence of SP$_{NK1}$ on firing frequency in the juvenile.

**Consequences for MN excitability**

The functional significance of SP for XII MN behavior, as proposed for MNs in general, is that the SP$_{NK1}$-mediated membrane depolarization and increased $R_N$ will increase excitability such that for a given input, MN output and muscle force will be enhanced (Rekling et al. 2000). Greater potentiation of repetitive firing by SP$_{NK1}$ and in particular the generation of initial spike doublets in juveniles is also significant due to the importance of spike pattern in determining muscle force production (Binder-Macleod 1995; Milano et al. 1992). That these effects on XII MN excitability are physiologically relevant is supported by the presence of SP-containing terminals in the XII nucleus (Connaughton et al. 1986; Gatti et al. 1999; Hinrichsen and Weston 1999; Tallaksen-Greene et al. 1993) that make synaptic contacts with MN dendrites (Gatti et al. 1996; Richardson and Gatti 2004); NK1 receptor immunoreactivity (Fig. 1) (Nakaya et al. 1994) and SP binding sites in the XII nucleus (Helke et al. 1986; Manaker and Zucchi 1998; Rodier et al. 2001); the presence of SP in raphe obscurus neurons that project to (Henry and Manaker 1998) and excite the XII MNs (Ptak et al. 2009); and the inhibition of endogenous XII inspiratory activity by SP$_{NK1}$ receptor antagonists (Yasuda et al. 2001). In addition, while the N-methyl-d-aspartate receptor dependent potentiation of phrenic MN inspiratory output by SP (Ptak et al. 2009) is not observed in XII MNs (Yasuda et al. 2001), SP does potentiate XII inspiratory output (Yasuda et al. 2001). Our demonstration that SP$_{NK1}$ currents remain constant during the first postnatal weeks, in spite of reduced NK1 receptor expression, suggests that SP will be an important modulator of XII MN output throughout the postnatal period when intrinsic membrane excitability is decreasing (Berger et al. 1996). Of particular significance is our observation that the relative contributions of the two main currents underlying the total SP$_{NK1}$ current change postnatally.

Defining the precise role of SP in modulating XII MN excitability, however, is difficult because like most MNs, XII MNs participate in multiple behaviors (Bartlett et al. 1990; Miller 2002), and each behavior is generated by a distinct neuronal network that activates the somatodendritic tree with a specific spatiotemporal pattern of input. SP inputs to the XII MN will have their own spatiotemporal dynamic as these inputs derive from raphe neurons (Dobbins and Feldman 1995; Manaker and Tischler 1993; Manaker et al. 1992) the activity of which changes with behavior and state (Jacobs and Fornal 1993; Veasey et al. 1995, 1997). The influence of SP on any behavior will therefore depend on the spatiotemporal overlap between primary and modulatory inputs, which is likely to differ between behaviors. Little is known about the conditions that evoke the release of SP from raphe neurons. The demonstration in vitro that SP release is frequency-dependent (Franck et al. 1993) further suggests that it may only be important during periods when raphe neuron discharge is high, such as during locomotion, or elevated states of arousal. The possibility that SP differentially modulates specific types of inputs is supported indirectly by evidence that, while NE and SP$_{NK1}$ occlude each other actions and have virtually identical effects on XII MN responses to somally injected current pulses, NE is approximately threefold more effective at potentiating endogenous inspiratory drive (Yasuda et al. 2001). These observations, combined with relatively sparse innervation of other airway MNs by SP containing terminals (Sun et al. 2003), suggest that SP may be more important in modulating nonrespiratory behaviors of XII MNs.

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**DISCLOSURES**

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

**REFERENCES**


MODULATION OF XII MOTONEURON EXCITABILITY BY SUBSTANCE P


