Published in final edited form as:

Neuroscience. 2012 May 17; 210: 1–20. doi:10.1016/j.neuroscience.2012.03.018.

Postnatal development of Na⁺-K⁺-2Cl⁻ co-transporter 1 (NKCC1) and K⁺-Cl⁻ co-transporter 2 (KCC2) immunoreactivity in multiple brain stem respiratory nuclei of the rat

Qiuli Liu and Margaret T.T. Wong-Riley

Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin, 53226, USA

Abstract

Previously, we reported that in rats, GABA_A and glycine receptor immunoreactivity increased markedly in multiple brain stem respiratory nuclei around postnatal days (P) 12–13, a critical period when abrupt neurochemical, metabolic, ventilatory, and electrophysiological changes occur in the respiratory network and when the system is under greater inhibition than excitation. Since Na⁺-K⁺-2Cl⁻ co-transporter 1 (NKCC1) and K⁺-Cl⁻ co-transporter 2 (KCC2) play pivotal roles in determining the responses of GABA_A and glycine receptors, we hypothesized that NKCC1 and KCC2 undergo significant changes during the critical period. An in-depth immunohistochemical and single neuron optical densitometric study of neurons in seven respiratory-related nuclei (the pre-Bötzinger complex [PBC], nucleus ambiguus [Amb], hypoglossal nucleus [XII], ventrolateral subnucleus of solitary tract nucleus [NTS_{VL}], retrotrapezoid nucleus/parafacial respiratory group [RTN/pFRG], dorsal motor nucleus of the vagus nerve [DMNX], and inferior olivary nucleus [IO]) and a non-respiratory cuneate nucleus (CN, an internal control) was undertaken in P0–21 rats. Our data revealed that: (1) NKCC1 immunoreactivity exhibited a developmental decrease from P0 to P21 in all eight nuclei examined, being relatively high during the first 1½ postnatal weeks and decreased thereafter. The decrease was abrupt and statistically significant at P12 in the PBC, Amb, and XII; (2) KCC2 immunoreactivity in these eight nuclei showed a developmental increase from P0 to P21; and (3) the significant reduction in NKCC1 and the greater dominance of KCC2 around P12 in multiple respiratory nuclei of the brain stem may form the basis of an enhanced inhibition in the respiratory network during the critical period before the system stabilizes to a more mature state.

Keywords

critical period; dorsal motor nucleus of the vagus nerve; hypoglossal nucleus; nucleus ambiguus; pre-Bötzinger complex; ventrolateral subnucleus of solitary tract nucleus

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the vertebrate central nervous system and acts on GABA_A and GABA_B receptors to mediate fast and relatively slow responses, respectively (Krnjević, 2010). GABA inhibitory transmission

© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Corresponding Author: Margaret T.T. Wong-Riley, Ph.D., Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, Tel.: 414-955-8467, Fax: 414-955-6517, mwr@mcw.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

plays an essential role in respiratory rhythmogenesis and pattern generation (Bonham, 1995; Pierrefiche et al., 1998; Haji et al., 2000) as well as responses to hypoxia, hypercapnia, respiratory depressants, or excitants (Kazemi and Hoop, 1991; Paton and Richter, 1995; Burton and Kazemi, 2000; Haji et al., 2000). Likewise, glycine is an inhibitory neurotransmitter important in mediating fast synaptic inhibition in the brain stem respiratory system of both neonatal and adult animals (Schmid et al., 1991; Paton and Richter, 1995; Shao and Feldman, 1997; Büsselberg et al., 2001; Dutschmann et al., 2000; Dutschmann and Paton, 2002; Fong et al., 2009).

The GABA_A receptor (GABA_AR) is a heteropentameric, Cl⁻-selective ligand-gated ion channel, whose activation allows chloride to pass through the transmembrane channel along its gradient (Kaila, 1994; Moulton, 2009). The glycine receptor (GlyR) is a pentameric, cysteine-loop ligand-gated chloride channel that is also dependent on the chloride gradient (Jentsch, 1996; Webb and Lynch, 2007). In the mammalian CNS, the precise regulation of the chloride gradient and the intracellular chloride concentration [Cl⁻]_i is achieved by cation-chloride co-transporters, including the Cl⁻ inward-directed Na⁺-K⁺-2Cl⁻ co-transporter 1 (NKCC1) and the neuron-specific Cl⁻ outward-directed K⁺-Cl⁻ co-transporter 2 (KCC2) (Delpire, 2000; Payne et al., 1996, 2003; Gamba, 2005). These co-transporters play crucial roles in regulating the strength and polarity of GABAergic and glycinergic neurotransmission (Hebert et al., 2004; Price et al., 2009; Fuchs et al., 2010; Viemari et al., 2011). In general, NKCC1 predominates in the neonate and facilitates depolarizing GABAergic and glycinergic transmission, whereas KCC2 tends to dominate in the adult and promotes hyperpolarizing GABAergic and glycinergic transmission (Rivera et al., 1999; Ben-Ari, 2002; Löhrke et al., 2005; Blaesse et al., 2009; Stil et al., 2011; Viemari et al., 2011). However, virtually nothing is known about the development of NKCC1 and KCC2 expressions in brain stem respiratory nuclei.

Previously, we reported that a transient neurochemical imbalance exists around postnatal day (P) 12 in rats, with enhanced expression of inhibitory neurochemicals (GABA, GABA_B, and glycine receptors) and reduced expression of excitatory neurochemicals (glutamate and *N*-methyl-D-aspartate [NMDA] receptor subunit 1), concomitant with attenuated cytochrome oxidase level (a metabolic marker of neuronal activity, Wong-Riley, 1989) in multiple brain stem respiratory nuclei (Liu and Wong-Riley, 2002, 2003, 2005; Wong-Riley and Liu, 2005, 2008). We also revealed that GABA_A receptor subunits switch from the neonatal $\alpha 3$ to the adult $\alpha 1$ form around P12 (Liu and Wong-Riley, 2004, 2006). Moreover, abrupt changes in serotonergic and NMDA receptor subunits occur at this time (Liu and Wong-Riley, 2008, 2010a, 2010b, 2010c). Importantly, our detailed, daily electrophysiological study indicates a striking change in synaptic transmission in hypoglossal motoneurons at P12–13 (Gao et al., 2011). Specifically, this is the only time point from P0 to P16 that a significant fall in miniature and spontaneous excitatory postsynaptic currents (mEPSCs and sEPSCs) and a significant rise in miniature and spontaneous inhibitory postsynaptic currents (mIPSCs and sIPSCs) are found, indicating a transient imbalance between heightened inhibition and suppressed excitation. By the same token, around P13, the animal's ventilatory and metabolic responses to hypoxia are also at their weakest (Liu et al., 2006, 2009). Thus, the end of the second postnatal week is a critical period of respiratory network development in the rat (Wong-Riley and Liu, 2005, 2008).

The predominance of inhibitory neurotransmission during the critical period led us to hypothesize that the expressions of the Cl⁻-importer NKCC1 and the Cl⁻-exporter KCC2 undergo significant changes around the critical period. To test such a hypothesis, we conducted an in-depth, immunohistochemical and single neuron optical densitometric analysis of NKCC1 and KCC2 in seven respiratory-related nuclei and one non-respiratory nucleus in rats from P0 to P21. The respiratory nuclei included the pre-Bötzinger complex

(PBC), nucleus ambiguus (Amb), hypoglossal nucleus (XII), ventrolateral subnucleus of the solitary tract nucleus (NTS_{VL}), retrotrapezoid nucleus (RTN)/parafacial respiratory group (pFRG), dorsal motor nucleus of the vagus nerve (DMNX), and the inferior olivary nucleus (IO). The non-respiratory cuneate nucleus (CN) is a relay in the somatosensory system with no known respiratory function and served as an internal control.

EXPERIMENTAL PROCEDURES

Tissue preparation

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996), and all protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee (approval can be provided upon request). All efforts were made to minimize the number of animals used and their suffering.

A total of 162 Sprague-Dawley rats, both male and female, from 13 litters were used. Rat pups were sacrificed at each of postnatal days P0, 2, 3, 4, 5, 7, 10, 11, 12, 13, 14, 17, and 21. They were deeply anesthetized with 4% chloral hydrate (1 ml/100 g IP; Fisher Scientific, Fair Lawn, NJ) and perfused through the aorta with 4% paraformaldehyde-4% sucrose in 0.1 M sodium phosphate buffered saline (PBS), pH 7.4. Brain stems were then removed, postfixed in the same fixative for 3 h at 4°C, cryoprotected by immersion in increasing concentrations of sucrose (10, 20, and 30%) in 0.1 M PBS at 4°C, then frozen on dry ice and stored at -80°C until use.

Two adult male *Nkcc1*^{-/-} mice of 129 Black Swiss background (Flagella et al., 1999) and one adult male wild type of the same strain were kindly provided by Dr. Gary Shull (University of Cincinnati) for our NKCC1 antibody testing. They were perfused and their brain stems processed as described for rats.

Characterization of antibodies

Table 1 includes a brief summary of the antibodies used in the present study. Both antibodies (anti-NKCC1 and anti-KCC2) have been well characterized and their specificities established by the manufacturer and previous investigators. The anti-NKCC1 mouse monoclonal antibody (mAb T4, Developmental Studies Hybridoma Bank, Iowa City, IA) was a purified immunoglobulin raised against a peptide at the C-terminus (MET-902 to SER-1212) of human NKCC1. By western blot analysis, this antibody specifically yielded a single band between 145 and 205 KDa in 23 cell types (Lytle et al., 1995), and failed to show immune-signals in brain tissue of NKCC1 knockout mice (Chen et al., 2005). This mAb T4 has been used by a number of investigative groups (e.g., Alvarez-Leefmans et al., 2001; Gilbert et al., 2007; Mykoniatis et al., 2010). We have tested this antibody in brain stem sections of *Nkcc1*^{-/-} mice and found no specific labeling as compared to wild type controls (Fig. 1). The anti-neuron-specific KCC2 mouse monoclonal antibody (75-013, UC Davis/NIH NeuroMab Facility, Davis, CA) was a purified immunoglobulin raised against the intracellular C-terminus (amino acid 932–1043) of rat KCC2. Its specificity was confirmed by a single band at the expected molecular size (140 – 150 KDa) in western blots (Bragin et al., 2009; Horn et al., 2010; and the manufacture's datasheet). This antibody has also been used by several groups (e.g., Lee et al., 2007; Hedstrom et al., 2008; Bragin et al., 2009; Horn et al., 2010).

Immunohistochemistry

Coronal sections (12-μm thickness) of frozen brain stems were cut with a Leica CM1900 cryostat (Leica Microsystems, Heidelberg, Nussloch, Germany). Seven or nine sets of

serial sections were mounted on gelatin-coated slides. In the same litter, sections from 3 rats at different ages were mounted on the same slides and processed together. Ages were grouped typically as follows: P2-10-21, P0-3-4-17, P5-7-14, and P11-12-13. All sections from all rats were processed under identical conditions (i.e., time, temperature, and concentration of reagents). They were blocked overnight at 4°C with 5% nonfat dry milk-5% normal goat serum-1% Triton X-100 in 0.1 M PBS (pH 7.4). Sections were then incubated at 4°C for 36 h in the primary antibodies diluted at the proper concentration in the same solution as used for blocking: 1:14,000 for mouse anti-NKCC1 or 1:500 for mouse anti-KCC2. Sections were rinsed 3 times, 5 min each, in PBS, then incubated in the secondary antibodies: 1:100 goat anti-mouse IgG-HRP (Bio-Rad, Hercules, CA), diluted in the modified blocking solution (without Triton X-100) for 4 h at room temperature. After rinsing twice with PBS and once with 0.1 M ammonium phosphate buffer (APB), pH 7.0, immunoreactivity was detected with 0.05% DAB-0.004% H₂O₂ in APB for 5 min. The reaction was then stopped with APB for 5 min, rinsed in PBS three times, dehydrated, and coverslipped. Control sections were processed without primary antibodies or with a non-immune serum in place of the primary antibodies.

One set of alternate sections was reacted for neurokinin-1 receptors, using protocols described previously (Liu and Wong-Riley, 2002).

For brain sections of *Nkcc1*^{-/-} and wild type mice, they were blocked overnight as above, then incubated in unconjugated affinity-purified Fab fragment of goat-anti-mouse IgG (H +L) (Jackson ImmunoResearch Labs, West Grove, PA) for 1 h at room temperature and rinsed three times in PBS before incubating in the primary antibodies and further processed as described above. This extra step reduces background labeling when mouse monoclonal antibodies are used against mouse tissues.

Semi-quantitative optical densitometry

The immunoreactivity of NKCC1 and KCC2 in the cell bodies of individual neurons in various nuclei was semi-quantitatively analyzed by optical densitometry performed with a Zeiss Zonax MPM 03 photometer, a ×25 objective, and a 2-μm-diameter measuring spot. White (tungsten) light was used for illumination, and all lighting conditions were held constant for all of the measurements. Since light intensity can directly affect optical densitometric values, a stepped density filter (Edmund Industrial Optics, Barrington, NJ) with 10-step increments of 0.1 (from 0.1 to 1) was used to precisely adjust the intensity of the light source to a standard value identical for all samples.

The boundary of each brain stem nucleus studied was determined with the aid of the Paxinos and Watson's "The Rat Brain Atlas" (Academic Press, New York, 1986). The PBC was identifiable with the neurokinin-1 receptor labeling (Gray et al., 1999), as described in our previous papers (Liu and Wong-Riley, 2002, 2003, 2005). The part of the nucleus ambiguus chosen for the present study (and our previous studies, Liu and Wong-Riley, 2003, 2005, 2008) was the semicompact formation and the rostral loose formation innervating upper airway muscles with pharyngolaryngomotor functions (Bieger and Hopkins, 1987). The RTN was originally described by Smith et al. (1989). The pFRG was identified in the neonate with electrophysiological approach and is located at the rostral ventrolateral medulla, ventrolateral to the facial nucleus and close to the ventral surface (Onimaru and Homma, 2003), apparently overlapping with the RTN (Feldman and Del Negro, 2006). Since it is not known if the RTN and pFRG are anatomically and functionally distinct (Feldman and Del Negro, 2006), we opted to refer to this region as 'RTN/pFRG'. For the remaining nuclei, measurements were taken from the central main portion of each nucleus.

The optical densitometric value of each labeled neuron in the various brain stem nuclei studied was an average reading of two to four spots on the cell body (excluding the nucleus). Only those neurons whose nuclei were clearly visible (i.e., sectioned through the middle of the cell body) were measured. To avoid measuring the same neuron more than once, values were taken from cells in sections at least 84 μm or 108 μm apart, as the largest neurons had a maximal diameter of 25–30 μm , with a maximal nuclear diameter of only about 10 μm . About 100 neurons in each brain stem nucleus were measured for each marker in each rat, and a total of about 700 (for NKCC1) or 1200 (for KCC2) at each age for each marker were measured. For statistical analyses, each sample's optical density value for each nucleus of each rat was the average of about 100 labeled neurons. Thus, the sample number of each time point in each nucleus for each marker was seven in Figures 6 and twelve in Figure 11. A total of 192,000 neurons were measured for the present study. Mean optical density values, standard deviations, and standard errors of the mean in each nucleus at each age were then obtained. Statistical comparisons were made among the age groups by using one-way analysis of variance (ANOVA) (to control for the type I comparison wise error rate) and, when significant differences were found, comparisons were made between successive age groups (e.g., P2 vs. P3, P3 vs. P4, and P5 vs. P7) by using Tukey's Studentized range test (*a post hoc* multiple comparisons, to control for the type I experiment wise error rate). Additional Tukey's tests were conducted between two groups that were not immediately adjacent to each other, and significant differences, if any, were presented in the Results section (but not shown in the graphs to minimize confusion). Significance was set at $P < 0.01$ for one-way ANOVA and $P < 0.05$ for Tukey's test.

RESULTS

NKCC1-immunoreactive (-ir) neurons in the brain stem nuclei

NKCC1 immunoreactivity (-ir) was clearly visible in subpopulations of neurons in all brain stem nuclei examined (Figs. 2–5). Immunoreaction product was present in cell bodies and proximal dendrites of labeled neurons as well as in the neuropil (mainly in dendritic processes). Before P11, the plasma membrane of labeled neurons showed clear immunoreaction product (see insets in Figs. 2–5). However, such labeling became lighter after P11, especially in the PBC, Amb and XII. The sizes of NKCC1-ir neurons increased with age and reached a relatively stable level by P11–P12 (Figs. 2–5). Some glial cells, much smaller in size than neurons, exhibited NKCC1-ir around P13 and later, but glial cells were not included in our optical densitometric analyses. Control sections had no specific immunoreactivity above background (data not shown). ANOVA indicated significant differences ($P < 0.01$) in NKCC1-ir among the ages in all eight nuclei examined. Tukey's Studentized range test that compared one age group with its adjacent younger age group revealed significant reductions at P12 for PBC, Amb, and XII ($P < 0.05$, as compared to the values at P11) (Fig. 6A–C).

NKCC1-ir neurons in the PBC—NKCC1-ir was observed in ~ 35% – 50% of the PBC neurons. They were multipolar, granular, or fusiform in shape and small and medium in size (Fig. 2A1–4). The size of small neurons ranged from 5.5 to 7.5 μm in diameter at P2 to 8 – 11 μm at P21, and medium-sized neurons ranged from 11 – 13.5 μm at P2 to 14 – 20.5 μm at P21. The expression of NKCC1 was relatively high from P2 to P11, and then decreased significantly at P12 ($P < 0.05$), followed by a plateau until P21 (Fig. 6A). P12 was the only time point in the first 3 postnatal weeks when a day-to-day significance was found. However, in comparing non-adjacent time points, Tukey's test revealed that the expression of NKCC1 in each of P14 to P21 rats was significantly lower than each of P0 – P11 rats ($P < 0.05$ – $P < 0.001$) (not shown in Fig. 6A).

NKCC1-ir neurons in the Amb—About 50% – 85% of Amb neurons demonstrated NKCC1-ir. These neurons were multipolar or oval in shape and mainly medium or small in size (Fig. 2B1–4). The size of small neurons ranged from 6 to 8.5 μm in diameter at P2 to 8.5 – 12 μm at P21, and medium-sized neurons ranged from 11.5 – 14 μm at P2 to 16 – 21 μm at P21. Occasionally, a few of large neurons (23 – 28 μm in diameter) can be observed at P21. NKCC1-ir was at relatively high levels from P0 to P11, but was significantly reduced at P12 ($P < 0.05$), followed by a plateau until P21 (Fig. 6B). The lowest value was at P21.

NKCC1-ir neurons in the XII—NKCC1-ir was present in ~ 40% – 65% of XII neurons. They were multipolar, oval, or fusiform in shape and mainly medium or large in size (Fig. 3A1–4). Medium-sized neurons ranged from 12 to 16 μm in diameter at P2 to 14.5 – 20.5 μm at P21, and large neurons ranged from 17 – 20.5 μm at P2 to 22 – 28 μm at P21. The developmental trend of NKCC1-ir was comparable to those of the PBC and Amb, with relatively high levels from P0 to P11 and a significant fall at P12 ($P < 0.05$), followed by a plateau until P21 (Fig. 6C).

NKCC1-ir neurons in the NTS_{VL}—NKCC1-ir was present in about 35% – 55% of the NTS_{VL} neurons. They were multipolar, granular, or oval in shape and mainly small in size (Fig. 3B1–4). The small neurons ranged from 4.5 – 7.5 μm in diameter at P2 to 5 – 13 μm at P21. The expression of NKCC1 was relatively high from P0 to P11, followed by a gradual decline until P21 (Fig. 6D). Whereas comparisons between any two adjacent age groups revealed no statistical significance, Tukey's test did show that the values at P17 or P21 were significantly lower than those at P0 ($P < 0.05$ for both). Likewise, values at P11 were significantly higher than those at P17 ($P < 0.05$) or at P21 ($P < 0.01$).

NKCC1-ir neurons in the RTN/pFRG—About 25% – 45% of neurons in the RTN/pFRG exhibited NKCC1-ir. They were small or medium in size and multipolar, fusiform, or granular in shape (Fig. 4A1–4). Small neurons ranged from 4.5 to 7 μm in diameter at P2 to 5.5 – 10 μm at P21, and the medium-sized neurons ranged from 10 to 12.5 μm in diameter at P2 to 12 – 15 μm at P21. The expression of NKCC1 was relatively high from P0 to P10, then declined gradually thereafter until P21 (Fig. 6E). No significant differences were found between any two adjacent age groups, but Tukey's test did find that the value at P21 was significantly lower than those at P2, P3, P4, P7, or P10 ($P < 0.05$ for all), that P12 and P14 were lower than P10 ($P < 0.05$), and that P14 or P17 was lower than P7 ($P < 0.05$ for both).

NKCC1-ir neurons in the DMNX—Approximately 35% – 60% of DMNX neurons were NKCC1-ir, and they were small or medium in size and multipolar, fusiform, or oval in shape (Fig. 4B1–4). Small neurons ranged from 5 to 6.5 μm in diameter at P2 to 6 – 8.5 μm at P21, and medium-sized neurons ranged from 8 to 10.5 μm in diameter at P2 to 9 – 13 μm at P21. The expression of NKCC1-ir was relatively stable from P0 to P13, with some small, statistically insignificant fluctuations, then declined gradually until P21 (Fig. 6F). Although no significant difference was revealed between any two adjacent age groups, Tukey's test did find that the value at P17 was lower than that at P7 ($P < 0.05$), and that the value at P21 was significantly lower than those at P7 ($P < 0.01$), P4, P5, and P10 ($P < 0.05$ for all).

NKCC1-ir neurons in the IO—About 50% – 70% of the IO neurons demonstrated NKCC1-ir. They were small or medium in size and oval, multipolar, or fusiform in shape (Fig. 5A1–4). Small neurons ranged from 5 – 7 μm in diameter at P2 to 6 – 9 μm at P21, and medium-sized neurons ranged from 8 – 10 μm in diameter at P2 to 10 – 14 μm at P21. The level of NKCC1 was relatively high from P0 to P13, then gradually decreased until P21 (Fig. 6G). No significant differences were found between any two adjacent age groups, but Tukey's test did reveal that the values at P17 or P21 were significantly lower than those of

individual day between P0 and P13 ($P < 0.05$ for comparisons with P17 and $P < 0.01$ for comparisons with P21).

NKCC1-ir neurons in the CN—About 25% – 40% of neurons in the CN demonstrated NKCC1-ir (Fig. 5B1–4). These labeled neurons were oval, multipolar, or granular in shape and mainly small in size. Small neurons ranged from 4 – 7.5 μm in diameter at P2 to 6 – 11 μm at P21, and medium-sized labeled neurons ranged from 8.5 – 11 μm at P2 to 12.5 – 15.5 μm at P21. The expression of NKCC1 followed a more gradual decrease with age (Fig. 6H). There were no significant differences between any two adjacent age groups, but Tukey's test did show that the values at P17 and P21 were significantly lower than those at P10 and P12 ($P < 0.05$). The value at P17 was also significantly lower than those at P2 and P7 ($P < 0.05$).

KCC2-immunoreactive (-ir) neurons in the brain stem nuclei

In general, KCC2-ir product was clearly visible in subpopulations of neurons in each of the brain stem nuclei examined (Figs. 7–10). Labeling was present in cell bodies and proximal dendrites of neurons as well as in the neuropil (mainly in dendritic processes). The plasma membrane of many labeled neurons showed clear immunoreaction product (see insets in Figs. 7–10). The sizes, developmental changes, shapes, and prevalence of KCC2-ir neurons were comparable to those of NKCC1-ir neurons, and will not be described in detail below. Control sections demonstrated no specific immunoreactive product above background (data not shown). ANOVA indicated significant differences ($P < 0.01$) in KCC2-ir among the ages in all eight nuclei examined.

KCC2-ir neurons in the PBC—KCC2-ir was visible in ~ 30% – 50% of neurons in the PBC (Fig. 7A1–4). The expression was the lowest at P0, increased gradually from P2 to P12, followed by a plateau until P21 (Fig. 11A). Although no significance was found between any two adjacent age groups, Tukey's test did yield significance between P0 and individual days from P4 to P21 ($P < 0.001$), between P2 and P7 – P21, between P3 and P7 – P13 ($P < 0.01$ – $P < 0.001$), and between P4 and P10 – P13 ($P < 0.05$ – $P < 0.01$).

KCC2-ir neurons in the Amb—Approximately 55% – 75% of neurons in the Amb exhibited KCC2-ir (Fig. 7B1–4). The expression was comparable to that in the PBC, with the lowest value at P0, increased gradually and significantly until P12, followed by a plateau until P21 (Fig. 11B). Tukey's test did not find significant differences between any two adjacent age groups, but it did reveal significance between P0 or P2 and individual days from P4 to P21 ($P < 0.01$ – $P < 0.001$), and between P3 and days between P7 and P14 ($P < 0.05$ – $P < 0.01$).

KCC2-ir neurons in the XII—KCC2-ir was observed in ~ 40% – 65% of neurons in the XII (Fig. 8A1–4). The expression was similar to those in the PBC and Amb, with a gradual rise from P0 (the lowest value) to P12, followed by a plateau thereafter until P21 (Fig. 11C). Although no significant differences were found between any two adjacent age groups, Tukey's test did yield significance between P0 and individual days from P3 to P21 ($P < 0.05$ – $P < 0.001$), as well as between P2 and P10, P12, or P13 ($P < 0.05$ for all).

KCC2-ir neurons in the NTS_{VL}—KCC2-ir was detected in about 30% – 50% of neurons in the NTS_{VL} (Fig. 8B1–4). The expression was the lowest at P0, increased gradually until P7, followed by a relative plateau until P21 (Fig. 11D). No significant differences were found between any two adjacent age groups, but Tukey's test did yield significance between P0 and individual days from P4 to P21 ($P < 0.05$ – $P < 0.001$), between P2 and individual days from P5 to P17 ($P < 0.01$ – $P < 0.001$), as well as between P3 and P13 or P17 ($P < 0.05$ for both).

KCC2-ir neurons in the RTN/pFRG—Approximately 35% – 55% of neurons in the RTN/pFRG exhibited KCC2-ir (Fig. 9A1–4). The developmental trend of KCC2-ir was comparable to that in the NTS_{VL}, with the lowest value at P0, followed by a gradual and significant rise from P2 to P7, then a plateau until P21 (Fig. 11E). Though no significant differences were found between any two adjacent age groups, Tukey's test did indicate significance between P0 and individual days from P3 to P21 ($P < 0.05$ – $P < 0.001$), and between P2 and P7 – P17 ($P < 0.05$ – $P < 0.01$).

KCC2-ir neurons in the DMNX—About 40% – 55% of neurons in the DMNX exhibited KCC2-ir (Fig. 9B1–4). The expression exhibited a developmental increase from P0 to P10, then plateaued until P21 (Fig. 11F). Tukey's test did not yield a day-to-day significance, but it showed that the value at P10 was significantly higher than those at P0 or P2 ($P < 0.001$ for both). Likewise, the value at P12 was significantly higher than those at P0 ($P < 0.05$) or at P2 ($P < 0.01$).

KCC2-ir neurons in the IO—About 45% – 65% of neurons in the IO were KCC2-ir (Fig. 10A1–4). The expression rose steadily during the first postnatal week, then plateaued until P21 (Fig. 11G). Tukey's test revealed that the values at P5 or P7 are significantly higher than that at P0.

KCC2-ir neurons in the CN—About 30% – 45% of neurons in the CN demonstrated KCC2-ir (Fig. 10B1–4). The immunoreactivity was relatively low at P0 and P2, then rose to a peak at P10, followed by a plateau until P21 (Fig. 11H). Tukey's test showed that the values at P7, P10, and P11 were significantly higher than those at P0 ($P < 0.05$ – $P < 0.01$), and the value at P10 was higher than that at P2 ($P < 0.05$).

To compare the developmental trends between NKCC1 and KCC2, the optical densitometric values were normalized such that the highest expression of each marker for each nucleus over the first 3 postnatal weeks was set at 1. Figure 12 illustrates the developmental decrease in the expression of NKCC1 against the developmental increase in the expression of KCC2 in all eight nuclei examined (Fig. 12A–H). The two trends intersected at or close to P11 in the PBC, Amb, XII, NTS_{VL}, and RTN/pFRG (Fig. 12A–E, respectively). The exact point of intersection was not as distinct in the other 3 nuclei. However, the expression of NKCC1 remained low whereas that of KCC2 remained high after P12 in all nuclei examined.

DISCUSSION

The present large-scale, in-depth, developmental study revealed for the first time that: (1) NKCC1 immunoreactivity exhibited a relatively high level during the first 1 ½ postnatal weeks, then fell precipitously and significantly at P12 in the PBC, Amb, and XII, but more gradually in the NTS_{VL}, RTN/pFRG, DMNX, IO and CN, followed by a plateau or further decline until P21, the oldest time point examined; (2) KCC2 immunoreactivity displayed a developmental increase, peaking at P12 in the PBC, Amb, and XII, or during the 2nd postnatal week in all of the respiratory nuclei examined, followed by a plateau at a relatively high level until P21; and (3) the two developmental trends intersected at or close to P11, and starting at P12, the expression of NKCC1 clearly fell whereas that of KCC2 remained high in multiple brain stem respiratory nuclei.

Technical limitations

The validity of immunohistochemistry is dependent mainly on the specificity of the antibody and is a reflection of relative protein amount. The antibodies used in the current study were monoclonal ones and were well documented for their specificity (see Experimental

Procedures and Fig. 1). With optical densitometric measurements of immunoreactive product in single neurons of well-identified brainstem nuclei, the present study was able to extend the analysis to a semi-quantitative level. This has the distinct advantage of localizing relative protein expressions in individual neurons rather than in mixed populations of neurons, glia, and endothelial cells as in immunoblots. The large number of animals used, the close time points of postnatal ages examined, and the 192,000 neurons measured all enhanced our confidence in the statistical significance of the present findings.

NKCC1 and KCC2 functions and developmental regulations

The postsynaptic actions of GABA and glycine are dependent on the transmembrane chloride gradient, which is regulated by NKCC1 and KCC2 (Hebert et al., 2004; Blaesse et al., 2009; Fuchs, et al., 2010; Viemari et al., 2011) via electroneutral transport mechanisms (Price et al., 2009). NKCC1 transports Cl^- together with Na^+ into the cell by utilizing the Na^+ gradient maintained by the Na^+/K^+ -ATPase, whereas KCC2 extrudes Cl^- by capitalizing on the K^+ gradient maintained also by the Na^+/K^+ -ATPase (Balena and Woodin, 2008; Blaesse et al., 2009; Price et al., 2009). A high level of $[\text{Cl}^-]_i$ sustained by NKCC1 renders GABA_AR- and GlyR-activated transmission depolarizing and excitatory, whereas a low level of $[\text{Cl}^-]_i$ maintained by KCC2 accounts for the hyperpolarizing and inhibitory effects of GABA_AR- and GlyR- activated transmission (Hübner et al., 2001; Ben-Ari, 2002; Yamada et al., 2004; Rivera et al., 2006; Fiumelli and Woodin, 2007; Blaesse et al., 2009; Viemari et al., 2011).

NKCC1 and KCC2 expressions are developmentally regulated (Rivera et al., 1999; Ben-Ari, 2002; Mikawa et al., 2002). In the embryonic and neonatal states, NKCC1 is the dominant cation-chloride co-transporter (Ben-Ari, 2002; Milawa et al., 2002; Yamada et al., 2004; Fiumelli and Woodin, 2007). This results in high $[\text{Cl}^-]_i$, depolarizing GABA_AR- and GlyR-mediated transmission, and the activation of voltage-dependent calcium channels that results in a serial cascade of calcium-sensitive signaling processes (Yuste and Katz, 1991; Leinekugel et al., 1995; Ganguly et al., 2001). Thus, during prenatal and early postnatal development, GABA acts as an excitatory neurotransmitter and a trophic factor involved in controlling morphogenesis and neuronal development (Ben-Ari, 2002; McCarthy et al., 2002; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005; Fiumelli and Woodin, 2007; Kahle et al., 2008; Blaesse et al., 2009). On the other hand, KCC2 expression is relatively low during embryonic and neonatal periods, but increases postnatally to eventually overcome the influence of NKCC1, thereby establishing the classic hyperpolarizing, inhibitory GABA_AR- and GlyR- mediated transmission characteristic of mature neurons (Ehrlich et al., 1999; Rivera et al., 1999; 2006; Ben-Ari, 2002; Galanopoulou, 2007; Viemari et al., 2011).

The switch in polarity of GABA_A- and glycine receptor-activated transmission from depolarizing to hyperpolarizing occurs at different time points among various regions reported thus far. In general, a chronological sequence along the caudorostral axis was proposed for the switch (Löhrke et al., 2005). It starts in the spinal cord prenatally (Delpy et al., 2008) or perinatally (Wu et al., 1992; Hübner et al., 2001), after P3 in the brainstem (Singer et al., 1998; Balakrishnan et al., 2003), then progresses to the cerebellum (Brickley et al., 1996), hippocampus (Ben-Ari et al., 1989; Rivera et al., 1999; 2005; Khazipov et al., 2004; Banke and McBain, 2006), and the cerebral cortex (Owens et al., 1996). However, the switch does not follow a strict chronological order even within a local area. For example, different subnuclei of the superior olivary complex exhibit switches that vary over almost two weeks (Löhrke et al., 2005). In the brain stem, the transition from excitation to inhibition for chloride-mediated effects on respiratory rhythmogenesis reportedly occurs around embryonic day 19 in the rat (Ren and Greer, 2006), and the GABA_A receptor-mediated reversal of current from depolarizing to hyperpolarizing occurs within the first

postnatal week in the mouse (Ritter and Zhang, 2000). However, in the hypoglossal nucleus of the rat, the reduction in $[Cl^-]_i$ from a high 33 mM to 8 mM and the maturation of the glycinergic transmission reportedly occurs during the first two postnatal weeks (Singer and Berger, 2000).

To date, less is known about the development of NKCC1 and KCC2 expressions in the brain stem. The present study provides a comprehensive analysis of this development in seven respiratory nuclei and one non-respiratory nucleus during the first three postnatal weeks in rats. Our data indicate that the expression of NKCC1 decreased with age but that of KCC2 increased with age in all eight nuclei examined. These findings are comparable to those reported in other regions of the brain, although only a few time points were examined in those studies (Mikawa et al., 2002; Hebert et al., 2004; Takayama and Inoue, 2006; Blaesse et al., 2009; Stil et al., 2009). We further revealed that at P12, a significant decrease in NKCC1 expression occurred against a maintained high level of KCC2 in the PBC, Amb, and XII. After P12, the expression of NKCC1 was clearly reduced and that of KCC2 was sustained at a high level in all eight nuclei examined. This strongly indicates that P12 is the definitive starting point in these nuclei when the action of KCC2 dominates over that of NKCC1, and when the Cl^- gradient is likely to support a stronger hyperpolarizing response for both GABA_A- and glycine receptors.

Functional relevance for the respiratory system

Of the respiratory nuclei investigated in the present study, the PBC is a presumed center of respiratory rhythmogenesis (Smith et al., 1991; 2000; Ramirez and Richter, 1996; Rekling and Feldman, 1998). The Amb, DMNX, and XII are involved in controlling upper airway muscles, the vagus nerve (cardio-respiratory interaction), and the tongue, respectively, during respiration (Taylor, 1994; St-John, 1998; Jordan, 2001). The IO may be involved in respiratory control via the cerebellum, which may play a role in coordinating the activities of ventilatory skeletal muscles with those of upper airway muscles (Harper et al., 2000; Kinney et al., 2001). The NTS_{VL} receives peripheral chemosensitive afferents (Finley and Katz, 1992) and is involved in respiratory regulation (Bonham, 1995). The RTN/pFRG is reportedly one of the major central chemoreceptive sites (Guyenet et al., 2005) as well as an additional site for respiratory rhythmogenesis (Onimaru and Homma, 2003).

Previously, we reported that a transient imbalance exists between enhanced expression of inhibitory neurochemicals (GABA, GABA_B, and glycine receptors) and attenuated expression of excitatory neurochemicals (glutamate and NMDA receptor subunits 1 and 2A), concomitant with reduced cytochrome oxidase activity in the PBC, Amb, XII, NTS_{VL}, DMNX, and IO at P12 in the rat (Liu and Wong-Riley, 2002; 2003; 2005; Wong-Riley and Liu, 2005; 2008). The ventilatory and metabolic responses to acute hypoxia are at their weakest at P13 (Liu et al., 2006; 2009). Recently, we discovered that the amplitude and charge transfer of miniature excitatory postsynaptic currents (mEPSCs) in XII motoneurons were significantly reduced at P12–13, whereas the amplitude, mean frequency, and charge transfer of miniature inhibitory postsynaptic currents (mIPSCs) were significantly increased at P12–13. Moreover, the amplitude and frequency of spontaneous EPSCs were significantly reduced at P12–13, but those of spontaneous IPSCs were significantly increased at P12–13 (Gao et al., 2011). These electrophysiological data match precisely in time those of our neurochemical findings of suppressed excitation and heightened inhibition at P12–13, the critical period of respiratory development in the rat. A marked reduction in NKCC1 expression against a high level of KCC2 at P12 in most of the respiratory-related nuclei examined in the present study should contribute significantly to the transient dominance of inhibition during the critical period (Wong-Riley and Liu, 2005; Liu et al., 2006; Gao et al., 2011). This is achieved by shifting the chloride reversal potential more negatively, thus enhancing the strength of inhibitory GABA_A- and glycine-mediated transmission (Balena

and Woodin, 2008; Kahle et al., 2008; Blaesse et al., 2009; Bray and Mynlieff, 2009) in these nuclei.

In addition to heightened inhibition and suppressed excitation during the critical period, we also found a switch in GABA_A receptor subunits from $\alpha 3$ to $\alpha 1$, a significant reduction in the expression of 5-HT1A, 1B, and 2A receptors, as well as of the 5-HT synthesizing enzyme tryptophan hydroxylase and serotonin transporter in multiple brain stem respiratory nuclei, all occurring at P12 (Liu and Wong-Riley, 2004; 2006; 2008; 2010a; 2010b). Additionally, NMDA receptors' subunit composition also underwent significant changes around the critical period (Liu and Wong-Riley, 2010c). These composite findings all point to the end of the 2nd postnatal week (P12–P13) as the critical period of postnatal respiratory development in the rat, when abrupt, unexpected changes occur that can tip the scale toward greater inhibition than excitation. Even small changes in GABA-mediated inhibition can profoundly alter neuronal excitation (Mody et al., 1994). Suppressed neuronal activity in the respiratory system can lead to attenuated upper airway patency and inadequate ventilatory response to exogenous insults, such as a weakened hypoxic response observed previously (Liu et al., 2006; 2009). After the critical period, the maturation of excitatory and inhibitory synapses will maintain the system at a more stabilized state.

Although the present findings suggest a switch in dominance from NKCC1 to KCC2 during the critical period, they do not address directly whether GABA_A and glycine receptors undergo a transition from depolarization to hyperpolarization at this time. If the functional switch should occur prenatally, as suggested by a number of previous reports (Dutschmann et al., 2000; Dutschmann and Paton, 2002), and indeed, synaptic inhibition is essential for the generation of cyclic motor activity, the postnatal trends of NKCC1 and KCC2 expressions can still indicate a further developmental drive toward greater negativity for the equilibrium potentials of GABA_A and/or glycine receptors. Future day-to-day developmental studies of these two receptors at the electrophysiological level should help clarify this point.

Research Highlights for Liu and Wong-Riley submitted to Neuroscience

- A critical period of postnatal respiratory development exists in rats around P12–13.
- NKCC1 expression decreases with age from P0 to P21, with marked reduction at P12.
- KCC2 expression in 7 brain stem respiratory nuclei increases with age from P0 to P21.
- A distinct reversal of dominance from NKCC1 to KCC2 in the nuclei starts at P12.
- The switch in NKCC1/KCC2 dominance potentiates inhibition during the critical period.

Acknowledgments

This study was supported by NIH grant HD048954. We thank Dr. Gary Shull for providing us with the Nkcc1 knockout and wild type mice and Dr. Vikram Prasad for facilitating this process.

Abbreviations

XII	hypoglossal nucleus
Amb	nucleus ambiguus
ANOVA	analysis of variance
APB	ammonium phosphate buffer
[Cl⁻]_i	intracellular chloride concentration
CN	cuneate nucleus
DMNX	dorsal motor nucleus of the vagus nerve
GABA	gamma-aminobutyric acid
IO	inferior olivary nucleus
-ir	immunoreactive
KCC2	K ⁺ -Cl ⁻ co-transporter 2
NKCC1	Na ⁺ -K ⁺ -2Cl ⁻ co-transporter 1
NMDA	<i>N</i> -methyl-D-aspartate
NTS_{VL}	ventrolateral subnucleus of the solitary tract nucleus
P	postnatal day
PBC	pre-Bötzinger complex
PBS	sodium phosphate buffered saline
R	receptor
RTN/pFRG	retrotrapezoid nucleus/parafacial respiratory group

REFERENCES

- Alvarez-Leefmans FJ, León-Olea M, Mendoza-Sotelo J, Alvarez FJ, Antón B, Garduño R. Immunolocalization of the Na(+)-K(+)-2Cl(-) cotransporter in peripheral nervous tissue of vertebrates. *Neurosci.* 2001; 104:569–582.
- Balakrishnan V, Becker M, Löhrke S, Nothwang HG, Güresir E, Friauf E. Expression and function of chloride transporters during development of inhibitory neurotransmission in the auditory brainstem. *J Neurosci.* 2003; 23:4134–4145. [PubMed: 12764101]
- Balena T, Woodin MA. Coincident pre- and postsynaptic activity downregulates NKCC1 to hyperpolarize E(Cl) during development. *Eur J Neurosci.* 2008; 27:2402–2412. [PubMed: 18430034]
- Banke TG, McBain CJ. GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development. *J Neurosci.* 2006; 26:11720–11725. [PubMed: 17093093]
- Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci.* 2002; 3:728–739. [PubMed: 12209121]
- Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol.* 1989; 416:303–325. [PubMed: 2575165]
- Bieger D, Hopkins DA. Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: the nucleus ambiguus. *J Comp Neurol.* 1987; 262:546–562. [PubMed: 3667964]
- Blaesse P, Airaksinen MS, Rivera C, Kaila K. Cation-chloride cotransporters and neuronal function. *Neuron.* 2009; 61:820–838. [PubMed: 19323993]

- Bonham AC. Neurotransmitters in the CNS control of breathing. *Respir Physiol.* 1995; 101:219–230. [PubMed: 8606995]
- Bragin DE, Sanderson JL, Peterson S, Connor JA, Müller WS. Development of epileptiform excitability in the deep entorhinal cortex after status epilepticus. *Eur J Neurosci.* 2009; 30:611–624. [PubMed: 19674083]
- Bray JG, Mynlieff M. Influx of calcium through L-type calcium channels in early postnatal regulation of chloride transporters in the rat hippocampus. *Dev Neurobiol.* 2009; 69:885–896. [PubMed: 19777558]
- Brickley SG, Cull-Candy SG, Farrant M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. *J Physiol.* 1996; 497:753–759. [PubMed: 9003560]
- Burton MD, Kazemi H. Neurotransmitters in central respiratory control. *Respir Physiol.* 2000; 122:111–121. [PubMed: 10967338]
- Büsselberg D, Bischoff AM, Paton JF, Richter DW. Reorganisation of respiratory network activity after loss of glycinergic inhibition. *Pflugers Arch.* 2001; 441:444–449. [PubMed: 11212206]
- Chen H, Luo J, Kintner DB, Shull GE, Sun D. Na(+)-dependent chloride transporter (NKCC1)-null mice exhibit less gray and white matter damage after focal cerebral ischemia. *J Cereb Blood Flow Metab.* 2005; 25:54–66. [PubMed: 15678112]
- Delpire E. Cation–chloride cotransporters in neuronal communication. *News Physiol Sci.* 2000; 15:309–312. [PubMed: 11390932]
- Delpy A, Allain AE, Meyrand P, Branchereau P. NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. *J Physiol.* 2008; 586:1059–1075. [PubMed: 18096599]
- Dutschmann M, Wilson RJ, Paton JF. Respiratory activity in neonatal rats. *Auton Neurosci.* 2000; 84:19–29. [PubMed: 11109986]
- Dutschmann M, Paton JF. Glycinergic inhibition is essential for co-ordinating cranial and spinal respiratory motor outputs in the neonatal rat. *J Physiol.* 2002; 543:643–653. [PubMed: 12205196]
- Ehrlich I, Lohrke S, Friauf E. Shift from depolarizing to hyperpolarizing glycine action in rat auditory neurons is due to age-dependent Cl[−] regulation. *J Physiol.* 1999; 520:121–137. [PubMed: 10517806]
- Feldman JL, Del Negro CA. Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci.* 2006; 7:232–242. [PubMed: 16495944]
- Finley JCW, Katz DM. The central organization of carotid body afferent projection to the brain stem of the rat. *Brain Res.* 1992; 572:108–116. [PubMed: 1611506]
- Fiumelli H, Woodin MA. Role of activity-dependent regulation of neuronal chloride homeostasis in development. *Curr Opin Neurobiol.* 2007; 17:81–86. [PubMed: 17234400]
- Flagella M, Clarke LL, Miller ML, Erway LC, Giannella RA, Andringa A, Gawenis LR, Kramer J, Duffy JJ, Doetschman T, Lorenz JN, Yamoah EN, Cardell EL, Shull GE. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. *J Biol Chem.* 1999; 274:26946–26955. [PubMed: 10480906]
- Fong AY, Zimmer MB, Milsom WK. The conditional nature of the “Central Rhythm Generator” and the production of episodic breathing. *Respir Physiol Neurobiol.* 2009; 168:179–187. [PubMed: 19505592]
- Fuchs A, Ringer C, Bilkei-Gorzo A, Weihe E, Roeper J, Schütz B. Downregulation of the potassium chloride cotransporter KCC2 in vulnerable motoneurons in the SOD1-G93A mouse model of amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol.* 2010; 69:1057–1070. [PubMed: 20838240]
- Galanopoulou AS. Developmental patterns in the regulation of chloride homeostasis and GABA(A) receptor signaling by seizures. *Epilepsia.* 2007; 48(Suppl 5):14–18. [PubMed: 17910576]
- Gamba G. Molecular physiology and pathophysiology of electroneutral cation–chloride cotransporters. *Physiol Rev.* 2005; 85:423–493. [PubMed: 15788703]
- Ganguly K, Schinder AF, Wong ST, Poo M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell.* 2001; 105:521–532. [PubMed: 11371348]

- Gao XP, Liu QS, Liu Q, Wong-Riley MTT. Excitatory-inhibitory imbalance in hypoglossal neurons during the critical period of postnatal development in the rat. *J Physiol.* 2011; 589:1991–2006. [PubMed: 21486774]
- Gilbert D, Franjic-Würtz C, Funk K, Gensch T, Frings S, Möhrle F. Differential maturation of chloride homeostasis in primary afferent neurons of the somatosensory system. *Int J Dev Neurosci.* 2007; 25:479–489. [PubMed: 17869474]
- Gray PA, Reikling JC, Bocchiaro CM, Feldman JL. Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the pre-Bötzinger complex. *Science.* 1999; 286:1566–1568. [PubMed: 10567264]
- Guyenet PG, Stornetta RL, Bayliss DA, Mulkey DK. Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp Physiol.* 2005; 90:247–257. [PubMed: 15728136]
- Haji A, Takeda R, Okazaki M. Neuropharmacology of control of respiratory rhythm and pattern in mature mammals. *Pharmacol Ther.* 2000; 86:277–304. [PubMed: 10882812]
- Harper RM, Woo MA, Alger JR. Visualization of sleep influences on cerebellar and brainstem cardiac and respiratory control mechanisms. *Brain Res Bull.* 2000; 53:125–131. [PubMed: 11033217]
- Hebert SC, Mount DB, Gamba G. Molecular physiology of cation-coupled Cl⁻-cotransport: the SLC12 family. *Pflügers Arch.* 2004; 447:580–593. [PubMed: 12739168]
- Hedstrom KL, Ogawa Y, Rasband MN. AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. *J Cell Biol.* 2008; 183:635–640. [PubMed: 19001126]
- Horn Z, Ringstedt T, Blaesse P, Kaila K, Herlenius E. Premature expression of KCC2 in embryonic mice perturbs neural development by an ion transport-independent mechanism. *Eur J Neurosci.* 2010; 31:2142–2155. [PubMed: 20529123]
- Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron.* 2001; 30:515–524. [PubMed: 11395011]
- Jentsch TJ. Chloride channels: a molecular perspective. *Curr Opin Neurobiol.* 1996; 6:303–310. [PubMed: 8794080]
- Jordan D. Central nervous pathways and control of the airways. *Respir Physiol.* 2001; 125:67–81. [PubMed: 11240153]
- Kahle KT, Staley KJ, Nahed BV, Gamba G, Hebert SC, Lifton RP, Mount DB. Roles of the cation-chloride cotransporters in neurological disease. *Nat Clin Pract Neurol.* 2008; 4:490–503. [PubMed: 18769373]
- Kaila K. Ionic basis of GABA_A receptor channel function in the nervous system. *Prog Neurobiol.* 1994; 42:489–537. [PubMed: 7522334]
- Kazemi H, Hoop B. Glutamic acid and gamma-aminobutyric acid neurotransmitters in central control of breathing. *J Appl Physiol.* 1991; 70:1–7. [PubMed: 1672687]
- Khazipov R, Khalilov I, Tyzio R, Morozova E, Ben-Ari Y, Holmes GL. Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. *Eur J Neurosci.* 2004; 19:590–600. [PubMed: 14984409]
- Kinney HC, Filiano JJ, White WF. Medullary serotonergic network deficiency in the sudden infant death syndrome: review of a 15-year study of a single dataset. *J Neuropathol Exp Neurol.* 2001; 60:228–247. [PubMed: 11245208]
- Krnjević K. When and why amino acids? *J Physiol.* 2010; 588:33–44. [PubMed: 19822549]
- Lee HH, Walker JA, Williams JR, Goodier RJ, Payne JA, Moss SJ. Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J Biol Chem.* 2007; 282:29777–29784. [PubMed: 17693402]
- Leinekugel X, Tseeb V, Ben-Ari Y, Bregestovski P. Synaptic GABA_A activation induces Ca²⁺ rise in pyramidal cells and interneurons from rat neonatal hippocampal slices. *J Physiol.* 1995; 487:319–329. [PubMed: 8558466]
- Liu Q, Wong-Riley MTT. Postnatal expression of neurotransmitters, receptors, and cytochrome oxidase in the rat pre-Bötzinger complex. *J Appl Physiol.* 2002; 92:923–934. [PubMed: 11842022]
- Liu Q, Wong-Riley MTT. Postnatal changes in cytochrome oxidase expressions in brain stem nuclei of rats: implications for sensitive periods. *J Appl Physiol.* 2003; 95:2285–2291. [PubMed: 12909612]

- Liu Q, Wong-Riley MTT. Developmental changes in the expression of GABAA receptor subunits alpha1, alpha2, and alpha3 in the rat pre-Botzinger complex. *J Appl Physiol.* 2004; 96:1825–1831. [PubMed: 14729731]
- Liu Q, Wong-Riley MTT. Postnatal developmental expressions of neurotransmitters and receptors in various brain stem nuclei of rats. *J Appl Physiol.* 2005; 98:1442–1457. [PubMed: 15618314]
- Liu Q, Wong-Riley MTT. Developmental changes in the expression of GABAA receptor subunits alpha1, alpha2, and alpha3 in brain stem nuclei of rats. *Brain Res.* 2006; 1098:129–138. [PubMed: 16750519]
- Liu Q, Wong-Riley MTT. Postnatal changes in the expression of serotonin 2A receptors in various brain stem nuclei of the rat. *J Appl Physiol.* 2008; 104:1801–1808. [PubMed: 18420721]
- Liu Q, Wong-Riley MTT. Postnatal changes in the expressions of serotonin 1A, 1B, and 2A receptors in ten brain stem nuclei of the rat: implication for a sensitive period. *Neurosci.* 2010a; 165:61–78.
- Liu Q, Wong-Riley MTT. Postnatal changes in tryptophan hydroxylase and serotonin transporter immunoreactivity in multiple brainstem nuclei of the rat: implications for a sensitive period. *J Comp Neurol.* 2010b; 518:1082–1097. [PubMed: 20127812]
- Liu Q, Wong-Riley MTT. Postnatal development of N-methyl-D-aspartate receptor subunits 2A, 2B, 2C, 2D, and 3B immunoreactivity in brain stem respiratory nuclei of the rat. *Neurosci.* 2010c; 171:637–654.
- Liu Q, Lowry TF, Wong-Riley MTT. Postnatal changes in ventilation during normoxia and acute hypoxia in the rat: implication for a sensitive period. *J Physiol.* 2006; 577:957–970. [PubMed: 17038423]
- Liu Q, Fehring C, Lowry TF, Wong-Riley MTT. Postnatal development of metabolic rate during normoxia and acute hypoxia in rats: implication for a sensitive period. *J Appl Physiol.* 2009; 106:1212–1222. [PubMed: 19118157]
- Löhrke S, Srinivasan G, Oberhofer M, Doncheva E, Friauf E. Shift from depolarizing to hyperpolarizing glycine action occurs at different perinatal ages in superior olivary complex nuclei. *Eur J Neurosci.* 2005; 22:2708–2722. [PubMed: 16324105]
- Lytle C, Xu JC, Biemesderfer D, Forbush B 3rd. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. *Am J Physiol.* 1995; 269(6 Pt 1):C1496–C1505. [PubMed: 8572179]
- McCarthy MM, Auger AP, Perrot-Sinal TS. Getting excited about GABA and sex differences in the brain. *Trends Neurosci.* 2002; 25:307–312. [PubMed: 12086749]
- Mikawa S, Wang C, Shu F, Wang T, Fukuda A, Sato K. Developmental changes in KCC1, KCC2 and NKCC1 mRNAs in the rat cerebellum. *Brain Res Dev Brain Res.* 2002; 136:93–100.
- Mody I, De Koninck Y, Otis TS, Soltesz I. Bridging the cleft at GABA synapses in the brain. *Trends Neurosci.* 1994; 17:517–525. [PubMed: 7532336]
- Moult PR. Neuronal glutamate and GABAA receptor function in health and disease. *Biochem Soc Trans.* 2009; 37:1317–1322. [PubMed: 19909268]
- Mykoniatis A, Shen L, Fedor-Chaiken M, Tang J, Tang X, Worrell RT, Delpire E, Turner JR, Matlin KS, Bouyer P, Matthews JB. Phorbol 12-myristate 13-acetate-induced endocytosis of the Na-K-2Cl cotransporter in MDCK cells is associated with a clathrin-dependent pathway. *Am J Physiol Cell Physiol.* 2010; 298:C85–C97. [PubMed: 19864322]
- Onimaru H, Homma I. A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci.* 2003; 23:1478–1486. [PubMed: 12598636]
- Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci.* 2002; 3:715–727. [PubMed: 12209120]
- Owens DF, Boyce LH, Davis MB, Kriegstein AR. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J Neurosci.* 1996; 16:6414–6423. [PubMed: 8815920]
- Paton JF, Richter DW. Role of fast inhibitory synaptic mechanisms in respiratory rhythm generation in the maturing mouse. *J Physiol.* 1995; 484:505–521. [PubMed: 7602541]
- Payne JA, Stevenson TJ, Donaldson LF. Molecular characterization of putative K-Cl cotransporter in rat brain. A neuron-specific isoform. *J Biol Chem.* 1996; 271:16245–16252. [PubMed: 8663311]

- Payne JA, Rivera C, Voipio J, Kaila K. Cation–chloride cotransporters in neuronal communication, development and trauma. *Trends Neurosci.* 2003; 26:199–206. [PubMed: 12689771]
- Pierrefiche O, Schwarzacher SW, Bischoff AM, Richter DW. Blockade of synaptic inhibition within the pre-Bötzinger complex in the cat suppresses respiratory rhythm generation in vivo. *J Physiol.* 1998; 509:245–254. [PubMed: 9547397]
- Price TJ, Cervero F, Gold MS, Hammond DL, Prescott SA. Chloride regulation in the pain pathway. *Brain Res Rev.* 2009; 60:149–170. [PubMed: 19167425]
- Ramirez JM, Richter DW. The neuronal mechanisms of respiratory rhythm generation. *Curr Opin Neurobiol.* 1996; 6:817–825. [PubMed: 9000025]
- Rekling JC, Feldman JL. Pre-Bötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu Rev Physiol.* 1998; 60:385–405. [PubMed: 9558470]
- Ren J, Greer JJ. Modulation of respiratory rhythmogenesis by chloride-mediated conductances during the perinatal period. *J Neurosci.* 2006; 26:3721–3730. [PubMed: 16597726]
- Represa A, Ben-Ari Y. Trophic actions of GABA on neuronal development. *Trends Neurosci.* 2005; 28:278–283. [PubMed: 15927682]
- Ritter B, Zhang W. Early postnatal maturation of GABA-mediated inhibition in the brainstem respiratory rhythm-generating network of the mouse. *Eur J Neurosci.* 2000; 12:2975–2984. [PubMed: 10971638]
- Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K⁺/Cl[−] co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature.* 1999; 397:251–255. [PubMed: 9930699]
- Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K⁺/Cl[−] co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature.* 2006; 397:251–255. [PubMed: 9930699]
- Schmid K, Bohmer G, Gebauer K. Glycine receptor-mediated fast synaptic inhibition in the brainstem respiratory system. *Respir Physiol.* 1991; 84:351–361. [PubMed: 1656503]
- Shao XM, Feldman JL. Respiratory rhythm generation and synaptic inhibition of expiratory neurons in pre-Bötzinger complex: differential roles of glycinergic and GABAergic neural transmission. *J Neurophysiol.* 1997; 77:1853–1860. [PubMed: 9114241]
- Singer JH, Berger AJ. Development of inhibitory synaptic transmission to motoneurons. *Brain Res Bull.* 2000; 53:553–560. [PubMed: 11165791]
- Singer JH, Talley EM, Bayliss DA, Berger AJ. Development of glycinergic synaptic transmission to rat brain stem motoneurons. *J Neurophysiol.* 1998; 80:2608–2620. [PubMed: 9819267]
- Smith JC, Morrison DE, Ellenberger HH, Otto MR, Feldman JL. Brainstem projections to the major respiratory neuron populations in the medulla of the cat. *J Comp Neurol.* 1989; 281:69–96. [PubMed: 2466879]
- Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. Pre-Bötzinger complex: a brain stem region that may generate respiratory rhythm in mammals. *Science.* 1991; 254:726–729. [PubMed: 1683005]
- Smith JC, Butera RJ, Koshiya N, Negro CD, Wilson CG, Johnson SM. Respiratory rhythm generation in neonatal and adult mammals: the hybrid pacemaker-network model. *Respir Physiol.* 2000; 122:131–147. [PubMed: 10967340]
- Stil A, Liabeuf S, Jean-Xavier C, Brocard C, Viemari JC, Vinay L. Developmental up-regulation of the potassium-chloride cotransporter type 2 in the rat lumbar spinal cord. *Neurosci.* 2009; 164:809–821.
- Stil A, Jean-Xavier C, Liabeuf S, Brocard C, Delpire E, Vinay L, Viemari JC. Contribution of the potassium-chloride co-transporter KCC2 to the modulation of lumbar spinal networks in mice. *Eur J Neurosci Eur J Neurosci.* 2011; 33:1212–1222.
- St-John WM. Neurogenesis of patterns of automatic ventilatory activity. *Prog Neurobiol.* 1998; 56:97–117. [PubMed: 9723132]
- Takayama C, Inoue Y. Developmental localization of potassium chloride co-transporter 2 in granule cells of the early postnatal mouse cerebellum with special reference to the synapse formation. *Neurosci.* 2006; 143:757–767.

- Taylor EW. The evolution of efferent vagal control of the heart in vertebrates. *Cardioscience*. 1994; 5:173–182. [PubMed: 7827254]
- Viemari JC, Bos R, Boulenguez P, Brocard C, Brocard F, Bras H, Coulon P, Liabeuf S, Pearlstein E, Sadlaoud K, Stil A, Tazerart S, Vinay L. Importance of chloride homeostasis in the operation of rhythmic motor networks. *Prog Brain Res*. 2011; 188:3–14. [PubMed: 21333799]
- Webb TI, Lynch JW. Molecular pharmacology of the glycine receptor chloride channel. *Curr Pharm Des*. 2007; 13:2350–2367. [PubMed: 17692006]
- Wong-Riley MTT. Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends Neurosci*. 1989; 12:94–101. [PubMed: 2469224]
- Wong-Riley MTT, Liu Q. Neurochemical development of brain stem nuclei involved in the control of respiration. *Respir Physiol Neurobiol*. 2005; 149:83–98. [PubMed: 16203213]
- Wong-Riley MTT, Liu Q. Neurochemical and physiological correlates of a critical period of respiratory development in the rat. *Respir Physiol Neurobiol*. 2008; 164:28–37. [PubMed: 18524695]
- Wu WL, Ziskind-Conhaim L, Sweet MA. Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J Neurosci*. 1992; 12:3935–3945. [PubMed: 1403091]
- Yamada J, Okabe A, Toyoda H, Kilb W, Luhmann HJ, Fukuda A. Cl⁻ uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. *J Physiol*. 2004; 557:829–841. [PubMed: 15090604]
- Yuste R, Katz LC. Control of postsynaptic Ca²⁺ influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron*. 1991; 6:333–344. [PubMed: 1672071]

Fig. 1.

NKCC1-ir neurons and neuropil in the brain stem of mice. A1 shows labeling in the region surrounding and including the nucleus ambiguus (Amb), which is marked by a rectangular box in A1 and enlarged in A2. The diagrammatic inset in A1 indicates the location of Amb in the medulla. B1 and B2 are comparable regions as A1 and A2, respectively, in the brain stem of an *Nkcc1*^{-/-} mouse, which shows no specific labeling with NKCC1 antibody. Scale bars: 40 μ m for A1 and B1, 10 μ m for A2 and B2. Diagrammatic insets in Figures 2–5 and 7–10 indicate the location of each nucleus in cross-sections of the medulla. The other insets in these figures are higher magnifications of representative labeled neurons (shown with arrows in the figures) highlighting the accumulation of immunoreaction product along the plasma membranes of the cell bodies. Scale bars: 10 μ m (3.33 μ m for insets) for all.

Fig. 2. NKCC1-ir neurons and neuropil in the pre-Bötzinger complex (PBC) (A) and nucleus ambiguus (Amb) (B) at postnatal days P2 (A1 and B1), P7 (A2 and B2), P12 (A3 and B3), and P21 (A4 and B4). The expression of NKCC1 in both nuclei was relatively high at P2 and P7, but was markedly reduced at P12 and remained low at P21.

Fig. 3. NKCC1-ir neurons and neuropil in the hypoglossal nucleus (XII) (A) and ventrolateral subnucleus of solitary tract nucleus (NTS_{VL}) (B) at P2, P7, P12, and P21. Immunoreactivity in the XII was relatively high at P2 and P7 but was markedly reduced at P12 and remained low at P21. Labeling in the NTS_{VL} was relatively high at P2 and P7, but was reduced at P12 and at P21.

Fig. 4. NKCC1-ir neurons and neuropil in the retrotrapezoid nucleus (RTN)/parafacial respiratory group (pFRG) (A) and dorsal motor nucleus of the vagus nerve (DMNX) (B) at P2, P7, P12, and P21. Immunoreactivity in the RTN/pFRG was relatively high at P2 and P7, but was reduced at P12 and plateaued at P21. Labeling in DMNX was relatively stable at P2 and P7, but was reduced at P12 and further reduced at P21.

Fig. 5. NKCC1-ir neurons and neuropil in the inferior olivary nucleus (IO) (A) and cuneate nucleus (CN) (B) at P2, P7, P12, and P21. Immunoreactivity in the IO was relatively stable at P2 and P7, but was slightly reduced at P12 and markedly reduced at P21. The labeling in the CN was relatively stable at P2, P7, and P12, and but was lower at P21.

Fig. 6.

Optical densitometric measurements of immunoreactive product for NKCC1 in individual neurons of the PBC (A), Amb (B), XII (C), NTS_{VL} (D), RTN/pFRG (E), DMNX (F), IO (G), and CN (H) from P0 to P21. Data points were presented as mean $\pm$ SEM. Note that the developmental trends for NKCC1 in all seven respiratory-related nuclei followed an age-dependent decrease, whereas that in the CN was a more gradual decrease. Note also that a significant fall in NKCC1 expression occurred at P12 for the PBC, Amb, and XII. This was the only time point when a day-to-adjacent day comparison with the Tukey's Studentized test showed a significance (*, $P < 0.05$). See text for details and for other comparisons. ANOVA yielded significant differences in the expression of NKCC1 among the ages in all eight nuclei examined ($P < 0.01$).

Fig. 7. KCC2-ir neurons and neuropil in the PBC (A) and Amb (B) at P2, P7, P12, and P21. The expression of KCC2 was relatively low in both nuclei at P2, but was higher at P7 and peaked at P12, followed by a plateau at P21.

Fig. 8. KCC2-ir neurons and neuropil in the XII (A) and NTS_{VL} (B) at P2, P7, P12, and P21. Immunoreactivity in both nuclei was relatively low at P2, rose at P7, peaked at P12 and remained high at P21.

Fig. 9. KCC2-ir neurons and neuropil in the RTN/pFRG (A) and DMNX (B) at P2, P7, P12, and P21. Immunoreactivity in both nuclei was relatively low at P2, increased at P7, and plateaued at P12 and P21.

Fig. 10. KCC2-ir neurons and neuropil in the IO (A) and CN (B) at P2, P7, P12, and P21. Immunoreactivity in both was relatively low at P2, rose at P7, and plateaued at P12 and P21.

Fig. 11.

Optical densitometric measurements of immunoreactive product for KCC2 in individual neurons of the PBC (A), Amb (B), XII (C), NST_{VL} (D), RTN/pFRG (E) DMNX (F), IO (G), and CN (H) from P0 to P21. Data points were presented as mean $\pm$ SEM. Note that there is an age-dependent increase in KCC2 expression in all eight nuclei examined, reaching a peak during the 2nd postnatal week and plateaued thereafter until P21. The trends were present but less distinct in the DMNX, IO, and CN. ANOVA yielded significant differences among the ages in all eight nuclei examined ($P < 0.01$). Tukey's test did not reveal any significant difference when comparisons were made between any two adjacent age groups. See text for details.

Fig. 12.

Comparisons of developmental trends between NKCC1 (triangle) and KCC2 (square) in eight brain stem nuclei over the first three postnatal weeks. Optical densitometric values (OD) were normalized such that the highest expression of each marker for each nucleus over the first 3 postnatal weeks was set at 1. Data points represent the mean values. Note that the trends of age-dependent decrease in NKCC1 expression and age-dependent increase in KCC2 expression intersected around P11 in the PBC (A), Amb (B), XII (C), NTS_{VL}(D), and RTN/pFRG (E), and that KCC2 expression has a clear dominance after P12 in all eight nuclei examined. See text for details.

Table 1

Primary antibodies used.

Antigen	Immunogen	Manufacturer, species, type Catalog number	Dilution used
Na–K–2Cl co-transporter 1 (NKCC1)	Human NKCC1, C-terminus (MET-902 to SER-1212)	Developmental Studies Hybridoma Bank (Iowa City, IA), mouse monoclonal IgG, mAb T4	1:14,000
K–Cl co-transporter 2 (KCC2)	Intracellular C-terminus of rat KCC2 (amino acids 932–1043)	UC Davis/NIH NeuroMab Facility (Davis, CA), mouse monoclonal IgG, #75-013	1:500