Developmental changes in the expression of GABA<sub>A</sub> receptor subunits α1, α2, and α3 in the rat pre-Bötzinger complex

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Liu, Qili, and Margaret T. T. Wong-Riley. Developmental changes in the expression of GABA<sub>A</sub> receptor subunits α1, α2, and α3 in the rat pre-Bötzinger complex. J Appl Physiol 96: 1825–1831, 2004. First published January 16, 2004; 10.1152/japplphysiol.01264.2003.—Previously, we reported that the pre-Bötzinger complex (PBC) exhibited a dramatic reduction in cytochrome oxidase activity at postnatal day (P) 12. This coincided in time with decreases in glutamate and NMDA receptor subunit 1 and increases in GABA, GABA<sub>B</sub>, glycine receptor, and glutamate receptor GluR2. To test our hypothesis that various α-subunits of GABA<sub>A</sub> receptors also undergo changes in their expression during postnatal development, as they do in other brain regions, we undertook an in-depth immunohistochemical study of GABA<sub>A</sub> receptor subunits α1, α2, and α3 in the PBC of P0 to P21 rats. We found that 1) GABA<sub>A</sub> α3-subunit was expressed at relatively high levels at P0, which then declined with age; 2) GABA<sub>A</sub> α1-subunit was expressed at relatively low levels at P0 but increased with age; 3) the developmental trends of subunits α1 and α3 intersected at P12; and 4) GABA<sub>A</sub> α2-subunit expression was moderate to light at P0 and remained quite constant during development, being lowest at P21. These findings suggest that the apparent switch in relative expressions of subunits α3 and α1 during development and the intersection of slopes around P12 may be associated with possible changes in GABA<sub>A</sub> receptor subtypes that would mediate different functional properties of GABA<sub>A</sub> transmission, such as primarily a less efficient inhibitory transmission before P12 and a more mature inhibitory effect at P12 and thereafter, as suggested by the kinetics of distinct postsynaptic potentials. This mechanism may contribute partially to the dramatic reduction in cytochrome oxidase activity within the PBC at P12, as shown previously.

Development; respiratory control; brain stem; inhibition; vulnerable period

THE PRE-BÖTZINGER COMPLEX (PBC) has been postulated as the center of respiratory rhythmogenesis (17, 54, 57, 62). Previously, we reported that the metabolic activity revealed by cytochrome oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, as a marker of neuronal functional activity; Ref. 67) in the rat PBC exhibited a plateau on postnatal days (P) 3–4 (33, 36) and a prominent decrease on P12 (33), despite a general increase in COX activity with age. These changes were correlated with a concomitant reduction in the expression of glutamate and NMDA receptor subunit 1 and an increase in GABA, GABA<sub>B</sub>, and glycine receptors. The glutamate receptor GluR2, which reduces the permeability of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors to Ca<sup>2+</sup> and decreases neuronal excitation, also exhibited an increase during those two periods (33). Our data suggest that, during the postnatal development of rats, there may be two critical periods, P3–4 and especially P12, when the respiratory system may be under a stronger inhibitory than excitatory drive and may render the animal more vulnerable to exogenous stressor(s). Additionally, most of the brain stem nuclei with known or suspected respiratory control functions also exhibited dramatic decreases in COX activities at P12 (35).

Carotid body denervation at the two presumed vulnerable windows (close to P3 and P11–P13), but not at other times, induced a distinct delay as well as prolongation of the metabolic maturational process (32). This lends further support to the possibility of two vulnerable windows in postnatal development of the rat PBC. Furthermore, the prominent rise-fall-rise pattern of COX 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 determined genetically but modifiable by functional deprivation.

The prominence of inhibitory neurochemical expression during the presumed critical period of respiratory development led us to probe the GABAergic system further. GABA<sub>A</sub> receptors mediate the majority of fast inhibitory synaptic interactions in the adult mammalian brain. During early neonatal development, however, GABA, acting on GABA<sub>A</sub> receptors, provides most of the excitatory drive, whereas excitatory glutamatergic synapses are quiescent or less efficient (11, 18). Thus neurons’ responses to GABA shift from depolarization to hyperpolarization in many brain areas during late embryonic and early postnatal periods. This switch was postulated to result from a change in intracellular chloride concentration brought about by the maturation of chloride transport (53) and may occur in the late embryonic or early postnatal periods, depending on the neurons in question.

We hypothesized that perhaps a switch in GABA<sub>A</sub> α-subunits was associated with possible changes in receptor subtypes that would mediate different functional properties of GABA transmission. We, therefore, performed an in-depth immunohistochemical study with antibodies against GABA<sub>A</sub> receptor subunits α1, α2, and α3 on the PBC of P0–P21 rats, and immunoreaction product was analyzed by optical densitometry. A preliminary report of the findings appeared elsewhere (34).

MATERIALS AND METHODS

Tissue preparation. A total of 75 Sprague-Dawley rats from five litters was used in accordance with National Institutes of Health and Medical College of Wisconsin regulations. Rats were killed at postnatal days (P) 0, 2, 3, 4, 5, 7, 10, 11, 12, 13, 14, 15, 17, 19, and 21;
at every time point, five rats from five different litters were used. Rats were deeply anesthetized with 4% chloral hydrate (0.1 ml/10 g ip, Fisher Scientific, Fair Lawn, NJ) and perfused through the aorta with warm buffered saline followed by cold (4°C) 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.35, and 4% sucrose. The brain stems were removed and postfixed by immersion in the same fixative for 3 h at 4°C. They were then cryoprotected in increasing concentrations of sucrose (10, 20, 30%) in 0.1 M PBS at 4°C, frozen in dry ice, and stored at −80°C until use.

**Immunohistochemistry.** Coronal sections of frozen brain stems were cut at 12-μm thickness with a cryostat. For each time point, four sets of serial sections [for neurokinin 1 receptor (NK1R) and GABA<sub>A</sub> receptor subunits α1, α2, and α3] were mounted on gelatin-coated slides. The sections 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). They were then incubated at 4°C for 36 h in the primary antibodies diluted at the appropriate concentrations in the same solutions as used for blocking. The dilutions were 1:10,000 for NK1R (Sigma Chemical, St. Louis, MO), 1:100 for GABA<sub>A</sub> receptor subunit α1 (Sigma Chemical), 1:500 for GABA<sub>A</sub> receptor subunit α2 (Chemicon, Temecula, CA), and 1:2,000 for GABA<sub>A</sub> receptor subunit α3 (Sigma Chemical). Sections were incubated in the secondary antibodies: goat anti-rabbit IgG-HRP (Bio-Rad Laboratories, Hercules, CA) at 1:100 dilution in the modified blocking solution (without Triton X-100) for 4 h at room temperature. Immunoreactivity was detected with 0.05% DAB-0.004% H<sub>2</sub>O<sub>2</sub> in PBS (pH 7.4) for 5 min, and the reaction was stopped with cold PBS (pH 7.4). Sections were then washed with cold 0.1 M PBS (pH 7.4) three times, dehydrated, and coverslipped.

**Quantitative densitometry.** Optical densitometric measurements of reaction product of immunohistochemistry 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. The white matter was used as an internal standard for measurements because of its very low level of immunoreactivity. Thus the white matter was set at zero for each section measured. The optical densitometric value of each neuron in the PBC was an average reading of two to four spots in the cytoplasm of cell bodies. Thirty to eighty neurons in the PBC for each rat, and a total of 150–350 neurons for each marker at each age, were measured. The mean optical density values and standard deviations of each marker at each age were then obtained. Statistical comparisons were made between successive age groups (e.g., P0 vs. P2, P2 vs. P3, P5 vs. P7) 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). Significance was set at P < 0.01 for one-way ANOVA and P < 0.05 for Tukey’s test.

**RESULTS**

**NK1R-immunoreactive neurons in the PBC.** NK1R was used as a marker to identify the PBC, which is known to lie ventral to the nucleus ambiguus, caudal to the retrofacial nucleus, and rostral to the anterior tip of the lateral reticular nucleus (19, 20, 66). The expression of NK1R in this study was similar to that in our previous reports (33, 36).

**GABA<sub>A</sub> receptor subunit α1-immunoreactive neurons in the PBC.** GABA<sub>A</sub> receptor subunit α1 expression was relatively low at P0, with light labeling of the cytoplasm and processes in small neurons. The expression in the cytoplasm increased with age, and, as neurons increased in size, there were more cell membrane and neuropil labeling (Fig. 1). Quantitative densitometry showed that the expression of subunit α1 was low in the PBC at birth and gradually increased with age, with a peak at P12 followed by a slight drop at P13 and a plateau between P14 and P19, then increasing again at P21 (see Fig. 4, A and D).

**GABA<sub>A</sub> receptor subunit α2-immunoreactive neurons in the PBC.** GABA<sub>A</sub> receptor subunit α2 expression was light to moderate in the cytoplasm with a few lightly labeled processes at P0 and a mild decrease during development, being lowest at P21 (Fig. 2). With age and increasing neuronal size, the
expression of subunit α2 decreased in the processes (Fig. 2), especially after P10. Quantitative densitometry showed that the expression of subunit α2 remained at relatively constant levels during development, with relatively higher levels between P0 and P10 and relatively lower levels from P11 to P21 (see Fig. 4, B and D).

**GABA<sub>A</sub> subunit α3-immunoreactive neurons in the PBC.** GABA<sub>A</sub> receptor subunit α3 was expressed at moderate to high levels at P0, mainly in the cytoplasm and membranes of neuronal cell bodies and sometimes of processes. With age and increasing size of neurons, the expression declined in the cytoplasm, processes, and nuclei (Fig. 3), and few processes

**Fig. 2.** GABA<sub>A</sub> receptor subunit α2-immunoreactive neurons in the PBC at P0 (A), P7 (B), P12 (C), and P21 (D). Immunoreactivity for GABA<sub>A</sub> receptor subunit α2 demonstrates a relatively constant level in the cytoplasm during postnatal development, with a slight reduction at P10 (not shown) and thereafter. Immunoreactivity in the processes decreased with age. Scale bar: 20 μm.

**Fig. 3.** GABA<sub>A</sub> receptor subunit α3-immunoreactive neurons in the PBC at P0 (A), P7 (B), P12 (C), and P21 (D). Immunoreactivity for GABA<sub>A</sub> receptor subunit α3 decreased in the cytoplasm, processes, and nuclei with increasing age. Immunoreactivity at P12 and P21 was dramatically lower than that at P0 and P7. Scale bar: 20 μm.
were observed after P10. Quantitative densitometry showed that the expression was higher in PBC at birth and then decreased with age, with a dramatic drop at P12 followed by a slight fluctuation between P14 and P19 and was lowest at P21 (Fig. 4, C and D).

Thus a dramatic drop in the expression of subunit α3 and a peak in the expression of subunit α1 both occurred at P12, forming a crossroads for the developmental trends of the two α-subunits. After P12, subunit α3 expression never reached higher than that at P12, and subunit α1 expression was maintained at relatively high levels similar to that at P12. P12 is, therefore, a very important time point in the postnatal development of GABA<sub>A</sub> receptors in the rat PBC.

**DISCUSSION**

The present data showed that 1) GABA<sub>A</sub> α3-subunit was expressed at relatively high levels at P0, which then declined with age; 2) GABA<sub>A</sub> α1-subunit, on the other hand, was expressed at relatively low levels at P0 but increased with age; 3) the developmental trends of GABA<sub>A</sub> receptor subunits α1 and α3 intersected at P12; and 4) GABA<sub>A</sub> α2-subunit expression was between light to moderate levels at P0 and remained at relatively constant levels during development, being lowest at P21. Our study indicated that, during the early stages of postnatal development of the PBC, α3 was a predominant α-subunit in GABA<sub>A</sub> receptors; however, α1 replaced α3 to become the predominant α-subunit in GABA<sub>A</sub> receptors after P12.

One possibility for the observed changes is that the antigenic properties of GABA<sub>A</sub> subunits change during development. However, there are no published or known documentations to date to support such a change. If the amino acid composition and/or availability of the epitope should vary with development, it would serve as an additional indication that GABA<sub>A</sub> receptor subunit composition undergoes alterations during development.

Depolarization induced by GABAergic and glycinergic neurotransmission has been observed in immature neural systems, such as the spinal cord (16, 18, 48, 56, 68), brain stem (25, 31, 41), olfactory bulb (59), hippocampus (4, 12, 13, 24), hypothalamus (10, 18), and the neocortex (14, 37, 51, 69). Such depolarization can result in action potentials when the depolarization reaches the spike threshold, and it often induces an increase in cytoplasmic Ca<sup>2+</sup> concentration (31). These events are postulated to function mainly as trophic signals (3) for facilitating synapse formation (5, 12, 27), regulating the rate of neurite outgrowth (39), promoting morphological differentiation (6, 29, 40), and/or increasing the motility of migrating neurons (2).

Most of the fast inhibitory synapses in the central nervous system express the GABA<sub>A</sub> receptor type that appears in a variety of structurally and functionally different subtypes (21). The native GABA<sub>A</sub> receptor is a heteropentameric protein, most often consisting of two α (α 1–6), two β (β1–3), and a fifth subunit, which can be either a third β, a γ (γ1–3), a δ, or a ρ (ρ1–2) (28). The functional characteristic of GABA<sub>A</sub> receptor is determined by the structure and assembly of different subunits. Developmental changes in the subunit composition of GABA<sub>A</sub> receptors appear to be more pronounced among the α-subunits than among the other subunits, and more
commonly a switch occurs from α2 (or α3, such as in the visual cortex) at earlier periods of life to α1 in the adult (8, 15, 22, 30). Even in the human brain, α1-subunit demonstrates a dramatic increase from birth to 4 mo of age (55). Thus subunit composition of α2/β2/γ2 may be a predominant GABA_A receptor subtype in the neonatal brain, whereas α1/β2/γ2 is the most prevalent subtype in the adult (7, 15, 22). It has been postulated that subunit composition with α2 (or α3) would contribute to the depolarizing GABA transmission (22), whereas the onset of α1-subunit expression in the maturing brain is an indicator of the expression of a new, prevalent GABA_A receptor subtype involved in synaptic inhibition (15). Although no direct information is available yet about the relationship between subunit composition and postsynaptic potentials mediated by GABA_A receptors, the fact that α2- and/or α3-subunit is expressed predominantly in neurons in which excitatory GABA_A receptors have been reported, in both the developing and adult brains (13, 41, 42, 56), supports that relationship. Furthermore, the increase in intracellular calcium observed in immature rat cortical neurons induced by GABA application disappears when GABA_A receptor α2-subunit is replaced by α1-subunit (31). Genesis of mature-type synapses accelerates dramatically after P12 (1), at which stage GABA is postulated to be the main inhibitory neurotransmitter (11).

The time course of switch from depolarizing to hyperpolarizing GABA transmission and the timing of the replacement of GABA_A receptor α2 (or α3)-subunit by α1-subunit vary among different species, different brain regions, and different neuronal types. Additionally, many studies did not follow a close time course to demonstrate the alterations. Thus previous data only offered a rough idea about the time course of switch. In rats, a switch from α2- to α1-subunits occurred during the first postnatal week in the mesencephalon and hypothalamus and during the second and third weeks in the thalamus, pallidum, and medulla (15). GABA_A receptor subunit mRNA studies (with in situ hybridization) demonstrated in the rat thalamus that the switch from α2 to α1 occurred between P6 and P12 (30). However, in the marmoset monkeys, this switch occurred before embryonic day 130 in the pallidum and around the first postnatal week in the thalamus (22). By means of subunit-specific pharmacology and electrophysiological recordings, a switch from α3- to α1-subunit was found in layers II–III neurons of the rat visual cortex between P6 and P21 (8). In mice, GABA (not glutamate) is the primary transmitter driving action potentials in the developing (P1–P4) hypothalamus and spinal cord neurons, whereas at P10 and thereafter glutamate is the predominant excitatory transmitter to drive action potentials in these two structures (18). Intracellular recordings showed that, between P0 and P8, most rat CA3 hippocampal neurons (~85–93%) displayed spontaneous giant depolarizing potentials, whereas at P11–P12, only 25% of the neurons showed these phenomena and no giant depolarizing potentials after P12 (4). Until P8–P12, GABA apparently mediates neuronal depolarization rather than hyperpolarization through GABA_A receptors (11).

For respiratory control systems, studies on the murine PBC showed that the reversal potential of GABA_A receptor subunits found in the present study may contribute to alterations in the kinetics of postsynaptic potentials mediated by GABA_A receptors, rather than to the reversal potential of GABA_A receptor mediated current, in affecting the function of GABA transmission. In the rat visual cortex, developmental changes in α-subunit expression coincide with alteration in the decay time constants of spontaneous inhibitory postsynaptic currents, with α3-subunit being responsible for slow decay time at early stages and α1-subunit for fast decay time in the adult (8). The same is true in the mouse superior colliculus (α3/α1) (25) and the rat thalamus (α2/α1) (49).

The switch in α-subunit expression in the PBC may be related to its functional maturation. Although there is no known switch from excitatory to inhibitory postsynaptic potentials in the rat PBC, functional changes in the rat PBC may still occur during postnatal development. We hypothesize that, during early stages of development (probably before P12 in the rat), the inhibition mediated by neonatal GABA_A receptor subtypes (with α3-subunit being the predominant one in the composition) would be less efficient, as GABAergic synaptic transmission is primarily constrained by the slow kinetics, slow decay time, and the saturation of postsynaptic GABA_A receptors. At P12 and thereafter, the inhibition mediated by adult GABA_A receptor subtypes (with α1-subunit being the predominant one in the composition) would dramatically increase in its efficiency due to its fast kinetics and faster decay time.

A fragile balance between excitation and inhibition maintains the normal functioning of the central nervous system. Small changes in GABA-mediated inhibition can profoundly alter neuronal excitability (43). Thus a dramatic increase in the efficiency of inhibition mediated by GABA_A receptor at P12 (based on the switch of GABA_A receptor α-subunits) may result in a transient imbalance between excitation and inhibition, i.e., increased inhibitory drives and decreased excitatory drives. This may partially contribute to reduced COX activity at P12 in the PBC (34). Transmitter systems other than GABA, such as glycine, glutamate, and serotonin, may also play important roles during the critical period formation, as they also undergo marked changes in gene expression during rat brain development (38, 44, 52, 63, 65). Indeed, reduced protein expression of glutamate and NMDA receptor subunit 1 and increased expression of glycine receptor and GluR2 occurred at P12, coincident with heightened expression of GABA and GABA_B receptor (33). Any ontogenetic effects of the GABAergic system most likely involve interactions with the other systems. At P13, the respiratory control network may have attained a greater balance between excitation and inhibition and continues to function with a more mature organization.

Thus an imbalance between excitatory and inhibitory drives transiently occurs at and around P12 in the rat, resulting in a markedly lower level of metabolic activity on P12. During such critical periods, the animal may be less able to overcome the detrimental effects of exogenous respiratory insults. If such periods exist in humans and if exogenous stressors are introduced during such critical periods in an infant that has some vulnerable attributes (such as prematurity, abnormal development, prenatal exposure to nicotine and other toxins, and/or genetic defects) and especially during sleep [when respiratory control system are suppressed (45–47, 50, 64)], it is possible...
that catastrophic events, such as sudden infant death syndrome, may result.

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REFERENCES


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