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ABUNDANT EXPRESSION OF GLUT1 AND GLUT3 IN RAT EMBRYO DURING THE EARLY ORGANOGENESIS PERIOD

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Summary: The developmental change of both GLUT1 and GLUT3 protein in rat embryonal and fetal brain was examined using Western blot analysis and immunohistochemistry. The brains were collected from fetuses (gestational days 10 to 20), newborn, and adult rats. On day 10, the levels of GLUT1 and GLUT3 expressions were twofold higher than those of adult levels, but thereafter decreased rapidly as the gestation progressed. The tissue distribution of GLUT1 and GLUT3 in embryo was apparently distinct. On day 10, GLUT1 was expressed in the neural tube, gut, heart and optic vesicle, while GLUT3 was expressed in the surface ectoderm and gut. Thus, high affinity glucose transporters may be required in the early organogenesis period because their energy requirement is completely dependent upon anaerobic glycolysis. GLUT3 may facilitate glucose transfer from amniotic fluid to the embryo and GLUT1 may supply glucose for use as an embryonal fuel.

Glucose is transported into cells by glucose transporters, for which the cDNA encoding seven facilitative glucose transporter isoforms (GLUT1-7) has been isolated and characterized (1). The glucose transporter isoforms demonstrate unique tissue-specific patterns of distribution and may be subject to different types of regulation (1, 2). GLUT1 (erythrocyte type) and GLUT3 (brain type) have a low Km for glucose and are characterized as insulin-independent (1). These glucose transporters are widely expressed at various levels in human tissues, and are most abundant in the brain (2, 3). The expression of these two glucose transporters in adult rodent brain is well observed

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by several investigators. GLUT1 is localized mainly in the blood-brain barrier (4, 5), and GLUT3 is localized primarily in the neurons (6, 7). Several studies have examined GLUT1 expression during fetal life (8, 9). However, a detailed time course of the quantification and localization of GLUT1 in the developing embryo has not been determined. Concerning GLUT3, little is known about the developmental change of this glucose transporter during whole gestational stage.

The aim of this study was to compare the expression and localization of both GLUT1 and GLUT3 in the developing embryonal rat brain.

Materials and Methods

Materials: Virgin Wistar rats were mated and pregnancy was confirmed the next morning by the presence of sperm on a vaginal smear. This time was designated as day 0 of gestation. On day 10, 11, 12, 14, 16, 18 and 20 of gestation, mothers were sacrificed and their embryos and fetuses were collected. Whole brains were harvested under a dissecting microscope and samples were frozen in liquid nitrogen for Western blot analysis or fixed in 4% paraformaldehyde (PFA) for immunohistochemical studies. Since embryos of day 10, 11, 12 were too small to permit brain removal, they were collected as whole specimens. Newborn and adult (12 weeks after birth, female) rat's brains also were harvested and stored in a similar manner.

Western blot analysis: Whole protein preparation: Samples were thawed separately in a homogenating buffer consisting of 20 mM HEPES, 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1% Triton X-100 with a dounce homogenizer (Kontes Co., NJ, U.S.A.). The insoluble material was removed by centrifugation at 12,000 X g for 10 min at 4°C.

Membrane preparation: Samples were homogenized separately in buffer A consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose and 1 mM PMSF with a dounce homogenizer. The homogenate was centrifuged at 900 X g for 10min at 4°C, and the resulting supernatant was centrifuged at 110,000 X g for 75 min at 4°C. The membrane pellet was solubilized in buffer A with 1% Triton X-100, subsequently, the insoluble material was removed by centrifugation at 14,000 X g for 10 min at 4°C.

Western blotting: Twenty micrograms of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to Immobilon-P membranes (Millipore, MA, U.S.A.), and these membranes were blocked using a solution of 5% powdered milk in TTBS (0.05% Tween 20, 10 mM Tris-HCl (pH 7.4), and 500 mM NaCl). The membranes were incubated for 2 hrs at room temperature with the 1:2500 dilution of a GLUT1 antiserum (10) in TTBS or 1:2000 dilution of a GLUT3 antiserum (11). Then, the membranes were incubated for 1 hr at room temperature with the 1:2000 dilution of a peroxidase-conjugated anti-rabbit immunoglobulin in TTBS. Visualization was facilitated by autoradiography following the addition of an enhancing chemiluminescence reagent (Amersham, IL, U.S.A.). Bands on the autoradiograms were quantified by scanning densitometry.

Immunohistochemistry: Samples were fixed in 4% PFA at 4°C for 2 hrs. Next, they were impregnated in sucrose solutions (10, 15, and 20%, for 2 hrs each) in phosphate-buffered saline (PBS, pH 7.4) at 4°C. The samples were then embedded in Tissue-Tek O.C.T. Compound (Miles, Inc., IN, U.S.A.) and frozen in liquid nitrogen. Sections (7 μm thick) were made with a cryostat, placed on glass slides coated with ovalbumin, and air-dried. After pretreatment with 0.3% H_2O_2 and 2% BSA,they were incubated with the 1:200 dilution of a GLUT1 antiserum or 1:200 dilution of a GLUT3 antiserum for 1 hr at room temperature. Then, samples were incubated with the peroxidase-labeled protein A (Sigma, MO, U.S.A.) at a concentration of 5 μg/ml for 30 min at room temperature. Immunolabels were stained by incubation with 3, 3'-diaminobenzidine with 0.03% H_2O_2. For control, 10% normal rabbit serum, omission of the first antibody, and PBS alone were used.

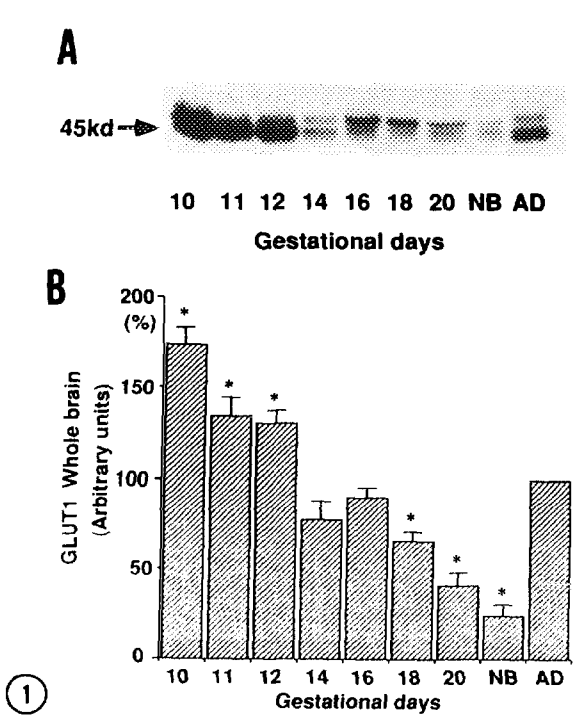
Statistical analysis: All data are presented as the mean ± SEM. The results were analyzed using Student's *t* test.

Results

We first isolated the whole cell lysate from embryonic and adult brains, then measured the amounts of GLUT1 and GLUT3 by semi-quantitative Western blot analysis. Antisera to GLUT1 immunoblotted a series of proteins of 45-55 kDa in rat whole brain (Fig. 1A). In the developing brain, high levels of GLUT1 expression were noted between days 10 to 12 of gestation, which subsequently decreased as the gestation progressed. The results of the quantification of GLUT1 by densitometry are presented in Figure 1B. On day 10, the levels of GLUT1 expressions were 1.7-fold higher than those of adult levels. An immunoblot of GLUT3 was not detected in whole brain lysate (Fig. 2A).

Second, we isolated the brain membranes and measured GLUT3 expression by Western blot analysis. Antisera to GLUT3 immunoblotted a series of proteins of 45-50 kDa in rat brain plasma membrane preparations (Fig. 2B). A quantification of the levels of GLUT3 in the brain membranes is presented in Figure 2C. The maximal relative abundance of GLUT3 was noted on day 10, and it rapidly decreased as the gestation progressed. The levels of GLUT3 expressions on day 10 were twofold higher than those of adult levels.

To clarify the localization of GLUT1 and GLUT3, we performed peroxidase-immunohistochemistry. The developmental changes of tissue localization on days 10, 11, 12 and 14 of gestation are presented in Figure 3 (A to L). On day 10 of gestation, high levels of GLUT1 were expressed in the neural tube, as well as in the heart tube, gut, and optic vesicle (Fig. 3A). GLUT3 was expressed principally in the surface ectoderm and gut tube (Fig. 3B). On day 11, GLUT1 expression was observed primarily in the



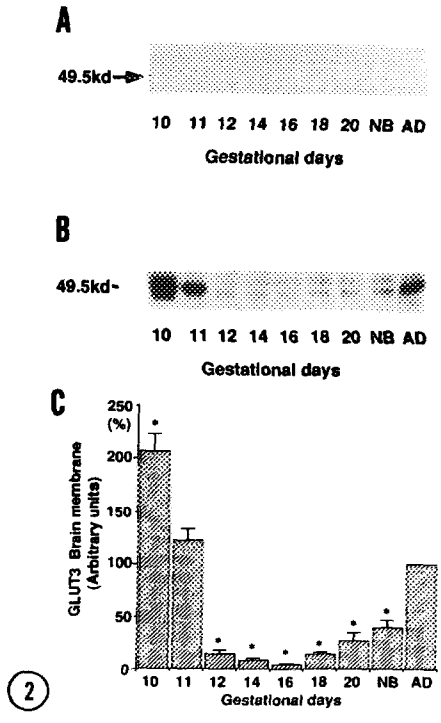


Fig. 1. GLUT1 protein expression in the developing rat brain [day10 to newborn (NB) and adult (AD)].

A: Representative Western blot analysis of whole brain lysate (20 μg of protein per lane).
B: Semi-quantification of GLUT1 expression measured by densitometry (% of adult as 100%). Each bar represents the mean ± SEM of four independent determinations.

* $p<0.05$ vs. adult.

Fig. 2. GLUT3 protein expression in the developing rat brain [day 10 to newborn (NB) and adult (AD)].

A: Representative Western blot of whole brain lysate (20 μg of protein per lane). No detectable band was found except for a trace band detected on day 10.

B: Representative Western blot of developing rat brain membranes (20 μg of protein per lane). C: Semi-quantification of GLUT3 expression measured by densitometry (% of adult as 100%). Each bar represents the mean ± SEM of four independent determinations.

* $p<0.05$ vs. adult.

neural tube and gut tube (Fig. 3D), and GLUT3 was still expressed in the surface ectoderm and gut tube (Fig. 3 E). On day 12, GLUT1 was localized to the neural tube (Fig. 3G), and GLUT3 was localized to the surface ectoderm (Fig. 3H). When the microvessels became evident on day 14, GLUT1 was specifically localized to this lesion (Fig. 3J). Weak GLUT3 expression could still be observed in the surface ectoderm (Fig. 3K). No specific staining was demonstrated on any control slide treated with 10%

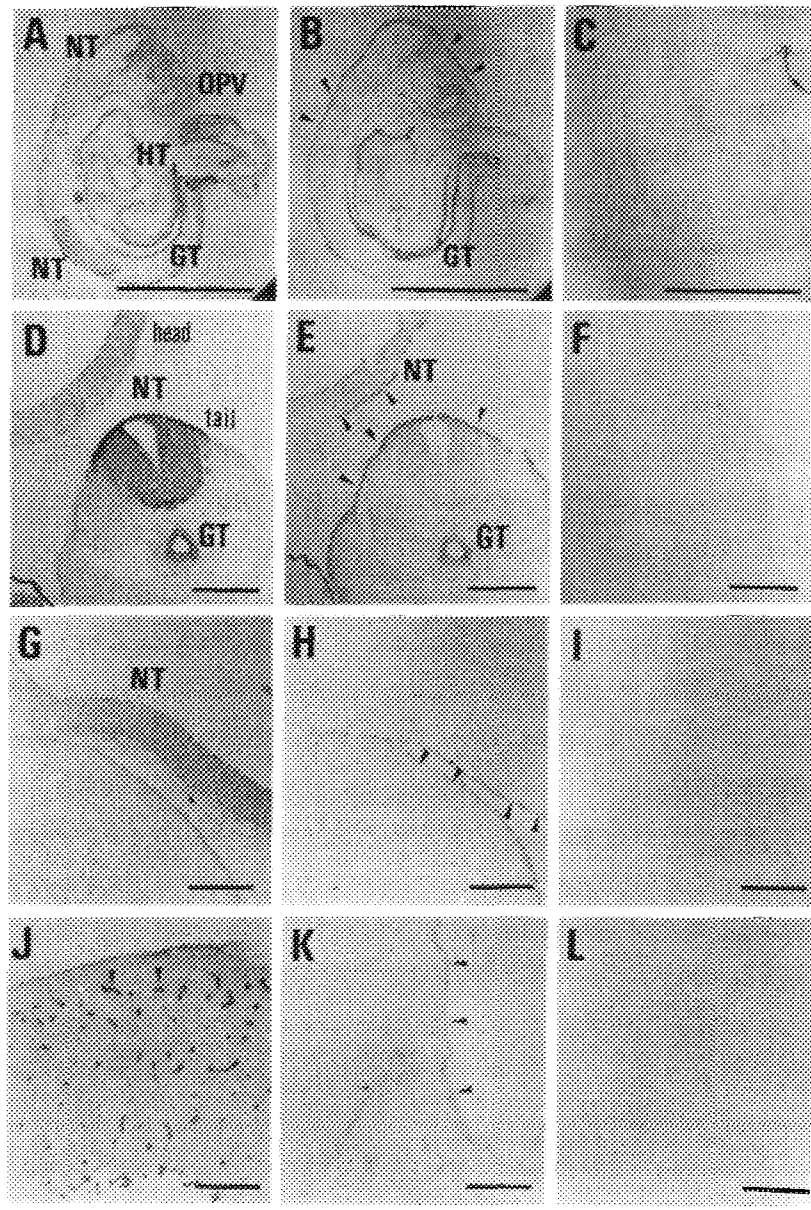


Fig. 3. Peroxidase-immunostaining of the developing embryonal and fetal brain (days 10 to 14 of gestation). A-C: day 10, D-F: day 11, G-I: day 12, J-L: day 14. A, D, G and J: With GLUT1 antiserum. B, E, H and K: With GLUT3 antiserum. C, F, I and L: With 10 % normal rabbit serum. A: Sagittal section of embryo on day 10. GLUT1 was expressed in the neural tube (NT), heart tube (HT), gut tube (GT), and optic vesicle (OPV). B: GLUT3 was expressed in the surface ectoderm (arrowheads) and gut tube. D: Day 11, GLUT1 was expressed in the neural tube of the head and tail of fetus. E: GLUT3 was expressed in the surface ectoderm (arrowheads) and gut tube. G: Day 12, GLUT1 was expressed in the neural tube. H: GLUT3 was expressed in the surface ectoderm (arrowheads). J: Forebrain of the day 14 fetus demonstrating the appearance of microvessels expressing GLUT1 (arrowheads). K: Forebrain of the day 14 fetus, GLUT3 was expressed in the surface ectoderm (arrowheads). *Scale bar*: A-C, 1 mm; D-L, 200 μm.

normal rabbit serum (Fig. 3C, F, I and L). On day 16 and 18, GLUT1 remained to localize to the microvessels, but the expression of GLUT3 was difficult to document (data not shown). On day 20 in preterm rats, GLUT1 was still expressed in the microvessels,while weak GLUT3 expression was observed in neuron-like cells (data not shown). In the newborn rat brain, again GLUT1 was expressed in the microvessels, and GLUT3 expression remained weak. In the adult rat brain, GLUT1 was expressed in the microvessels, while abundant GLUT3 was expressed in neurons (data not shown).

Discussion

We demonstrated that high levels of GLUT1 and GLUT3 are expressed in embryos (day 10), but that this expression decreases rapidly as gestation progresses. Both GLUT1 and GLUT3 expression appears to increase following birth.

We previously have reported high levels of expression of the GLUT1 in rat embryos during organogenesis (12, 13). Other ontogenic studies performed in rat and rabbit brains also have shown that GLUT1 expression is high in fetal life, but decreases following birth (8, 14). In our results, days 11 to 14, GLUT1 and GLUT3 decreases rapidly as the chorioallantoic circulation develops. During organogenesis, the embryo is absolutely dependent on anaerobic glycolysis (15); a very high rate of glucose utilization because only 2 mol of ATP have been synthesized by per mole of glucose. The embryo gets its nutrients (glucose and amino acids) and oxygen via direct diffusion prior to the establishment of the chorioallantoic circulation (16, 17). After the establishment of chorioallantoic circulation (day 11), glucose utilization decreases because of the appearance of TCA cycle (18). Therefore, high affinity glucose transporters are required in early organogenesis period.

In the present study, distinct localizations of GLUT1 and GLUT3 were noted from days 10 to 14. GLUT1 was localized to the neural tube, gut, heart, and optic vesicles, while GLUT3 was localized to the surface ectoderm and gut. Smith and Gridley have reported that GLUT3 is mainly localized to the yolk sac and surface ectoderm of an embryo (19). Although both GLUT1 and GLUT3 manifest a high affinity for glucose, GLUT3 has a lower Km for glucose (20). Thus, GLUT3 (which has the highest affinity for glucose) is important for glucose transfer from the amniotic fluid to the embryo,while GLUT1 is responsible for supplying glucose for use as an embryonal fuel.

In the present study, both GLUT1 and GLUT3 increased after birth. GLUT1 and GLUT3 localized to microvessels and to neurons, respectively. These results are agreement

with findings in previous report (21, 22). Therefore, both GLUT1 and GLUT3 may not be important in late gestational stage, and again required for brain development postnatally.

In other organs such as heart, intestine, and kidney, results of ontogenic studies have been reported. In the heart, the glucose transporter changes from GLUT1 to GLUT4 during postnatal development (23). In contrast, GLUT2 and GLUT5 appear during the mid-gestational stage in the intestine and kidney (24, 25). Therefore, the glucose transporters are developmentally regulated and demonstrate organ specificity. Little is known, however, about the factors which regulate the expression of glucose transporters. On a molecular basis, the GLUT1 gene has enhancer elements responsive to growth factor and oncogenes (26). Therefore, we speculate that the glucose transporters are at least regulated by oncogenes and/or growth factors. Further examination of the factors which regulate developmental changes of the glucose transporters is necessary.

In the preimplantation embryo, high affinity glucose transporters are abundantly expressed (19). Taken together with our results, GLUT1 and GLUT3 play an important role prior to the development of the chorioallantoic circulation. In fetal life, when the nutrients come from mother via umbilical cord, GLUT 1 and GLUT3 may not be responsible for brain development.

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