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Dynamics of Changes in the Expression of the GLUR2 Subunit of the Ionotropic Glutamate Receptor in the Ventrolateral Part of the Solitary Tract Nucleus during the Early Postnatal Period in Health and Prenatal Serotonin Deficiency

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Experiments on Wistar rats (n = 18) were performed to study changes in the expression of ionotropic glutamate receptor subunits (GluR2) in the respiratory subnuclei (ventral and lateral) of the solitary tract nucleus (STN). Observations were made during the early postnatal period (days 5, 10, and 20, 5–6 animals per group) in normal conditions and with prenatal decreases in serotonin levels due to inhibition of tryptophan hydroxylase with parachlorophenylalanine. Immunocytochemical detection of GluR2 revealed significant increases in GluR2 expression in the respiratory subnuclei of the STN in the early postnatal period (a 2-fold increase in the lateral subnucleus, 2.6-fold in the ventral). Prenatal serotonin deficiency altered GluR2 expression in the respiratory subnuclei of the STN. There was a significant delay in GluR2 expression in the early period, which increased to control levels by two weeks after birth, though GluR2 expression by juvenile age was lower (twofold) than in controls. Impairments to the glutamatergic receptor networks in the respiratory nuclei may be the basis of respiratory dysfunction.

Keywords: solitary tract nucleus, respiratory subnuclei, ionotropic glutamate receptors, serotonin.

One of the main functions of the solitary tract nucleus (STN) is to integrate information coming from the internal organs [3], where respiratory, cardiovascular, and other afferent spikes are mediated by glutamate [5]. The ventral and lateral subnuclei constitute groups of dorsal respiratory nuclei and are part of the bulbar respiratory center. A significant proportion of the nuclei in these nuclei are inspiratory [3], and regulation of the interactions between these are mediated mainly by the release of glutamate and γ-aminobutyric acid (GABA). Transmission of excitation is generally mediated by the actions of glutamate on two main ionotropic receptors – NMDA (binding N-methyl-D-aspartate) and AMPA (binding 2-α-amino-3-hydroxy -5-methyl-4-isoxazolepropionic acid). AMPA receptors are tetramers consisting of subunits whose combinations determine the structure, ion selectivity, permeability, and kinetics of receptor activation [22]. The calcium permeability of AMPA-dependent ion channels is determined by the presence of (GluR2) subunits in the receptor, and a calcium current through synapses during active synaptogenesis is required for the formation and specialization of the structural and functional properties of neural networks [9, 12]. However, there are virtually no data on changes in GluR2 expression in the respiratory subnuclei of the STN during the early postnatal period (i.e., the period during which the respiratory system matures in mammals and humans).

In the STN, serotonin (5-hydroxytryptamine; 5-HT), along with other neurotransmitters, is also involved in glutamate neurotransmission from visceral afferent terminals [17]. The subnuclei of the STN contain significant numbers of serotonin fibers, which have been shown to run from the medullary raphe nuclei [20]. Spontaneous serotonin release from the terminals of serotoninergic fibers has been shown to occur in the STN, transmitter release being controllable

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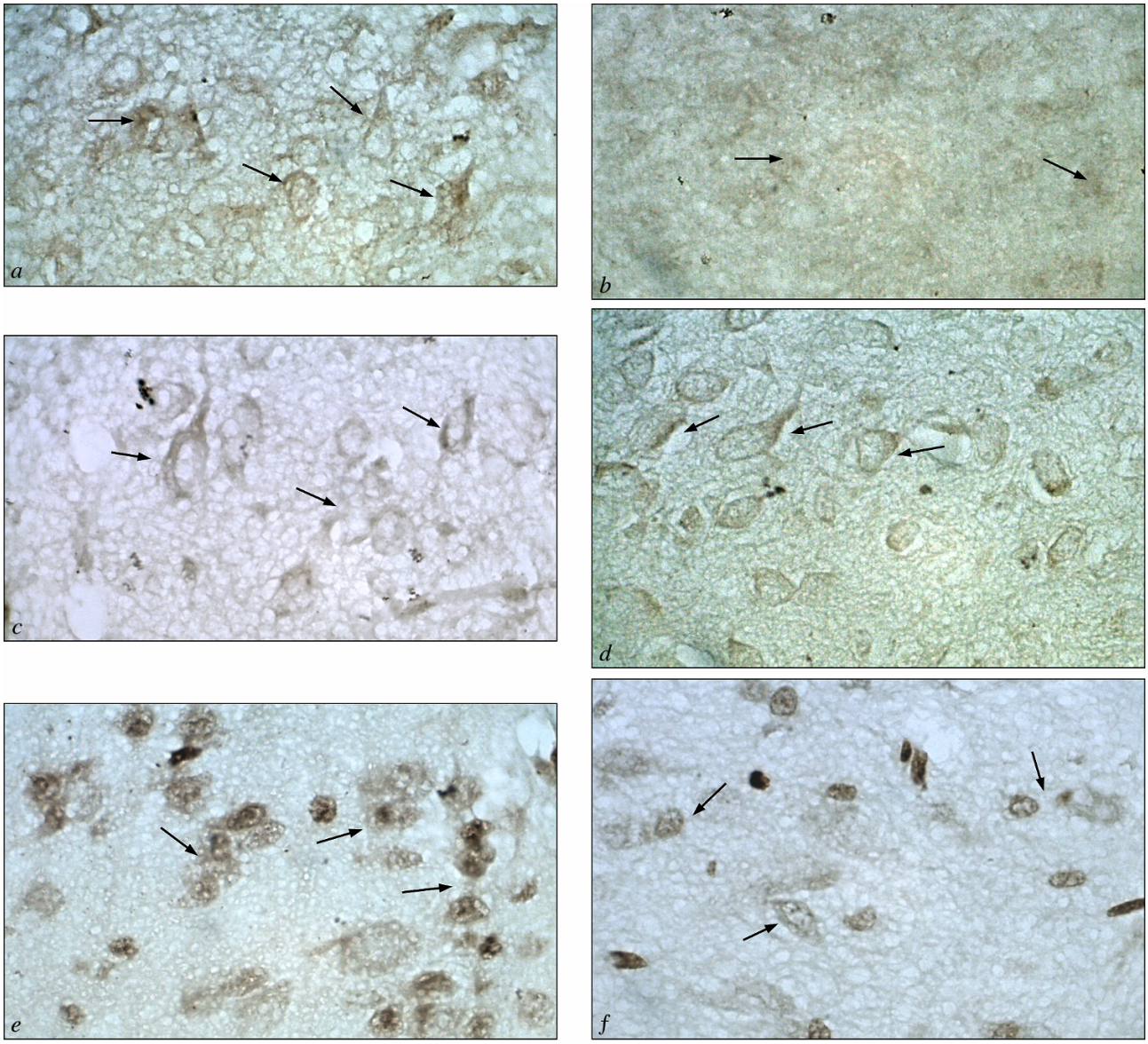

Fig. 1. Lateral subnucleus of the solitary tract nucleus on days 5 (*a*, *b*), 9 (*c*, *d*), and 20 (*e*, *f*) in controls (*a*, *c*, *e*) and after prenatal serotonin deficiency (*b*, *d*, *f*). *a*, *b*) Levels of GluR2 expression in experimental animals is lower than in controls; *c*, *d*) GluR2 expression in experimental animals is essentially the same as that in controls; *e*) a significant number of GluR2-expressing neurons; *f*) a small number of GluR2-expressing neurons; arrows show immunopositive neurons, processes, and granules; immunohistochemical reaction for GluR2. Objective ×100, ocular ×10.

via the serotonin reuptake system [7]. The STN has more intense expression of 5-HT receptors than any other part of the brain. These are presynaptic receptors activated by 5-HT and induce glutamate release from afferent terminals which in turn leads to excitation of postsynaptic neurons in the STN [11, 14, 16]. However, it is not known whether prenatal changes in serotonin levels affect the expression of ionotropic GluR2-containing receptors and, thus, the excitability of the neural network. Thus, the aim of the present work was to study changes in the expression of GluR2 in the respiratory subnuclei (ventral and lateral) of the STN in the early postnatal period in health and in conditions of prenatal reductions in serotonin content.

Materials and Methods. Studies were carried out on Wistar lab rats (n = 18). Animal keeping and all experimental procedures were conducted in compliance with the "Regulations for Studies Using Experimental Animals" (USSR Ministry of Health Decree No. 755 of August 12, 1977). Endogenous serotonin levels were reduced by inhibition of tryptophan hydroxylase (the enzyme synthesizing serotonin) with parachlorophenylalanine (PCPA) (Sigma, USA), given i.p. at a dose of 400 mg/kg to females on day 9 of

pregnancy (to induce long-lasting decreases in endogenous serotonin levels to 50–80% during the period at which the STN and serotonin system form in fetuses). Newly born rat pup brains were studied on days 5, 10, and 20. Controls consisted of animals of the same developmental ages born to intact females (each experimental and control group consisted of 5–6 pups of each age). Specimens were fixed in zinc-ethanol-formaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin by standard methods; serial sections of thickens 5 μm were cut from the medulla oblongata at the level of the bregma 11.88–12.00 [15].

An immunocytochemical reaction was used to detect the ionotropic glutamate receptor using mouse monoclonal antibodies to GluR2 (Abcam, USA). Secondary reagents for detecting GluR2 were from an EnVision+ System HRP Labelled Polymer Anti-Rabbit kit (DakoCytomation, USA). Reaction product was visualized using DAB+ chromogen (Dako, Denmark). The cellular localizations of GluR2 are the cytosol, the endoplasmic reticulum membrane, and cell contacts – synapses and postsynaptic membrane. Immunocytochemical reaction conditions were standardized and all procedures with histological sections of the medulla oblongata from control and experimental animals were performed simultaneously. After immunocytochemical reactions, some sections were counterstained with thionine (Serva, USA, Germany) and embedded in synthetic resin (thermo Fisher Scientific, USA).

Morphological analysis and quantitative studies were performed using digital images of serial sections obtained using a Leica DME light microscope (Leica, Germany) and a Leica EC3 digital camera (Leica, Germany). The numbers of immunopositive neurons per standard area of 0.1 mm^2 were counted and the densities of plexuses of terminal processes in the neuropil were determined; the densities of granules and large granules (the latter presumptively being terminal synaptic structures and groups of these) were evaluated [10]. Assessments of the optical density of reaction product in the neuron cytoplasm and neuropil were performed using images obtained with a digital video camera and VideoTest Master Morphology software (VideoTest, St. Petersburg). The arithmetic mean and its error were calculated and the critical significance level was $p < 0.05$; statistical analysis was run by ANOVA (Statistica 7.0, Statsoft Inc., USA).

Results. *Lateral subnucleus of the STN*. On day 5, control animals showed 16.3 ± 1.1 neurons per unit area with weakly staining cytoplasm (optical density 0.0751) (Fig. 1, *a*). On day 9, the number of immunopositive neurons persisted, at 15.7 ± 1.4, though reaction intensity increased slightly (0.0857) (see Fig. 1, *c*). On day 20, the number of immunopositive neurons increased twofold, to 31.6 ± 0.9, while reaction intensity was virtually unaltered (0.0872) (Fig. 1, *e*). The neuropil at all study time points contained weakly immunopositive processes forming a loose network.

On day 5, the lateral subnucleus of experimental animals contained 3.1 ± 0.3 neurons per unit area with weakly immunopositive cytoplasm (0.0491) (see Fig. 1, *b*). There were almost no immunopositive processes in the neuropil. On day 9, the number of immunopositive neurons was 12.4 ± 1.2 and the staining intensity of the cytoplasm and network of processes in the neuropil was essentially unaltered (0.0489) (see Fig. 1, *d*). On day 20, the number of immunopositive neurons was 16.2 ± 1.5, which was two times smaller than that in controls; the intensity of the immune reaction in the cytoplasm and processes was weak (0.0504) (see Fig. 1, *f*).

Ventral subnucleus of the STN. On day 5, the number of neurons with weakly immunopositive cytoplasmic reactions in control animals in the ventral subnucleus was 15.5 ± 1.6 per unit area and staining intensity was 0.0714. On day 9, the number of immunopositive neurons was 18.5 ± 1.3 and cytoplasmic reaction intensity was 0.0796, which were essentially the same as on day 5. On day 20, the number of immunopositive neurons increased sharply, to 47.6 ± 1.8 (a 2.6-fold increase) and staining intensity showed a minor increase (0.0841). In the neuropil, weakly stained processes formed a loose network at all time points.

On day 5, immunopositive neurons were not seen in the ventral subunit in experimental animals. Occasional processes were stained in the neuropil. On day 9, the number of immunopositive neurons was 19.7 ± 1.2 and the staining intensity of the neuron cytoplasm was weak (0.0412).

On day 20, there were 17.6 ± 1.8 immunopositive neurons, which was 2.4 times smaller than the number in controls; the intensity of the immune reaction was unaltered (0.0498).

Discussion. These studies showed that the two respiratory subnuclei in the STN during the early postnatal period contained essentially identical levels of expression of GluR2-containing receptors, as evidenced by both the numbers of GluR2-expressing neurons and the intensity of the immunochemical reaction. By juvenile age, there were significant increases in the expression of GluR2 – by factors of two in the lateral subnucleus and 2.6 in the ventral subnucleus; reaction intensity showed only minor increases (1.1-fold).

During embryogenesis, STN neurons have been shown to appear early, between developmental days 10 and 14, and the afferent fibers of the solitary tract start to appear in the solitary tract nucleus on day 15 [2, 18]; weak excitatory synaptic activity here is detected on day 18 [23]. Our experiments showed that reactions for GluR2 also appeared on day 18 and the locations of staining essentially coincided with the points of reactions for synaptophysin. Furthermore, the locations of synaptic structures on detection of GluR2 were confirmed by simultaneous detection of glutamatergic terminals [13]. Observations showed that between day 18 of intrauterine development and the onset of the postnatal period, the density of GluR2-immunopositive structures increased about threefold; in the first week after birth and to day 21 there was a further twofold increase [4].

The results obtained here confirm these data and provide evidence that by juvenile age, the respiratory subnuclei

of the STN undergo significant increases in GluR2 expression (by factors of 2–2.6) as compared with the level in the early neonatal period. AMPA receptors have been shown to be tetramers consisting of different combinations of four main subunits, termed GluR1, GluR2, GluR3, and GluR4. AMPA receptors not containing GluR2 are highly permeable for calcium ions [12]; the presence of a single GluR2 subunit in a heteromeric AMPA receptor ensures its impermeability for calcium ions [21].

Studies have shown that during early development and the formation of synaptic networks, the first AMPA receptors to be expressed do not contain the GluR2 subunit and that this subunit is subsequently gradually included in the composition of the receptor. During neural development, this process is common to other areas of the brain [8, 12, 19]. It has been suggested that during the early periods (early synaptogenesis), the intracellular calcium flux through AMPA-dependent ion channels is important in the formation and maturation of synaptic structures and emphasizes the role of calcium in processes stimulating the transformation of these synapses [6, 12, 19], which then determine the structural and functional properties of neural networks. Furthermore, the presence of GluR2 in AMPA receptors affects not only the permeability of AMPA-dependent channels for calcium ions, but also creates the molecular foundation for the development of synaptic plasticity [12].

Excitatory glutamate ionotropic GluR2-containing receptors have been shown to appear in the STN at the end of the prenatal period [13]. The results of the present study show that at the early stages of the perinatal period, GluR2 expression in the respiratory subnuclei of the STN starts to increase, while during the postnatal period there is a significant increase by juvenile age. It is likely that the first three weeks of postnatal development is a critical period for the formation and maturation of the glutamatergic synaptic network in the subnuclei of the STN.

Serotonin has been shown to play a role in glutamate transmission in the STN. Its excitatory action is mediated via activation of its own ionotropic receptors (5-HT_3 receptors) located mainly presynaptically on the afferent terminals of solitary tract fibers [14, 16], which leads to glutamate release which, in turn, activates ionotropic postsynaptic AMPA receptors and excites STN neurons [11].

The results of these studies show that prenatal serotonin deficiency alters GluR2 expression in the respiratory subnuclei of the STN in the early postnatal period. During the early period, there is either a delay in GluR2 expression or a low level of GluR2 expression. On day 9, GluR2 expression increases and corresponds essentially to the level in controls, though it does not change by juvenile age, remaining at half the control level.

This decrease in GluR2 expression is probably due to impairment in the processes synthesizing repair proteins and decreases in their quantity, which leads to impaired formation of synapses and arousal of the glutamatergic receptor network during the postnatal period; an additional cause may be a decrease in the number of postsynaptic targets for the afferent fibers of the solitary tract, which results from structural impairments to the development of the STN itself.

On the other hand, a prenatal reduction in serotonin content can lead to decreases in the quantity of serotoninergic fibers running from the medullary raphe nuclei, thus inducing a secondary drop in the level of serotonin released in the STN and impairment to the development of the innervation of the respiratory organs and a reduction in the number of afferent fibers running from them to the STN and, as a result, a sharp reduction in the content of serotonin receptors located on their terminals and taking part in glutamate release.

These studies showed that the first three weeks of the postnatal period of development are critical for the formation of the excitatory receptor network in the respiratory subnuclei of the STN. This decrease in expression of excitatory ionotropic GluR2-containing glutamate receptors induced by prenatal serotonin deficiency [1] can undoubtedly disturb the normal balance of arousal and inhibition in the respiratory subnuclei in the early periods of development and lead to respiratory dysfunction.

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