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Glutamine Synthetase in Rat Brain Cells

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Objective. To use an immunohistochemical method to study glutamine synthetase (GS)-synthesizing brain cells. **Materials and methods.** Enzyme was detected on frontal rat brain sections ($n = 10$) using mouse monoclonal antibodies. Specimens were analyzed by light and confocal laser microscopy. **Results.** GS was found to be expressed in all areas of the brain, mainly by two types of cells, with different structures and topographies. The main type of cell with immunopositive reactions to glial fibrillary acidic protein were identical to astrocytes. The other structural and locational type of cell differed from typical astrocytes. **Conclusions.** The data obtained here provide evidence that GS is not a selective marker for any particular population of rat brain cells.

Keywords: brain, glia, glutamine synthetase, immunohistochemistry.

Glutamine synthetase (GS) is the key enzyme in the metabolism of the most important excitatory neurotransmitter, i.e., glutamate. The results of early immunohistochemical studies [11] showed that GS is present exclusively in astrocytes and is absent from other brain cells. Later studies showed it to be present in oligodendrocytes [6, 7], though it has been suggested that the data obtained from these studies result from the use of poorly purified antigens and that the conclusions were due to differences in interpretations of the data. The intracellular distribution of GS in astrocytes [3, 12] is currently the best studied, while data on the observation of this protein in other nervous tissue cells in mammals are fragmentary and contradictory [5, 8]. Data on the localization of an enzyme as important as GS, which is involved in regulating synaptic processes and intercellular interactions are needed for developing new approaches to determining the in situ functional activity of brain cells and for improving the criteria for assessing the status of the glutamatergic system of the brain using histological studies.

The aim of the present work was to carry out an immunohistochemical study of GS-synthesizing rat brain cells.

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Materials and Methods. Experiments were performed using whole brains from adult male Wistar rats ($n = 10$). Animals were sacrificed in compliance with the international regulations of the Helsinki Declarations on the Humanitarian Treatment of Animals. The study was approved by the ethics committee (No. 1/14 of April 21, 2014). Rat brains were fixed in zinc-ethanol-formaldehyde, which provides the high level of antigen preservation required for optimum results from immunocytochemical reactions [1, 10]. Fixed brains were sectioned in the frontal plane at the level of the anterior hippocampus, dehydrated, and both parts of the brain were embedded in paraffin by standard methods. Frontal sections of thickness 5 μm were cut from these blocks on a Leica RM2125RT rotary microtome (Leica, Germany). Immunocytochemical detection of GS was with mouse monoclonal antibodies (clone GS-6, diluted 1:400, Chemicon, USA). Secondary reagents for light microscopy were from an EnVision+ System Labelled Polymer-HRP Anti-Mouse kit (Dako, Denmark). Thermal antigen demasking in modified citrate buffer (S1700, Dako, Denmark) for 25 min was performed before reactions. Once immunohistochemical reactions were under way, some sections were counterstained with alum hematoxylin. Secondary antibodies for confocal laser microscopy consisted of biotinylated donkey antimouse Fab

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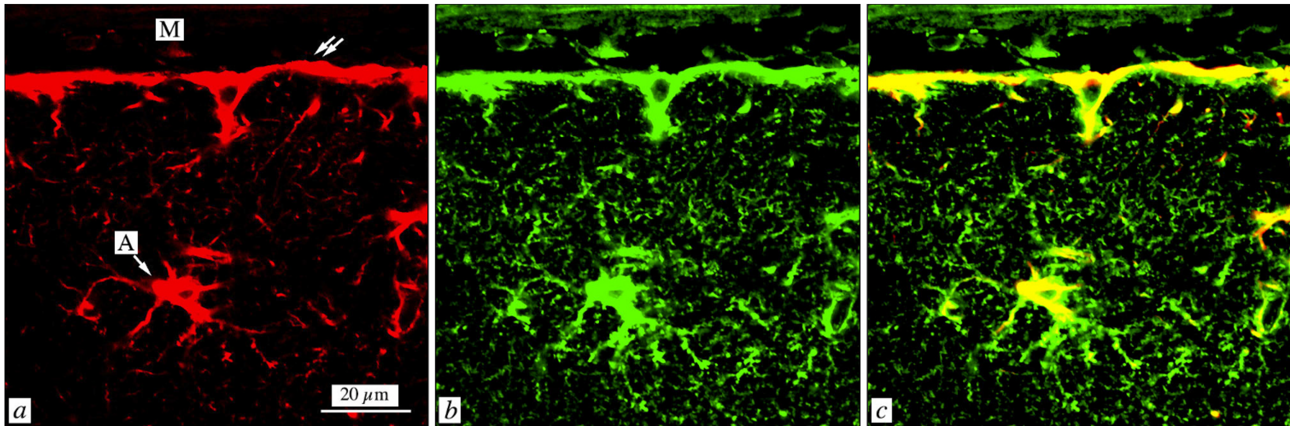

Fig. 1. Combined localization of two proteins in astrocytes in the superficial layers of the rat cerebral cortex. *a*) Positive reaction in wedge-shaped astrocytes forming the superficial glial delimiting membrane (double arrows) and in an astrocyte (A) in layer I of the cortex. M – meninges. Double immunocytochemical reactions for glutamine synthetase (*a*), visualized with fluorochrome Cy2 (green), and glial fibrillary acidic protein (*b*), visualized with the fluorochrome TRITC (red). Combined localization of markers result in structures staining yellow (*c*). Confocal laser microscopy. The size of the Z series was 7.8 μm and the number of optical slices was 27. Reconstruction run in the 3D module of Zen 2011 (Zeiss, Germany) in the "Maximum Advanced" mode.

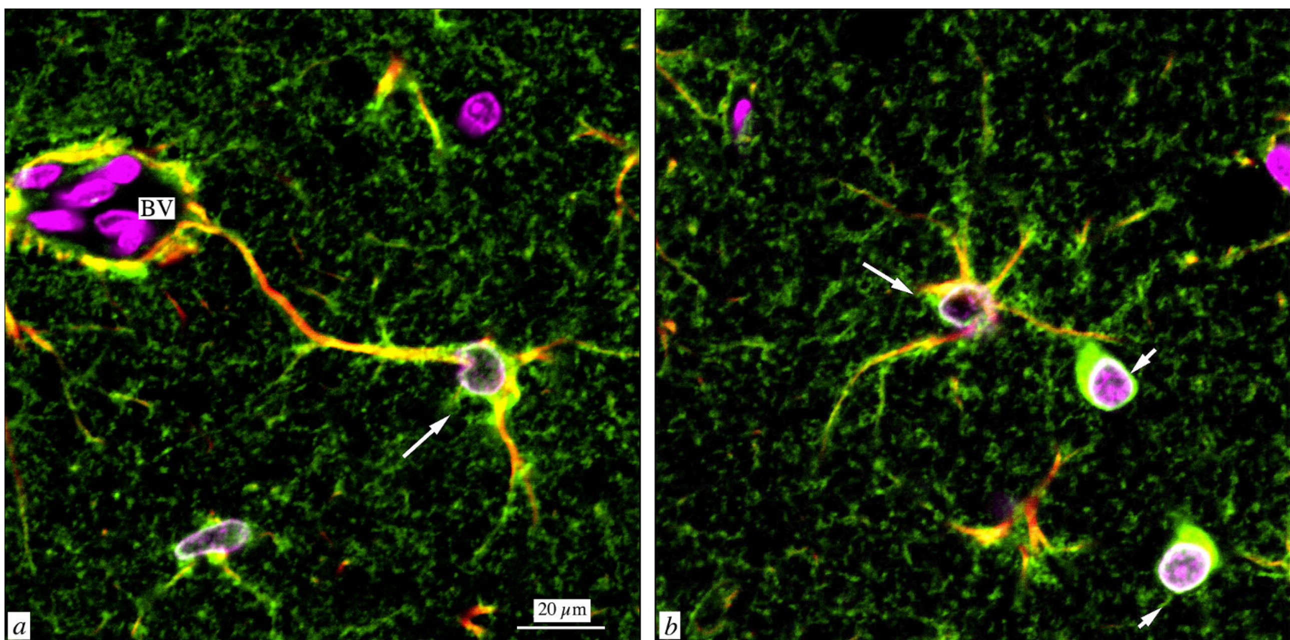

Fig. 2. Glutamine synthetase in cells in hippocampal field CA1 (*a*) and field CA3 (*b*). BV – blood vessel. Long arrow – astrocyte; short arrows – oligodendrocytes. Double immunocytochemical reaction for glutamine synthetase (visualized using fluorochrome Cy2, green) and glial fibrillary acidic protein visualized using fluorochrome tetramethylrhodamine isothiocyanate (red). Structures are stained yellow when both markers are present. Cell nuclei are stained with the fluorescent dye ToPro3 (violet). Confocal laser microscopy: *a*) magnitude of Z series 4.8 μm, number of optical sections 17.6; *b*) magnitude of Z series 2.7 μm, number of optical sections 10. Reconstruction in the 3D module of ZEN 2011 (Zeiss, Germany) in the "Maximum Advanced" mode.

immunoglobulin fragments (Jackson ImmunoResearch, USA, diluted 1:200) and streptavidin conjugated with fluorescent dye carbocyanin (Cy2, Jackson ImmunoResearch, USA, diluted 1:50). The astroglial nature of some of the cells detected was confirmed by double immunofluorescence reactions using the most selective astrocyte marker [2] – glial fibrillary acidic protein (GFAP). Primary antibodies were polyclonal rabbit anti-GFAP antibodies (Dako, Denmark) and secondary antibodies were pig antirabbit antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) diluted 1:25. Cell nuclei were stained with the fluorescent dye ToPro3. Analysis of specimens by microscopy in transmitted light and photography at low (×10) and high (×100) magnifications were performed using a Leica

DM750 microscope and an ICC50 digital camera (Leica, Germany). Sections treated with fluorochrome-conjugated antibodies were examined under an LSM-710 confocal laser microscope (Zeiss, Germany) and three-dimensional reconstruction was performed using ZEN-2011 software (Zeiss, Germany). Excitation of Cy2, TRITC, and ToPro3 fluorescence was with lasers with wavelengths of 488, 561, and 633 nm, respectively. Measurements were made using LSM Image Browser software (Zeiss, Germany).

Results. Studies of specimens at low magnification allowed differences in the distributions of immunoreactive cells in different parts of the brain to be seen. Thus, the greatest staining intensity was seen in the superficial glial delimiting membrane, the deep layers of the cerebral cortex, including the piriform cortex, and the white matter of the brain. Of the neural centers, the strongest reactions to GS were found in the lateral nucleus of the septum, the habenula, the striatum, and the hippocampus. Less marked reactions were seen in the outer layers of the neocortex, the thalamic nuclei, and the hypothalamus, as well as in cells forming the walls of the cerebral ventricles, the subventricular zone, and the vascular plexus. However, the presence of a weak immunopositive cytoplasmic reaction in the cells of the vascular plexus had was not convincingly confirmed in studies of specimens using confocal laser microscopy.

Examination of specimens at high magnification revealed a monolayer of wedge-shaped immunopositive cells at the brain surface (Fig. 1). The wide bases of these cells faced the surface, while the apex gave rise to a small number of relatively long (some up to 60 μm), nonbranching, fibrous (maximum wave amplitude less than 3 μm) processes of thickness 0.7–0.9 μm running into the depth of the tissue.

Detailed investigation of other areas with high immunoreactivity revealed two different types of intensely staining cells. In the zones corresponding to the gray matter of the cortex and neural centers, the main type was stellate cells with centrally located immunopositive nuclei and bright rims of perinuclear cytoplasm with 3–4 large radially directed processes of thickness 3–4 μm, which gave rise to smaller processes. These cells were located between neurons, not infrequently surrounding them with their bodies or processes, and also surrounding blood vessels (Fig. 2, *a*). The white matter of the brain contained a small number of immunoreactive cells of this type. The double immunofluorescence reaction (GFAP/GS) showed that some of these cells were immunonegative for GS. The other type consisted of round cells without processes (or with single processes) with clearly stained cytoplasm and immunonegative nuclei. In zones corresponding to the gray matter and neural centers, these cells were distributed diffusely and individually (see Fig. 2, *b*) or grouped into pairs. The number of these cells was greater in the white matter, where they were located in groups of 3–5 cells in the form of chains extending along immunonegative nerve fibers.

Discussion. The data obtained here identify the distribution of GS in rat CNS cells. Distributions in different parts of the brain were nonuniform. Clearly outlined GS-positive cells with processes, seen in different parts of the brain and involved in forming superficial and perivascular glial delimiting membranes, allowing for their structures and positions, should be identified as astrocytes. The assignment of these cells to the astrocyte population was also confirmed by their immunopositive reaction for GFAP. The second type of GS-positive cell, without processes (or with single processes) were not, in terms of their morphological characteristics and locations, typical astrocytes. The sizes of these cells and the shapes of their nuclei indicated that the second type of GS-immunopositive cells could not be microglial cells or neurons, but could represent an atypical population of astrocytes or oligodendrocytes. However, while the abundance of GS-positive astrocytes in the zones containing large neurons is explained by their role in the glutamate/glutamine cycle [4, 9], the importance of their presence in the white matter remains to be understood. It is possible that GS-positive astrocytes and oligodendrocytes in the white matter have a role in eliminating excess glutamate to prevent local excitotoxic damage to nerve fibers. None of the areas studied contained GS in neuron perikarya, which is not consistent with data reported by other authors [5], who have found GS in neurons in the hippocampus, substantia nigra, and hypothalamic nuclei. However, this can be explained by the use of other, less specific antibodies to detect GS and use of a different study system - the human brain. The detection of GS-negative astrocytes is no less interesting. If we accept the fact of GS expression by these cells, this observation may indicate that GS-negative astrocytes are in an altered functional state in which the synthesis of this enzyme is suppressed. In addition, negative immunocytochemical reactions in some astrocytes can be explained by the fact that GS in these cells is either not expressed at all, or is formed in such small quantities that it could not be detected by the methods used here.

Thus, the data obtained in these studies provide evidence that GS is expressed by two cell populations: typical astrocytes and another cell population which may consist of atypical astrocytes or, less likely, oligodendrocytes. Thus, we do not believe that this protein can be regarded as a selective marker for just one cell type.

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The authors have no conflicts of interests.

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