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MORPHOLOGICAL BASICS FOR EVOLUTION OF FUNCTIONS

Formation of GABA-Aα1 and GABA-B1 Receptor-Mediated Inhibitory Network in the Ventrolateral Part of the Solitary Tract Nucleus during the Early Postnatal Period under Normal Conditions and Prenatal Serotonin Deficiency

L. I. Khozhai

Pavlov Institute of Physiology, Russian Academy of Sciences , St. Petersburg, Russia
e-mail: astarta0505@mail.ru

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Abstract—Time course of formation of the inhibitory receptor network (GABA-Aα1 and GABA-B1) in the respiratory subnuclei (ventral and lateral) of the solitary tract (NTS) during the early postnatal period was studied on laboratory Wistar rats under normal conditions and prenatal serotonin deficiency. It was found that in normal rats the maturation of the inhibitory receptor networks in both NTS subnuclei occurs within the first three postnatal weeks. Some features of their formation were noticed. The dynamics of changes in intensity of GABA-Aα1 expression in the ventral and lateral subnuclei proceeds in a similar way. During the first postnatal week, GABA-Aα1 expression is low. In the neuropil, the network of GABA-Aα1-containing presynaptic terminals and synapses is poorly developed. Within the second week, the number of GABA-Aα1-expressing neurons increases in both subnuclei with a simultaneous rise in the density of the network of terminals and synaptic structures. By the end of the third week, the number of GABA-Aα1-expressing neurons decreases, but the network density continues to increase. GABA-B1 expression in the ventral and lateral subnuclei occurs also simultaneously, although with some distinctions. During the first postnatal week, intensity of GABA-B1 expression is weak. In the neuropil, few GABA-B1-containing terminals form a loose network with sporadic synaptic structures. During the second week, expression of the receptor increases, being particularly considerable in the ventral subnuclei. Simultaneously, the density of the presynaptic terminals increases. By the end of the third week, the number of GABA-B1-expressing neurons in the ventral subnuclei decreases, while in the lateral subnuclei remains almost intact and the network density increases. The data obtained show that prenatal serotonin deficiency leads to malformation and impaired maturation of the GABAergic inhibitory neuronal network in the ventrolateral part of NTS.

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Key words: ventral and lateral respiratory subnuclei, solitary tract nucleus, GABA-Aα1 receptor, GABA-B1 receptor, terminal, synapse.

INTRODUCTION

The ventrolateral part (ventral and lateral subnuclei) of the solitary tract nucleus (NTS) is incorporated into the bulbar respiratory center, which is regulated by a compound mechanism including a set of "classical" neurotransmitters (GABA, serotonin, glycine, glutamate, peptides) and their

specific receptors. A major inhibitory neurotransmitter is γ-aminobutyric acid (GABA). In adult animals, GABAergic NTS neurons are scattered diffusely throughout its caudal part, while its ventrolateral part was shown to contain GABAergic terminals and synapses [1–3].

Data obtained in physiological experiments demoNTSrated that GABA transmission exerts a strong inhibitory effect on activity of NTS neurons [4] and, moreover, plays an important role in sustaining normal respiratory rhythm and inspiratory pattern both in adult and neonatal animals [5, 6].

A considerable role in GABA transmission that provides inhibitory effects is played by the receptor link represented by two classes of receptors, GABA-A and GABA-B, suggested to be activated by GABA itself [7]. GABA-A receptors are ionotropic, and their activation results in hyperpolarization of the cell membrane due to influx of chlorine ions [8]. GABA-A receptors were found in the medulla oblongata during the late prenatal period prior to maturation of presynaptic terminals. GABA-A receptors are believed to be essential for activity of the respiratory neuronal network [2]. The second group of GABA receptors is represented by metabotropic GABA-B receptors, having a heterodimeric structure and consisting of two subunits, GABA-B1 and GABA-B2, activated by lower concentrations of GABA than GABA-A receptors [9, 10]. It was established that these subunits are alike and functionally inseparable, since in the absence of GABA-B1 subunits GABA-B receptor does not respond to the effect of agonists [11]. It was also found that GABA-B receptors, localized to the postsynaptic membrane, are involved in the activation of G protein-coupled K^+ channels by inducing hyperpolarization of the neuronal membrane in the form of a slow inhibitory postsynaptic current [12, 13].

The ventrolateral part of the solitary tract nucleus is thought to play a central role in controlling the respiratory function [2], however, it is little known at present about the formation in this part of the local inhibitory GABAergic receptor network during the early postnatal period, when the respiratory function in mammals and humans undergo maturation. It was determined that in adult animals the GABAergic and serotoninergic (5-HT) systems influence many neuronal populations in the medulla oblongata, controlling thereby general neurotransmitter homeostasis in this area [14, 15]. It was shown that not only GABA but serotonin also directly regulates activity of respiratory neurons in the NTS ventrolateral part via the projections from the caudal 5-HTergic raphe nuclei with the involvement of the 5-HT1A and 5-HT1B receptor link [14–16]. However, it is extremely little known about the effect of serotonin on the formation of the inhibitory receptor network during the early developmental period. In this connection, the purpose of this work was to study the dynamics of the formation of the inhibitory GABAergic receptor network in the early postnatal period under normal conditions and prenatal serotonin deficiency.

MATERIALS AND METHODS

The work was carried out on laboratory Wistar rats kept and handled in compliance with the Regulations for conducting works with the use of experimental animals (Order no. 755 of 12.08.1977 of the USSR Ministry of Public Health). The endogenous serotonin level was reduced by inhibiting tryptophan hydroxylase (serotonin-synthesizing enzyme) with the aid of p-chlorophenylalanine (pCPA) (Sigma, USA). At a dose of 400 mg/kg, pCPA was injected intraperitoneally to female rats at day 9 of gestation, resulting in 50–80% reduction in the serotonin level during the formation of the own fetal serotoninergic system. The brain was studied in neonate rat pups on postnatal days 5, 9, and 20 (P5, P9 and P20). As a control, the respective age animals obtained from intact females were used. By 5–6 pups, both control and experimental, were taken for each age group. The material was fixed in zinc/ethanol/formaldehyde in PBS (pH 7.4), conventionally paraffin embedded, and serially sectioned. Transverse sections of the medulla oblongata, 5 μm thick, were cut at the level between −12.24 mm and −12.36 mm relative to bregma [17]. Immunocytochemical reactions for neurons expressing GABA-Aα1 and GABA-B1 receptors were conducted using rabbit polyclonal antibodies to GABA-A Receptor alpha 1 and GABA-B Receptor 1 (Abcam, USA), respectively. The secondary reagents for GABA-A Receptor alpha 1 and GABA-B Receptor1 came from

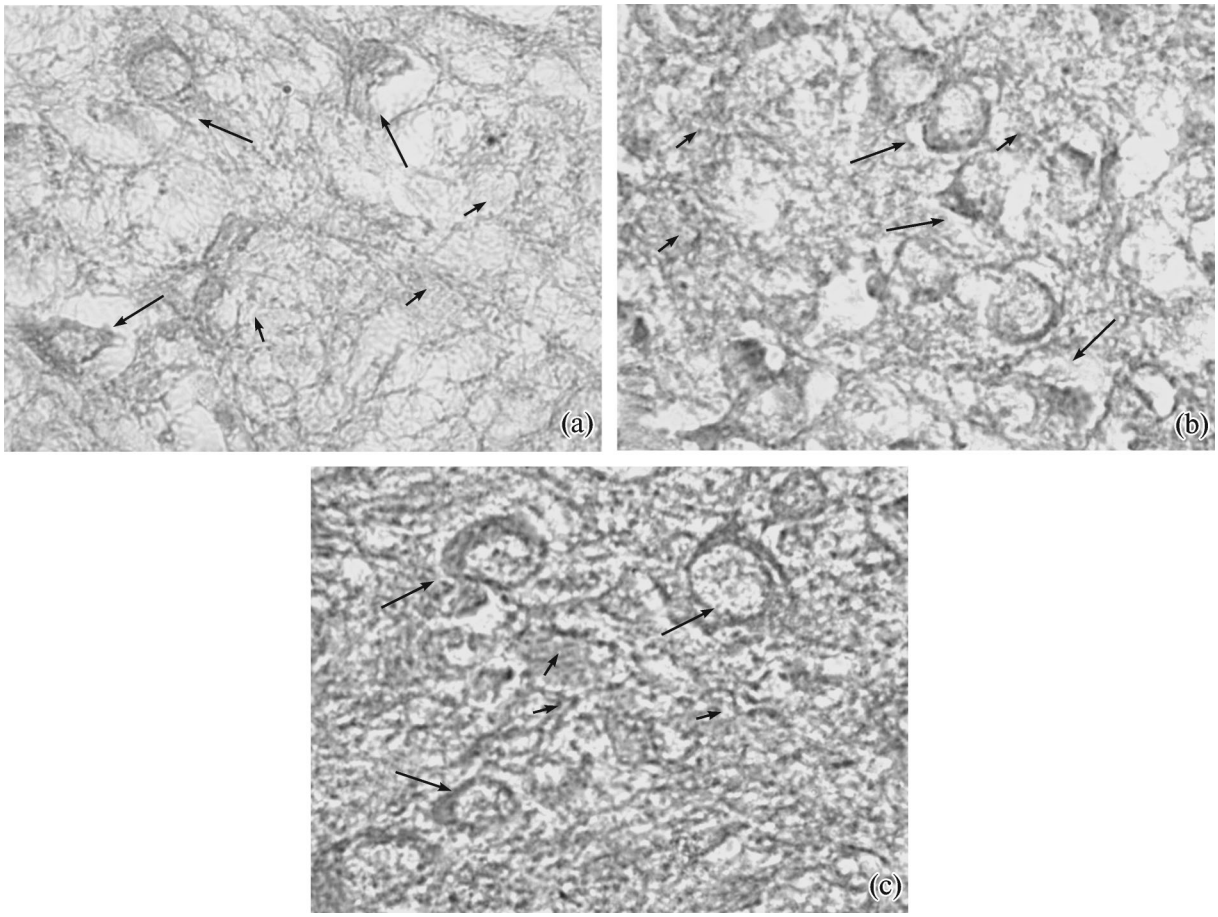

Fig. 1. Rat medulla oblongata, ventral NTS subnucleus in control. Immunocytochemical reaction for GABA-Aα1 receptor: (a)—P5, (b)—P9, (c)—P20; immunopositive neurons (*long arrows*), network of presynaptic terminals and granules (*short arrows*). Magn.: oc. ×10, ob. ×100.

a LSAB2 System-HRP kit (Dako, Denmark). A chromogen DAB+ (Dako, Denmark) was used to visualize the reaction product. After immunocytochemistry, a part of sections were additionally stained with Mayer's hematoxylin (Bio-Optica, Italy) or thionin (Serva, USA, Germany) and mounted in a Permount (Termo, USA). The conditions for immunocytochemistry were standardized, while all immunostaining procedures were synchronized both for experimental and control materials.

Morphological analysis and quantification procedures were conducted on digital serial sections obtained with the use of a Leica DME light microscope (Leica, Germany) and Leica EC3 digital camera (Leica, Germany).

Count of GABA-Aα1 and GABA-B1 immunopositive neurons was performed on 10 serial sections of the medulla oblongata obtained from 3–4 animals of each experimental age both in experimental and control groups. On a standard section square equal to 0.105 mm^2 at an objective magnification of ×40, the mean number of immunopositive neurons (± SEM), density of the network of immunoreactive terminals in the neuropil, distribution of granules thought to be synaptic structures, granule aggregations, and the number of varicosities indicative of terminal immaturity [18] were counted. Statistical data processing was fulfilled using applied computer programs Statistica 6.0, ImageScope Color and ORIGIN50. Significance of differences was determined by a Student's *t*-test value. Differences were considered significant at $p < 0.05$.

RESULTS

Distribution of GABA-Aα1 immunopositive neu-

rons, terminals and synaptic structures in the ventral NTS subnucleus in control rats. On P5, 4.1 ± 0.8 immunopositive neurons were revealed in the subnuclei per section. The neuropil contains the loose network of immunoreactive terminals and large solitary granules. No granules were found on the neuronal cell bodies. On P9, the number of immunopositive neurons per section rises up to 11.0 ± 0.6 (by 2.7 times), while the reaction intensity in the cytoplasm is quite high. The neuropil contains a network of immunoreactive terminal processes and large granules. On P20, the number of immunopositive neurons dwindles to 7.2 ± 0.8 per section. In the neuropil, the immunoreactive processes form a dense network; a plenty of large granules are present around (Fig. 1).

Distribution of GABA-Aα1 immunopositive neurons, terminals and synaptic structures in the lateral NTS subnucleus in control rats. On P5 stage, there are 7.6 ± 0.3 neurons per section. The neuropil exhibits a loose network of immunoreactive processes and few large granules. Solitary granules are present on the neuronal cell bodies. On P9, 8.7 ± 0.5 immunopositive neurons are revealed per section. In the neuropil, the network of immunopositive terminals is denser than at the previous stage; the number of large granules increases, and they appear on the cell bodies of most neurons in the amount of 2 to 5. On P20, in the subnuclei there are 5.2 ± 0.4 immunopositive neurons per section. In the neuropil, the dense network of GABA-Aα1-containing terminal processes and large granules appear. From 2 to 4 large granules reside on the bodies of uNTSained neurons.

Distribution of GABA-B1 immunopositive neurons, terminals and synaptic structures in the ventral NTS subnucleus in control rats. On P5, the ventral subnucleus contains 4.2 ± 0.6 neurons per section with a very weak immunostaining of the cypolasm. In the neuropil, there is a loose network of immunoreactive processes and solitary granules observed both on the neuronal cell bodies and on the processes. On P9, the number of immunopositive neurons increases approximately by 4 times, averaging 16.0 ± 0.7 cells per section. In the neuropil, the density of the network of terminals and granules increases, while the granules on the neuronal cell bodies are sporadic. On P20, the number of immunopositive neurons in the subnuclei decreases (roughly by 1.5 times), however, still remains high enough, averaging 10.3 ± 0.4 cells per section. The network of immunmopositive processes and large granules becomes even denser than at the previous stage under study.

Distribution of GABA-B1 immunopositive neurons, terminals and synaptic structures in the lateral NTS subnucleus in control rats. On P5, 5.1 ± 0.3 neurons in the lateral subnucleus exhibit a weak immune reaction of the cytoplasm. In the neuropil, there is a loose network of immunopositive processes and few granules, both on the processes and cell bodies. On P9, the number of immunopositive neurons increases up to 8.4 ± 0.5 per section. In the neuropil, the density of the network of immunopositive terminals increases; the same does the number of granules, but on the cell bodies they are sporadic. On P20, 9.6 ± 0.3 immunoreactive neurons per section are present in the subnucleus. The network density in the neuropil increases considerably.

Distribution of GABA-Aα1 immunopositive neurons, terminals and synaptic structures in the ventral NTS subnucleus in rats exposed to prenatal serotonin deficiency. On P5, the ventral subnucleus contains 2.7 ± 0.4 immunopositive neurons per section with a very weak immunoreactivity of the cytoplasm. The neuropil exhibits a loose network of terminals with solitary large granules lacking on the neuronal cell bodies, and no varicosities. On P9, 11.2 ± 0.6 immunopositive neurons per section are revealed. In the neuropil, the density of immunoreactive terminals and large granules is higher than on P5, while granules are solitary and lacking on the neuronal cell bodies. On P20, there are 6.7 ± 0.7 weakly immunopositive neurons per section. The neuropil contains a loose network of immunoreactive terminals and few large granules (Fig. 2).

Distribution of GABA-Aα1 immunopositive neurons, terminals and synaptic structures in the lateral NTS subnucleus in rats exposed to prenatal serotonin deficiency. On P5, the lateral subnucleus has 1.8 ± 0.6 immunopositive neurons per section. The neuropil contains a loose network of immunoreactive terminals free of any varicosities and few large granules. The neuronal cell bodies bear no granules. On P9, the number of immunopositive neurons increases up to 8.7 ± 0.6 per section. In

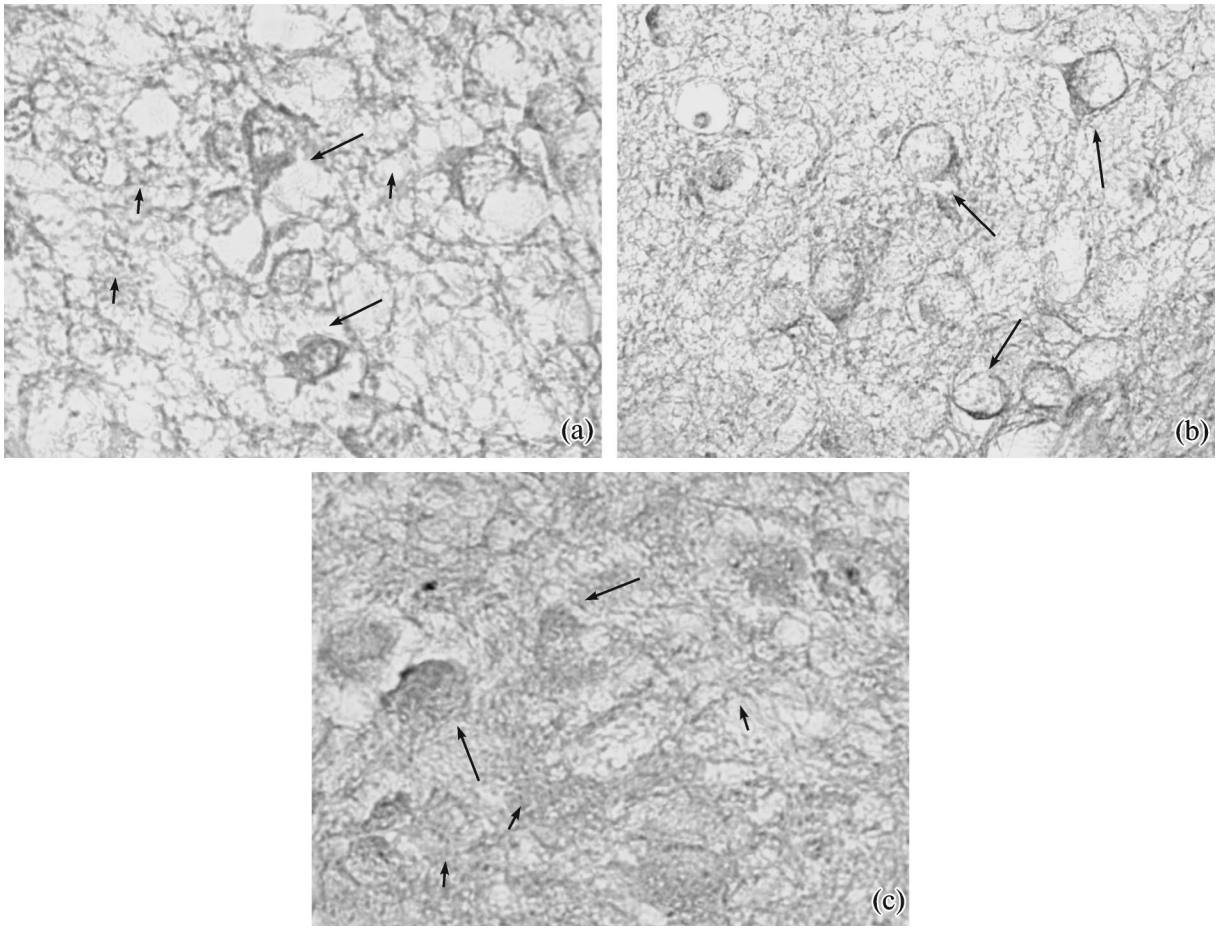

Fig. 2. Rat medulla oblongata, ventral NTS subnucleus in rat exposed to prenatal serotonin deficiency. Immunocytochemical reaction for GABA-Aα1 reaction: (a)—P5, (b)—P9, (c)—P20; immunopositive neurons (*long arrows*), network of presynaptic terminals and granules (*short arrows*). Magn.: oc. ×10, ob. ×100.

the neuropil, the density of the network of terminals increases, while few granules are spaced singly, lacking on the cell bodies. On P20, the number of immunopositive neurons dwindles to 5.7 ± 0.6 per section. The neuropil has a loose network of terminals and sparse large granules.

Distribution of GABA-B1 immunopositive neurons, terminals and synaptic structures in the ventral NTS subnucleus in rats exposed to prenatal serotonin deficiency. On P5, the ventral subnucleus contains 2.3 ± 0.4 immunopositive neurons per section with a small volume of the stained perinuclear cytoplasm. In the neuropil, there is a very loose network of terminals with multiple varicosities and solitary large immunoreactive granules. On P9, the ventral subnucleus has 4.8 ± 0.7 immunopositive neurons per section. The volume of cytoplasm is small, while neurons are retarded in their development versus their control counterparts. The neuropil contains a loose betwork of terminals with varicosities and sparse large granules. On P20, there are 5.7 ± 0.6 immunopositive neurons per section. In the neuropil, the loose network of immunoreactive terminals lacks any varicosities, while large granules are few, being located mainly on the processes.

Distribution of GABA-B1 immunopositive neurons, terminals and synaptic structures in the lateral NTS subnucleus in rats exposed to prenatal serotonin deficiency. On P5, 3.3 ± 0.2 immunopositive neurons with a very weakly stained cytoplasm are present per section. In the neuropil, there is a loose network of immunoreactive terminals with varicosities and sparse large granules (2–3 on cell bodies and in the neuronal cytoplasm). On P9, 4.7 ± 0.6 immunopositive neurons are revealed per section. Neurons look developmentally retarded and have a small volume of cytoplasm. In

the neuropil, there is a loose network of immunoreactive terminals, some of which have varicosities. The large granules are few (1–3 on cell bodies). On P20, there are 6.0 ± 0.8 immunopositive neurons per section. The neuropil has a loose network of immunoreactive terminals bearing sparse large granules.

DISCUSSION

Our results show that in rats maturation of the inhibitory receptor (GABA-Aα1 and GABA-B1) networks in the ventrolateral part of NTS occurs during the first three postnatal weeks, while each week is characterized by its distinctive features. Changes in intensity of GABA-Aα1 expression in the ventral and lateral subnuclei in the early postnatal period proceed in a similar way. During the first week, there occurs weak GABA-Aα1 expression, as evidenced by a small number of GABA-Aα1-immunopositive neurons. In the neuropil a loose network of terminals and solitary receptor-expressing synaptic structures can be revealed. During the second week, the number of immunopositive neurons in both subnuclei increases approximately by 2.5 times, while the density of the network of GABA-Aα1-containing terminals and synaptic structures rises. By the end of the third week, the number of GABA-Aα1-expressing neurons decreases approximately by 1.5 times, and a dense network of terminals and synaptic structures forms in the neuropil.

Our study demoNTSrates that during the early postnatal weeks expression of GABA-B1 receptorsoccurs simultaneously both in the ventral and lateral NTS subnuclei, but changes in the intensity of expression during this period occur dissimilarly. In the first week, both subnuclei exhibit a low intensity of GABA-B1 expression, as evidenced by an equally small number of GABA-B1-immunoreactive neurons. The neuropil also contains a loose network of terminals and single synaptic structures containing GABA-B1. During the second week, the number of GABA-B1-expressing neurons increases: by 4 times in the ventral subnuclei and by 1.6 times in the lateral one. At the same time, the density of receptor-containing terminals and synaptic structures rises in the neuropil of both subnuclei. Be the end of the third week, the number of GABA-B1-expressing neurons in the ventral subnuclei dwindles by 1.5 times, remaining almost intact in the lateral subnuclei. Simultaneously, the density of the network of terminals and synaptic structures in both subnuclei considerably increases.

Similar dynamics of GABA-A and GABA-B receptor expression during the early postnatal period occurs in the gustatory subnuclei situated in the NTS rostral part. We found a gradual increase in the number of neurons expressing both receptors and in the density of the network of terminals and synaptic structures. Although changes in expression of both receptors occur almost synchronously, it was noticed that maturation of GABA-A receptors occur a little earlier than that of GABA-B receptors. Synapses containing GABA-A receptors are believed to be the earliest in their emergence in neonatal rats [19, 20]. Such an early maturation of GABA-A synapses is consistent with the formation of physiological functions during this developmental period [21]. The results of the present study show that fluctuations in GABA-Aα1 receptor expression during the first three postnatal weeks occur almost simultaneously in both subnuclei as compared to GABA-B1 expression. A sharp increase in the number of receptor-expressing neurons during the second week may be explained by the intense formation in the neuropil of a dense network of GABA-A- and GABA-B-containing terminals and synaptic structures during this period.

It is believed that formation and maturation of GABA receptors is determined by the influence of GABA itself on these processes [20]. It was shown that in the rostral NTS part during the early postnatal weeks GABA is present in the cytoplasm of many neurons and processes, while the neuropil contains few GABA-containing synaptic structure [22]. The excess of GABA and deficit of GABAergic terminals is thought to be indicative of the fact that during this period GABA fulfills not a neurotransmitter but neurotrophic function [19]. It was demoNTSrated that in the rostral NTS part the total volume of GABA decreases by P20, while the number of synaptic structures, including GABAergic synapses, increases up to their level in adult animals [22]. Presumably, during the first postnatal days the GABAergic synaptic structures

in the medulla oblongata are immature. These observations were supported electrophysiologically [13, 19, 23].

Maturation of GABA receptors is believed to be crucial for the transition from non-neurotansmitter to neurotransmitter GABA functions [19]. An electron microscopic study showed that in the rostral NSP part GABA-A and GABA-B receptors can already be revealed in neonate animals, but the formation of the receptor-containing synaptic structures occurs after birth. During the first postnatal week, GABA-A receptors begin grouping in the nascent GABAergic synapses into clusters, whereas GABA-B receptors still remain scattered throughout the neuronal cytoplasm. Later, by P10, GABA-B receptors are also located in the synaptic structures [19]. These data support the viewpoint that during the early perinatal period in rats GABA-A receptors are predominant, being responsible for GABA transmission. Besides, it was established that in the gustatory NTS subnuclei there is a group of neurons, in which both GABA-A and GABA-B receptors are colocalized [4, 23–25].

The results of this study show that prenatal serotonin deficiency leads to malformation and impaired maturation of the GABAergic receptor network in the ventrolateral NTS part. In animals, developing under serotonin deficiency, the number of GABA-Aα1- and GABA-B1-expressing neurons in both subnuclei is considerably lower during the first postnatal week than in control. The network of immunopositive terminals in the neuropil is extremely poorly developed; sometimes terminal endings have varicosities thought to indicate immaturity of terminals [18]; synaptic structures are sporadic, and a general retardation of neurogenesis is evident. During the second postnatal week, the number of both GABA-Aα1- and GABA-B1-expressing neurons insignificantly increases, and the synaptic structures appear (this occurs more actively in the ventral subnucleus). By the end of the third week, the number of GABA-Aα1- and GABA-B1-expressing neurons, network of terminals, and paucity of receptor-containing synaptic structures remains intact.

The results of physiological observations allow suggesting that the early postnatal stages are critical to the developing respiratory system in mammals and humans [5, 6, 19]. Apparently, this may be due to a decrease in the level of some monoamines (specifically, serotonin and its receptors) in the stem respiratory nuclei. For example, by the end of the second week (P12–14) in normal rats there was found a sharp decrease in the level of tryptophan hydroxylase, a key enzyme of serotonin synthesis, as well as in HT(2A) receptors, mediating serotonin transmission in these nuclei, and serotonin transporter [14, 15, 25].

In view of our data, it should be pointed out that in the respiratory NTS subnuclei, agaiNTS the background of impaired formation of local inhibitory GABAergic receptor networks due to prenatal serotonin deficiency, the decrease in the level of serotonin and its receptors established to occur in normal rats by the end of the second week (i.e. during maturation of the inhibitory network) [14, 15, 25], may lead to even a stronger imbalance in neurotransmitter levels in the respiratory subnuclei. As a result, this may induce an appreciable dysregulation of the respiratory function in the early postnatal period, the causative factor of respiratory failure and a sudden infant death syndrome.

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