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POSTNATAL DEVELOPMENT OF N-METHYL-D-ASPARTATE RECEPTOR SUBUNITS 2A, 2B, 2C, 2D, AND 3B IMMUNOREACTIVITY IN BRAIN STEM RESPIRATORY NUCLEI OF THE RAT

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Abstract—Previously, we reported that a critical period in respiratory network development exists in rats around postnatal days (P; P12–P13), when abrupt neurochemical, metabolic, and physiological changes occur. Specifically, the expressions of glutamate and *N*-methyl-D-aspartate (NMDA) receptor (NR) subunit 1 in the pre-Bötzinger complex (PBC), nucleus ambiguus (Amb), hypoglossal nucleus (XII), and ventrolateral subnucleus of solitary tract nucleus (NTS_{VL}) were significantly reduced at P12. To test our hypothesis that other NR subunits also undergo postnatal changes, we undertook an in-depth immunohistochemical study of NR2A, 2B, 2C, 2D, and 3B in these four respiratory nuclei in P2–P21 rats, using the non-respiratory cuneate nucleus (CN) as a control. Our results revealed that: (1) NR2A expression increased gradually from P2 to P11, but fell significantly at P12 in all four respiratory nuclei (but not in the CN), followed by a quick rise and a relative plateau until P21; (2) NR2B expression remained relatively constant from P2 to P21 in all five nuclei examined; (3) NR2C expression had an initial rise from P2 to P3, but remained relatively constant thereafter until P21, except for a significant fall at P12 in the PBC; (4) NR2D expression fell significantly from P2 to P3, then plateaued until P12, and declined again until P21; and (5) in contrast to NR2D, NR3B expression rose gradually from P2 to P21. These patterns reflect a dynamic remodeling of NMDA receptor subunit composition during postnatal development, with a distinct reduction of NR2A expression during the critical period (P12), just as NR1 did in various respiratory nuclei. There was also a potential switch between the neonatal NR2D and the more mature NR3B subunit, possibly around the critical period. Thus, during the critical period, NMDA receptors are undergoing greater adjustments that may contribute to attenuated excitatory synaptic transmission in the respiratory network.

Key words: critical period, glutamate receptors, hypoglossal nucleus, nucleus ambiguus, nucleus tractus solitarius, pre-Bötzinger complex.

A critical period of postnatal development in the respiratory network of rats was characterized in our previous studies (Liu and Wong-Riley, 2002, 2003, 2005; Liu et al., 2003, 2006, 2009; Wong-Riley and Liu, 2005, 2008). This is a narrow window at the end of the second postnatal (P) week (around P12–P13), when abrupt neurochemical, metabolic, and physiological changes occur in the brain stem respiratory network. During this time, there is a transient imbalance between the expressions of excitatory and inhibitory neurochemicals, such that the levels of inhibitory neurotransmitters (GABA) and receptors ($GABA_B$ and glycine receptors) are significantly increased, whereas those of the excitatory ones (glutamate and *N*-methyl-D-aspartate (NMDA) receptor subunit 1) precipitously fall. At the same time, there is a sudden decrease in the activity of a metabolic marker of neuronal activity, cytochrome oxidase, in various respiratory nuclei (Liu and Wong-Riley, 2002, 2003, 2005; Liu et al., 2003; Wong-Riley and Liu, 2005, 2008), and the animals' ventilatory and metabolic responses to hypoxia are also at their weakest (Liu et al., 2006, 2009).

To explore additional mechanisms that might underlie the critical period, we analyzed subunit expressions of $GABA_A$ receptors that mediate the majority of fast inhibitory synaptic interactions in the adult mammalian brain. We found that the developmental trend of $\alpha 3$ subunit decreases with age, whereas that of $\alpha 1$ increases with age, and the two intersect at P12 (Liu and Wong-Riley, 2004, 2006). Subunit switches might underlie a change in $GABA_A$ receptor subtype that may mediate a transition from a less efficient inhibitory transmission before P12 to a more mature one at P12 and thereafter, as suggested by a change in the kinetics of postsynaptic potentials during postnatal development in the thalamus and the visual cortex (Okada et al., 2000; Bosman et al., 2002).

Possible switches in subunit composition of the excitatory glutamatergic NMDA receptors have not been explored in brain stem respiratory nuclei. The present study aimed at testing our hypothesis that subunits of NMDA receptors (NR) undergo distinct changes during postnatal development, especially during the critical period in rats. An in-depth immunohistochemical study of NR2A, NR2B, NR2C, NR2D, and NR3B were conducted in P2–21 rats in the pre-Bötzinger complex (PBC, postulated as the center of respiratory rhythmogenesis; Smith et al., 1991, 2000; Rekling and Feldman, 1998), the nucleus ambiguus (Amb, which controls the upper airway muscles; Jordan, 2001), the hypoglossal nucleus (XII, which controls the tongue muscles associated with airway patency; Horner, 2007), and the ventrolateral subnucleus of solitary tract nucleus [NTS_{VL}, which receives peripheral chemosensitive affer-

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Abbreviations: Amb, nucleus ambiguus; ANOVA, analysis of variance; APB, ammonium phosphate buffer; CN, cuneate nucleus; -ir, immunoreactive; GABA, gamma aminobutyric acid; NMDA, *N*-methyl-D-aspartate; NR, NMDA receptor; NTS_{VL}, ventrolateral subnucleus of solitary tract nucleus; P, postnatal day; PBC, pre-Bötzinger complex; PBS, sodium phosphate buffered saline; R, receptor; SIDS, sudden infant death syndrome; XII, hypoglossal nucleus.

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ents (Finley and Katz, 1992) and is involved in respiratory modulation (Paton et al., 1991; Bonham, 1995)]. The non-respiratory cuneate nucleus (CN, a relay in the somatosensory system with no known respiratory function) was chosen as an internal control. Results were compared with those of NR1 analyzed previously (Liu and Wong-Riley, 2002, 2005).

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 132 Sprague–Dawley rats, both male and female, from 11 litters were used. Rat pups were sacrificed at each of postnatal days P2–P5, P7, P10–P14, P17, and P21 (i.e. 12 time points, with 11 rats per time point for NR2A and 2B, and five rats per time point for the other NR subunits). Rats 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-4% sucrose in 0.1 M sodium phosphate buffered saline (PBS), pH 7.4. Brain stems were then removed and postfixed in the same fixative for 3 h at 4 °C. They were subsequently 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.

Antibody characterization

Table 1 shows a brief summary of the antibodies used in the present study. All five antibodies (anti-NR2A, anti-NR2B, anti-NR2C, anti-NR2D, and anti-NR3B) have been well characterized and their specificities have been established by the manufacturers and previous investigators. The amino acid sequence of each of the synthetic peptides bore no sequence homology with any other peptides, and there was no cross-reactivity with any other known proteins. The anti-NR2A polyclonal antibody (AB1555P, Chemicon, Temecula, CA, USA) was a purified immunoglobulin raised against a C-terminal fusion protein of rat NR2A (aa 1253–1391). By Western blots it recognized a 180 kDa band in rat brain membranes, and did not react with NR2B or 2C. Immunolabeling was blocked by preadsorption of the antibody with the immunogen. The anti-NR2B polyclonal antibody (AB1557P, Chemicon) was a purified immunoglobulin raised against a C-terminal fusion protein of NR2B. It showed specific immunolabeling of the 180 kDa NR2B and no reactivity to NR2A or NR2C by western blots. The immunolabeling was blocked by preadsorption of the antibody with the C-terminal fusion protein used to generate the antibody. The anti-NR2C polyclonal antibody (sc-50437, Santa Cruz Biotech, Santa Cruz, CA, USA) was raised against amino acids 21–100, mapping near the N-terminus of NR2C of human origin. By Western blots it recognized a 135 kDa band. The anti-NR2D monoclonal antibody (MAB5578, clone 1G9.39A5, Chemicon) was raised against the C-terminal protein of rat NR2D and reacted with a band at ~145 kDa by Western blots. The anti-NR3B polyclonal antibody (07-351, Upstate, Temecula, CA, USA) was a purified immunoglobulin raised against amino acids 916-930 of mouse NR3B that recognized NR3B at ~98 kDa by Western blots.

Immunohistochemistry

Coronal sections (12-μm thickness) of frozen brain stems were cut with a Leica CM1900 cryostat (Leica Microsystems, Heidelberger, Nussloch, Germany). Seven 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 so that they might be processed together. Ages were grouped typically as follows: P2–10–21, P3–4–17, P5–7–14, and P11–12–13. The first three sects showed the developmental trends, whereas the fourth set concentrated on the critical period. Having three distinct ages on the same slide ensured that any changes observed between ages were intrinsic to the animals and not as a result of unforeseen variations of tissue processing between slides. 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 (Table 1) in the same solution as used for blocking. Sections were rinsed three times, 5 min each, in PBS, then incubated in the secondary antibodies: 1:100 goat anti-rabbit immunoglobulin G-horse radish peroxidase (IgG-HRP) (Bio-Rad) for NR2A, 2B, 2C, and 3B, and 1:100 goat anti-mouse IgG-HRP (Bio-Rad) for NR2D, 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% H_2O_2 in APB for 5 min, and the reaction was stopped with APB for 5 min and then 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. All routine chemicals are from Sigma (St. Louis, MO, USA).

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

We used a non-respiratory nucleus, the CN, as a negative control. CN is known for its relay function in somatosensory transduction but is not involved in respiratory functions.

Semi-quantitative optical densitometry

The immunoreactivity of different markers in the cytoplasm of neurons in various nuclei studied was semi-quantitatively ana-

Table 1. Primary antibodies used

Antigen	Immunogen	Manufacturer, species, type, catalog number	Dilution used
NE2A	C-terminal fusion protein of rat NR2A, aa 1253–1391	Chemicon (Temecula, CA, USA), rabbit polyclonal, #AB1555P	1:500
NR2B	C-terminal fusion protein of NR2B (30 kDa)	Chemicon (Temecula, CA, USA), rabbit polyclonal, #AB1557P	1:1000
NR2C	Amino acid 21–100 mapping near the N-terminus of human NR2C	Santa Cruz Biotech (Santa Cruz, CA, USA), rabbit polyclonal, #sc-50437	1:600
NR2D	Recombinant protein from rat NR2D	Chemicon (Temecula, CA, USA), mouse monoclonal, #MAB5578	1:300
NR3B	Amino acid 916–930 of mouse NR3B	Upstate (Temecula, CA, USA), rabbit polyclonal, #07–351	1:200