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Changes in the Expression of GAT_1 (GABA transporter) in the Ventrolateral Part of the Solitary Tract Nucleus in Prenatal Serotonin Deficiency in Rats

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Objectives. To study the expression of GAT_1 (GABA transporter) at the early periods of postnatal development in the ventrolateral part of the solitary tract nucleus in prenatal serotonin deficiency in rats. **Materials and methods.** The endogenous serotonin level in the embryonic period was decreased by inhibition of tryptophan hydroxylase with para-chlorophenylalanine (PCPA) (Sigma, USA). GAT-1 transporter was detected using primary rabbit polyclonal antibodies (anti-GABA transporter 1, GAT_1) (AbCam, UK). **Results.** From the early neonatal period to the beginning of juvenile age, the neuropil and the processes, terminals, and synaptic structures of the lateral and ventral parts of the solitary tract nucleus of control rats showed a gradual increase in GAT_1 expression in experimental animals. The level of expression of GAT_1 transporter was significantly greater in the lateral and ventral parts during postnatal weeks 1 and 2 than in controls, though by the end of week 3, i.e., by the beginning of juvenile age, it decreased to become significantly lower than in controls. **Conclusions.** The respiratory areas of the solitary tract nucleus in control rats in the first three weeks of postnatal development showed a gradual increase in GAT_1 expression. Serotonin deficiency during the prenatal period led to impaired expression of GAT_1 in the early postnatal period. These changes can lead to changes in GABA transmission, which may in turn be the cause of imbalance between inhibitory and excitatory effects in the respiratory center in the early postnatal period and, thus, may be the basis of the development of respiratory dysfunctions at early age.

Keywords: solitary tract nucleus, serotonin, GAT_1 (GABA transporter), early postnatal period.

Introduction. The lateral and ventral groups of neurons (subnuclei) are located in the caudal part of the solitary tract nucleus (STN), which is part of the dorsal respiratory group of neurons and is included as part of the bulbar respiratory center [3]. Regulation of the functions of the STN subnuclei is mediated by several neurotransmitters: GABA, serotonin, glycine, glutamate, etc., along with neuropeptides, and the corresponding receptor components. The main inhibitory neurotransmitter in the CNS in mammals is GABA. In adult animals, GABAergic neurons are distributed diffusely and are seen throughout the area of the caudal part of the STN. GAT_1 is one of the main GABA transporters and is an Na^+-dependent neurotransmitter reuptake protein located on the plasma membrane of neurons and neuroglial cells [1, 2]. Both GABA and serotonin (5-HT) regulate overall neurotransmitter homeostasis in this area via the projections of neurons in the caudal serotoninergic raphe nuclei and the receptor component (5-HTRA and 5-HTRB receptors) [11, 14]. Changes in the balance of the contents of these biologically active substances in the nuclei of the respiratory center can induce respiratory dysfunction [12]. Very little is as yet known of the effects of serotonin on establishment of the elements of the inhibitory GABAergic network and mechanisms of control of GABA transmission in respiratory neurons during early postnatal development.

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TABLE 1. Changes in GAT_1 Levels (immune reaction product optical density D) in the Lateral Subnucleus of the STN in the Early Postnatal Period in Rats in Controls and in Prenatal Serotonin Deficiency

Location	Developmental period					
	P5		P10		P20	
	D, arb. units					
	control	experiment	control	experiment	control	experiment
Synaptic structures	0.164 ± 0.004	0.170 ± 0.006	0.181 ± 0.007	0.204 ± 0.004*	0.300 ± 0.006	0.175 ± 0.005*
Neuropil (cell processes and terminals)	0.032 ± 0.002	0.046 ± 0.004*	0.052 ± 0.006	0.060 ± 0.003	0.091 ± 0.004	0.023 ± 0.007*

*Here and Table 2: significant differences compared with controls, $p \le 0.05$.

Thus, the aim of the present work was to study the expression of the transporter GAT_1 in the ventrolateral part of the solitary tract nucleus at early postnatal periods in health and prenatal serotonin deficiency.

Materials and Methods. Studies were carried out on Wistar laboratory rats (n = 21) from the animal house of the Pavlov Institute of Physiology, Russian Academy of Sciences. Animal keeping and all experimental procedures were conducted in compliance with the recommendations of Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes. Experimental protocols (No. 04/02 of February 4, 2019) were approved by the Commission for the Keeping and Use of Laboratory Animals, Pavlov Institute of Physiology. Endogenous serotonin levels during the embryonic period were suppressed by inhibition of tryptophan hydroxylase with para-chlorophenylalanine (PCPA) (Sigma, USA). PCPA at a dose of 400 mg/kg was given to female rats i.p. on day 9 of pregnancy. The brains of newborn rat pups were removed, fixed in zinc-ethanol-formaldehyde in phosphate buffer pH 7.4, embedded in paraffin by standard methods, and serial transverse sections of the medulla oblongata of thickness 6–7 μm were cut at the level of the bregma – 12.24–12.36 mm [13]. The lateral and ventral subnuclei of the STN were studied. Investigations were carried out on postnatal days 5, 10, and 20 (n = four animals in each group; P5, P10, and P20). Controls consisted of rat pups at the same developmental ages obtained from intact females (n = 3 for each time point). Immunohistochemistry reactions for detection of GAT_1 were run using primary rabbit polyclonal antibodies (anti-GABA transporter 1; GAT_1) (AbCam, UK). Secondary reagents for GAT_1 were from the EnVision+ System HRP Labelled Polymer Anti-Rabbit kit (DakoCytomation, USA). Reaction product was visualized using DAB+ chromogen (Dako, Denmark). Sections were embedded in synthetic Permount medium (Thermo, USA).

Reaction product optical density in the neuropil was evaluated using a general image analysis system to extract parts of the network of immunopositive processes and terminals and groupings of small granules and large granules, which are regarded as synaptic terminal structures and their groupings [10]. The GAT_1 level was expressed in relative optical density units. Immunoreactivity was determined quantitatively using an image analysis system including an Olympus CX31 light microscope (Japan), a VideoZavr Standard VZ-C31Sr color digital camera, and Videozavr Multimetr 2.3 software (developed by ATM Praktika, St. Petersburg). The mean reaction product optical density was determined in relative units at a magnification of ×1000. Measurements were made on 10 serial sections taken from 3–4 animals of each of the groups studied. Data were processed mathematically in Statistica 8.0 (StatSoft Inc., USA). All values are given as arithmetic means ± error of the mean. Significant differences between mean values were identified using Student's t test and one-way ANOVA. Differences were taken as significant at $p < 0.05$.

Results. *Distribution of GAT_1 in the lateral subunit of the STN in control animals*. GAT_1 levels in the synaptic structures of control rat pups gradually increased during development: by a factor of 1.1 from P5 to P10 and a factor of 1.7 from P10 to P20. In the neuropil, the network of immunopositive processes and terminals showed a 1.6-fold increase in the GAT_1 level from P5 to P10 and a 1.7-fold increase from P10 to P20 (Table 1).

Distribution of GAT_1 in the lateral subunit of the STN in animals with prenatal serotonin deficiency. GAT_1 levels in synaptic structures in experimental animals showed changes. Thus, the level increased 1.5-fold from P5 to P10 but decreased 1.2-fold from P10 to P20 (see Table 1).

In the neuropil, the network of immunopositive processes and terminals showed a 1.3-fold increase in the GAT_1 level from P5 to P10 and a significant 2.6-fold decrease from P10 to P20 (see Table 1).

Distribution of GAT_1 in the ventral subnucleus of the STN in control animals. The GAT1 level in synaptic structures in control rat pups also increased gradually with development: by a factor of 1.5 from P5 to P10 and by a factor of 3 from P10 to P20 (Table 2).

In the neuropil, the network of immunopositive processes and terminals showed an increase in the GAT_1 level by a factor of 8 from P5 to P10 and by a factor of 6.4 from P10 to P20 (see Table 2).

Distribution of GAT_1 in the ventral subnucleus of the STN in animals with prenatal serotonin deficiency. Synaptic

TABLE 2. Changes in GAT_1 Levels (immune reaction product optical density D) in the Ventral Subnucleus of the STN in the Early Postnatal Period in Rats in Controls and in Prenatal Serotonin Deficiency

Location	Developmental period					
	P5		P10		P20	
	D, arb. units					
	control	experiment	control	experiment	control	experiment
Synaptic structures	0.050 ± 0.006	0.110 ± 0.006*	0.072 ± 0.008	0.280 ± 0.008*	0.210 ± 0.005	0.168 ± 0.005*
Neuropil (cell processes and terminals)	0.0020 ± 0.0004	0.030 ± 0.002*	0.020 ± 0.003	0.090 ± 0.007*	0.102 ± 0.008	0.032 ± 0.004*

structures at the early stages of postnatal development in experimental animals showed changes in GAT_1 levels. Thus, there was a significant increase, by a factor of 2.6, from P5 to P10 and a 1.6-fold decrease from P10 to P20 (see Table 2). In the neuropil, processes and synapses showed a 2.8-fold increase in GAT_1 by the second week of postnatal development and a 2.89-fold decrease by the end of the third week, i.e., there was no difference between GAT_1 levels at P5 and P20 (see Table 2).

Discussion. In mammals, GABA, as an inhibitory neurotransmitter, is known to play an important role in regulating neuron excitability. In synaptic inhibitory neurotransmission, reuptake of neurotransmitter from the synaptic cleft or intercellular space is mediated by transporters, and the effectiveness of neurotransmission is determined by the rate of transmitter reuptake. Changes in these processes can lead to functional impairments to the CNS [2]. Four classes of transporter protein are known for GABA: GAT_1, GAT_2, GAT_3, and GAT_4 (or BGT-1, betaine), of which GAT_1 is regarded as one of the main transporters in synaptic neurotransmission [9].

The results obtained here showed that the level of GAT_1 expression in processes, terminals, and synaptic structures in the neuropil of the lateral and ventral subnuclei of the STN in control animals increased gradually from the early neonatal periods to the onset of juvenile age. The level of GAT_1 expression in the lateral subnucleus was significantly greater than that in the ventral.

Studies of animals developing in conditions of prenatal serotonin deficiency showed that both subnuclei of the STN showed altered levels of GAT_1 expression at all time points. The data showed that GAT_1 expression in synapses, processes, and terminals increased significantly in the first two weeks and decreased by the beginning of juvenile age.

GAT_1 expression levels in synapses on the one hand and the network of processes and terminals on the other in the ventral subnucleus were greater than those in controls by factors of 2.0 and 15.5 in the first week of postnatal development, by factors of 3.5 and 5.5 in the second week, and were lower by factors of 1.3 and 3.2 at the end of the third week. In the lateral subnucleus, GAT_1 expression levels in synapses and in the network of processes and terminals at postnatal weeks 1 and 2 were essentially no different from those in controls, while at the end of week 3 they were significantly lower, by factors of 1.7 and 4.0, respectively.

GABA is known to be found not only in synapses, but also in the extracellular space. The source of extracellular GABA may be its diffusion from the synaptic cleft, i.e., neurotransmitter spillover [6, 7]. Spillover can probably be regarded as a process stimulating active neurotransmitter reuptake from the extracellular space [8] and, thus, requiring increased synthesis of transporter protein. This may be confirmed by data obtained from studies of the synthesis of surface proteins showing that increases in GAT_1 transporter expression correlate with increases in GABA transport [2, 4].

The increase in the level of GAT_1 expression seen in the subnuclei of the STN of the experimental animals in the present studies are probably due to GABA spillover, which may in turn occur when synapse structure changes and the process of GAT_1 internalization is slowed and the expression of postsynaptic receptors is impaired. Changes in GABA transporter expression were noted as a result of impairment to metabolic processes (phosphorylation) [5, 7], osmotic stress in response to high levels of neuron activity, the actions of hypoxia/ischemia on the brain, and trauma [5, 12]. Furthermore, changes in the expression of different GABA transporters have been found in Alzheimer's disease. Increases in GAT_4 expression were seen in the dentate fascia and in hippocampal fields C2 and C3, with decreases in the entorhinal cortex [8].

The decreases in the level of GAT_1 expression by the beginning of the juvenile period seen in the present studies may be a consequence of the decrease in expression of GABA itself in the experimental animals; it remains possible the there is a limit on receptor protein synthesis and impaired synaptogenesis. These observations show that there are specific changes in GABA transporter expression in various brain formations in response to adverse factors and during neurodegenerative disease, though the mechanisms of these changes are unknown.

Conclusions. It should be noted that the lateral and ventral subnuclei of the STN in rats, starting from the early neonatal period and lasting to the beginning of juvenile age, showed a gradual increase in the level of expression of GABA transporter GAT_1. Prenatal serotonin deficiency led to impairment of GAT_1 transporter expression during the

postnatal period. This may cause alterations in GABA transmission, which in turn lead to imbalance between inhibitory and excitatory effects in the respiratory subnuclei at the early periods of postnatal development and provides the basis for the development of respiratory impairments. These results aid our understanding of the mechanisms regulating GAT_1 expression and its release and internalization when respiratory dysfunction occurs. Furthermore, the process of GAT_1 expression may be a target for pharmacological correction of inhibitory effects.

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