Neuroscience 165 (2010) 61–78

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

Q. LIU AND M. T. T. WONG-RILEY*

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

Abstract—A critical period in respiratory network development occurs in the rat around postnatal days (P) 12–13, when abrupt neurochemical, metabolic, and physiological changes were evident. As serotonin and its receptors are involved in respiratory modulation, and serotonergic abnormality is implicated in sudden infant death syndrome, we hypothesized that 5-HT receptors are significantly downregulated during the critical period. This was documented recently for 5-HT_{2A}R in several respiratory nuclei. The present study represents a comprehensive analysis of postnatal development of 5-HT_{1A}R and 5-HT_{1B}R in 10 brain stem nuclei and 5-HT_{2A}R in six nuclei not previously examined. Optical densitometric analysis of immunohistochemically-reacted neurons from P2 to P21 indicated four developmental patterns of expression: (1) Pattern I: a high level of expression at P2-P11, an abrupt and significant reduction at P12, followed by a plateau until P21 (5-HT_{1A}R and 5-HT_{1B}R in raphé magnus [RM], raphé obscurus [ROb], raphé pallidus [RP], pre-Bötzinger complex [PBC], nucleus ambiguus [Amb], and hypoglossal nucleus [XII; 5-HT_{1A}R only]). (2) Pattern II: a high level at P2-P9, a gradual decline from P9 to P12, followed by a plateau until P21 (5-HT_{1A}R and 5-HT_{1B}R in the retrotrapezoid nucleus (RTN)/parafacial respiratory group (pFRG)). (3) Pattern III: a high level at P2-P11, followed by a gradual decline until P21 (5-HT_{1A}R in the ventrolateral subnucleus of solitary tract nucleus [NTS_{VL}] and the non-respiratory cuneate nucleus [CN]). (4) Pattern IV: a relatively constant level maintained from P2 to P21 (5-HT_{1A}R in the commissural subnucleus of solitary tract nucleus (NTS_{COM}); 5-HT_{1B}R in XII, NTS_{VL}, NTS_{COM}, and CN; and 5-HT_{2A}R in RM, ROb, RP, RTN/pFRG, NTS_{VL}, and NTS_{COM}). Thus, a significant reduction in the expression of 5-HT_{1A}R, 5-HT_{1B}R, and 5-HT_{2A}R in multiple respiratory-related nuclei at P12 is consistent with reduced serotonergic transmission during the critical period, thereby rendering the animals less able to respond adequately to ventilatory distress.

Key words: critical period, development, hypoglossal nucleus, nucleus ambiguus, pre-Bötzinger complex, respiratory nuclei.

*Corresponding author. Tel: +1-414-456-8467; fax: +1-414-456-6517.
E-mail address: mwr@mcw.edu (M. T. T. Wong-Riley).

Abbreviations: Amb, nucleus ambiguus; ANOVA, analysis of variance; APB, ammonium phosphate buffer; CN, cuneate nucleus; ir, immunoreactive; NTS, solitary tract nucleus; NTS_{COM}, commissural subnucleus of solitary tract nucleus; NTS_{VL}, ventrolateral subnucleus of solitary tract nucleus; P, postnatal day; pAb, polyclonal antibodies; PBC, pre-Bötzinger complex; PBS, sodium phosphate buffered saline; pFRG, parafacial respiratory group; R, receptor; RM, nucleus raphé magnus; ROb, nucleus raphé obscurus; RP, nucleus raphé pallidus; RTN, retrotrapezoid nucleus; SIDS, sudden infant death syndrome; XII, hypoglossal nucleus.

It is well established that 5-hydroxytryptamine (5-HT), an indoleaminergic neurotransmitter, is involved in respiratory modulation (Halliday et al., 1995; Hilaire and Duron, 1999; Hodges and Richerson, 2008). Brain stem serotonergic neurons are distributed mainly in medullary raphé nuclei and the ventrolateral medulla (parapyramidal area) (Holtman et al., 1984; Connelly et al., 1989; Smith et al., 1989; Thor and Helke, 1989; Manaker and Tischler, 1993). These serotonergic neurons innervate all of the brain stem nuclei that are involved in respiratory control as well as phrenic motor nuclei in the spinal cord (Steinbusch, 1981; Holtman et al., 1984; Connelly et al., 1989; Voss et al., 1990; Hodges and Richerson, 2008). Serotonin's neuromodulatory effects on respiration are mediated by various receptor subtypes, such as 5-HT_{1A}R, 5-HT_{1B}R, and 5-HT_{2A}R (Zifa and Fillion, 1992; Bonham, 1995; Hilaire and Duron, 1999; Hodges and Richerson, 2008). These effects vary according to neuronal populations, pre- or post-synaptic sites, developmental stages, and environmental conditions (Zifa and Fillion, 1992; Hodges and Richerson, 2008). A developmental abnormality in the medullary 5-HT system has been implicated in certain pathological events occurring postnatally, such as in sudden infant death syndrome (SIDS) and obstructive sleep apnea (Hilaire et al., 1993; Panigrahy et al., 2000; Narita et al., 2001; Kinney, 2005).

Previously, we reported that a sudden drop in neuronal metabolic activity concomitant with reduced expression of excitatory neurochemicals (glutamate and NMDA (N-methyl-D-aspartate) receptors) and heightened expression of inhibitory neurochemicals (GABA, $GABA_B$ receptors, and glycine receptors) occurred at postnatal day (P) 12 in multiple brain stem respiratory nuclei of the rat (Liu and Wong-Riley, 2002, 2005; Wong-Riley and Liu, 2005). Both ventilatory and metabolic responses to hypoxia were also weakest at this time (P13; Liu et al., 2006, 2009). Thus, the end of the second postnatal week is a critical period of postnatal respiratory development in the rat.

Since 5-HT_{1A}R, 5-HT_{1B}R, and 5-HT_{2A}R all play an important role in respiratory modulation, we hypothesized that their expressions also undergo distinct changes around the critical period. Our recent investigation indicated that 5-HT_{2A}R expression was significantly reduced at P12 in the pre-Bötzinger complex (PBC), nucleus ambiguus (Amb), and hypoglossal nucleus (XII) (Liu and Wong-Riley, 2008; Wong-Riley and Liu, 2008). The present study represents a comprehensive, in-depth analysis of postnatal development of 5-HT_{1A}R and 5-HT_{1B}R in 10 brain stem

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2009.09.078

nuclei and 5-HT_{2A}R in six nuclei not previously examined. These nuclei included the respiratory-related ones (the PBC, Amb, XII, the ventrolateral subnucleus of the solitary tract nucleus (NTS_{VL}), the commissural subnucleus of the solitary tract nucleus (NTS_{COM}), and the retrotrapezoid nucleus (RTN)/parafacial respiratory group (pFRG)), serotonergic neuronal groups (nucleus raphé magnus [RM], nucleus raphé obscurus [ROb], and nucleus raphé pallidus [RP]), and a non-respiratory nucleus (the cuneate nucleus or CN) as a reference. CN is known for its relay function in somatosensory transduction but not with any respiratory function.

EXPERIMENTAL PROCEDURES

Tissue preparation

A total of 130 Sprague–Dawley rats from 16 litters were used. 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. Rat pups were sacrificed at each of Ps 2–5, 7–17, and 21 (i.e. 16 time points), with 10 rats from 10 different litters for each day from P10 to P14, eight rats from eight different litters each day for P2–5, P7, P17, and P21, and six rats from six different litters each day for P8, P9, P15, and P16. 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 in 0.1 M sodium phosphate buffered saline (PBS), pH 7.4, with 4% sucrose. After perfusion, brain stems were removed and immersion fixed in the same fixative for 3 h at 4 °C. They were then cryoprotected in increasing concentrations of sucrose (10%, 20%, and 30%) in 0.1 M PBS at 4 °C, frozen on dry ice, and stored at −80 °C until use.

Characterization of antibodies

Table 1 includes a brief summary of the antibodies used in the present study. All three antibodies (anti-5-HT_{1A}R, anti-5-HT_{1B}R, and anti-5-HT_{2A}R) have been well characterized and their specificities 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 proteins. Moreover, the specificity of AB5406 antibodies against 5-HT_{1A}R has been verified by preadsorption with the immunogen peptide, which effectively abolished the specific immunolabeling (Zhang et al., 2004). Huo et al. (2009) localized the same antibodies at the electron microscopic level to dendrites and cell bodies of neurons in the ventrolateral orbital cortex, where 5-HT_{1A}R-agonists and antagonists elicited specific behavioral response. The AB5410 antibodies against 5-HT_{1B}R was effectively used by Clark et al. (2002) to colocalize 5-HT_{1B}R immunoreactivity with GFP-labeled plasmid expressing 5-HT_{1B}R in the same dorsal raphé neurons. The same antibodies were documented in Western blots by Carruthers et al. (2001) and the labeling was abolished by preadsorption with the immunogen peptide. The s.c.-15074 affinity purified IgG against 5-HT_{2A}R was documented by Siddiqui et al. (2006) by Western blots to a single band of 52 kDa in HT1376 cells as well as in the rat brain. Li et al. (2006) also localized a single band for s.c.-15074 on immunoblot in rat limbs.

Immunohistochemistry

Coronal sections of frozen brain stems were cut at 12 μm thickness with a cryostat. Six sets of serial sections were mounted on gelatin-coated slides. Sections from 3 to 4 rats at different ages were mounted on the same slides so that they might be processed together. Ages were typically grouped as follows: P2–10–21, P3–4–5–17, P7–8–9, P11–12–13, and P14–15–16. All sections from all animals 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 (or rabbit for 5-HT_{2A}R) 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: 1:200 anti-5-HT_{1A}R polyclonal antibodies (pAb); 1:200 anti-5-HT_{1B}R pAb; and 1:100 anti-5-HT_{2A}R pAb. These dilutions were found to be optimal for immunohistochemistry of brain stem nuclei under study in postnatal developing rats. Sections were rinsed 3 times, 5 min each, in PBS, then incubated in the secondary antibodies: 1:100 goat anti-guinea pig IgG-HRP (Chemicon, Temecula, CA, USA) for 5-HT_{1A}R and 5-HT_{1B}R, 1:100 rabbit anti-goat IgG-HRP (Chemicon) for 5-HT_{2A}R, 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% 3,3′-diaminobenzidine-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 either without the primary antibodies or with a non-immune serum in place of the primary antibodies.

For estimates of the percentage of immunoreactive neurons in a specific nucleus, alternate sections were processed with Nissl, which stained all neuronal cell bodies. Another set of alternate sections were reacted for neurokinin receptor subunit 1 with protocols described previously (Liu and Wong-Riley, 2002).

Table 1. Primary antibodies used

Antigen	Immunogen	Manufacturer, species, type, catalog number	Dilution used
Serotonin 1A receptor	A synthetic peptide corresponding to a region located in the large third intracellular loop of the rat and mouse serotonin 1A receptor protein, with sequence specific to 5-HT_{1A}R.	Chemicon (Temecula), guinea pigs polyclonal, AB5406.	1:200
Serotonin 1B receptor	A synthetic peptide corresponding to a region located in the large third intracellular loop of the rat and mouse serotonin 1B receptor protein, with sequence specific to 5-HT_{1B}R.	Chemicon (Temecula), guinea pigs polyclonal, AB5410.	1:200
Serotonin 2A receptor	A synthetic peptide mapping within an internal region of human serotonin 2A receptor protein (accession number P28223), with sequence specific to 5-HT_{2A}R.	Santa Cruz Biotech (Santa Cruz), goat polyclonal, affinity purified IgG, sc-15074.	1:100