Glycine cleavage system in neurogenic regions

Prof. Z. Molnar

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Akiko Ichinohe[1,2], Shigeo Kure[1], Sumiko Mikawa[3], Takatoshi Ueki[3], Kanako Kojima[1], Kazuko Fujiwara[4], Kazuie Iinuma[2], Yoichi Matsubara[1], and Kohji Sato[3]

Departments of [1]Medical Genetics, and [2]Pediatrics, Tohoku University School of Medicine, Sendai 980-8574, Japan

[3]Department of Anatomy and Neuroscience, Hamamatsu University School of Medicine, Hamamatsu, 431-3192, Japan

[4]Institute for Enzyme Research, Tokushima University School of Medicine, Tokushima, 770-8503, Japan

Correspondence should be addressed to Kohji Sato

Department of Anatomy and Neuroscience, Hamamatsu University School of Medicine, Hamamatsu, 431-3192, Japan

Tel & Fax: +81-53-435-2288

E-mail address: ksato@hama-med.ac.jp

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Abstract

The glycine cleavage system (GCS) is the essential enzyme complex for degrading glycine and supplying 5,10-methylenetetrahydrofolate for DNA synthesis. Inherited deficiency of this system causes non-ketotic hyperglycinemia, characterized by severe neurological symptoms and frequent association of brain malformations. Although high levels of glycine have been considered to cause the above-mentioned problems, the detailed pathogenesis of this disease is still unknown. Here we show that GCS is abundantly expressed in rat embryonic neural stem/progenitor cells in the neuroepithelium, and this expression is transmitted to the radial glia-astrocyte lineage, with prominence in postnatal neurogenic regions. These data indicate that GCS plays important roles in neurogenesis, and suggest that disturbance of neurogenesis induced by deficiency of GCS may be the main pathogenesis of non-ketotic hyperglycinemia.

Introduction

Glycine is an important amino acid involved in central nervous system function. Besides its metabolic roles, glycine serves as a neurotransmitter at many inhibitory synapses in the spinal cord and brain stem (Betz, 1991). It is also a co-agonist of glutamatergic neurotransmission at the NMDA subtype of glutamate receptors (Kemp & Leeson, 1993). The importance of glycine in the central nervous system is further highlighted by the existence of a congenital metabolic disorder, non-ketotic hyperglycinemia. Non-ketotic hyperglycinemia is caused by an inherited deficiency of the glycine cleavage system (GCS, EC 2.1.2.10) and characterized by accumulation of a large amount of glycine in body fluids, particularly in cerebrospinal fluid (Tada et al., 1969; Hayasaka et al, 1987). Individuals with this disorder develop severe neurological symptoms such as coma, respiratory distress, and intractable seizures within the first few days of life. The disorder is frequently associated with brain malformations, such as agenesis of the corpus callosum, gyral malformations, and cerebellar hypoplasia (Press et al., 1989; Dobyns, 1989; Nissenkorn et al., 2001). These developmental abnormalities indicate that adequate GCS activity and precise regulation of glycine concentration are indispensable for normal brain development.

GCS catalyzes the direct cleavage of glycine to form one molecule each of 5,10-methylenetetrahydrofolate, carbon dioxide, and ammonia. It consists of 4 proteins referred to as P-protein, H-protein, T-protein, and L-protein (Kikuchi, 1973). P, T, and H-proteins are encoded by GLDC, AMT, and GCSH genes, respectively. In patients with non-ketotic

hyperglycinemia, specific mutations have been identified in the GLDC gene (Kure et al., 1992), AMT gene (Nanao et al.,1994), and GCSH gene (Kure et al., 2002), providing unequivocal evidence that deficiency of the GCS causes non-ketotic hyperglycinemia. GCS is considered to be the major pathway for the catabolism of glycine not only in mammals, but also in birds, reptiles, amphibians and fish (Kikuchi, 1973; Yoshida & Kikuchi, 1973). In addition, GCS produces 5,10-methylenetetrahydrofolate as a donor of one-carbon units, which is essential for the synthesis of DNA in cell proliferation (Fleming & Copp, 1998).

Recently, glycine receptors and NMDA receptors have been known to be expressed in the developing brain well before synaptogenesis occurs, suggesting that they could mediate signaling unrelated to classical neurotransmission (Nguyen et al., 2001). Since these two types of receptors require glycine and extracellular glycine concentration is regulated by the activity of GCS, GCS would also be involved in this type of signaling. Although we have previously reported that GCS exists in astrocytes in the adult rat brain (Sato et al., 1991), little attention has been paid to GCS in the developing brain. In the present study, we examine GCS expression in the developing rat brain and show that GCS is initially expressed in neural stem/progenitor cells, and that this expression is transmitted to the radial glia-astrocyte lineage, with prominence in postnatal neurogenic regions.

Materials and methods

Animals.

Under diethylether anesthesia brain samples were isolated from Wistar rats. All experiments conformed to the Guidelines for Animal Experimentation at Hamamatsu University School of Medicine on the ethical use of animals.

In situ hybridization.

Distributions of P-, T-, and H-protein mRNAs were determined with *in situ* hybridization using ^{35}S-labeled antisense oligonucleotides as previously reported (Sakata et al., 2001).

Immunohistochemistry.

Rabbit polyclonal anti-chicken P-protein antibody (Sato et al., 1991) was used. The specificity of the antibody has been already confirmed in our previous study (Sato et al., 1991). For immunoperoxidase staining, sections were immersed in a preincubation solution containing 1 % bovine serum albumin and 0.3% Triton X-100 in 0.01 M PBS for overnight, and then incubated with anti P-protein (diluted 1:500) antibody in the above solution for 48 h at 4 °C, followed by incubation with dextran-polymered goat anti-rabbit IgG with peroxidase complex for 2 hr using Envision+ kit (DAKO, Carpenteria, USA). Immunoreaction was visualized with 3,3'-diaminobenzidine (DAB) (DAKO). For immunofluoresence, sections immunoreacted with anti P-protein (1:1000) antibody overnight were incubated with rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 1 hr. For double immunofluorescence, we used mouse anti-nestin antibody (1:200;

Chemicon, Temecula, USA), guinea pig anti-GLAST antibody (1:2000; Chemicon), mouse anti-GFAP antibody (1:200; Chemicon) or mouse anti-MAP2 antibody (1:200; Chemicon). Then sections were incubated with FITC-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hr. Each step was followed by washing with 0.01 M PBS.

Preparation of primary neurosphere cultures.

From E17 fetuses, striata were excised and collected in PBS. Isolated striata were then triturated by pipette in DMEM/F-12, 1:1 v/v (Invitrogen Corp., Carlsbad, CA) supplemented with 6 g/L glucose. Dissociated cells were resuspended in serum-free medium containing: DMEM/F-12 (1:1 v/v; Invitrogen), the N2 supplement (Invitrogen), B27 (1 % v/v, Invitrogen) and 20 ng/ml epidermal growth factor (Upstate biotechnology, New York). Cells were maintained in passage in non-adherent T25 culture dishes. The cultured cells were harvested by centrifugation at 700 x g for 10 min and stored in –80 °C until RT-PCR and GCS enzymatic analyses.

RT-PCR analysis.

Total RNA samples were purified from neurosphere cells with RNeasy kit (Qiagen GmbH, Hilden, Germany). One microgram of total RNA was reverse transcribed primed with oligo dT primers using Superscript II reverse transcriptase (Invitrogen). An 1 μl aliquot of the cDNA reaction mixture was added to 30μl of PCR reaction mixture containing: 0.5 □M each of primers, 2.5 mM each of dNTPs, 2 mM of $MgCl_2$, and 1.5 U of Ex Taq polymerase (Takara, Tokyo, Japan). The sequences of PCR primers were as follows; P-protein forward primer, 5’-GACAGATTCTGTGATGCTATG-3’, reverse primer,

5’-GATCCGAGCAATGGTTGGCCA-3’; T-protein forward primer, 5’-TGACAATGCAGCGGACAGTC-3’, reverse primer, 5’-TCACACGACAGCCAGACACAC-3’; H-protein forward primer, 5’-GAACCTTGGATCATGTCGCTGC-3’, reverse primer, 5’-TTCTATGTGGAAGCCATGGTC-3’; nestin forward primer, 5’-AATCTTTTCAGATGTGGGAG-3’; reverse primer, 5’-GTCAAGACGCTAGAAGAGCA-3’. The thermal cycle consisted of denaturing at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; this was repeated 35 cycles in PCR2400 thermal cycler (PE Biosystems, Foster City, CA).

Assay for GCS activity.

Overall activity of the GCS was determined by decarboxylation reaction of [1-^{14}C]glycine (Amersham, Arlington Height, IL) as described (Kure et al., 1991). Frozen cell pellets harvested from neurosphere cell cultures and frozen tissues were thawed and homogenized in 400 μl of 20 mM potassium phosphate buffer (pH 7.0) containing 0.25 M sucrose, 1 mM dithiothreitol, and 300 μl of the homogenate was used for the decarboxylation reaction. The protein concentration of the homogenate was determined by the method of Bradford.

Results

GCS in the embryonic brain and embryonic neurosphere cultures.

At E15, P-protein mRNA was very intensely expressed in neuroepithelium around the lateral ventricle, aqueduct and fourth ventricle (Fig. 1A). Interestingly, T- and H-protein mRNAs were also very intensely expressed in the above-mentioned neuroepithelium (Fig. 1B and C). At E18, very intense expressions of P-, T-, and H-protein mRNAs were continuously observed in neuroepithelium throughout the brain (Fig. 1D-F). In addition, the cortical plate and external granular layer of the cerebellum also intensely expressed P-, T-, and H-protein mRNAs (Fig. 1D-F). At E15, the rat cerebral cortex consists of the preplate and ventricular zone (Fig. 1G). P-protein like immunoreactivity (P-IR) was observed in both layers, with relatively intenser in the preplate (Fig. 1G). Double immunofluorescence with a marker for neural stem/progenitor, nestin (Lendahl et al., 1990), clearly showed that GCS is expressed in neural stem/progenitor cells in the ventricular zone (Fig. 1H-J). At E18, intense P-IR was seen in the marginal zone, cortical plate, cortical subplate and ventricular zone, while P-IR was not detected in the intermediate cortical layer (Fig. 1K). Interestingly, in the cortical plate, intense P-IR was seen on radial processes that extend from the cortical subplate to the marginal zone (Fig. 1L). The immunopositive processes were reactive for a marker for radial glial cells, glutamate transporter GLAST (Fig. 1M-O; Shibata et al., 1997), while negative for a neuronal marker, MAP-2 (Fig. 1P-R), showing that the P-IR positive cells in the cortical plate were radial glial cells. Taken together, these data show that GCS is initially expressed in neural stem/progenitor cells and this expression is transmitted to radial glial cells in the embryonic rat cortex.

To directly prove that neural stem/progenitor cells express functional GCS, we measured GCS activity in embryonic neurosphere cultures. First, we examined expression of nestin mRNA, a marker for neural stem/progenitor cells, and those of mRNAs encoding the proper components of GCS (Fig. 1S). Specific cDNA bands were identified with the expected sizes only in each mixture with reverse transcriptase (RTase +) (Fig. 1S), indicating that embryonic neurosphere cells express these genes. We then measured GCS activity in embryonic neurosphere cultures, and compared with that in adult liver (Fig. 1T). Interestingly, embryonic neurosphere cultures showed incredible high GCS activity (8.5 ± 0.5 nmole $^{14}CO_2$ formed/mg protein/hr), which is equivalent to the activity of the adult liver (7.5 ± 0.5 nmole/mg protein/hr), where the highest GCS activity has been reported (Kure et al, 1991), indicating that rat embryonic neurosphere cultures possess the highest level of GCS activity.

GCS in the postnatal rat brain

At P7, very intense P-protein mRNA expression was observed in the subventricular zone, rostral migratory stream, dentate gyrus of the hippocampus, and cerebellum (Fig. 2A). At P14 and adult stage, its intense expression was continuously observed in the above-mentioned regions (Fig. 2B and C). In contrast, P-protein mRNA expression in the other parts was generally low. These data clearly show that P-protein mRNA is preferentially expressed in neurogenic regions in the postnatal rat brain. As we have already reported that GCS is expressed in astrocytes throughout the adult rat brain (Sato et al., 1991), in the present study we further investigated the cell type of GCS-positive cells in the following neurogenic regions.

Rostral migratory stream. Intense P-IR was detected throughout the

rostral migratory stream at P7 (Fig. 2D). P-IR was seen as tiny puncta in small cell bodies and their processes surrounding the nests of migrating cells (arrows in Fig. 2E). Double immunofluorescence with GLAST, a marker for specialized astrocyte (Shibata et al., 1997), showed that GCS is expressed in specialized ensheathing astrocytes in the rostral migratory stream (Fig. 2F-H).

External granular layer. In the P7 cerebellum, high signals for P-protein mRNA were observed in the external granular layer (EGL) and Purkinje cell layer (Fig. 2I). Coincident with the expression pattern, intense P-IR was detected with immunohistochemistry also in the external granular layer and Purkinje cell layer (Fig. 2J). Interestingly, intense P-IR was seen on radial processes that extend from small cell bodies in the Purkinje cell layer to the external granular layer (Fig. 2J). Double immunofluorescence with a marker for Bergmann glia, GLAST, indicated that the P-IR positive cells are Bergmann glias (Fig. 2J-L) .

Subventricular zone. P-protein mRNA was intensely expressed in the subventricular zone at adult stage (Fig. 3A). Immunopositive cells for P-protein were also prominently detected in the subventricular zone (Fig. 3B). P-IR positive cells were small and extending relative short processes with tiny puncta (Fig. 3C). Double immunofluorescence with a marker for astrocyte, GFAP, showed that the P-IR positive cells are astrocytes (Fig. 3C-E).

Dentate gyrus. Prominent hybridization signals for P-protein mRNA were detected in the dentate gyrus, especially in the inner surface of the granule cell layer (Fig. 3F). Intense P-IR positive cells were aligned in a monolayer along the inner surface of the granule cell layer (Fig. 3G), and extending long processes toward the molecular layer (Fig. 3H). Double immunofluorescence with GFAP exhibited that the P-IR positive cells are

astrocytes (Fig. 3I-K). Taken together, these data indicate that GCS is very intensely expressed in radial glias and astrocytes in the postnatal neurogenic regions.

To know whether regional GCS activities reflect the above-mentioned GCS distribution patterns, we measured GCS activities in the rat cerebral cortex and brain stem at E17 and adult (Fig.3L). GCS activities in the E17 cerebral cortex, adult cerebral cortex, fetal brain stem, and adult brain stem were 3.7 ± 0.2, 0.9 ± 0.3, 1.7 ± 0.3, and 0.6 ± 0.2 nmole/mg protein/hr, respectively. Interestingly, GCS activities at E17 was significantly higher than those in adult in both regions ($p<0.01$), coincident with the present finding that GCS expression is generally intenser in the embryonic brain. In contrast, in the liver GCS activity at E17 (2.8 ± 0.3 nmole/mg protein/hr) was significantly lower than that in adult (7.5 ± 0.5 nmole/mg protein/hr, $p<0.01$). These results indicate that expression level of GCS is well coincident with its regional activity, and that the embryonic brain requires higher GCS activity than the adult brain.

Discussion

GCS was expressed very intensely and ubiquitously in neural stem/progenitor cells in the neuroepithelium. We believe that GCS in the neuroepithelium may have two roles. First, GCS may be indispensable in supplying proliferating cells with 5,10-methylenetetrahydrofolate as a one-carbon donor, which is essential for the synthesis of DNA in cell proliferation (Fleming & Copp, 1998). Since neuroepithelial stem cells proliferate rapidly, a large amount of 5,10-methylenetetrahydrofolate should be supplied via GCS in these cells. Interestingly, the neuroepithelium also expresses very high levels of folate-binding proteins, which are needed to uptake folate as a source of 5,10-methylenetetrahydrofolate (Finnell et al., 1997; Piedrahita et al., 1999). In addition, it has been demonstrated that folate deficiency affects proliferation of neural stem cells (Mattoson & Shea, 2003) and the association of neural tube defects and inadequate folate intake has already been established (Whitehead et al., 1995, Fleming & Copp, 1998). Thus, we speculate that embryonic neural stem cells transport folate for *de novo* synthesis of sufficient nucleosides for proliferation, and that GCS may be indispensable for this process. Second, GCS may be needed to maintain an adequate extracellular glycine concentration so that glycine receptors and/or NMDA receptors expressed in neural stem/progenitor cells can maintain normal function. Behar et al. have reported that PCR confirmed the presence of NMDA receptors in the neuroepithelium (Behar et al., 1999). In addition, some reports have shown that cells in neurospheres express functional NMDA and glycine receptors (Nguyen, 2001, 2002; Sah, 1997), supporting the possibility that GCS is involved in signals mediated by these receptors in the neuroepithelium.

Interestingly, we also observed very high expression of GCS in glial cells in the subventricular zone, rostral migratory stream, external granular layer of the cerebellum, and dentate gyrus of the hippocampus. Since these regions are known to be areas where neurogenesis still persists postnatally (Gage, 2000; Lois et al., 1996), we speculate that GCS is somehow involved in postnatal neurogenesis. Among these regions, the neurogenesis of the hippocampal dentate gyrus has been intensively investigated (Cameron et al., 1995, Arvidsson et al., 2001, Kitayama et al., 2003). In the rat dentate gyrus, neuronal progenitor cells reside and proliferate in the subgranular zone. Cameron et al. have reported that in the adult dentate gyrus, activation of NMDA receptors rapidly decreased the number of cell synthesizing DNA, whereas blockade of NMDA receptors rapidly increased the number of cells in the S phase identified with ^{3}H-thymidine (Cameron et al., 1995), indicating that NMDA receptors regulate neurogenesis in the adult hippocampal dentate gyrus. As shown in the Fig. 3 F-H, we found that astrocytes situated in the subgranular zone expressed intense P-IR, suggesting that GCS controls extracellular glycine concentrations, which might be important for regulating NMDA receptor functions in neurogenesis. Similarly, in the cerebellar external granular layer, the involvement of NMDA receptors in neurogenesis has been reported (Farrant et al., 1994). Thus, high GCS expression in astrocytes adjacent to neutral stem/progenitor cells might be needed to control NMDA receptors expressed in neural stem/progenitor cells in the hippocampal dentate gyrus and cerebellar external granular layer. However, there remains the possibility that GCS supplies stem/progenitor cells with 5,10-methylenetetrahydrofolate in these regions.

Because all postnatal neurogenic regions intensely express P-protein, GCS seems to play pivotal roles to keep a suitable condition for nurturing neural stem/progenitor cells in postnatal neurogenic regions.

The congenital malformations reported in non-ketotic hyperglycinemia are parenchymal volume loss, agenesis or hypogenesis of corpus callosum, gyral malformations, ventricular enlargement, and cerebellar hypoplasia (Press et al., 1988; Dobyns, 1989). These hypoplastic abnormalities suggest reduced cellular proliferation in prenatal brains of these individuals. How do these hypoplastic abnormalities occur? As mentioned-above, GCS seems to supply 5,10-methylenetetrahydrofolate for nucleoside synthesis in the neuroepithelium. Since neuroepithelial stem cells proliferate rapidly, enough 5,10-methylenetetrahydrofolate should be supplied via GCS in these cells for their proliferation. In addition, GCS seems to regulate extracellular glycine concentration to control signaling mediated by NMDA and glycine receptors in the neuroepithelium. Furthermore, the involvement of NMDA receptors and glycine receptors in early brain development is well accepted (Nguyan et al., 2001, 2002). Thus, we believe GCS deficiency causes the short supply of 5,10-methylenetetrahydrofolate and also disturbs signalings mediated by NMDA receptors and glycine receptors in the embryonic brain, resulting in impaired proliferation and differentiation of neural stem/progenitor cells. Animal models with conditionally regulated GCS activities will be needed to further explore the pathogenesis and to establish effective treatments for the individuals with this disorder.

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Abbreviations

GCS, glycine cleavage system; IR, immunoreactivity; PB, phosphate buffer; PBS, phosphate-buffered saline

Figure Legends

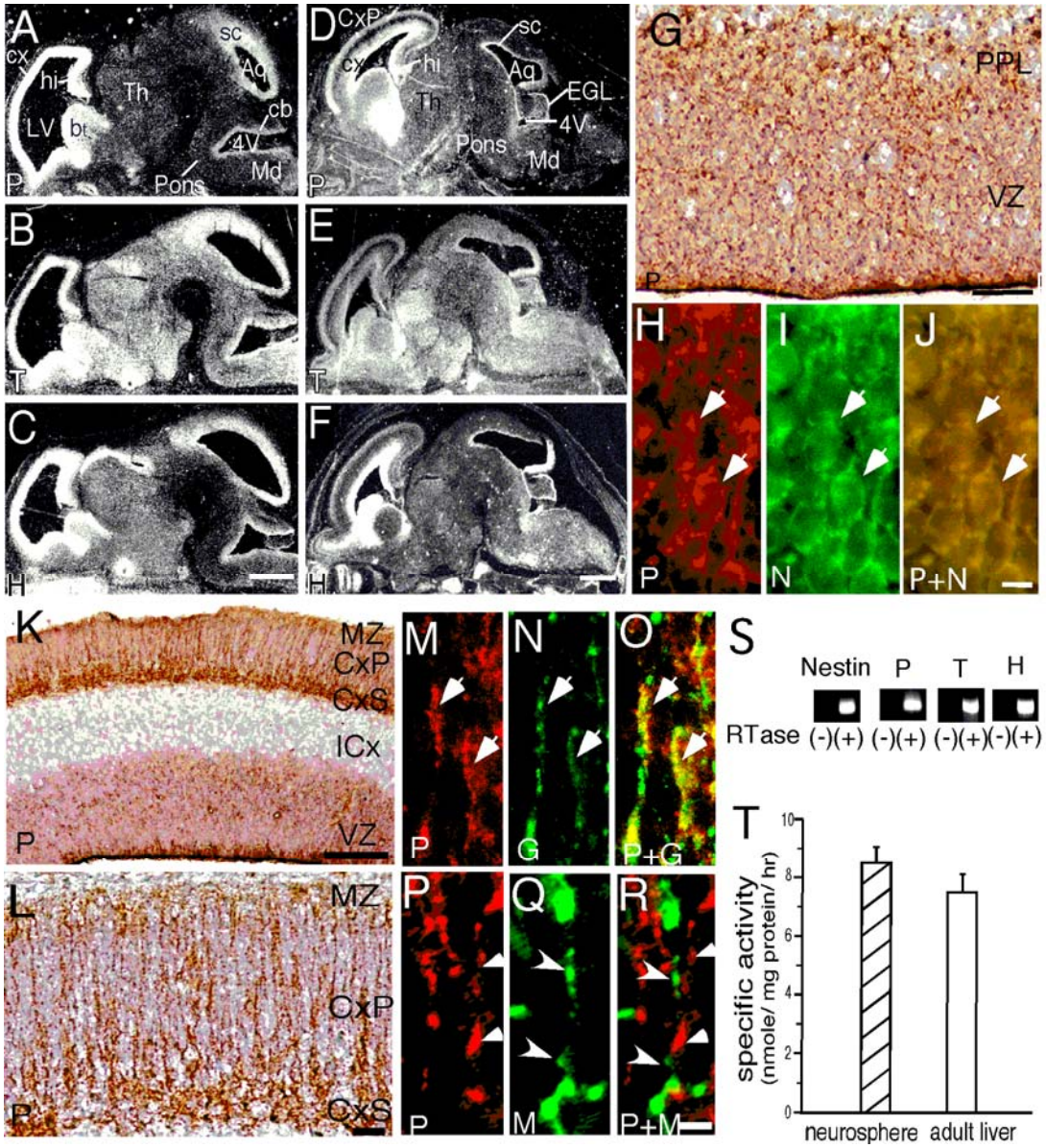

Fig. 1. Embryonic expressions of P-protein mRNA (A, D), T-protein mRNA (B, E), and H-protein mRNA (C, F) at E15 (A, B, C), and E18 (D, E, F). P-IR positive cells in the cortical neuroepithelium at E15 (G). Double-staining study showing that most cells with P-IR (H) are also positive for nestin (I) at a merged photomicrograph (J). P-IR positive cells in the E18 cerebral cortex (K, L). Double-staining study showing that P-IR positive processes (arrows in M, triangles in P) are positive for GLAST (arrows in N), but negative for MAP-2 (arrowheads in Q) at merged photomicrographs (O, R). RT-PCR detection of mRNAs encoding nestin, P-, T-, H-proteins using total RNA extracted from E17 rat neurosphere cells (S). A single band of the corresponding size is obtained for each mRNA: nestin-amplicon (200 bp), P-amplicon (236 bp), T-amplicon (668 bp), H-amplicon (546 bp), only when reverse transcriptase is added (RTase +). Specific activity of GCS in E17 neurosphere cells, compared to that of liver homogenates from adult rat (T). Data are mean±SD (bars) values of three independent experiments. 4V, 4th ventricle; Aq, aqueduct; bt, basal telencephalic neuroepithelium; cb, cerebellar neuroepithelium; cx, cortical neuroepithelium; CxP, cortical plate; CxS cortical subplate; EGL, external granular layer; hi, hippocampal formation neuroepithelium; ICx, intermediate cortical layer; LV, lateral ventricle; Md, medulla oblongata; MZ, marginal zone; sc, superior colliculus neuroepithelium; PPL, preplate; Th, thalamus; VZ, ventricular zone. Scale bars, 1 mm (C, F), 50 μm (G), 10 μm (J), 100 μm (K), 20 μm (L), 5 μm (R).

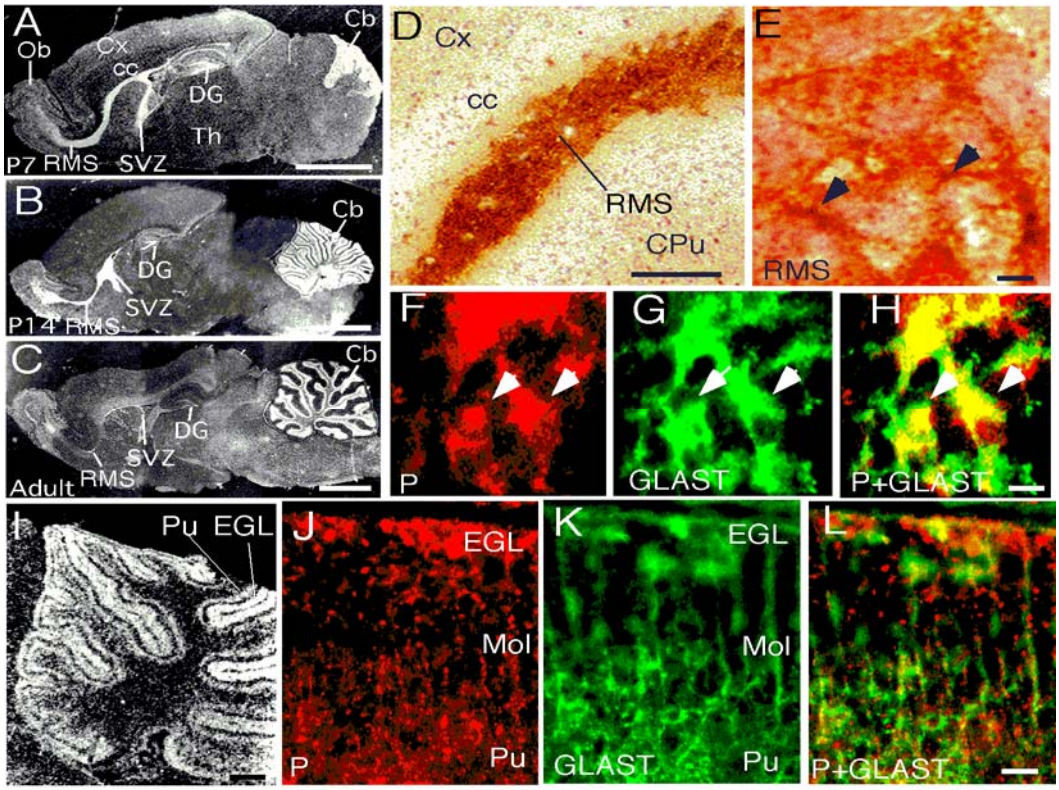

Fig. 2. Expression of P-protein mRNA at P7 (A), P14 (B), and adult stage (C). P-IR in the rostral migratory stream at P7 (D, E). Double-staining study showing that P-IR positive cells in the rostral migratory stream (F) are also positive for GLAST (G) at a merged photomicrograph (H). Expression of P-protein mRNA in the cerebellum (I). Double-staining study showing that P-IR cells in the cerebellar cortex (J) are also positive for GLAST (K) at a merged photomicrograph (L). Cb, cerebellum; cc, corpus callosum; CPu, caudate putamen; Cx, cerebral cortex; DG, dentate gyrus; EGL, external granular layer; Mol, molecular cell layer; Ob, olfactory bulb; Pu, Purkinje cell layer; RMS, rostral migratory stream; SVZ, subventricular zone; Th, thalamus. Scale bars, 2 mm (A-C), 500 μm (D, I), 20 μm (E, H, L).

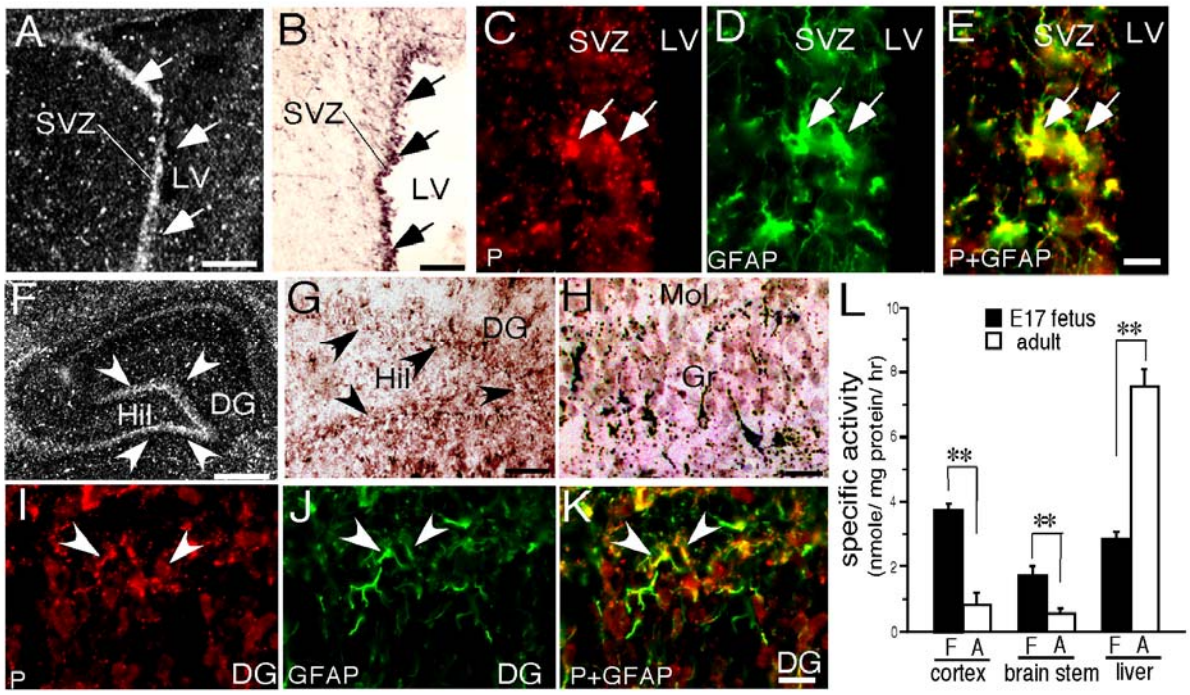

Fig. 3. P-protein mRNA expression in the subventricular zone of the lateral ventricle (A) at adult stage, and immunoperoxidase staining for P-protein in the same region (B). Double-staining study showing that P-IR positive cells in the subventricular zone (C) are also positive for GFAP (D) at a merged photomicrograph (E). P-protein mRNA expression in the adult dentate gyrus (F-H). Double-staining study showing that P-IR positive cells in the dentate gyrus (I) are also positive for GFAP (J) at a merged photomicrograph (K). Specific activity of GCS in cerebral cortex, brain stem and liver homogenates from E17 fetus and adult rat (L). Data are mean±SD (bars) values of three independent experiments; all **$p<0.01$ (fetus vs. adult) using unpaired t-test. DG, dentate gyrus; Gr, granule cell layer; LV, lateral ventricle; Mol, molecular layer; Hil, hilus of the dentate gyrus; SVZ, subventricular zone; Scale bars, 500 μm (A, F), 200 μm (B, G), 10 μm (E, H, K).

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