ISSN 1819-7124, Neurochemical Journal, 2022, Vol. 16, No. 1, pp. 58–67. © Pleiades Publishing, Ltd., 2022.
Russian Text © The Author(s), 2022, published in Neirokhimiya, 2022, Vol. 39, No. 1, pp. 59–69.

EXPERIMENTAL ARTICLES

Morphological Changes in GABAergic Structures of the Rat Brain during Postnatal Development

V. A. Razenkova[a, 1] and D. E. Korzhevskii[a]

[a] *Institute of Experimental Medicine, St. Petersburg, Russia*

Received April 7, 2021; revised April 27, 2021; accepted May 14, 2021

Abstract—The aim of the study was to investigate brain GABAergic neurons and synaptic terminals in early postnatal development and aging. Wistar rat brain slices at different stages of postnatal development (from postnatal day 7 to 24 months) were used. Immunohistochemical staining of GABA synthesizing enzyme (glutamate decarboxylase isoform 67 or GAD67) was performed to reveal GABAergic structures. We described morphological changes that occur in the GABAergic system during postnatal development. In particular, it has been shown that synaptic terminals are predominantly localized in cortical layer 1 in 7-day-old animals. Our findings suggest that Cajal–Retzius cells are not GABAergic. GABAergic neurons were found in young and old animals in the subventricular zone. We found that the epithelial layer of choroid plexus contains GAD67 by the end of the first month of postnatal development. In this regard, these epithelial cells may be a source of extrasynaptic GABA, which enters the cerebrospinal fluid and then nervous tissue of the brain.

Keywords: gamma-aminobutyric acid, glutamate decarboxylase, development, brain, immunohistochemistry, confocal laser microscopy

DOI: 10.1134/S181971242201010X

INTRODUCTION

The GABAergic structures of the brain are mainly inhibitory interneurons and their synaptic terminals. These neurons are a heterogeneous set of cells that selectively modulate the activity of the neural circuits of the central nervous system [1, 2]. It seems that these cells are formed from progenitors in the embryonic subpallium, in the area of the ganglion eminence. GABAergic neurons develop under the regulation of transcription factors typical of the subpallium (the Dlx, Ascl1, Gsx1, and Gsx2 gene families); however, their diversity is influenced by additional transcription factors that limit the differentiation of progenitors only into certain types of interneurons [3–7]. Despite the fact that the ganglion eminence is considered the main source of GABAergic cells, a number of authors suggest the presence of progenitor cells in the subventricular zone of the lateral ventricles of the brain during postnatal ontogeny [2, 8–10].

During postnatal development, the final formation of the elements of the inhibitory system takes place. The migration processes occurring in embryonic development are replaced by the processes of differentiation and maturation. At the end of the first week after birth, functional synaptic connections begin to form, and the maturation of GABAergic interneurons occurs in the first month of postnatal development [11–13].

It is known that the GABAergic system of the brain is involved in the structural and functional organization of the central nervous system during development [1, 14, 15]. In the early stages of development, prior to the formation of synapses, GABA is released extrasynaptically. Extrasynaptic GABA release creates the chemotaxis necessary for the correct migration of phenotypically different populations of neuronal precursors during corticogenesis, and partial or complete blocking of GABA release leads to malformations of cerebral blood vessels and pathologically affects the migration and positioning of cortical interneurons [16, 17].

Despite the fact that there is a large amount of information about the formation of the GABAergic system, the data on the role of GABA in development are contradictory. In particular, this refers to the regulation of the process of synaptogenesis that occurs in the first month of postnatal development. It has been shown that in the brain of young animals, in the absence of glutamate, GABA can act as an excitatory neurotransmitter. Activation of $GABA_A$ receptors and voltage-gated calcium channels leads to spontaneous depolarization of neurons, which is necessary for the activity-dependent formation of synapses [18–20]. However, other authors challenge these assumptions drawing attention to the fact that these data were obtained on acute brain slices which have damaged cells, which can lead to erroneous results [21, 22]. Their observations cast doubt on previous findings about the role of GABA in synaptogenesis.

[1] Corresponding author; address: ul. Akademika Pavlova 12, St. Petersburg, 197022 Russia; e-mail: valeriya.raz@yandex.ru.

Thus, despite the fact that the GABAergic system has a significant contribution towards maintenance of central nervous system functions both during development and in adulthood, the features of the formation of GABAergic interneurons and their connections during early postnatal development, as well as during normal aging remain controversial. Therefore, the aim of this study was to study GABAergic neurons and synaptic terminals in early postnatal development and aging.

MATERIALS AND METHODS

The study was carried out on paraffin sections of the brain of male Wistar rats at different stages of postnatal development: postnatal days 7 (P7) and 30 (P30), mature (5–6 months) and old (23–24 months) animals ($n = 5$ for each group). All procedures involving animals were carried out in accordance with the guidelines established by the Directive 86/609/EEC on the protection of Animals used for Experimental and other scientific purposes (Strasbourg, 1986) and "Regulations of work with the use of experimental animals" (order no. 755 of August 12, 1977, Ministry of Health, USSR). The study was approved by the local ethics committee of the Institute of Experimental Medicine (report no. 1/20 dated February 27, 2020). The material was fixed in zinc-ethanol-formaldehyde [23] and embedded in paraffin according to the standard technique. Frontal sections with a thickness of 5 μm were made and mounted onto "Superfrost Ultra Plus" adhesive slides (Menzel Gläser, Germany). After dewaxing and rehydration of the preparations, thermal unmasking of the antigen was carried out in a modified citrate buffer (S1700, Agilent, United States) for 24 min. Inhibition of endogenous peroxidase was carried out by treating the sections with a 3% aqueous solution of hydrogen peroxide for 10 min.

To identify GABAergic structures of the brain, we used a polyclonal rabbit antibody to the enzyme of GABA synthesis, glutamate decarboxylase isoform 67 (GAD67) at a dilution of 1: 300 (E10260, Spring Bioscience, United States). Incubation in the primary antibody was carried out for three days at a temperature of 27.5°C. The secondary antibody was Goat anti-rabbit HRP Conjugate from the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (ab236466, Abcam, United Kingdom). Chromogen 3'3-diaminobenzidine from the DAB + kit (Agilent, United States) was used for light microscopy to detect the reaction product. Some of the sections were stained with alum hematoxylin. For confocal laser microscopy, after incubation with a secondary antibody (30 min at a temperature of 27.5°C), a goat anti-horseradish peroxidase antibody conjugated with the Cy3 fluorochrome (Jackson ImmunoResearch, United States) was applied to the sections [24]. The fluorescent nuclear dye SYTOX Green at a concentration of 0.5 μmol (Invitrogen, United States) was added directly to the antibody solution, and the sections were incubated for 30 min at a temperature of 27.5°C. To establish a positive control of antibodies, we used preparations of the rat cerebellum, where the localization of GAD67-positive neurons is well known [25]. When setting a negative control, Antibody Diluent (Abcam, Great Britain) was applied instead of a solution of primary antibodies to one of the sections of the treated series of preparations. The resulting preparations were analyzed using a Leica DM750 light microscope (Leica, Germany) and a Zeiss LSM 800 scanning confocal microscope equipped with an Airyscan system (Carl Zeiss AG, Germany). Plan-Apochromat 20×/0.8 M27 and Plan-Apochromat 63×/1.40 Oil DICM27 (oil immersion) objectives were used. To excite Cy3 and SYTOX Green fluorescence, lasers with a wavelengths of 561 and 488 nm, respectively, were used. The obtained images were processed using ZEN-2012 software (Carl Zeiss AG, Germany) and ImageJ (Wayne Rasband (NIH), United States). To analyze the age-related dynamics of the number of GABAergic interneurons, cells were counted in four fields of view, 325 × 244 μm in size for every case. After that the number of GAD67-positive cells was divided by the total number of cells to calculate the percentage. The average intensity of the red channel fluorescence in each image was estimated according to the method described by Shihan et al. [26]. The average fluorescence intensity was presented in the form of conventional units of pixel brightness for 8-bit images (0 to 255). Statistical processing was performed using the GraphPad Prism 8 software (GraphPad Software, United States). Data were presented as mean ± mean error. On the basis of checking for conformity with the normal distribution using the Shapiro–Wilk test, a one-way Kruskal–Wallis analysis of variance was used to compare the data. Subsequent group comparisons were performed using Dunn's post-hoc test. The differences were considered significant at $p < 0.05$.

RESULTS

The preliminary part of the study consisted of testing the possibility of visualizing synapses using an immunohistochemical reaction for GAD67 on brain preparations of mature rats using a Zeiss LSM 800 confocal microscope. When using the Plan-Apochromat 63×/1.40 Oil DICM27 lens with the diameter of the confocal diaphragm (pinhole), equal to one Airy unit (1 AU), it turned out to be possible to obtain images with both the bodies of GAD67-positive neurons and GABAergic synaptic terminals (Figs. 1a, 1b). A decrease in the pinhole aperture to about half of the Airy disk (0.56 AU) made it possible to increase the axial and lateral resolution of the confocal microscope and visualize specifically GABAergic presynapses (Fig. 1c). To check the suitability of the obtained image for further analysis, the Range Indicator profile was used, which transforms the original image so that

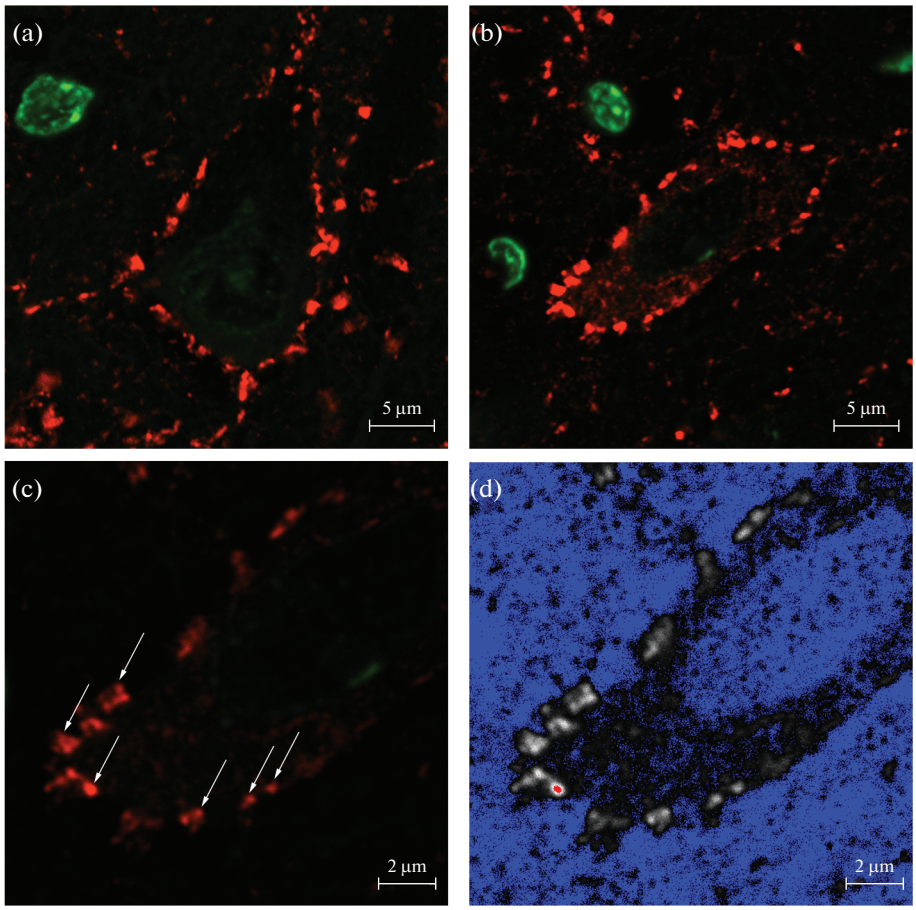

Fig. 1. Synaptic terminals on the bodies of neurons in the brain of adult rats. Immunohistochemical staining for GAD67 (red) and SytoxGreen-stained nuclei (green). (a) Pyramidal neuron of the cortical layer III; (b) cortical GABAergic interneuron; (c) cortical GABAergic interneuron, high magnification; (d) cortical GABAergic interneuron, high magnification, Range Indicator profile. Arrows indicate GABAergic synaptic terminals.

pixels with a minimum brightness value (0) are colored blue, and those with a maximum brightness value (255) are colored red (Fig. 1d). With certain settings of the scanning mode of the confocal microscope, it is possible to obtain images in which some objects will have brightness values of 255 (i.e., overexposure), which can be used for specific purposes. In the present work, a similar scan setting was used to obtain images with clear visualization of not only the synaptic terminals of GABAergic cells but also the bodies of the interneurons themselves (see Fig. 1b). The need for such a device adjustment is due to the fact that the cytoplasm of interneurons is characterized by weaker values of the GAD67 signal compared to presynapses.

During the main immunohistochemical study, it was revealed that on the 7th day of postnatal development, the ventral region of the brain and layer I of the neocortex are intensely stained (Fig. 2a). The intense coloration of layer I of the neocortex is due to the accumulation of a large number of GABAergic terminals. In this case, the bodies of GABAergic interneurons are located in the underlying layers (V–VI). It was shown that the presence of not only synaptic terminals but also cell bodies with a pronounced GAD67 cytoplasmic reaction, is characteristic of layer I of the cingulate cortex. The enzyme is evenly distributed over the cytoplasm of the perikaryon of neurons (but not detected in the nucleus), cell processes are not detected or can only be traced at a short distance from the cell body. In subcortical structures, an enhanced response was observed in the olfactory tubercle, hypothalamus, and globus pallidus. The septal zone of the brain is characterized by uneven staining of the lateral septal nucleus. In the subventricular zone, cells with a distinct cytoplasmic reaction to GAD67 were detected (Fig. 3a).

Despite the presence of GAD67-positive axon terminals detected in all brain structures by the end of the first week of postnatal development, fully formed GABAergic axosomatic presynapses on interneurons were not observed at this time.

On the 30th day of postnatal development, a clear layering of the cortex appears due to intense staining of

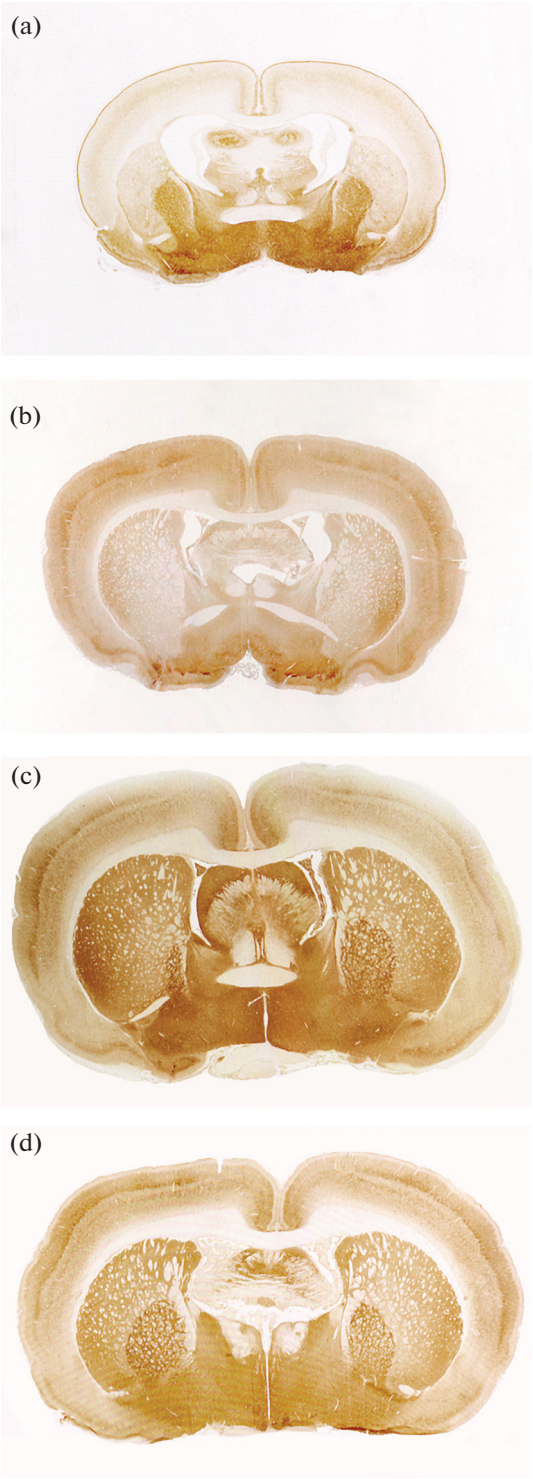

Fig. 2. Distribution of GABAergic structures in the brain at different stages of postnatal development. Immunohistochemical staining for GAD67. (a) Day 7 of postnatal development; (b) day 30 of postnatal development; (c) an adult animal; (d) an old animal.

the synaptic terminals of GABAergic interneurons concentrated on the bodies and processes of pyramidal cells in layers III and V (Fig. 2b). The insular cortical zone is characterized by weak staining while maintaining the laminar distribution of GAD67. No accumulation of GABAergic terminals in the first layer of the cortex characteristic of the 7th postnatal day was observed at this time. The bodies of GAD67-positive neurons were located in all layers of the cortex. Synaptic endings appear on the bodies of interneurons. In contrast to P7, in P30 animals both caudate putamen and globus pallidus exhibited medium-intensity staining. This period is also characterized by the highest percentage of GABAergic interneurons both in the cortex and in the subcortical structures (Table 1). GAD67-positive structures in the subventricular zone were not detected at this time (Fig. 3c). Interestingly, one-month-old animals develop a response to GAD67 in the choroid plexus (Fig. 4b). The enzyme was found in the apical part of epithelial cells. The average fluorescence intensity was low and totaled 68.71 ± 1.58 conventional units of brightness.

In adult animals (5–6 months), like in 30-day-old animals, an enhanced reaction was observed in layers III and V of the cortex. In the neuropil of layers I and IV of the cortex, there was a lower density of GABAergic terminals. The cingulate and sensory zones of the cortex are distinguished by a high intensity of staining, the rest of the cortex (with the exception of the insular) are characterized by medium intensity. The bodies of GABAergic neurons have a varied morphology and are located in all layers of the cortex. However, the presence of neuronal bodies in layer I of the cortex was observed only in certain areas: mainly in the cingulate cortex and the secondary motor cortex. Due to the unequal intensity of the immunohistochemical reaction, it was possible to distinguish between globus pallidus and caudate-putamen at the anatomical level (Fig. 2c). In subcortical structures, two populations of GABAergic interneurons can also be identified: cells with strong GAD67 staining and cells with moderate GAD67 staining. In the subventricular zone, there were no cells with a positive immunohistochemical reaction to GAD67. At this age, accumulation of the enzyme was also observed in the apical part of epithelial cells (Fig. 4c), while the average fluorescence intensity was the highest among the studied ages (183.4 ± 3.05 conventional units of brightness).

In old rats (23–24 months), as well as in P30 and adult animals, the distribution of the enzyme makes it possible to clearly distinguish the layers of the cortex (Fig. 2d). The different intensity of staining of the cortical areas, characteristic of earlier periods (P30 and adult animals), viz., high-intensity staining in the cingulate and sensory regions, medium intensity staining of the motor and piriform regions, and weak staining of the insular cortex, is preserved. In I layer of certain areas of the cerebral cortex, namely in the cingulate, motor and sensory areas, bodies of GABAergic neurons were detected. The different intensities of staining of caudate-putamen and globus pallidus were preserved (see Fig. 2). In old animals, as in 7-day-old ani-

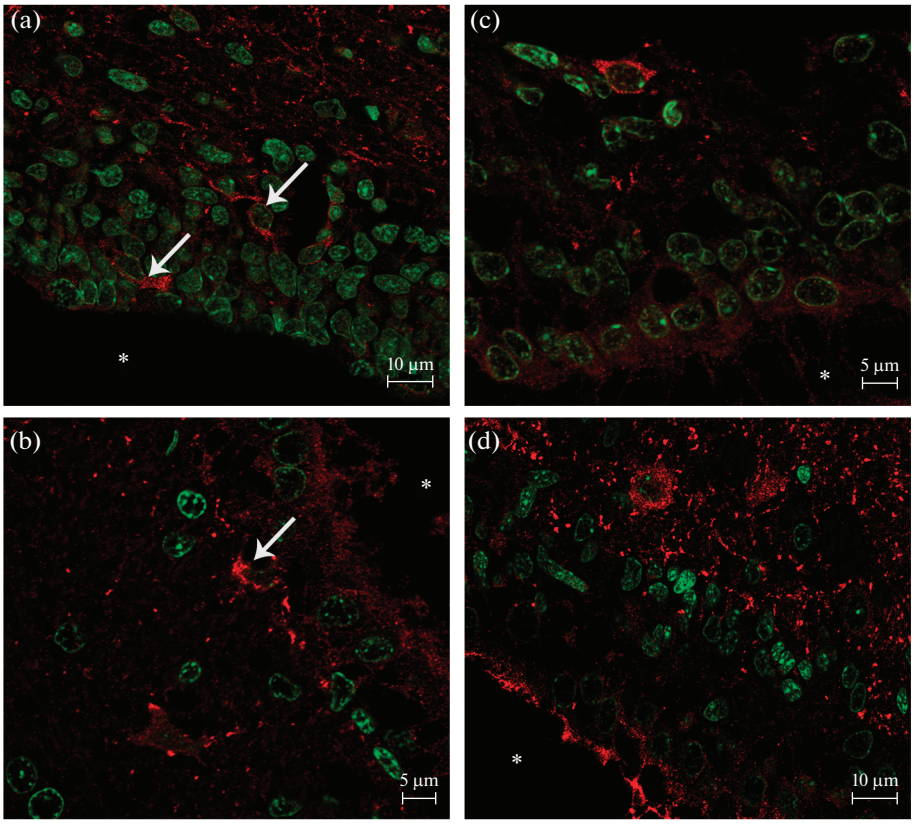

Fig. 3. GABAergic neurons of the subventricular zone. Immunohistochemical staining for GAD67 (red) and SytoxGreen-stained nuclei (green). (a) Day 7 of postnatal development; (b) an old animal; (c) day 30 of postnatal development; (d) an adult animal. The arrow points to the GABAergic neurons in the subventricular zone, the asterisk to the ventricular cavity.

mals, GABAergic cells can be seen in the subventricular zone (Fig. 3b). At this time, the average intensity of GAD67 fluorescence in the choroid plexus decreases to 53.01 ± 1.16 conventional units of brightness. The differences in this parameter were statistically significant for all the studied periods ($p < 0.05$).

DISCUSSION

A preliminary study showed the possibility of detecting the bodies of GABAergic neurons and axonal terminals in rat brain slices using confocal laser scanning microscopy. Despite the fact that synaptic terminals, due to their small size, are traditionally studied using electron microscopy, their observation in the brain is also possible at the light level using antibodies to proteins selectively localized in the region of synaptic contacts. The most easily visualized are large synapses, for example, the mossy fiber terminals in the hippocampus and cerebellum [27]. The identification of smaller structures is feasible due to the fact that a high concentration of antigen in a small volume makes it possible to obtain a signal with a high intensity of fluorescence, which can be recorded by a confocal microscope as a discrete structure if the location of these structures is not closer than the resolution of the microscope. In works on the study of the morphology of synapses, performed using electron microscopy [28–30], it can be seen that GABAergic synaptic terminals are approximately 0.3 to 2 μm in size. Since the lateral resolution of the Zeiss LSM 800 confocal microscope for the Plan-Apochromat 63 ×/1.40 Oil DICM27 objective and the 561 nm laser (which was used to excite the Cy3 fluorescence) is about 0.16 μm,

Table 1. The percentage of GAD67-positive cells in the cortex and in the subcortical structures of the brain at different ages (mean ± standard error (the number of values used to calculate the mean)). *p-value < 0.01

Zone	P7	P30	Sexually mature	Old
Cortex	6.96 ± 0.3 (20)	15.76 ± 0.89* (20)	7.21 ± 0.58 (20)	7.96 ± 0.49 (20)
Striatum	4.38 ± 0.13 (20)	6.25 ± 0.31* (20)	4.12 ± 0.57 (20)	3.72 ± 0.27 (20)

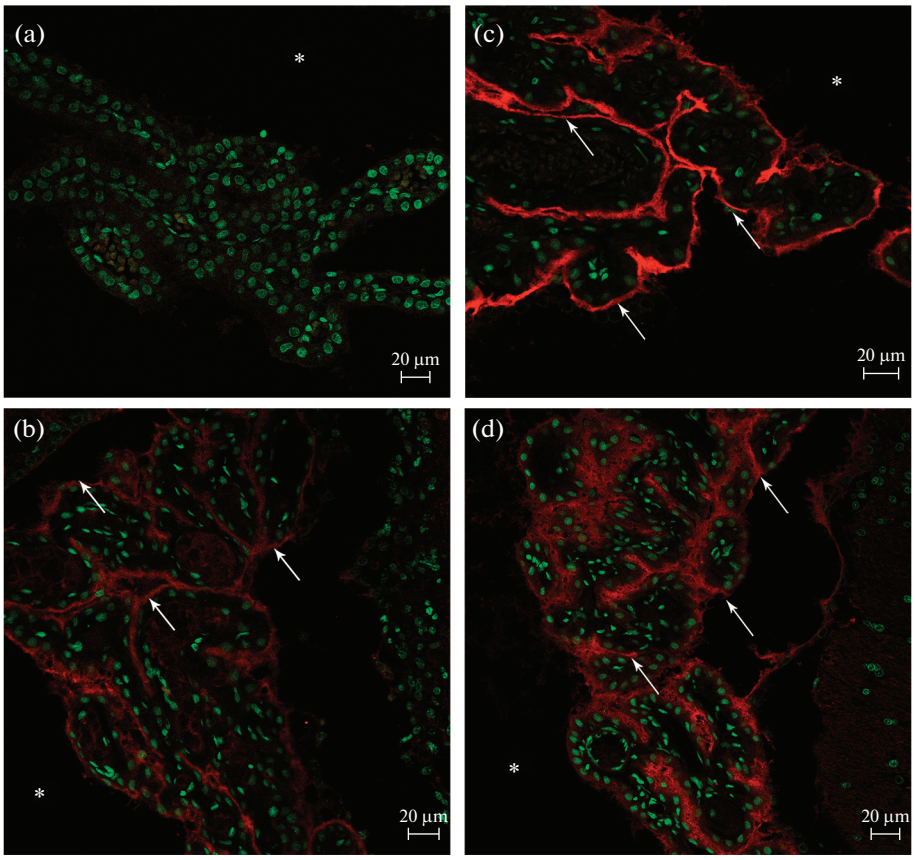

Fig. 4. GAD67 in the choroid plexus. Immunohistochemical staining for GAD67 (red) and SytoxGreen staining of nuclei (green). (a) Day 7 of postnatal development, no reaction; (b) day 30 of postnatal development; (c) adult animal, intense reaction; (d) old animal. The arrow indicates the accumulation of the enzyme in the apical part of the epithelial cells, the asterisk indicates the cavity of the ventricle.

it is technically possible to detect synapses. The present study confirms the possibility of identifying discrete GAD67-immunopositive structures that are axosomatic synapses (see Fig. 1).

During development, GABAergic interneurons undergo various molecular, structural, and functional changes that affect the state and functioning of the central nervous system. GABAergic brain cells are formed in the subpallium and populate the cerebral cortex during tangential migration during embryogenesis [2, 31]. These cells are located in all layers of the cortex and differ not only morphologically but also in their biochemical and electrophysiological properties, and the intercellular contacts they form [32, 33]. This diversity is necessary for clear modulation of the activity of the main efferents of the cortex, pyramidal neurons, and other cells. The high diversity of morphological, molecular, and physiological characteristics of GABAergic cells has led to the development of a complex nomenclature of inhibitory neurons in the cerebral cortex [34–36].

In the layer I of the cortex in 7-day-old rats, a large number of GABAergic structures were observed in all areas of the cortex, except for cingulate and piriform. The layer I of the cerebral cortex is the only layer that does not contain excitatory cells. It is known that GABAergic Martinotti cells project their axons into the first layer establishing synaptic connections with the dendrites of pyramidal cells. It was found that the majority of inhibitory synapses during the first week of postnatal development are created by Martinotti cells precisely on the apical dendrites of pyramidal cells [37], the processes of which reach layer I of the cortex. In the cortex of the developing brain, layer I also contains reelin-producing Cajal–Retzius cells, which regulate the migration of cortical neurons during the embryonic and early postnatal stages of development [38]. Since the Cajal–Retzius cells express $GABA_A$ receptors both during embryonic and postnatal development, and somatostatin-containing Martinotti cells differentiate early enough, it was suggested that the GABAergic terminals observed in the first layer are involved in the formation of brain columns due to the regulation of the Cajal–Retzius cells [39–41]. In addition, since in the adult brain reelin-containing cells are GABAergic interneurons, it was suggested that the

Cajal–Retzius cells of rats undergo phenotypic transformation into neocortex interneurons [42, 43]. Studies in mice, however, have shown that these cells die by apoptosis during the first two weeks of postnatal development [44, 45]. Since no GAD67-positive cells were found in 7-day-old animals in layer I of different parts of the neocortex, our data indicate that the Cajal–Retzius cells of rats, at least at the end of the first week of postnatal development, are not GABAergic.

It seems that two types of GABAergic interneurons are located in the first layer of the cortex of the adult brain: neurogliaform cells and single-bouquet cells. Single-bouquet cells regulate the work of interneurons in layers II/III of the cortex forming unidirectional axonal synaptic terminals on them [46]. Neurogliaform cells are remarkable in that they exert their inhibitory effect not only through synaptic transmission, but also through extrasynaptic GABA release [47]. It is noteworthy that GAD67-positive cells in the first layer of the cortex were most often observed in the cingulate zone at all stages of development. This corresponds to the concept of the complex structure of this area of the cortex [48, 49]. The peculiarities of the cellular composition of the first layer in different zones of the cortex of adult animals may be associated with the functional specialization of the structures of the first layer of the cortex.

Despite our data that the first GABAergic synapses can be observed in the cortex at early stages, synaptic terminals on interneurons were observed by the end of the first month of postnatal development in this work. In mice, the first synapses begin to form on days 5–8, and the peak of synaptogenesis falls on day 10 [37]. In rats, active maturation of GABAergic synapses also occurs by the end of the first postnatal week. It has been shown that the frequency of spontaneous postsynaptic currents from GABAergic cells acquires statistically significant nonzero values only to P7, and the ratio of the frequencies of glutamate/GABA signals exceeded one until the 11th day of postnatal development, which indicates earlier development of glutamatergic conductivity than GABAergic [50]. Presumably, this is due to the formation of thalamocortical connections, which are formed after the first week of development [51]. Thus, the development of GABAergic innervation is due to the need to process sensory information. Using behavioral tests, it was found that in the second week of life, neonatal rats begin to explore the environment [50]. Therefore, it seems logical that by the middle of the second week of postnatal development, after the full development of motor and sensory functions, an increase in synaptogenesis occurs, which continues for a long period of up to 30 days.

On the 30th day of postnatal development, the highest content of GABAergic interneurons among all ages was also recorded, both in the cortex and in the subcortical structures. In recent studies on the distribution of GABA receptors, an increase in the density of $GABA_B$ receptors has been found from P30 to P90 [52]. Since there is an increase in the density of postsynapses, one would expect an increase in the number of presynapses due to either the appearance of new connections between neurons, or an increase in the number of GABAergic neurons themselves. This suggests the involvement of GABAergic neurons in the late stages of brain development.

Immunohistochemical staining for GAD67 revealed weak reactivity in the area of the insular cortex in rats of all ages. GABA and dopamine are considered key neurotransmitters in this area of the cortex. GABAergic interneurons of the insular cortex receive signals from the axons of dopaminergic neurons in the ventral tegmental region [53]. Since studies have shown that dopaminergic stimulation can affect the levels of GAD67 mRNA synthesis [54], it can be assumed that low levels of the enzyme in the insular cortex may be associated with dopamine control.

The low GAD67 content could also be associated with the predominance of long-range interneurons over interneurons that form connections within the insular cortex. Since GAD67, unlike other enzymatic markers of neurons such as tyrosine hydroxylase and choline acetyltransferase [55], is not evenly distributed over the cytoplasm of the cell, the intensity of staining seems to be less than in other areas of the cortex. However, to date, there is insufficient information on the interneuronal composition and their connections in this region to draw a conclusion about a decrease in the intensity of GAD67 staining due to the predominance of long-range neurons in the insular area of the cortex.

It has been shown that the ventral structures of the brain are characterized by the highest staining intensity. By the 30th postnatal day, the sharp difference in the intensity of staining begins to decrease. Nevertheless, in the subcortical structures of sexually mature animals, caudate putamen exhibits a lower intensity of staining than globus pallidus, which is also preserved during aging. These observations are consistent with data on the subpallial origin of most GABAergic interneurons in the brain [2]. The same conclusion was made by Davila et al., who observed a similar ratio of staining intensities for calbindin-positive cells in the dorsal and ventral parts of the claustrum complex. They also noted calbindin-positive neurons with intense and weak staining and suggested that the intensity of staining for calbindin may be associated with the phenotype of GABAergic neurons [56].

At the early stages, GAD67-positive cells were observed in the subventricular zone. Since it is assumed that the subventricular zone serves as an additional source of precursor cells for GABAergic neurons [8], the presence of these cells can be expected not only at the postnatal but also at the embryonic stages of brain development. Indeed, experiments on mice have shown that GABA-positive

cells can be observed in the subventricular zone in late prenatal (E14-E19) and early postnatal development (P0–P8) [15]. GABAergic cells were also observed in the ventricular zone during embryogenesis (E14–E20) in Wistar rats [57]. GAD67-positive cells in the subventricular zone are not observed at a later stage, but they are present in old animals. Apparently, this is due to the differentiation of cells in the neurogenic zone in response to neurodegenerative processes occurring during aging under conditions of inhibition of migration.

In our study, attention was first drawn to the ontogenetic dynamics of GAD67 immunoreactivity in choroid plexus. It was previously described that the choroid plexus of the brain of mouse embryos may be immunostained against another enzyme of GABA synthesis, GAD65 [58]. In contrast, GAD67 was not found at this stage of development. Although in some publications it can also be observed that the apical part of the choroid plexus epithelial cells of the brain of adult animals contains GAD67 [59], the authors do not focus on this, since this area is not their area of interest. From this it can be concluded that the presence of GAD67 in this zone is not an artifact of immunohistochemical staining. This is also evidenced by the fact that, in the negative control preparations, no immune activity was observed either from the choroid plexus or from other structures.

Therefore, the question arises whether the cells of the choroid plexus can produce extrasynaptic GABA. Since the localization of the enzyme is close to the apical surface, and it is also known that GABA is contained in the cerebrospinal fluid (CSF), it can be assumed that the production of GABA in the choroid plexus is produced for further transport of the amino acid to the CSF. It was proposed that GABA enters the CSF from the blood [60]. However, it is known that transthyretin (prealbumin), a blood serum product, does not enter the CSF from the blood, but is synthesized in the epithelial cells of the choroid plexus [61]. In addition, it was found that the level of GABA transport in the CSF decreases in adult rats compared to young animals [60]. This may indirectly indicate that the synthesis of GABA in the apical part of the choroid plexus epithelial cells by means of GAD67 occurs and increases with age. A decrease in fluorescence intensity with aging indicates a decrease in the amount of the enzyme in old animals.

CONCLUSIONS

This study characterizes the morphological changes occurring in the GABAergic system during postnatal development. (1) The predominant localization of GABAergic synaptic structures observed in layer I of the cortex of 7-day-old animals, may be associated with the provision of the neuronal migration process. (2) Putative Cajal-Retzius GABAergic cells in rats are not detected by immunohistochemical reaction for GAD67, therefore, they are not GABAergic. (3) The presence of GAD67-positive cells in the subventricular area of the brain at the early stages of postnatal development and in old animals indicates the presence of progenitor cells of GABAergic neurons and their differentiation within this area of the brain. Their local appearance during aging may be associated with a slowdown in the migration process. (4) Epithelial cells of the choroid plexus of the brain by the end of the first month of development may be a source of extrasynaptic GABA entering the cerebrospinal fluid.

FUNDING

The work was carried out within the state assignment of the Institute of Experimental Medicine.

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of interest. The authors declare that they have no conflicts of interest.

Ethical approval. During the study, all applicable international, national and institutional (report no. 1/20 of February 27, 2020 of the local ethical committee of the Institute of Experimental Medicine) principles of animal care and use were followed.

REFERENCES

1. Kubota, Y., *Curr. Opin. Neurobiol.,* 2014, vol. 26, pp. 7–14.
2. Lim, L., Mi, D., Llorca, A., and Marin, O., *Neuron,* 2018, vol. 100, no. 2, pp. 294–313.
3. Wang, B., Long, J.E., Flandin, P., Pla, R., Waclaw, R.R., Campbell, K., and Rubenstein, J.L.R., *J. Comp. Neurol.,* 2013, vol. 521, no. 7, pp. 1561–1584.
4. Long, J.E., Cobos, I., Potter, G.B., and Rubenstein, J.L.R., *Cereb. Cortex,* 2009, vol. 28, pp. 3797–3815.
5. Wang, Y., Neubauer, F.B., Luscher, H.R., and Thurley, K., *Eur. J. Neurosci.,* 2010, vol. 31, no. 9, pp. 1582–1594.
6. Sandberg, M., Flandin, P., Silberberg, S., Su-Feher, L., Price, J.D., Hu, J.S., Kim, C., Visel, A., Nord, A.S., and Rubenstein, J.L.R., *Neuron,* 2016, vol. 91, no. 5, pp. 1260–1275.
7. Liu, Z., Zhang, Z., Lindtner, S., Li, Z., Xu, Z., Wei, S., Liang, Q., Wen, Y., Tao, G., You, Y., Chen, B., Wang, Y., Rubenstein, J.L., and Yang, Z., *Cereb. Cortex,* 2019, vol. 29, no. 6, pp. 2653–2667.
8. Shmakova, A.A., Balatskiy, A.V., Kulebyakina, M.A., Schaub, T., Karagyaur, M.N., Kulebyakin, K.Y., Rysenkova, K.D., Tarabykin, V.S., Tkachuk, V.A., and Semina, E.V., *Russ. J. Dev. Biol.,* 2021, vol. 52, no. 1, pp. 53–63.
9. Al-Jaberi, N., Lindsay, S., Sarma, S., Bayatti, N., and Clowry, G.J., *Cereb. Cortex,* 2015, vol. 25, no. 3, pp. 631–645.

10. Radonjic, N.V., Ayoub, A.E., Memi, F., Yu, X., Maroof, A., Jakovcevski, I., Anderson, S.A., Rakic, P., and Zecevic, N., *Cell Res.,* vol. 9, no. 6, pp. 2139–2151.
11. Okaty, B.W., Miller, M.N., Sugino, K., Hempel, C.M., and Nelson, S.B., *J. Neurosci.,* 2009, vol. 29, no. 21, pp. 7040–7052.
12. Rice, F.L., Gomez, C., Barstow, C., Burnet, A., and Sands, P., *J. Comp. Neurol.,* 1985, vol. 236, no. 4, pp. 477–495.
13. Vincent, S.L., Pabreza, L., and Benes, F.M., *J. Comp. Neurol.,* 1995, vol. 355, no. 1, pp. 81–92.
14. Foster, A.C. and Kemp, J.A., *Curr. Opin. Pharmacol.,* 2006, vol. 6, pp. 7–17.
15. Le Magueresse, C. and Monyer, H., *Neuron,* 2013, vol. 77, no. 3, pp. 388–405.
16. Behar, T.N., Schaffner, A.E., Scott, C.A., O'Connell, C., and Barker, J.L., *J. Neurosci.,* 1998, vol. 18, no. 16, pp. 6378–6387.
17. Li, S., Kumar, P., Joshee, S., Kirschstein, T., Subburaju, S., Khalili, J.S., Kloepper, J., Du, C., Elkhal, A., Szabo, G., Jain, R.K., Kohling, R., and Vasudevan, A., *Cell Res.,* 2018, vol. 28, no. 2, pp. 221–248.
18. Oh, W.C., Lutzu, S., Castillo, P.E., and Kwon, H.B., *Science,* 2016, vol. 353, no. 6303, pp. 1037–1040.
19. Wang, D.D. and Kriegstein, A.R., *J. Physiol.,* 2009, vol. 587, no. 9, pp. 1873–1879.
20. Ben-Ari, Y., *Epileptic Disord.,* 2006, vol. 8, no. 2, pp. 91–102.
21. Bregestovski, P. and Bernard, C., *Front. Pharmacol.,* 2012, vol. 3, no. 65, p. 5.
22. Zilberter, M., *J. Neurosci.,* 2016, vol. 36, no. 40, pp. 10242–10244.
23. Korzhevskii, D.E., Sukhorukova, E.G., Kirik, O.V., and Grigorev, I.P., *Eur. J. Histochem.,* 2015, vol. 59, no. 3, pp. 5–9.
24. Gusel'nikova, V.V. and Korzhevskii, D.E., *Neurosci. Behav. Physiol.,* 2018, vol. 48, no. 2, pp. 153—156.
25. Greif, K.F., Erlander, M.G., Tillakaratne, N.J.K., and Tobin, A.J., *Neurochem. Res.,* 1991, vol. 16, no. 3, pp. 235–242.
26. Shihan, M.H., Novo, S.G., Le Marchand, S.J., Wang, Y., and Duncan, M.K., *Biochem. Biophys. Reports,* 2021, vol. 25, p. 100916.
27. Kolos, E.A., Grigor'ev, I.P., and Korzhevskii, D.E., *Morfologiya,* 2015, vol. 147, no. 1, pp. 78–82.
28. Nakamoto, K.T., Mellott, J.G., Killius, J., Storey-Workley, M.E., Sowick, C.S., and Schofield, B.R., *Front. Neuroanat.,* 2014, vol. 8, no. 108, pp. 1–13.
29. Cesa, R., Morando, L., and Strata, P., *Proc. Natl. Acad. Sci. USA,* 2008, vol. 105, no. 48, pp. 18988–18993.
30. Peters, A., *J. Neurocytol.,* 2002, vol. 31, nos. 3–5.
31. Lodato, S. and Arlotta, P., *Annu. Rev. Cell Dev. Biol.,* 2015, vol. 31, pp. 699–720.
32. Feldmeyer, D., Qi, G., Emmenegger, V., and Staiger, J.F., *Neuroscience,* 2018, vol. 368, pp. 132–151.
33. Tremblay, R., Lee, S., and Rudy, B., *Neuron,* 2016, vol. 91, no. 2, pp. 260–292.
34. Ascoli, G.A., Alonso-Nanclares, L., Anderson, S.A., Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., Buzsáki, G., Cauli, B., DeFelipe, J., Fairén, A., Feldmeyer, D., Fishell, G., Fregnac, Y., Freund, T.F., Gardner, D., Gardner, E.P., Goldberg, J.H., Helmstaedter, M., Hestrin, S., Karube, F., Kisvárday, Z.F., Lambolez, B., Lewis, D.A., Marin, O., Markram, H., Mũoz, A., Packer, A., Petersen, C.C.H., Rockland, K.S., Rossier, J., Rudy, B., Somogyi, P., Staiger, J.F., Tamas, G., Thomson, A.M., Toledo-Rodriguez, M., Wang, Y., West, D.C., and Yuste, R., *Nat. Rev. Neurosci.,* 2008, vol. 9, no. 7, pp. 557—568.
35. Defelipe, J., López-Cruz, P.L., Benavides-Piccione, R., Bielza, C., Larrañaga, P., Anderson, S., Burkhalter, A., Cauli, B., Fairén, A., Feldmeyer, D., Fishell, G., Fitzpatrick, D., Freund, T.F., González-Burgos, G., Hestrin, S., Hill, S., Hof, P.R., Huang, J., Jones, E.G., Kawaguchi, Y., Kisvárday, Z., Kubota, Y., Lewis, D.A., Marín, O., Markram, H., McBain, C.J., Meyer, H.S., Monyer, H., Nelson, S.B., Rockland, K., Rossier, J., Rubenstein, J.L.R., Rudy, B., Scanziani, M., Shepherd, G.M., Sherwood, C.C., Staiger, J.F., Tamás, G., Thomson, A., Weng, Y., Yuste, R., and Ascoli, G.A., *Nat. Rev. Neurosci*, 2013, vol. 14, no. 3, pp. 202—216.
36. Mihaljević, B., Benavides-Piccione, R., Bielza, C., Larrañaga, P., and DeFelipe, J., *Sci. Data,* 2019, vol. 6, no. 1, p. 221.
37. Favuzzi, E., Deogracias, R., Marques-Smith, A., Maeso, P., Jezequel, J., Exposito-Alonso, D., Balia, M., Kroon, T., Hinojosa, A.J., Maraver, E.F., and Rico, B., *Science,* 2019, vol. 363, no. 6425, pp. 413—417.
38. Kohno, T., Honda, T., Kubo, K.I., Nakano, Y., Tsuchiya, A., Murakami, T., Banno, H., Nakajima, K., and Hattori, M., *J. Neurosci.,* 2015, vol. 35, no. 11, pp. 4776—4787.
39. Cheng, Q., Yeh, P.W.L., and Yeh, H.H., *Eur. J. Neurosci.,* 2006, vol. 24, no. 8, pp. 2145–2151.
40. Gonchar, Y., Wang, Q., and Burkhalter, A.H., *Front. Neuroanat.,* 2008, vol. 1, p. 3.
41. Wang, Y., Toledo-Rodriguez, M., Gupta, A., Wu, C., Silberberg, G., Luo, J., and Markram, H., *J. Physiol.,* 2004, vol. 561, no. 1, pp. 65–90.
42. Imamoto, K., Karasawa, N., Isomura, G., and Nagatsu, I., *Neurosci. Res.,* 1994, vol. 20, no. 1, pp. 101–105.
43. Sarnat, H.B. and Flores-Sarnat, L., *Semin. Pediatr. Neurol.,* 2002, vol. 9, no. 4, pp. 302–308.
44. Chowdhury, T.G., Jimenez, J.C., Bomar, J.M., Cruz-Martin, A., Cantle, J.P., and Portera-Cailliau, C., *Front. Neuroanat.,* 2010, vol. 4, no. 10, p. 8.
45. Derer, P. and Derer, M., *Neuroscience,* 1990, vol. 36, no. 3, pp. 839–856.
46. Jiang, X., Wang, G., Lee, A.J., Stornetta, R.L., and Zhu, J.J., *Nat. Neurosci.,* 2013, vol. 16, pp. 210–218.
47. Oláh, S., Füle, M., Komlósi, G., Varga, C., Báldi, R., Barzó, P., and Tamás, G., *Nature,* 2009, vol. 461, no. 7268, pp. 1278–1281.
48. Fedosikhina, L.A., *Neurosci. Behav. Physiol.,* 1986, vol. 16, no. 4, pp. 340–345.

49. Grigor'ev, I.P., Alekseeva, O.S., Kirik, O.V., Sufieva, D.A., and Korzhevskii, D.E., *Morfologiya,* 2018, vol. 154, no. 5, pp. 7–12.

50. Naskar, S., Narducci, R., Balzani, E., Cwetsch, A.W., Tucci, V., and Cancedda, L., *Nat. Commun.,* 2019, vol. 10, no. 1, pp. 1–12.

51. Molnar, Z. and Blakemore, C., *Trends. Neurosci.,* 1995, vol. 18, no. 9, pp. 389–397.

52. Behuet, S., Cremer, J.N., Cremer, M., Palomero-Gallagher, N., Zilles, K., and Amunts, K., *Front. Neuroanat.,* 2019, vol. 13, no. 100, p. 14.

53. Ohara, P.T., Granato, A., Moallem, T.M., Wang, B.R., Tillet, Y., and Jasmin, L., *J. Neurocytol.,* 2003, vol. 32, no. 2, pp. 131–141.

54. Retaux, S., Trovero, F., and Besson, M.-J., *Eur. J. Neurosci.,* 1994, vol. 6, no. 12, pp. 1782–1791.

55. Kolos, E.A. and Korzhevskii, D.A., *Neurochem. J.,* 2016, vol. 10, no. 1, pp. 47–52.

56. Davila, J.C., Real, M.A., Olmos, L., Legaz, I., Medina, L., and Guirado, S., *J. Comp. Neurol.,* 2005, vol. 481, no. 1, pp. 42–57.

57. Cobas, A., Fairen, A., Alvarez-Bolado, G., and Sanchez, M.P., *Neuroscience,* 1991, vol. 40, no. 2, pp. 375–397.

58. Tochitani, S. and Kondo, S., *PLoS One,* 2013, vol. 8, no. 2, p. 7.

59. Andrews, W.D., Barber, M., Nemitz, M., Memi, F., and Parnavelas, J.G., *Brain Struct. Funct.,* 2017, vol. 222, no. 5, pp. 2217–2233.

60. Al-Sarraf, H., *Dev. Brain Res.,* 2002, vol. 139, no. 2, pp. 121–129.

61. Vieira, M. and Saraiva, M.J., *Biomol. Concepts,* 2014, vol. 5, no. 1, pp. 45–54.