Neuroscience 210 (2012) 1–20

POSTNATAL DEVELOPMENT OF NA^+–K^+–$2CL^-$ CO-TRANSPORTER 1 AND K^+–CL^- CO-TRANSPORTER 2 IMMUNOREACTIVITY IN MULTIPLE BRAIN STEM RESPIRATORY NUCLEI OF THE RAT

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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 [retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG)], dorsal motor nucleus of the vagus nerve [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–P21 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.

Key words: critical period, dorsal motor nucleus of the vagus nerve, hypoglossal nucleus, nucleus ambiguus, pre-Bötzinger complex, ventrolateral subnucleus of solitary tract nucleus.

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Abbreviations: 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; -ir, immunoreactive; KCC2, K^+–Cl^- co-transporter 2; NKCC1, Na^+–K^+-$2Cl^-$ co-transporter 1; NMDA, *N*-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; XII, hypoglossal nucleus.

0306-4522/12 $36.00
doi:10.1016/j.neuroscience.2012.03.018

GABA is a major inhibitory neurotransmitter in the vertebrate CNS and acts on $GABA_A$ and $GABA_B$ receptors to mediate fast and relatively slow responses, respectively (Krnjević, 2010). GABA inhibitory transmission 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; Moult, 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 serotoninergic and NMDA receptor subunits occur at this time (Liu and Wong-Riley, 2008, 2010a,b,c). Importantly, our detailed, daily electrophysiological study indicates a striking change in synaptic transmission in hypoglossal motoneurons at P12–P13 (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 on request). All efforts were made to minimize the number of animals used and their suffering.

A total of 162 Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA), both male and female, from 13 litters were used. Rat pups were sacrificed at each of postnatal days 0, 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, i.p.; Fisher Scientific, Fair Lawn, NJ, USA) and perfused through the aorta with 4% paraformaldehyde and 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, USA) 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 (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 with wild type controls (Fig. 1). The anti-neuron-specific KCC2 mouse monoclonal antibody (75-013, UC Davis/NIH NeuroMab Facility, Davis, CA, USA) 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 (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, Heidelberger, Nussloch, Germany). Seven or nine sets of serial sections were mounted on gelatin-coated slides. In the same litter, sections from three 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, and 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 three times, 5 min each, in PBS, then

Table 1. Primary antibodies used

Antigen	Immunogen	Manufacturer, species, type/catalog number	Dilution used
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
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