Postnatal changes in cytochrome oxidase expressions in brain stem nuclei of rats: implications for sensitive periods

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Liu, Qiuli, and Margaret T. T. Wong-Riley. Postnatal changes in cytochrome oxidase expressions in brain stem nuclei of rats: implications for sensitive periods. J Appl Physiol 95: 2285–2291, 2003. First published August 8, 2003; 10.1152/japplphysiol.00638.2003.—Previously, we reported that cytochrome oxidase (CO) activity in the rat pre-Bötzinger complex (PBC) exhibited a plateau on postnatal days (P) 3–4 and a prominent decrease on P12 (Liu and Wong-Riley, J Appl Physiol 92: 923–934, 2002). These changes were correlated with a concomitant reduction in the expression of glutamate and N-methyl-D-aspartate receptor subunit 1 and an increase in GABA, GABA_B, glycine receptor, and glutamate receptor 2. To determine whether changes were limited to the PBC, the present study aimed at examining the expression of CO in a number of brain stem nuclei, with or without known respiratory functions from P0 to P21 in rats: the ventrolateral subnucleus of the solitary tract nucleus, nucleus ambiguus, hypoglossal nucleus, nucleus raphe obscurus, dorsal motor nucleus of the vagus nerve, medial accessory olivary nucleus, spinal nucleus of the trigeminal nerve, and medial vestibular nucleus (MVe). Results indicated that, in all of the brain stem nuclei examined, CO activity exhibited a general increase with age from P0 to P21, with MVe having the slowest rise. Notably, in all of the nuclei examined except for MVe, there was a plateau or decrease at P3–P4 and a prominent rise-fall-rise pattern at P11–P13, similar to that observed in the PBC. In addition, there was a fall-rise-fall pattern at P15–P17 in these nuclei, instead of a plateau pattern in the PBC. Our data suggest that the two postnatal periods with reduced CO activity, P3–P4 and especially P12, may represent common sensitive periods for most of the brain stem nuclei with known or suspected respiratory control functions.

hypoglossal nucleus; ventrolateral subnucleus of the solitary tract nucleus; nucleus ambiguus; nucleus raphe obscurus; respiratory control

THE RESPIRATORY CONTROL SYSTEM of mammals exhibits relatively mature functional activity at birth, as breathing is necessary for maintaining blood-gas homeostasis after birth. However, the system undergoes postnatal development before achieving the “adult” form weeks or months after birth (10). Knowledge of normal postnatal development of the respiratory control system may shed light on respiratory pathological events, such as occurs in sudden infant death syndrome (SIDS).

In the last decade, the triple risk model was proposed to explain the pathogenesis of SIDS (12, 20). Three overlapping factors must occur at the same time: 1) a vulnerable infant, 2) a critical developmental period in homeostatic control, and 3) an exogenous stressor or stressors. Ninety percent of SIDS occurs in the first 6 mo of life, with a peak at 2–4 mo, implying strongly that a vulnerable period exists in postnatal development (20).

Previously, we employed cytochrome oxidase (CO), the terminal enzyme of the mitochondrial respiratory chain, as a marker of neuronal functional activity (49) to investigate postnatal development of the pre-Bötzinger complex (PBC), the presumed center of respiratory rhythmogenesis (15, 35, 39, 45). We found that, against a general increase in CO with age from postnatal days (P) 0–21, there was a plateau or reduction of CO activity on P3–P4 and a dramatic drop of enzyme levels on P12 (22, 24). This was accompanied by a concomitant decrease in excitatory neurotransmitter glutamate and its N-methyl-D-aspartate receptor subunit 1 and an increase in inhibitory neurotransmitters GABA, its receptors (GABA_B), and glycine receptors. The glutamate receptor GluR2, which reduces the permeability of DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid receptors to Ca2+ and thereby reduces neuronal excitation, also exhibited an increase during those two periods (22). These findings suggest that, during postnatal development of the rat, there may be two critical periods, P3–P4 and especially P12, when its respiratory system may be under a stronger inhibitory than excitatory drive and may render the animal more vulnerable to respiratory insults.

Carotid body denervation at the two presumed vulnerable windows (close to P3 and P11–P13), as opposed to other times, induced a distinct delay as well as prolongation of the metabolic maturational process (23). This lends further support to the possibility of two vulnerable windows in postnatal development of the rat PBC. In addition, the prominent rise-fall-rise pattern seen in normal animals between P11 and P13 was retained, albeit delayed and prolonged, in both sham
and carotid body denervated animals, suggesting strongly that it is a maturational process programmed genetically but modifiable by functional deprivation (23).

In the present study, we wished to determine whether various brain stem nuclei with or without respiratory functions underwent a similar postnatal developmental trend in CO activity as in the PBC. The nuclei examined were as follows: the ventrolateral subnucleus of the solitary tract nucleus (NTS; NTSVL), nucleus ambiguus (Amb), hypoglossal nucleus (XII), nucleus raphe obscurus (ROb), dorsal motor nucleus of the vagus nerve (DMNX), medial accessory olivary nucleus (IOMa), spinal nucleus of the trigeminal nerve (SPV), and medial vestibular nucleus (MVe).

**MATERIALS AND METHODS**

_Tissue preparation._ A total of 120 Sprague-Dawley rats from 12 litters were employed, in accordance with the National Institutes of Health and Medical College of Wisconsin regulations. Litter size averaged 8–13 pups. Each litter covered the earlier or later part of the time points studied, i.e., every day from P0 to P17, P19, and P21; and, at every time point, six rats from six different litters were used. Rats were deeply anesthetized with 4% chloral hydrate (0.1 ml/10 g ip) and perfused through the aorta with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4, and 4% sucrose. Brain stems were removed and postfixed in 0.1 M PBS, pH 7.4, and 4% sucrose in 0.1 M PBS (pH 7.4) at 37°C until use.

_CO histochemistry._ Coronal sections of frozen brain stems were cut at 12-μm thickness with a cryostat, mounted on gelatin-coated slides, and processed for CO histochemistry. Briefly, the sections were incubated in 0.05% 3,3′-diaminobenzidine (Sigma), 0.02% cytochrome c (type III, Sigma), and 4% sucrose in 0.1 M PBS at 37°C in the dark for 3 h. After incubation, the sections were washed with cold 0.1 M PBS (pH 7.4) three times, for 5 min each. The slides were then air-dried, dehydrated, and coverslipped.

_Quantitative densitometry._ Optical densitometric measurements of reaction product of CO histochemistry were 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. For each section measured, the white matter with very low levels of CO activity was set at zero as an internal standard. The optical densitometric value of each neuron measured in the brain stem nuclei was an average reading of two to four spots in the cytoplasm. Only those neurons whose nuclei are 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 50 μm apart, as the largest neurons have a maximal diameter of 25–30 μm, with a maximal nuclear diameter of 10 μm. Thirty to eighty neurons in each of the nuclei studied for each rat and a total of 180–2286 for each rat were counted. The mean optical density values and standard deviations of each nucleus at each age were then measured. The mean optical density values and standard deviations of each nucleus at each age were then obtained. Statistical comparisons were made by using both one-way ANOVA (to control for the type I comparisonwise error rate) and Tukey’s Studentized range test (to control for the type I experimentwise error rate), to be carried out between each successive pair of age groups. Significance was set at $P < 0.01$ for one-way ANOVA and $P < 0.05$ for Tukey’s test.

**RESULTS**

The locations of the brain stem nuclei examined are shown in Fig. 1. CO-reactive neurons in various nuclei exhibited dark, moderate, or light intensities of CO labeling. They also assumed multiple shapes, such as multipolar, oval, round, fusiform, or pyramidal. In general, neurons in all of the brain stem nuclei examined exhibited an increase in CO activity with age that coincided with an increase in cell size. In the neuropil, there was an increase in CO activity with a decrease in the number of CO-reactive processes with age.

_CO-reactive neurons in the NTSVL._ In general, there were 10–15 CO-reactive neurons in each section with high, moderate, or sometimes low levels of CO. These neurons were small or medium in size, and multipolar, fusiform, or pyramidal in shape (Fig. 2A). The neuropil

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exhibited rather high levels of CO after P7. NTSVL exhibited a developmental trend in CO activity similar to that in the PBC (see Refs. 22 and 23), i.e., against a general increase in CO activity with age, there was a plateau at P3–P4 and a prominent rise-fall-rise pattern between P11 and P13 (22–24). In addition, there was a fall-rise-fall pattern at P15–P17 not observed in the PBC (Fig. 3A).

CO-reactive neurons in the Amb. There were ~10–12 CO-reactive neurons in each section that were medium to small in size and mainly multipolar in shape. Medium-sized neurons tended to exhibit higher CO activity than small ones (Fig. 2B). The neuropil in Amb exhibited low levels of CO activity until after P14, when the level became more moderate. The developmental trend of CO activity in Amb (Fig. 3B) was similar to that in the NTSVL.

CO-reactive neurons in the XII. XII was composed of mainly medium and large neurons that were multipolar or fusiform in shape, with moderate to rather high levels of CO activity (Fig. 2C). There was a fast rise in CO activity from P0 to P7 (except for a decrease at P4), followed by fluctuations thereafter. A distinct rise-fall-rise pattern was seen at P11–P13 and a fall-rise-fall pattern at P15–P17 (Fig. 3C), similar to the trends after P9 in NTSVL and Amb.

CO-reactive neurons in the ROb. ROb was composed of mainly small neurons that were fusiform or multipolar in shape, with moderate or low levels of CO activity (Fig. 2D). The neuropil also exhibited relatively low CO levels. The developmental trend of CO had a pattern similar to those in NTSVL and Amb, except for a plateau at P2–P3 (rather than at P3–P4 as in NTSVL and Amb) (Fig. 3D).
CO-reactive neurons in the DMNX. CO-reactive neurons in DMNX exhibited low or moderate levels of CO activity. They were small and medium in size and fusiform or multipolar in shape (Fig. 2E). The neuropil in DMNX had rather low CO levels. The developmental trend of CO activity in DMNX was similar in pattern to those in NTSVL and Amb, except for a decrease at P3 (rather than a plateau at P3–P4) and a relatively lower CO level at P10–P11 (as compared with P9) (Fig. 3E).

CO-reactive neurons in the IOma. IOma was composed of mainly medium and small neurons with moderate to high levels of CO activity. They were multipolar, oval, or fusiform in shape (Fig. 2F). The neuropil in IOma also had moderate to high levels of CO. IOma neurons exhibited a sharp rise in CO activity from P0 to P5 (except for a plateau at P4), followed by a plateau at P6–P7 and a fall at P8. It then assumed a pattern similar to those in NTSVL and Amb, except for a dramatic increase at P14 (Fig. 3F).

CO-reactive neurons in the SPV. Neurons in SPV exhibited moderate to relatively high levels of CO activity. They were medium and small in size and multipolar, oval, or fusiform in shape (Fig. 2G). The neuropil in SPV had moderate levels of CO. As in IOma, SPV neurons had a fast rise in CO activity from P0 to P6 (except for a plateau at P4), followed by small
fluctuations and a pattern that was similar, although not identical, to those in NTSvL and Amb (Fig. 3G).

**CO-reactive neurons in the MVe.** MVe was composed of mainly small- and medium-sized neurons that were fusiform or multipolar in shape, with low to moderate levels of CO activity (Fig. 2H). The neuropil in MVe exhibited a moderate level of CO. MVe neurons underwent a slow increase in CO activity with age, with some plateau and limited fluctuations (Fig. 3H). The pattern of CO development was not comparable to those of the other nuclei examined.

**DISCUSSION**

In the brain stem, many nuclear groups contribute to the control of respiration. There are three main groups that are known to play a major role: 1) the ventral respiratory group, including PBC, Amb, nucleus retroambiguus, and the Bötzinger complex; 2) the dorsal respiratory group, consisting primarily of NTSvL; and 3) the pontine respiratory group, including medial and lateral parabrachial nuclei and Kölliker-Fuse nucleus (5, 17). The respiratory control network generates a basic rhythm, most likely from the PBC (15, 35, 39, 45), which is modified by the network under the influence of peripheral and central chemosensory afferents and higher brain structures. In turn, the network sends effenter signals to motor units for controlling inspiratory and expiratory muscles and upper airway muscles (5, 7, 17, 45). NTSvL relates peripheral chemosensitive afferents (13), as well as contributing to respiratory regulation (7). Amb, DMNX, and XII are involved in controlling upper airway muscles, the vagus, and the tongue, respectively, during respiration (17, 18). Rob consists of mainly serotoninergic neurons (20) that are thought to be involved in respiratory control (34) and central chemosensitivity (3, 21). The inferior olivary nucleus is connected mainly with the cerebellum, which may play a role in coordinating ventilatory skeletal muscles with upper airway muscles (20). SPV relates mainly to somatic sensations of the head and face, and it could be involved in some defensive reflexes, such as sneezing (28). MVe is concerned primarily with the function of balance and equilibrium, but it may play a role in regulating respiratory activity during movement (50). In short, most, if not all, of the brain stem nuclei examined may play some roles in influencing respiration. However, the degree of influence may be less prominent for IOma, SPV, and least for MVe. Thus these three nuclei are generally not included in the traditional respiratory control system (5, 7, 11, 45, 46). In the present study, we consider IOma, SPV, and MVe as nonrespiratory nuclei.

The respiratory control system undergoes postnatal development mainly within the first 2 wk of life in rats and mice. The time course, however, has not been explored in detail. The pattern of respiratory rhythm is adultlike at and after P15 in the mouse (32). The suppression of respiratory rhythmic activity after the blockade of synaptic inhibition occurs after, but not before, P15 (33), and the ventilatory response to hypercapnia is lower at P5 than after P16 (1). Dramatic changes in both the motor pattern of rhythmic hypoglossal neurons and the sensitivity of the respiratory rhythm to the blockade of glycine receptors also occur during the first 2 wk of life (31).

Relatively few reports have addressed the issue of postnatal development of the respiratory control nuclei. Most of the synaptic development in the NTS and Amb occurs postnatally (38). In XII, the total number of synapses is increased significantly from birth to P20 (843%), followed by a significant decrease in the adult (30%) (29). Glutamate binding densities in NTS, DMNX, inferior olive nucleus, and SPV peak at P9 and then decline gradually to reach levels close to that of the adult by P23–P30 (37). Less is known about the possible existence of critical periods of respiratory control system during postnatal development.

Studies on regional CO activity in the rat revealed that, in many brain structures including the brain stem, CO activity exhibits a significant increase between P10 and P14 and between P21 and P35 (6). CO concentration of the rat brain pool increases with age, with most of the changes occurring between P5 and P25 (9). These studies, however, did not reveal CO activity at the cellular level.

Our data indicate that, in most brain stem nuclei examined (except for MVe), CO activity exhibited a fast rise from P0 to P5–P7 (with a plateau or decrease at P3–P4), followed by minor fluctuations until P9, then assumed a trend similar to that in the PBC, with a prominent rise-fall-rise pattern at P11–P13. However, instead of the plateau between P15–P17 as in the PBC (22, 23), these nuclei had a fall-rise-fall pattern during that time, with the swing being less prominent in SPV and XII after P9. MVe exhibited a slower rise in CO activity with age, with some plateau and smaller fluctuations. Compared with our previous data (22–24), most brain stem nuclei examined followed a developmental trend in CO activity from P0 to P14 that was quite similar to that of the PBC, with a plateau or decrease at P3–P4 and a prominent rise-fall-rise pattern at P11–P13. We postulate that these nuclei (with varying contributions to respiratory functions) underwent a developmental trend in metabolic activity that was controlled by a mechanism similar to that in the PBC. Such mechanism probably involves the establishment and maturation of synapses. Among these nuclei, SPV and XII probably matured earlier than the others, as they exhibited gentler fluctuations in CO activity after P9. The fall-rise-fall pattern at P15–P17 found in most nuclei differed from the plateau seen in the PBC. This might have resulted from three possibilities: 1) pre- and/or postsynaptic mechanisms that exerted greater inhibitory influence on P15 in the brain stem nuclei that either did not involve the PBC or had achieved a more balanced state in the PBC; 2) other intrinsic functions that called for an abrupt adjustment of metabolic demands in the brain stem nuclei that either did not affect the PBC or affected it in a different way; and/or 3) the pacemaker neurons in the PBC may be protected from vulnerability during this
phase of postnatal development because the excitatory and inhibitory neurochemical composition has reached a more balanced state in this nucleus, and the nucleus is more mature functionally at this time.

What factors would contribute to such a postnatal developmental pattern in CO activity? Previously, we have suggested that a transient imbalance between excitatory and inhibitory drives (i.e., a lower excitatory drive and/or a higher inhibitory drive) might have accounted for a plateau at P3–P4 and a dramatic decrease in CO activity at P12 in the PBC of rats (22). The same may be true for most of the nuclei in the present study. Postnatal development of the respiratory control system may involve a transition from a predominantly excitatory mechanism to a greater reliance on inhibitory mechanisms (4, 33, 45) and a final adjusted balance between the two. For example, PBC neurons in adult animals in vivo (40, 42) exhibit larger inhibitory hyperpolarizations than in neonatal in vitro systems (8, 36, 43). Receptors of GABA and glycine in other brain regions and other species undergo subunit switches during development, such as from α2 to α1 (for GABA_A receptor) and α2 to α1 (for glycine receptor) (2, 14, 16, 44). Perhaps a switch in subunits is associated with changes in receptor subtypes that would mediate different functional properties of inhibitory neurotransmitters, such as primarily an excitatory effect in early life and an inhibitory effect thereafter (41, 51).

This hypothesis would address, at least in part, why CO activity in the brain stem nuclei exhibit a plateau or a decrease during postnatal development, especially at P12. In addition, one cannot exclude the possibility that programmed cell death plays a role in the postnatal development of CO activity in brain stem nuclei.

It is of interest that CO activity in ROb exhibited a plateau at P2–P3, rather than at P3–P4, as in most of the other nuclei studied. This may imply that ROb is one of the original sites that prompt the plateau of CO activity in the brain stem nuclei. In addition to respiratory control and central chemosensitivity, raphe also functions as one of the controllers of sleep and wakefulness (19). It is well known that sleep states can influence respiratory functions. For example, many changes in the respiratory system occur during sleep, including a reduction in respiratory motor output associated with the loss of wakefulness, increased upper airway resistance, blunted protective reflexes (such as load compensation) (30, 47), apnea during non-rapid eye movement sleep (25, 26), and lower CO2 sensitivity in non-rapid eye movement sleep (27). Thus, if the sleep-wakefulness system undergoes a developmental trend in CO activity similar to that in the respiratory control system, it would render the system to be at a higher risk in failing to overcome the stresses of respiratory insults during sleep and especially at the vulnerable period of postnatal development.

Results from our previous studies (22, 23) and the present one are consistent with our hypothesis that two vulnerable periods exist in the postnatal development of respiratory control system in the rat. In addition to the PBC, most of the brain stem nuclei examined also had a plateau or reduction of CO activity at P3–P4 and a prominent reduction at P12. Whether they relate to the maturation of the respiratory control network is unknown at present. It is interesting, however, that the nuclei that are more directly involved with respiratory control, the NTS_VL and Amb, exhibited the most dramatic fall in CO activity on P12, whereas those nuclei with possibly the least critical role in respiratory control, such as the SPV and especially the MVe, showed the least reduction of CO on that day. Taken together, the brain stem as a whole is operating at a markedly lower level of metabolic activity on P12 than on days immediately preceding or following it. During such critical periods, the animal may be less able to overcome the detrimental effect of exogenous respiratory insults. If such periods exist in human, and if exogenous stressors are introduced during such critical periods in an infant who has some vulnerable attributes, such as prematurity, abnormal development, prenatal exposure to nicotine and other toxins, and/or genetic defects, it is possible that catastrophic events, such as SIDS, may result.

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DISCLOSURES

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