FEBS Letters 346 (1994) 161–164

FEBS 14069

Developmental expression of GLUT3 glucose transporter in the rat brain

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Received 20 April 1994

Abstract

The ontogeny of the GLUT3 glucose transporter gene and protein expression was studied in rat brain. Northern blot analysis using total RNA from rat brains at different developmental stages revealed that the levels of GLUT3 mRNA were very low during the embryonic stage and increased towards the postnatal stage. Immunohistochemistry using a specific antibody showed that the expression of GLUT3 protein was barely detectable in the embryonic stage, but was clearly detected on the plasma membrane of neuronal cells from 10 days after birth to the adult. Expression of GLUT3 mRNA and protein in the cerebral neuronal cell cultures was also examined during the maturation of neurons. GLUT3 glucose transporter of primary neuronal cultured cerebral cortical neurons was only detected in mature neurons after they were cultured for 14 days. These results indicate that GLUT3 plays an important role in glucose homeostasis postnatally in neurons of the rat brain.

Key words: Facilitated-diffusion glucose transporter; GLUT3; Nervous system development; Primary neuronal cell culture; Glucose transporter

1. Introduction

Glucose is the major substrate for brain metabolism and it sustains more than 90% of the energy requirements of this organ. By contrast, in the postnatal stage brain tissue uses ketones as its primary energy source, which subsequently converts to carbohydrate metabolism as the animals approach maturity [1,2]. Consistent with this phenomenon, the activity of several glycolytic enzymes is low in newborn rat brains, but increases progressively throughout the first few days-to-weeks after birth [3]. Thus, the utilization of glucose in the brain is closely related to the developmental stages of brain. The first step of glucose metabolism in the brain is the transportation of the sugar across the plasma membrane by facilitated diffusion. At least five isoforms of glucose transporter (GLUT1–GLUT5) have been identified, and the expression of the isoforms is to some extent tissue specific [4]. In adult rodent brain, GLUT1 is concentrated in endothelial cells of the blood–brain barrier [5–7], and GLUT3 is present on the plasma membrane of neurons as previously described [8,9]. The developmental regulation of GLUT1 glucose transporter has been extensively studied [10–13], and GLUT1 has been primarily localized to vasculature in brain sections [12,13]; however, the regulation of glucose transporter expressed in neurons during development has not yet been determined. In an attempt to further understand glucose metabolism in neurons, we studied the developmental expression of GLUT3 mRNA and protein in the rat brain.

2. Materials and methods

2.1. Animals

Pregnant female Wistar rats were fed food and water ad libitum. A group of pregnant rats was anesthetized with diethyl ether and the fetuses of embryonic day 15, 17 and 18 were immediately removed, and brains were collected. Neonatal pups delivered by pregnant rats were housed together with their mothers. They were killed by decapitation at either 1, 8, 10, 14, or 60 days of age, and brains were removed.

2.2. Northern blot analysis

RNA was isolated using a single-step method by acid guanidinium thiocyanate/phenol/chloroform extraction [14]. The concentration of the RNA was determined by the absorbance at 260 nm, and the RNA solutions were stored in ethanol at −20°C until use. The integrity of the RNA and the accuracy of the quantification were confirmed by the ethidium bromide staining of the 28 S and 18 S ribosomal RNA bands after gel electrophoresis. RNA samples were subjected to Northern blot analysis, using methods that we have previously described [8,9]. Briefly total RNA (15 μg) was separated in a 1.5% agarose, 0.66 M formaldehyde gel, blotted on nitrocellulose filters, and hybridized with ^{32}P-labeled rat GLUT3 full-length cDNA, which we had previously isolated from rat brain [9]. Filters were washed under high stringency conditions (0.1 × SSC, 0.1% SDS, 55°C) and autoradiographed with intensifying screens for 7 days at −70°C.

2.3. Antibody

Antibodies against rat GLUT3 were prepared using a synthetic peptide containing the 17 amino acid (GVELNSMQPVKETPGNA) carboxy-terminus of this protein; the specificity has been well characterized previously [9].

2.4. Immunohistochemistry

Brains were dissected from fetuses or pups of timed pregnant Wistar rats, or 2-month-old adult rats. Dissected brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were sectioned at 5 μm as previously described [9]. Sections were pretreated with 0.3% H_2O_2 prior to normal goat serum incubation to reduce a non-specific binding. Sections were then incubated in a 1:500 dilution of rat GLUT3 antiserum for 20–24 h at 4°C. The immunohistochemical staining was performed according to the avidin-biotin peroxidase (Dako, Denmark) method of Hsu [15].

2.5. Immunoblots

Primary neuronal cells cultured for 1 or 14 days were detached by

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SSDI 0014-5793(94)00455-5

scraping, and washed three times with PBS. They were lysed by sonication in a solution of 40 mM Tris-HCl (pH 7.4), 2 mM EDTA, 250 mM sucrose, 1 mM PMSF, and 1% Triton X-100, and mixed with Laemmli sample solution. Immunoblot analysis of total cell lysates was performed essentially as previously described [9]. 50 μg samples of cell lysates were resolved by SDS gel-electrophoresis using a 9% gel, electrically transferred to nitrocellulose filter, and blocked with 5% non-fat milk at 37°C for 1 h followed by incubation for 3 h at room temperature with 1:500 dilutions of the primary antiserum. Immunoreactive bands were visualized by alkaline phosphatase-coupled goat anti-rabbit IgG secondary antibody (DAK0), using substrates Nitro blue tetrozolium and 5-bromo-4-chloro-3-indolyl-phosphate (WAKO Ind. Ltd.).

2.6. Primary rat brain neuronal cell cultures

Primary cultures of rat brain neuronal cells were prepared essentially as described previously [16]. Briefly, timed 17-day pregnant Wistar rats were anesthetized, and the fetuses were removed. After the brain tissue pieces were digested with trypsin and DNase I at 37°C, the dissociated cells were collected. The cells were cultured in a medium containing 5% (v/v) pre-colostrum newborn calf serum, and 90% DF medium, 1:1 mixture of Dulbecco's modified Eagle's (DME) and Hank's F12 media (Gibco) supplemented with 15 mM HEPES-NaOH buffer (pH 7.2), 30 nM selenium (Wako), 1.9 mg/dl sodium bicarbonate, 50 units/ml penicillin G and 100 μg streptomycin sulfate. The medium was changed at 2- to 3-day intervals, and the medium was supplemented with 1 μM cytosine arabinoside (Sigma) at day 2 of culture and then at every other medium change. Under these conditions, greater than 98% of the cells were neurons which reacted positively against anti-neurofilament monoclonal antibody (data not shown).

3. Results

Total rat brain mRNAs were prepared from rats ranging in age from embryonic day (E) 15 to postnatal day (P) 60 (adult). These RNAs were subjected to Northern blot analysis using rat GLUT3 cDNA probe. Similar to previous studies [8,9], this cDNA probe hybridized to a single transcript of ~4.0 kb, and the relative amounts of GLUT3 mRNAs varied considerably with the age of the rats (Fig. 1). The levels of GLUT3 mRNAs were very low during the embryonic days and 1 day after birth, then increased dramatically and maintained high levels until adult. Fig. 2 shows the immunohistochemical staining with a specific antibody against rat GLUT3 in the rat cortex of E18, P1, P10, and P60. In the E18 brains, where neurons, astrocytes, or oligodendrocytes could not be determined using morphological criteria, sections exposed to GLUT3 antibody exhibited no specific immunoreactivity. In the P1 section, a light perinuclear (cytoplasmic) staining to oval cells was observed. The immunoreactive parenchymal cells were round-to-oval, lacked distinct processes, and consequently were difficult to characterize in terms of glial or neuronal differentiation. On P10 (Fig. 2C), the cellular GLUT3 immunoreactivity exhibited plasma membranes of neuron-like cells, and cytoplasmic staining became less prominent. In the P60 section (Fig. 2D), GLUT3 immunoreactivity was clearly present on the cell surface membrane of neuronal cell bodies as previously shown [9].

We also examined the expression of GLUT3 in primary rat brain neuronal cells during the maturation of neurons in culture. Cerebral neurons prepared from

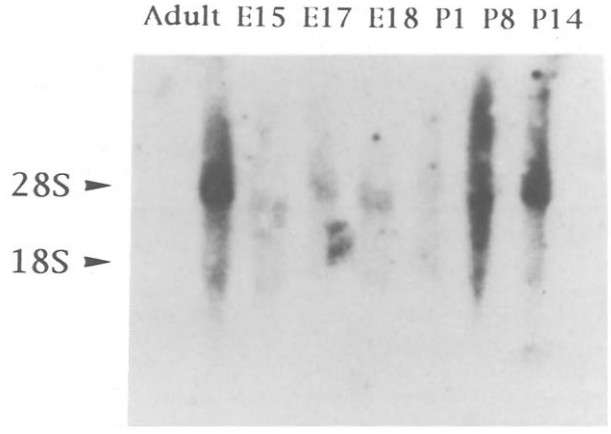

Fig. 1. Northern blot analysis of rat brain total RNA at different developmental ages. Total rat brain RNA (15 μg/lane) at different developmental ages (E, embryonic; P, postnatal) was size fractionated on a 1.5% agarose, 0.66 M formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled rat GLUT3 cDNA probe. A representative autoradiograph is shown of RNA-blot analysis of three separate experiments demonstrating a 4.0 kb GLUT3 transcript.

17-day-old rat embryos were cultured for 1 or 14 days. Since Northern blot analysis of 20 μg total RNA could not detect any signal of rat GLUT3 mRNA from neurons in culture (data not shown), the RT-PCR procedure using specific oligonucleotide primers based on the rat GLUT3 nucleotide sequence [9] was performed. As shown in Fig. 3, only mature neurons cultured for 14 days expressed GLUT3 mRNA. We also studied the expression of GLUT3 protein by immunoblot analysis. Consistent with the result of RT-PCR, an immunoreactive band of approximately 60 kDa was detected only in mature neurons.

4. Discussion

The results of this study show that the expression of GLUT3 was very low during the embryonic stage, but increased dramatically after birth, indicating that the developmental expression of GLUT3 correlated with both the maturation of neurons and alterations in the substrate required for metabolic energy.

At the E18 developmental stage, neither Northern blot nor immunohistochemical analysis detected any signal of GLUT3. Since some expression of other glucose transporter isoforms was observed in brain parenchyma at this stage [10], unidentified glucose transporter species may be expressed within brain parenchymal cells. At P1, GLUT3 mRNA could not be detected by Northern blot analysis, while GLUT3 immunoreactivity was weakly detectable in the cytoplasma, but not plasma membrane, in contrast to the prominent localization of GLUT3 to the plasma membrane of adult rat neurons. The meaning of the different cellular localization of GLUT3 protein

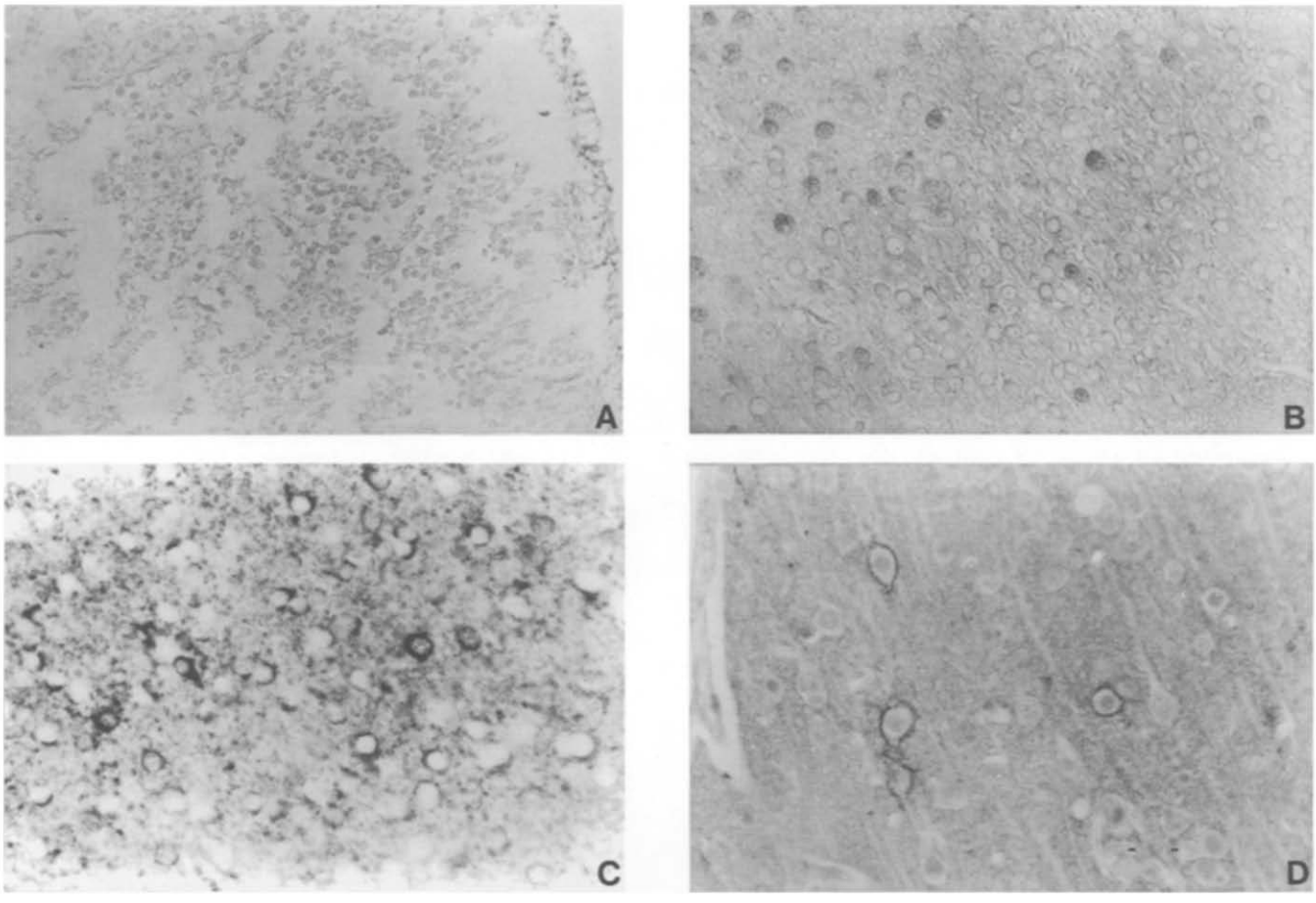

Fig. 2. Immunohistochemistry of rat brain cortex at different developmental ages. On E18 (A), GLUT3 immunoreactivity was not detected in parenchymal cells. On P1 (B), GLUT3 immunoreactivity was weakly observed in the cytoplasma rather than the plasma membrane. On P10 (C) and P60 (D), the distribution of GLUT3 was mainly on the cell surface membrane of neuron-like, or neuronal cell bodies.

during developmental stages is at present unknown. GLUT3 may transport some substrates intracellulary other than glucose on this stage. In fact, it is known that GLUT1 transports not only glucose but also dehydroascorbic acid [17], and we are now investigating the functions of the GLUT3 glucose transporter in detail.

Previously, low glucose influx into the immature rat brain, as compared to adult, was reported to be due to a difference in transport capacity (V_{max}) rather than a difference in the transport affinity constant (K_m) for glucose [18,19], suggesting a reduced number of the same isoforms of glucose transporter protein in the immature brain. Our data showing the lower expression of GLUT3 in brain cells at the earlier developmental stages agrees with these studies. Furthermore, Smith and Gridly [20] reported that mouse GLUT3 was also not detectably expressed in neuroepithelium at early embryonic stages of mouse. In contrast to the stages of P < 1, the levels of GLUT3 mRNA and protein dramatically increased after P8. On P10, strong GLUT3 immunoreactivity was observed in plasma membranes of neuron-like cells. It is known that cortical maturation is accompanied by the rapid growth of axons and dendrites by synaptogenesis, which accelerates at approximately P10 [21]. It is likely that this growth results in the increase of GLUT3 expression at this stage. Thus, evidence for both the expression of GLUT3 mRNA increased and the prominent distribution of GLUT3 protein to the plasma membranes of neurons observed in the postnatal stages indicates that developmental expression of GLUT3 is closely related to the increased requirements of glucose as the energy source in neurons. Finally, we examined the expression of GLUT3 glucose transporter in neuronal cell cultures during maturation, because the functions of primary cultured cerebral cortical neurons resembled those in the developing cerebral cortex in vivo [16,22,23]. In the immature neurons cultured for 1 day, GLUT3 mRNA and protein could not be detected, while they were clearly expressed in the mature neurons cultured for 14 days. This result also supports in vivo findings that GLUT3 expression is correlated to the maturation of neurons.

In conclusion, GLUT3 transcription must be turned on postnatally in neurons, suggesting the important role

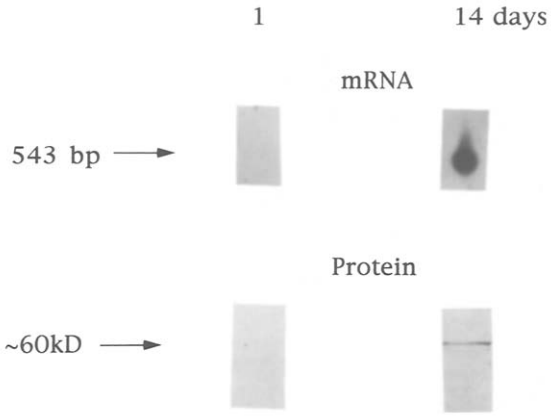

Fig. 3. Expression of rat GLUT3 mRNA and protein in primary rat brain neuronal cell cultures. Neuronal cells were prepared from rat embryos, and cultured for 1 or 14 days. The RT-PCR procedure was performed using 1 μg of total RNA and specific oligonucleotide primers as described in section 2. PCR product (543 bp DNA fragment) was separated in a 1.2% agarose gel, transfered to nitrocellulose filter, and hybridized with ^{32}P-labeled rat GLUT3 cDNA probe. Total cell lysates from neuronal cell cultures (50 μg of protein per lane) were separated by SDS 10% polyacrylamide gel electrophoresis and immunoblotted with anti-rat GLUT3 antibody. Antibody binding was detected using an alkaline phosphatase-conjugated secondary antibody. Rat GLUT3 protein was recognized as a single band of ~ 60 kDa.

of GLUT3 in glucose homeostasis postnataly in matured neurons.

Acknowledgements: We would like to thank Ms. H. Sato for her assistance in the immunohistochemical work. This work was supported in part by a Grant-in-Aid for Scientific Research (B-04454366) from the Ministery of Education, Science and Culture, Japan.

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