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MORPHOLOGICAL BASICS FOR EVOLUTION OF FUNCTIONS

Expression of Serotonin Transporter in the Dorsal Raphe Nucleus during the Early Postnatal Period in the Normal State and under Prenatal Deficiency of the Serotonergic System in Rats

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Abstract—Expression of the serotonin transporter protein (5-NTT) in the dorsal raphe nucleus (DNR) during the early postnatal period was investigated in laboratory Wistar rats. Immunocytochemical labeling showed that during the first 3 postnatal weeks the intensity of 5-NTT expression in DNR of control animals changes. During the earliest postnatal stages, most of DNR subnuclear neurons (dorsal, DNR-d; ventral, DNR-v; lateral, DNR-lat) were found to intensely express 5-NTT. 5-NTT localization sites were revealed on the membrane surface of neuronal cell bodies and their processes in neuropil. On P10, the number of 5-NTT expressing neurons and 5-NTT binding sites decreases. At this time, the 5-NTT binding sites were shown to undergo redistribution becoming very few on neuronal cell bodies and dendrites, but rather densely packed in the axonal membrane. The number of 5-NTT expressing neurons and density of 5-NTT localization sites in neuropil gradually increases with age. The reduction in the serotonin level in all DNR regions during prenatal development leads to the reduction both in the number of 5-NTT expressing neurons and 5-NTT localization sites during the early postnatal period. This tendency was shown to persist with age.

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Key words: serotonin, serotonin transporter, dorsal raphe nucleus, early postnatal period.

INTRODUCTION

The serotonergic system is one of the first brain neurotransmitter systems to differentiate during embryogenesis. At this time, serotonin (5-HT) serves in mammals a powerful morphogen which controls multiple histogenetic processes (neurogenesis, migration, neural differentiation, synaptogenesis etc.) [1–5]. Although the significance of its contribution to CNS development is well known, the degree of its activity at different stages of ontogenesis is studied insufficiently. Supposedly, it is regulated by different factors, primarily by the transmembrane serotonin transporter protein (5-NTT) responsible for serotonin reuptake from the synaptic cleft providing thereby a precise regulation of the extracellular serotonin concentration and serotonin signaling [6].

Is has been established that both in fetuses and adult animals in the dorsal raphe nucleus there is intense 5-NTT expression attributed mainly to serotonergic neurons [6], while considerable differences have been revealed in the distribution of 5-NTT localization sites over the membrane of

neuronal cell bodies and their processes. In adult animals, 5-NTT localization sites on neuronal cell bodies and dendrites are very few, whereas in embryos and fetuses during the prenatal period they are much more numerous on neuronal cell bodies and large dendrites of serotonergic neurons in the dorsal raphe nucleus (DNR) [6]. It is generally believed that in synaptic terminals extracellular serotonin is reabsorbed presynaptically, whereas in cell bodies and dendrites this occurs postsynaptically [6, 7, 13]. Probably, this feature underlies both effective serotonin reuptake from the extracellular space and the functional regulation of serotonergic neurons at early developmental stages, yet before the establishment of excitatory inputs [7, 8]. At early postnatal stages, in mammals and humans there occurs the development and maturation of many brain structures and their functions regulated by serotonin. However, almost no information is available now on the peculiarities of 5-NTT expression and distribution of its localization sites in the raphe nuclei during this period.

Thus, the aim of this study was to explore the expression of the transmembrane serotonin transporter protein in the DNR subnuclei during the early postnatal period in the normal state as well as under prenatal functional deficiency of the serotonergic system.

MATERIALS AND METHODS

This research was conducted on laboratory Wistar rats. Animal keeping and all experimental procedures were implemented in compliance with "Regulations of work using experimental animals" (order no. 755 of 12.08.1977 of the USSR Ministry of Public Health). To reduce the endogenous serotonin level, the method of tryptophan hydroxylase inhibition by para-chlorphenylalanine (PCPA) (Sigma; USA) was used. PCPA at a dosage of 400 mg/kg was injected to female rats intraperitoneally one time on day 16 of gestation (E16). When choosing a dosage, the literature data were considered showing that at this dosage PCPA induces both in adults and developing fetuses a long-term decrease in the endogenous serotonin level (by 50–80%) over 5–6 days [9–11].

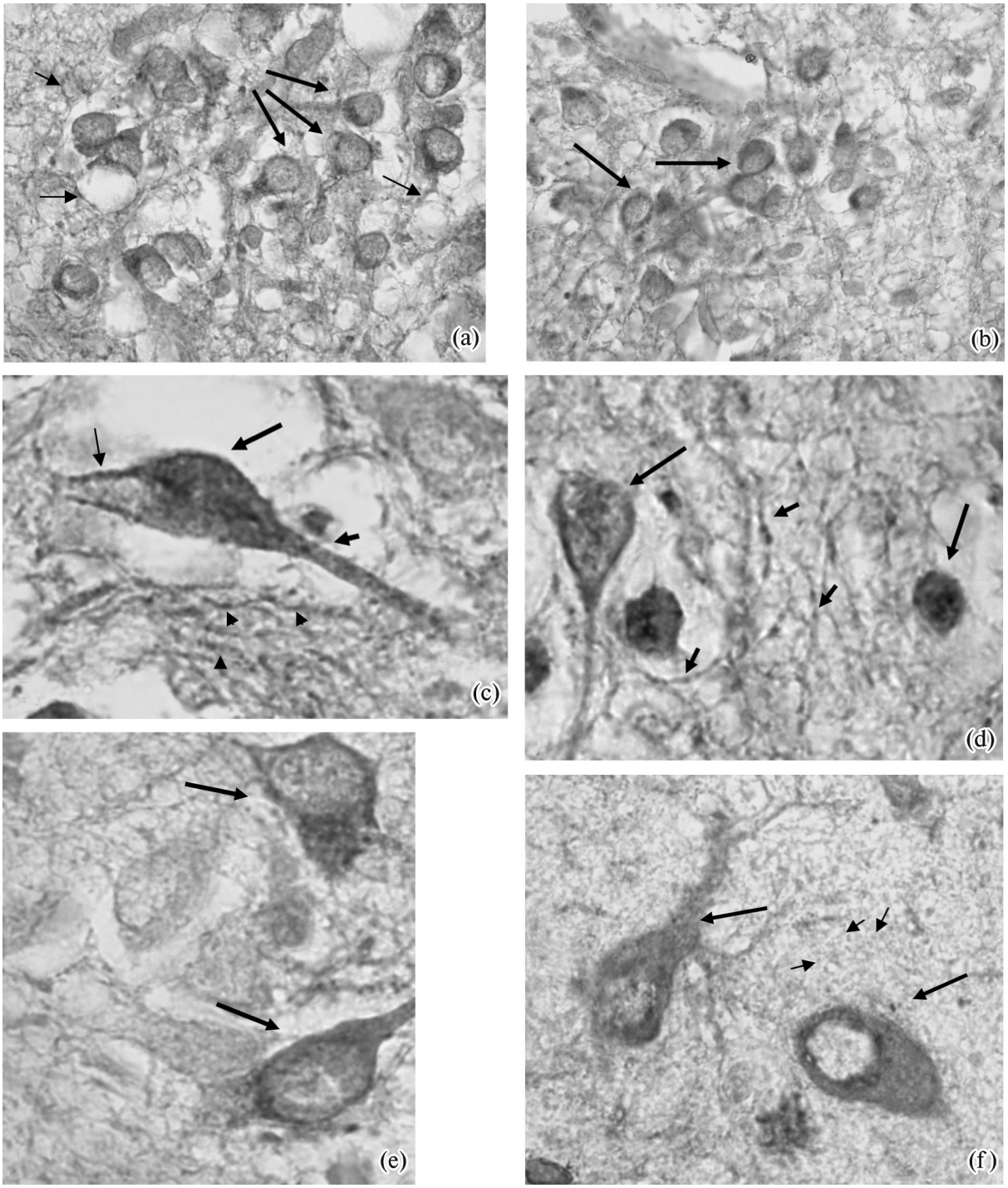
The brain in rat pups was studied on postnatal days 5, 10 and 20 (P5, P10, P20) ($n = 5$ for each age group). As a control, same-age animals obtained from females injected with physiological solution at respective stages of gestation were used. Material was fixed in zinc-ethanol-fomaldehyde (pH 7.4) for 24 h, conventionally embedded in paraffin and used for serial sectioning (transverse sections, 4–5 μm thick, at Bregma levels (–7.92)–(–8.04) mm. Expression of serotonin transporter was studied in the DNR subnuclei: dorsal (DNR-d), ventral (DNR-v) and lateral (DNR-lat).

Immunocytochemical reaction for 5-NTT was conducted with the use of primary anti-Serotonin transporter antibodies (AbCam; UK) and secondary reagents from the LSAB2 System-HRP kit (Dako; Denmark). To visualize the reaction product, DAB+ chromogen (Dako; Denmark) was used. After immunocytochemistry, a part of sections were additionally stained with Mayer's hematoxylin (Bio-Optica, Italy) or thyonin (Serva; USA, Germany). Conditions of the immunocytochemical reaction were standardized, and all the immunolabeling procedures on brain section obtained from animals at different developmental stages (both control and experimental) were performed simultaneously. When analyzing the results of the immunocytochemical reaction, the immunolabeling of the neuronal cytoplasm, presence of terminal varicosities and immunoreactive granules on the membrane of cell bodies and their processes (putative 5-NTT localization sites [3]) were taken into consideration.

Morphological analysis was carried out on histological sections passing through the central part of the dorsal raphe nucleus. In every animal, 15 sections were examined. Quantification was conducted on digital images of serial sections obtained using a Leica DME light microscope and Leica EC3 digital camera (Leica; Germany). The mean number of immunopositive neurons per square unit and its ratio to the total number of neurons on this square, expressed as percentage, were calculated. The standard frame (0.016 mm^2) was taken as a square unit.

RESULTS

Expression of serotonin transporter in subnuclei of the dorsal raphe nucleus in control animals. On postnatal day 5, virtually all neurons in DNR-d,



The rat dorsal raphe nucleus at early postnatal stages of development in control (a, c, d, f) and after prenatal serotonin deficiency (b, e). Immunocytochemistry for serotonin transporter. (a) Dorsal subnucleus on P5; immunopositive neurons (*long arrows*), terminal varicosities (*short arrows*); (b) dorsal subnucleus on P5; reduction in the number of immunopositive neurons (*arrows*) and their compact spacing; (c) dorsal subnucleus on P10; immunopositive neuron (*long arrow*), dendrite with immunopositive cytoplasm (*short bold arrow*), axon with unlabeled cytoplasm and granules on its axolemma (*short thin arrow*), terminal varicosities (*arrowheads*) in neuropil; (d) dorsal subnucleus on P10; immunopositive neurons (*long arrows*), terminal varicosities (*short arrows*); (e) dorsal subnucleus on P10; immunopositive neurons (*arrows*); (f) dorsal subnucleus on P20; immunopositive neurons (*long arrows*), small dust-like grains (*short arrows*) in neuropil. Mag.: oc. ×10, ob. ×100.

DNR-v and DNR-lat are 5-NTT immunopositive (Fig. 1a), while the neuronal and dendritic cytoplasm is intensely stained. In DNR-d, on the surface of immunopositive neurons, there are few immunopositive granules, although lacking at the same locations in DNR-v and DNR-lat. In neuropil of DNR-d and DNR-v, a rather dense network of slender terminal processes is detected bearing multiple varicosities (Fig. 1a). In neuropil of DNR-lat, by contrast, the network of slender immunopositive processes is loose, while the processes also have multiple varicosities. On the surface of the processes, both dendritic and axonal, there are immunopositive granules.

On P10, about 50% of neurons in DNR-d, DNR-v and DNR-lat are immunopositive. The cytoplasm of dendrites, not axons, is noticeably stained (Fig. 1c). Immunoreactive granules on the membrane of neuronal cell bodies are missing, being present on the processes, although scattered more sparsely than at the previous stage.

In neuropil, the density of the network of slender, poorly immunolabeled processes is much lower than on P5. Terminal varicosities are also fewer.

On P20, most neurons (about 80%) in DNR-d, DNR-v and DNR-lat are immunopositive. On the surface of cell bodies there are no granules. In neuropil, there is a network of immunolabled processes. In DNR-d and DNR-lat, the terminal varicosities can be rarely seen, but, at the same time, there is a great deal of very small (dust-like) immunoreactive grains (Fig. 1f). In DNR-v neuropil, there is a dense network of immunolabeled processes, multiple terminal varicosities and granules, although in lesser quantities than on P5.

Expression of serotonin transporter in subnuclei of the dorsal raphe nucleus in animals developed under deficiency of the serotonergic system. On P5, neurons in DNR-d and DNR-v are spaced more compactly versus control (Fig. 1b), probably, indicative of a retardation in neuropil development. Meanwhile, the number of neurons showing intense immunopositive labeling of the cytoplasm is significantly reduced (about 50%), and in DNR-lat only 25% of neurons are immunopositive versus control. On the surface of neuronal cell bodies there are almost no immunopositive granules.

Neuropil has rather a dense network of processes, while a part of them are poorly immunolabeled. Varicosities are virtually missing, and granules encrusting the processes are very few.

On P10, about 30% of neurons in DNR-d and DNR-v are immunopositive versus control, while the cytoplasm is stained rather intensely. Immunopositive granules on the surface of cell bodies are absent.

Neuropil contains few immunolabeled processes and almost no granules and varicosities (Fig. 1e). In DNR-lat of experimental animals, immunopositive neurons make up about 25%, that is much less than in control. In neuropil, the slender terminal processes with the sparse immunopositive granules on their surface form a loose network. Terminal varicosities occur infrequently.

On P20, in DNR-d and DNR-v there are much less (about 40%) immunolabeled neurons versus control. The surfaces of cell bodies bear no immunoreactive granules. In DNR-d, a part of neurons show a deformation of the cell body (it is elongated, with corrugated cytoplasm). In DNR-lat of experimental animals, the number of immunopositive neurons is intact and, as at the previous stage, they constitute about 25% of the total number of neurons in this subnucleus. Immunopositive granules on the surface of neuronal cell bodies are absent.

In DNR neuropil, the density of the plexus of processes is much lower than in control, and terminal varicosities are seldom to occur. On the surface of some processes there are immunopositive granules and small (dust-like) grains.

DISCUSSION

Our study shows that in control animals the intensity of serotonin transporter expression in DNR changes during the first three postnatal weeks. At earliest postnatal stages, a major part of DNR subnuclear neurons (in DNR-d, DNR-v and DNR-lat) intensely express 5-NTT. 5-NTT localization sites occur on the membrane surface of neuronal cell bodies and their processes in neuropil. On P10, however, the number of 5-NTT expressing neurons, as well as the density of the network formed by immunolabeled processes and the number of terminal varicosities and granules in neuropil, decreases. These findings are consis-

tent with observations of other authors indicating a sharp decrease in the number of 5-NTT localization sites on P10 in the lateral paragigantocellular nucleus of the parapyramdial region [12]. Probably, these facts reflect a general state of the serotonergic system at this postnatal stage. The axonal cytoplasm has been found to exhibit no immunopositive labeling at this time, in contrast to the cytoplasm of dendrites and neuronal cell bodies. The redistribution of 5-NTT localization sites has also been noticed: while on cell bodies and dendrites they are very sparse, on the axonal plasma membrane they occur in numbers. Similar presence of 5-NTT in the neuronal cytoplasm and processes localization site distribution, as well as the distribution pattern of 5-NTT localization sites have been detected in adult animals [7, 13]. These data allows the conclusion that both during the early postnatal period (by P10) and sexual maturity animals share the same distribution pattern of 5-NTT localization sites.

We demonstrated that by the end of the 3rd postnatal week (P20) the number of 5-NTT expressing neurons increases again, while the density of the network of immunopositive processes in neuropil becomes, respectively, higher; the number of varicosities and granules also increases, but they occur in lesser quantity than at the earlier postnatal stage (P5). At the same time, very small (dust-like) immunopositive grains appear suggested to represent presynaptic 5-NTT biding sites [7, 8].

The results of the study show that the serotonin level has a considerable effect on 5-NTT expression. Its reduction during the prenatal development leads to a reduction both in the number of 5-NTT expressing neurons and the sites of its localization in all DNR regions (DNR-d, DNR-v and DNR-lat) during the early postnatal period. In DNR-d and DNR-v at early stages there occurs a retardation of the neuropil development, probably, indicative of retarded neurogenesis and, as a result, delayed development of processes. We revealed that most sensitive to serotonin deficiency are the DNR-lat subnuclear neurons where 5-NTT expression was noticeably reduced. In all the DNR subnuclei, versus control, the density of the network of immunolabeled neuronal processes, as well as the number of immunopositive granules, are reduced, while terminal varicosities are virtually absent. With age, this pattern persists.

At present it is well known that 5-NTT expression in mammals is initiated in early ontogenesis and is associated mainly with serotonergic neurons both in the rostral and caudal groups of the serotonergic system. Being responsible for reuptake of serotonin from the extracellular space, 5-NTT is a major factor which regulates its extracellular level and transmission [6, 14]. The current CNS interneuronal transmission paradigm suggests the two ways in interaction among neurons: via "synaptic" transmission, when signal (neurotransmitter) transmission is mediated via a synaptic contact, and "extrasynaptic" ("volume") tansmission, when there is a diffuse 3D signal propagation in the intercellular fluid by means of the so-called "open" synapses structurally representing terminal varicosities where neurotransmitter can be synthesized, stored and then released [15–17]. "Volume" transmission, unlike "synaptic", is commonly believed to be not restricted spatially, propagating by diffusion in the intercellular medium (including the cerebrospinal fluid) and, consequently, reaching a greater number of targets. Recently, in studies of the "volume" transmission mechanisms, the vesicular microstructures have been discovered suggested to be information protein carriers able to easily reach target cells in the CNS [13, 15, 17]. Presumably, a major type of signal transmission in serotonergic neurons is exactly "volume" (extrasynaptic) transmission regulated by 5-NTT with its predominant "axonal" localization [13]. However, there is an opinion that with age "volume" serotonin transmission is supplemented by its "synaptic" form [15].

Our data on a sharp reduction in 5-NTT expression specifically on postnatal day 10, hence, on alterations in the serotonin transmission level in the serotonin-regulated CNS structures, may indicate a critical time point for the impact of adverse factors.

The previously established facts of intense 5-NTT expression during the prenatal period [6], as well as the results of this study indicating (a) its continued expression during the early postnatal period, (b) sharp fluctuations in the 5-NTT expression intensity and (c) redistribution of 5-NTT localization sites on the neuronal parts during the first 3 postnatal weeks, (d) the dependence

of the 5-NTT expression intensity on the endogenous serotonin level, allow two suggestions. On the one hand, during the early postnatal period the 5-NTT function may essentially change emphasizing the significance of this period in the complex regulation of serotonin transmission. On the other hand, the above findings emphasize the importance of 5-NTT as such because its expression in this specific period may be a precondition for the normal development and maturation of the brain structures and their serotonin-regulated functions.

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