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Distribution of immunoreactive glutamine synthetase in the adult human and mouse brain. Qualitative and quantitative observations with special emphasis on extra-astroglial protein localization

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ABSTRACT

Glutamine synthetase catalyzes the ATP-dependent condensation of ammonia and glutamate to form glutamine, thus playing a pivotal role in glutamate and glutamine homoeostasis. Despite a plethora of studies on this enzyme, knowledge about the regional and cellular distribution of this enzyme in human brain is still fragmentary. Therefore, we mapped fourteen post-mortem brains of psychically healthy individuals for the distribution of the glutamine synthetase immunoreactive protein. It was found that glutamine synthetase immunoreactivity is expressed in multiple gray and white matter astrocytes, but also in oligodendrocytes, ependymal cells and certain neurons. Since a possible extra-astrocytic expression of glutamine synthetase is highly controversial, we paid special attention to its appearance in oligodendrocytes and neurons. By double immunolabeling of mouse brain slices and cultured mouse brain cells for glutamine synthetase and cell-type-specific markers we provide evidence that besides astrocytes subpopulations of oligodendrocytes, microglial cells and neurons express glutamine synthetase. Moreover, we show that glutamine synthetase-immunopositive neurons are not randomly distributed throughout human and mouse brain, but represent a subpopulation of nitrergic (i.e. neuronal nitric oxide synthase expressing) neurons. Possible functional implications of an extra-astrocytic localization of glutamine synthetase are discussed.

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Introduction

Being the main excitatory and inhibitory neurotransmitters in the adult CNS, glutamate, aspartate and GABA are of crucial importance for normal brain functioning and are involved in a variety of brain pathologic processes. Glutamine serves as the precursor molecule for the synthesis of glutamate, aspartate and GABA (Holten and Gundersen, 2008). At glutamatergic synapses, glutamate released from neurons is taken up from the extracellular space by astrocytes and metabolized with ammonia and ATP to synthesize glutamine by the enzyme glutamine synthetase (GS; aka glutamate-ammonia ligase, EC 6.3.1.2). Glutamine is released from astrocytes into the extracellular space, from where it is transported to glutamatergic neurons. In glutamatergic neurons, glutamine is hydrolyzed to glutamate and ammonia by the enzyme phosphate activated glutaminase. An alternative route supplies GABAergic neurons with extracellular glutamine. GS is thus central

Abbreviations: CC, corpus callosum; Cdm, caudate nucleus (medial part); CG, cingulate cortex; Cla, claustrum; DAPI, 4′,6-diamidin-2-phenylindole; DIV, days in culture (in vitro); DMEM, Dulbecco's modified Eagle's medium; EGP, external globus pallidus; Ent, entorhinal cortex; FCS, fetal calf serum; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; IBA1, ionized calcium binding adaptor molecule 1; IG, insular gyrus; MFG, medial frontal gyrus; MTG, medial temporal cortex; nNOS, neuronal nitric oxide synthase; NG2, chondroitin sulfate proteoglycan; NS-1, cell clone oligo NS-1; OFG, orbitofrontal gyri; Pan-NF, pan-neurofilament (cell marker); PFA, paraformaldehyde; PrG, precentral gyrus; PVN, paraventricular nucleus; Pu, putamen; SN, septal nuclei; SCN, suprachiasmatic nucleus; SFG, superior frontal gyrus; SON, supraoptic nucleus; STG, superior temporal gyrus.

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to glutamate, aspartate and GABA metabolism, as well as to brain ammonia detoxification (for recent reviews see Bernstein et al., 2013; Dadsetan et al., 2013). Although commonly regarded a highly specific marker enzyme of astrocytes (as reviewed in Anlauf and Derouiche, 2013), GS has also been observed in extra-astroglial locations, both in normal and pathologic conditions (gray and white matter oligodendroglial cells in vivo, in cell culture and in oligodendrogliomas: Cammer, 1990; D'Amelio et al., 1990; Fressinaud et al., 1991; Tansey et al., 2009; Miyake and Kitamura, 1992; Baas et al., 1998; Baumann and Pham-Dinh, 2001; Takasaki et al., 2010; Zhuang et al., 2011; Bernstein et al., 2013; Liu et al., 2013 and others), microglia (for overview see Olabarria et al., 2011); ependymal cells (Graff et al., 1993) and even neurons (Carter, 1981; Robinson, 2000, 2001; Smalla et al., 2008; Fernandes et al., 2010, and according to data from the "The Human Protein Atlas", version 12, 2013; http://www.proteinatlas.org/). However, despite difficulties to functionally interpret the occurrence of GS outside astroglia, there are serious objections with regard to the specificity of extra-astroglial localizations of GS (oligodendroglia: possible misinterpretation of cell morphology; neurons: preparation and/or storage artifact, Anlauf and Derouiche, 2013). When analyzing GS expression in post-mortem brains of suicide victims, individuals with schizophrenia and control cases (Bernstein et al., 2013) we saw a considerable number of GS-immunolabeled neurons being scattered throughout the brains, which we initially decided to ignore because of the known concerns. However, the high reproducibility of neuronal GS immunostaining prompted us to systematically map human brains for the regional distribution and cellular GS immunoreactivity in psychically healthy controls. We herein demonstrate that (i) GS immunoreactivity is robustly and specifically expressed not only in a vast majority of astroglial cells, but also in numerous oligodendroglial cells, ependymal cells and certain neurons, and (ii) that neurons expressing GS are not randomly distributed throughout the CNS, but represent a subpopulation of nitrergic (i.e. neuronal nitric oxide synthase expressing) neurons instead.

Materials and methods

Human brain tissue

All brains were obtained from the Magdeburg brain collection. Case selection procedures, the acquisition of personal data, autopsies, and the handling of autoptic material were all conducted in strict accordance with the Declaration of Helsinki, and were approved by the responsible Magdeburg Ethics Committee.

The brains of fourteen human subjects (eight females, six males; mean age 55, 4 ± 6.0 years) were investigated. All individuals died of natural causes. Special attention was given to exclude all cases with chronic liver disease because of the possible influence of hyperammonemic state on cerebral glutamine synthetase expression (Brusilow et al., 2010). The brains were from subjects without a history of neurologic or neuropsychiatric disorders. Brains showing qualitative neuropathological changes due to neurodegenerative disorders such as Alzheimer's, Pick's or Parkinson's disease, or due to tumors, inflammatory, traumatic or vascular processes including lacunar infarctions, were eliminated by an experienced neuropathologist (Prof. Dr. Gerhardt, Essen).

Tissue collection and preparation

Brains were removed within 8–48 h after death and processed as previously described (Bernstein et al., 1998a,b). Briefly, brains were fixed into 10% paraformaldehyde for at least 110 days. After separation of the brainstem, brains were divided by coronal cuts into three coronal blocks comprising the frontal lobe anterior to the genu of the corpus callosum, the fronto-temporo-parietal lobe extending over the whole length of the corpus callosum, and the occipital lobe. After embedding all of the brain sections in paraffin, serial coronal sections of the middle block were cut (20 μm) and mounted. Every 50th section was Nissl and myelin stained (Heidenhain/Woelke). For immunostainings whole brain frontal sections were collected at intervals of about 1.8 cm from the level 2 cm rostral to the splenium to the posterior splenium, and from the central portion of the raphe nuclei to the central portion of the olivary nuclei. Individual volume shrinkage factors (VSF) were calculated by the formula VSF = $[A_1/A_2]^{3/2}$, A_1 being the cut area of the tissue before, A_2 after the embedding in paraffin. Mean shrinkage factor was 2.20. For morphological orientation and anatomical nomenclature the human brain atlas of Mai et al. (2007) was used.

Immunohistochemical staining procedures

Glutamine synthetase immunohistochemistry

To immunolocalize GS, we employed a well-characterized, monospecific polyclonal antiserum generated in rabbits against human GS (Prestige Antibody 007316; Lot 81287; from Sigma–Aldrich, Munich, Germany). Antigen demasking was carried out by boiling the sections for 4 min in 10 mM citrate buffer (pH 6.0). After the preincubation of the sections with methanol/H_2O_2 to suppress endogenous peroxidases and repeated washing with phosphate-buffered saline (PBS), the primary GS antibody was used at a dilution of 1:500 for 72 h at 4 °C. Sections were then incubated with a biotinylated anti rabbit IgG (Amersham Bioscience, Buckinghamshire, GB), followed by the streptavidin horse radish complex for the application of the streptavidin–biotin technique (Amersham). The chromogen 3,3′-diaminobenzidine was used to visualize the reaction product. Subsequently, ammonium nickel sulfate hexahydrate was added to enhance the immunoreaction (Bernstein et al., 2013). For control purposes, the primary antiserum was replaced by either buffer or normal serum. Further control experiments involved the application of the GS antiserum after preabsorption with glutamine synthetase protein (recombinant human glutamine synthetase, charge number CE02; from Novoprotein, Shanghai, China). When these controls were done the investigated regions did not show any specific immunostaining (as exemplified in Fig. 2H).

Neuronal nitric oxide synthase (nNOS) immunohistochemistry

Neuronal NOS (nNOS) immunoreactivity was revealed using a monoclonal antibody (N2280 from Sigma–Aldrich, Munich, Germany). The working dilution was 1:100 in PBS. The subsequent steps of the procedure were as previously described for other antigens (Bernstein et al., 2012). Specificity controls involved replacement of the primary antiserum either by a buffer or with normal serum. Specificity of the primary antibody was further demonstrated by immunoabsorption with a blocking peptide (SA-227, Biozol, Eching, Germany).

For reasons of comparison we also employed a polyclonal antiserum against nNOS with known staining properties (Eurodiagnostica, Stockholm). The staining protocol for this antiserum was as recently described for human brain material (Bernstein et al., 2014).

Double immunostaining for GS and nNOS

The double immunolabeling procedure for GS and nNOS involved the use of the monoclonal anti nNOS antibody (diluted 1:100 in PBS) and the application of the avidin–biotin method. 3,3′-Diaminobenzidine was used as the chromogen, which yielded a reaction product of golden-brownish color. After repeated washings of the sections with PBS the polyclonal antiserum against GS

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