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# Glucose transport in developing rat brain: Glucose transporter proteins, rate constants and cerebral glucose utilization

Susan J. Vannucci, Lisa B. Seaman, Robert M. Brucklacher and Robert C. Vannucci

*Department of Pediatrics (Pediatric Neurology), The Pennsylvania State University School of Medicine, The Milton S. Hershey Medical Center, Hershey, PA 17033, USA*

## Abstract

Developing rat brain undergoes a series of functional and anatomic changes which affect its rate of cerebral glucose utilization (CGU). These changes include increases in the levels of the glucose transporter proteins, GLUT1 and GLUT3, in the blood-brain barrier as well as in the neurons and glia. 55 kDa GLUT1 is concentrated in endothelial cells of the blood-brain barrier, whereas GLUT3 is the predominant neuronal transporter. 45 kDa GLUT1 is in non-vascular brain, probably glia. Studies of glucose utilization with the 2-$^{14}$C-deoxyglucose method of Sokoloff *et al.*, (1977), rely on glucose transport rate constants, $k_1$ and $k_2$, which have been determined in the adult rat brain. The determination of these constants directly in immature brain, in association with the measurement of GLUT1, GLUT3 and cerebral glucose utilization suggests that the observed increases in the rate constants for the transport of glucose into ($k_1$) and out of ($k_2$) brain correspond to the increases in 55 kDa GLUT1 in the blood-brain barrier. The maturational increases in cerebral glucose utilization, however, more closely relate to the pattern of expression of non-vascular GLUT1 (45 kDa), and more specifically GLUT3, suggesting that the cellular expression of the glucose transporter proteins is rate limiting for cerebral glucose utilization during early postnatal development in the rat. (Mol Cell Biochem **140**: 177–184, 1994)

*Key words:* GLUT1 protein, GLUT3 protein, glucose transport, cerebral glucose utilization, brain, development

## Introduction

Normal cerebral oxidative metabolism requires a constant supply of glucose to meet the energy demands of the brain. During development the brain undergoes a series of functional and anatomic changes that affect its energy requirements and thus its rate of cerebral glucose utilization (CGU). The delivery of glucose from the blood to the brain involves transport of this nutrient across the endothelial cells of the blood-brain barrier and the plasma membranes of the neurons and glia. This process is mediated by facilitative glucose transporter proteins. To date the multi-gene family of facilitative glucose transporter proteins has seven members, named GLUT1–7, for the order in which they were cloned (for review see Pessin and Bell, 1992 and Bell *et al.*, 1993), of which two proteins, GLUT1 and GLUT3, have been detected in rat brain.

In whole brain, two distinct forms of GLUT1 are detected: a higher Mr 55 kDa form which is localized to the endothelial cells of the cerebral microvessels [3–5], and a 45 kDa form present in the non-vascular fraction of brain [5]. The difference between these two forms relates to their extent of glycosylation [6]. GLUT1 mRNA has been detected in all non-neuronal elements of rat brain [7] and one study has detected GLUT1 by immunohistochemistry in glia but not neurons [8]. Thus it is likely that the 45 kDa GLUT1 is the major glial glucose transporter. GLUT3 is expressed primarily by neurons [9, 10] and, in cultured neurons is expressed at 6–10 times the concentration of GLUT1 [11]. GLUT3 mRNA in rat brain demonstrates both neuronal and regional

*Address for offprints:* S. J. Vannucci, Department of Pediatrics, Milton S. Hershey Medical Center, P.O. Box 850, Hershey, PA 17033, USA

specificity [7]. We have recently demonstrated that the expression of GLUT1 and GLUT3 are developmentally regulated in rat brain [12] coincident with increases in reported rates of cerebral glucose utilization [13].

The quantitative measurement of cerebral glucose utilization according to the 2-$^{14}$C-deoxyglucose method of Sokoloff *et al.* [14], involves an understanding of the kinetics of blood-brain barrier transport of both glucose and 2-deoxyglucose (2-DG), as well as their rates of phosphorylation by hexokinase. These values are combined to form the 'lumped constant', an integral part of the Sokoloff equation, which is a function of the transport and phosphorylation constants for the tracer and for glucose. The transport constants, defined in detail in Sokoloff *et al.* [14] are: $k_1$, the initial unidirectional clearance of glucose (or any hexose), from the blood to the brain, measured in ml $g^{-1}$ $min^{-1}$, and $k_2$, the fractional clearance of glucose from the brain to the blood, which is calculated from $k_1$ and the steady-state distribution of the non-metabolized 3-O-methylglucose, as $min^{-1}$. These values have been determined for the adult rat brain under several conditions [14, 15], however all of these measurements predated an appreciation of the involvement of different glucose transporter isoforms. Given the different patterns of the developmental increases in both GLUT1 and GLUT3 in rat brain and the fact that blood-brain barrier transport is generally assumed to be limiting to metabolism in the immature brain [16], we decided to examine the relationships between the rate constants for glucose transport into ($k_1$) and out of ($k_2$) the brain, the rate of cerebral glucose utilization, and the glucose transporter proteins, GLUT1 and GLUT3, during this period of maturation.

## Materials and methods

### *Animals*

Timed, pregnant Wistar rats (Charles River Laboratories) were allowed to deliver spontaneously and pups were removed from their dams at 1, 7, 14, 21 and 30 days of age and either decapitated for the determination of the levels of the glucose transporter proteins or administered radioisotope for the determination of either $k_1$ or $k_2$, or for the direct determination of cerebral glucose utilization. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Hershey Medical Center Animal Care Committee.

### *Analysis of GLUT1 and GLUT3 proteins*

Animals were killed by decapitation and the brains were rapidly removed for the preparation of either total membranes from discrete forebrain regions or the preparation of cerebral microvessels as described [12]. Briefly, for the preparation of total membranes, samples were homogenized in 5 volumes of TES [20 mM Tris, 1 mM EDTA, and 255 mM sucrose, pH 7.4] with protease inhibitors aprotinin, leupeptin, and pepstatin, 1 μg/ml each, and phenylmethylsulfonyl fluoride, $10^{-4}$ M, and centrifuged at 150,000 × g for 20 min. Membrane pellets were resuspended in TES and stored frozen. Isolated cerebral microvessels were prepared from pooled cortical shells, according to the method of Betz *et al.* [17] and vessel-free cortical (neuronal/glial) membranes prepared by centrifugation of the initial 1,000 g supernatant at 150,000 g [5, 18]. GLUT1 and GLUT3 proteins were measured in total membrane samples, isolated microvessels and vessel-free neuronal/glial membranes by Western blot analysis and quantitated as previously described [12].

### *Determination of rate constants, $k_1$ and $k_2$*

The individual rate constants for the transport of glucose into ($k_1$) and out of ($k_2$) brain were determined directly in 7, 14 and 21 day old animals according to the recommendations of Fuglsang *et al.* [19], using the radioactive tracers $^{14}$C-glucose and $^{14}$C-3-O-methylglucose (Amersham, Arlington Heights, IL). Because of their small size, separate animals were used for each time point on the saturation curves at 7 and 14 days whereas the entire blood curve was determined on individual animals with an arterial catheter at 21 days. Labeled tracer, 0.5 μCi/g, was injected s.c. and whole blood and brain samples were obtained a 0, 15, 30, 60 seconds and 2, 3, 4, 5 and 10 min for the determination of $k_1$, and samples processed for liquid scintillation counting. $K_1$ was then calculated according to the equation:

$$k_1 = [aV(T) - V(0)] / \theta\,(T)$$

Where: V(T) = apparent volume of distribution
V(0) = initial distribution volume
θ(T) = apparent exposure time of brain, measured as the time integral of blood radioactivity, normalized, as a function of time.

Thus, a V(T) = [dpm/g brain at each t × brain water content, ml/g] / [dpm/ml blood at each t], and a plot of a V(T) vs t (normalized) yields a line with a slope of $k_1$ in ml/g/min and a Y intercept of V(θ) in ml/g.

The subsequent determination of $k_2$ involved the determination of the steady-state distribution of [$^{14}$C]-3-O-methylglucose. The isotope was injected as above and whole blood and brain samples taken at timed intervals over 40 min and processed for liquid scintillation counting. $K_2$ was then calculated according to the equation:

$k_2 = k_1$ (glucose) / V, where

V = dpm/g brain at steady-state × water content / dpm/ml blood at steady-state. Values for the rate constants in the 1 day old rat were taken from the paper of Fuglsang *et al.* [19] and all values were then calculated as a percentage of the adult value as reported in that paper.

*Measurement of cerebral glucose utilization (CGU)*

CGU was measured in 7 and 14 day old rat pups by a modification of the Sokoloff equation, as previously described by us in the immature rat [20, 21]. Briefly, 7 or 14 day old rats were injected s.c. with 2.5 or 5 μCi 2-deoxy-[U$^{14}$C]-glucose (Amersham, Arlington Heights, IL), after which 2 or more animals were decapitated at either 2, 5, 10, 20, 30, 45, 60, 75 or 90 min. A blood sample was taken at decapitation (trunk blood) and used for determination of plasma radioactivity (liquid scintillation counting) and glucose concentration (Glucostat, Beckman Instruments) for the calculation of the integrated 2-DG/glucose specific activity (dpm/mmol) over 90 min. Decapitated heads were immediately frozen in liquid nitrogen to stop intermediary metabolism and brains were removed and powdered at –20°C. Neutralized perchloric acid extracts were prepared [22], and passed over an ion-exchange column, formate form (Biorad Econocolumn, Richmond, CA) for the separation of free (non-phosphorylated) 2-DG from phosphorylated 2-DG, to obtain the percentage 2-DG metabolized. Brain glucose (mM/kg) was measured in the neutralized perchloric acid extracts as previously described [22]. The lumped constant (LC) of the Sokoloff equation was determined with the Pardridge normogram [23] for adult rat brain which relates the LC to the blood and brain glucose values. Preliminary studies in 7 day old rats in which values obtained using all of the rate constants determined directly in the pups were compared with values obtained using the LC from the Pardridge normogram demonstrated an insignificant difference between the two calculations. Thus the measured values for the percentage of 2-DG phosphorylated can be substituted for the rate constants as previously described [20, 21] and the equation for cerebral glucose utilization becomes:

$$\text{CGU } (\mu\text{mol}/100\text{ g/min}) = \frac{C_i^*(T) \times ([^{14}\text{C}]\text{-2-DG-6-P/total } [^{14}\text{C}])}{(\text{LC}) \int_0^T \text{Cp}^*/\text{Cp}\, dt}$$

where Ci* (T) = tracer concentration in brain at T (dpm/g)
Cp* = tracer concentration of tracer in plasma (dpm/ml)
Cp = glucose concentration in plasma (μmol/ml) and
LC = lumped constant

## Results and discussion

*Blood-brain barrier transport in postnatal rats*

The delivery of glucose from the blood to the brain first relies on transport across the endothelial cells of the blood-brain barrier. Kinetically, this process can be described by the rate constants $k_1$, for the transport of glucose into brain, and $k_2$, for the transport of glucose from brain back to blood, as explained in detail by Sokoloff *et al.* [14]. Functionally, this process is mediated by the 55 kDa GLUT1 protein at both the luminal and abluminal sides of the endothelial cell membrane. The increased level of expression of this protein during ontogeny of the blood-brain barrier, from E19 through adult, is shown in Fig. 1. Although it has often been assumed that the blood-brain barrier is 'leaky' in the immature animal, it has now been clearly demonstrated that rat brain endothelial cells express barrier properties as early as E16 [24] and that capillary fenestrations are no longer present at the end of gestation [25]. Although not all capillaries are perfused in the rat brain prior to P10, those that are perfused have tight

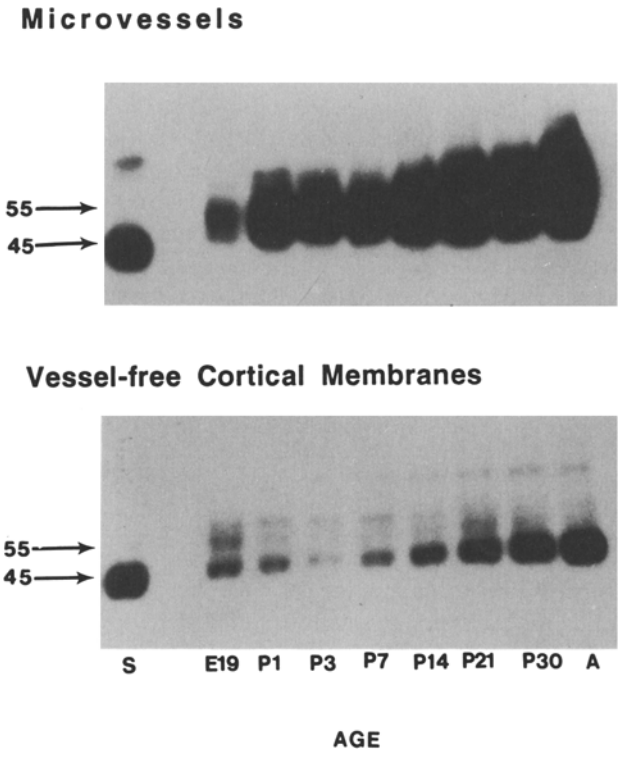


*Fig. 1.* GLUT1 protein in cerebral microvessels and neuronal/glial membranes in immature rat brain. Isolated microvessels and vascular-free neuronal/glial membranes were prepared from pooled cortical shells of fetal (E19), postnatal (P1–P30), and adult (A), rats, and 15 μg aliquots were subjected to Western blot analysis for GLUT1. S, brain 'standard'. Approximate molecular weights are indicated in the margin. Reprinted with permission from Vannucci, 1994 [12].

junctions consistent with barrier function [26] and therefore clearly need a system to transport glucose. The level of GLUT1 in these vessels (per mg of microvessel protein) is unchanged during the first week of life but then increase sharply during the next 2 weeks to attain near adult levels by P21. The capacity of this, as well as other, nutrient transport systems in the blood-brain barrier appears to vary with both the metabolic requirements of the brain and the nutritional state of the animal [27, 28], and the initial low levels are likely reflective of both the low cerebral energy demands as well as the high levels of circulating ketone bodies in the suckling pups which provide an alternative fuel to glucose [13, 16, 29].

To more specifically determine the extent to which the observed maturational increases in GLUT1 concentration in the cerebral microvessels relate to alterations in the rates of glucose transport into, and out of, brain, the rate constants describing these functions ($k_1$ and $k_2$, respectively) were measured directly in 7, 14 and 21 day old animals. The results of these measurements are presented in Table 1, where they are expressed as a percent of the value for the adult rat, as reported by Fuglsang *et al.* [19]. Fuglsang *et al.* [19] also performed these measurements in the neonatal (P1) rat, and their values, re-calculated as a percent of adult, are included as well. A comparison of the relative increases in these rate constants with the levels of GLUT1/microvessel protein at the same ages reveals quite a good agreement. The difference between the rate constants at P1 and the higher level of microvessel GLUT1 protein could relate to the fact that the constants were obtained from the study of Fuglsang *et al.* [19] and not determined by us. An alternate explanation, however, could be that although the concentration of GLUT1 (per mg microvessel protein) is already nearly half its adult value at P1, the total amount is still probably lower since the most rapid period of angiogenesis occurs later, between P5 and P9 [24]. Clearly, the total capacity of this system to transport glucose must depend on both the concentration of active transporter in the microvascular membranes and the total amount of vessels. This latter value increases from 80 vessels/mm$^2$ of cortex during the first week of life in the rat to 250–300 vessels/mm$^2$ by 15–21 days [26]. Following this period, i.e. from P7 through P21, the concentration of GLUT1 in the microvessels of the blood-brain barrier increases in the same proportion, relative to adult, as do the rate constants for glucose transport across this barrier.

*Table 1.* Glucose transport rate constants, cerebral glucose utilization and glucose transporter proteins in postnatal rat brain

| Age (days) | k1 | k2 | CGU | GLUT1 | | GLUT3 |
|---|---|---|---|---|---|---|
| | | | | Microvessel | | Non-vessel |
| 1 | 32# | 23# | 27# | 45 | 16 | 10 |
| 7 | 40 | 53 | 12 | 41 | 18 | 20 |
| 14 | 63 | 70 | 28 | 65 | 21 | 26 |
| 21 | 93 | 106 | 65* | 90 | 40 | 79 |
| 30 | – | – | 79* | 95 | 80 | 93 |

Rate constants and CGU were measured directly in postnatal rats as described in Methods except where noted, and expressed as a per cent of the reported value for adult rat brain: $k_1$ (ml g$^{-1}$ min$^{-1}$), 0.186; $k_2$ (min$^{-1}$), 0.363 [19]; CGU, 103 μmol/100 g/min [13], and represent 2–5 determinations. GLUT1 and GLUT3 proteins were determined by immunoblot analysis of cerebral cortical membranes (n=5 for each age), isolated cerebral microvessels and vascular-free cortical membranes (n=2 preparations for each age) and expressed as a per cent of the corresponding adult value. # denotes data of Fuglsang *et al.* [19]; * denotes data of Nehlig *et al.* [13].

*GLUT1 and GLUT3 expression in postnatal rat brain*

Once across the blood-brain barrier, glucose must be transported into the cellular elements of the brain, i.e. the neurons and glia, before it can be utilized. Unfractionated cerebral cortical membranes were prepared from postnatal rats (P1–P30) and adult rats. As can be seen in Fig. 2A, both molecular weight species of GLUT1 protein are detected in these membranes, i.e. the 55 kDa band (vascular) and the 45 kDa band (neuronal/glial). The developmental changes in both forms of GLUT1 are apparent in this immunoblot, with the total GLUT1 at P1 being quite low, relative to the adult, and consisting mostly of the high-molecular weight form. This may well relate to the existence of a higher molecular weight form of GLUT1 in differentiating cells at this early time point, as has also been demonstrated for L6 muscle cells during differentiation [30]. An additional explanation is that the microvascular GLUT1 represents a greater proportion of the total, relative to the parenchyma. Although the numbers of vessels/mm$^2$ is low at this early time, the amount of extracellular space is relatively high (20% in the newborn cf. <1% by P15–P21 [26]), and the concentration of 45 kDa GLUT1 in the non-vascular membranes is low (Fig. 1, bottom). This distribution between the two forms changes by P7 however at which time GLUT1 in these membranes is detected equally in both the 55 kDa and 45 kDa bands. As total GLUT1 approaches adult levels the 45 kDa form constitutes by far the greatest proportion which is indicative of the growth of the brain parenchyma such that any given tissue sample will be composed of mostly cellular, and not vascular, elements. The increased concentration of 45 kDa GLUT1 with the growth of the brain parenchyma is also apparent from the immunoblot of vascular-free cortical membranes shown in the bottom panel of Fig. 1. The quantitation of all of the analyses for cortical samples at each of these ages (n=5 for each age, relative to adult, n=3) is presented in Fig. 2B, in conjunction with a similar analysis for three other forebrain regions, i.e. hippocampus, thalamus and hypothalamus. As can be seen from this figure, total GLUT1 remains low through P14. What does change during this period, however, is the relative

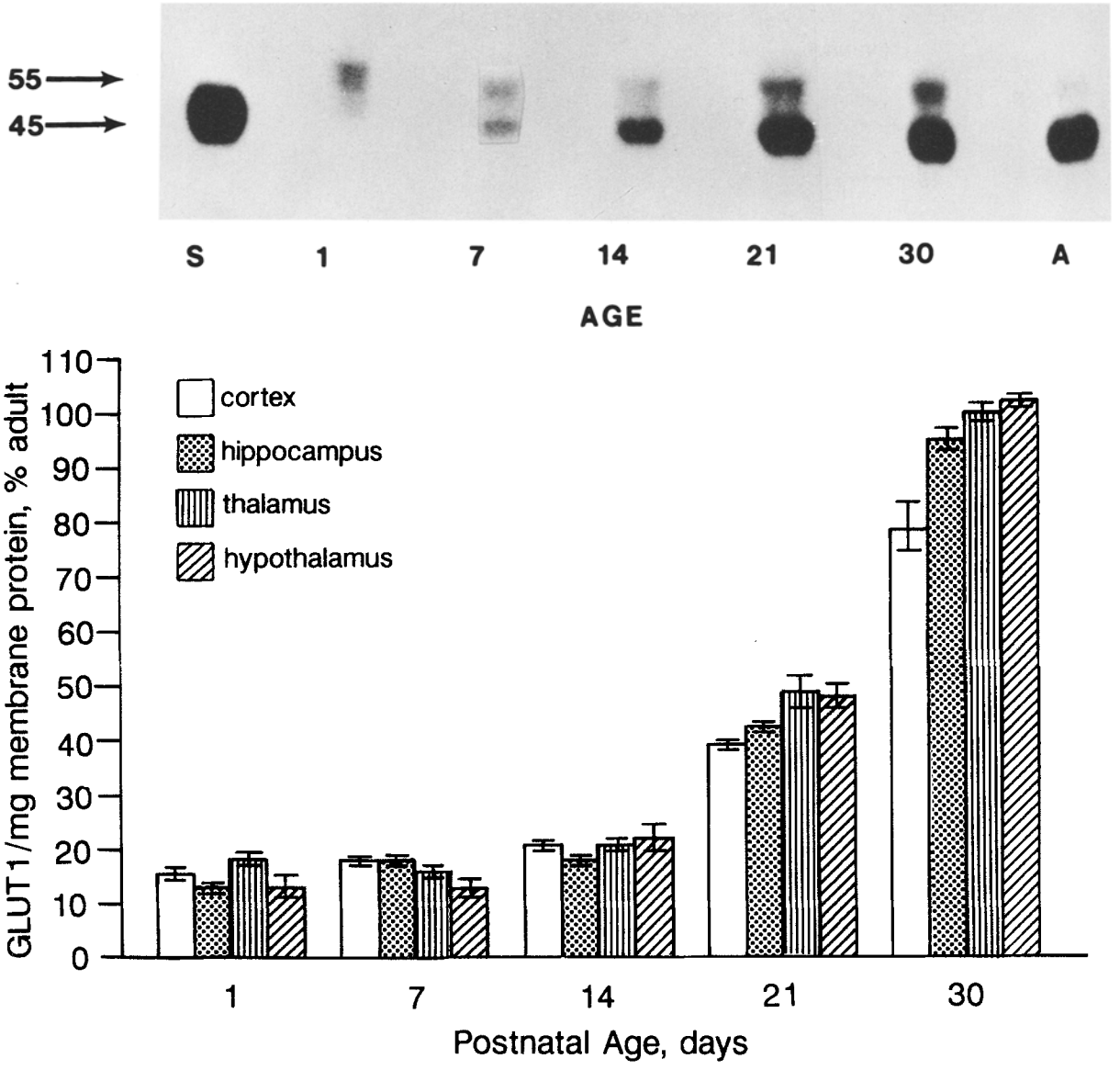


*Fig. 2.* GLUT1 protein expression in immature rat brain. A: Western blot analysis of cerebral cortical membranes from rats of increasing postnatal age (1–30 days), adults (A), and brain 'standard' (S), 15 μg protein/lane. Approximate molecular weights are indicated in the margin. B: Total GLUT1 concentration per mg membrane protein, measured in cerebral cortex, hippocampus, thalamus and hypothalamus in five rat pups at each age. Values for all samples were normalized to the brain 'standard', and the average adult value (n=3) for each region was set at 100%. Concentrations were calculated as percentages of the adult value for each region and are presented as mean ± SEM (bars). Reprinted with permission from Vannucci, 1994 [12].

contributions of the 55 kDa and 45 kDa forms of GLUT1 protein in the total membrane sample. The same pattern as is depicted in the immunoblot in Fig. 2A was seen for all regions (data not shown), i.e. the 55 kDa band predominates at P1, the 2 bands are evenly detected at P7 and the 45 kDa band predominates by P14 and thereafer. There is a substantial increase (2-fold) in total GLUT1 between 14 and 21 days and this value is doubled again in the 30 day old brain, at which time essentially adult levels are reached.

The pattern of expression of the neuronal glucose transporter, GLUT3, with development is quite different from that displayed for GLUT1. Figure 3A depicts the Western blot analysis of the same membrane samples shown in Fig. 2A but here analyzed for GLUT3, and the corresponding regional analysis and quantitation is depicted in Fig. 3B. As can be seen, GLUT3 is detected as a single band, or sometimes a doublet, of Mr 45 kDa. The expression of this transporter isoform is very low in the neonatal (P1) brain but increases linearly during the first 3 weeks of life to reach near adult expression by P21 and certainly by P30. This observation is in good agreement with the developmental pattern of GLUT3 mRNA in neurons coincident with terminal differentiation and migration. The period between birth and 21–30 days in the rat is characterized by significant alterations in

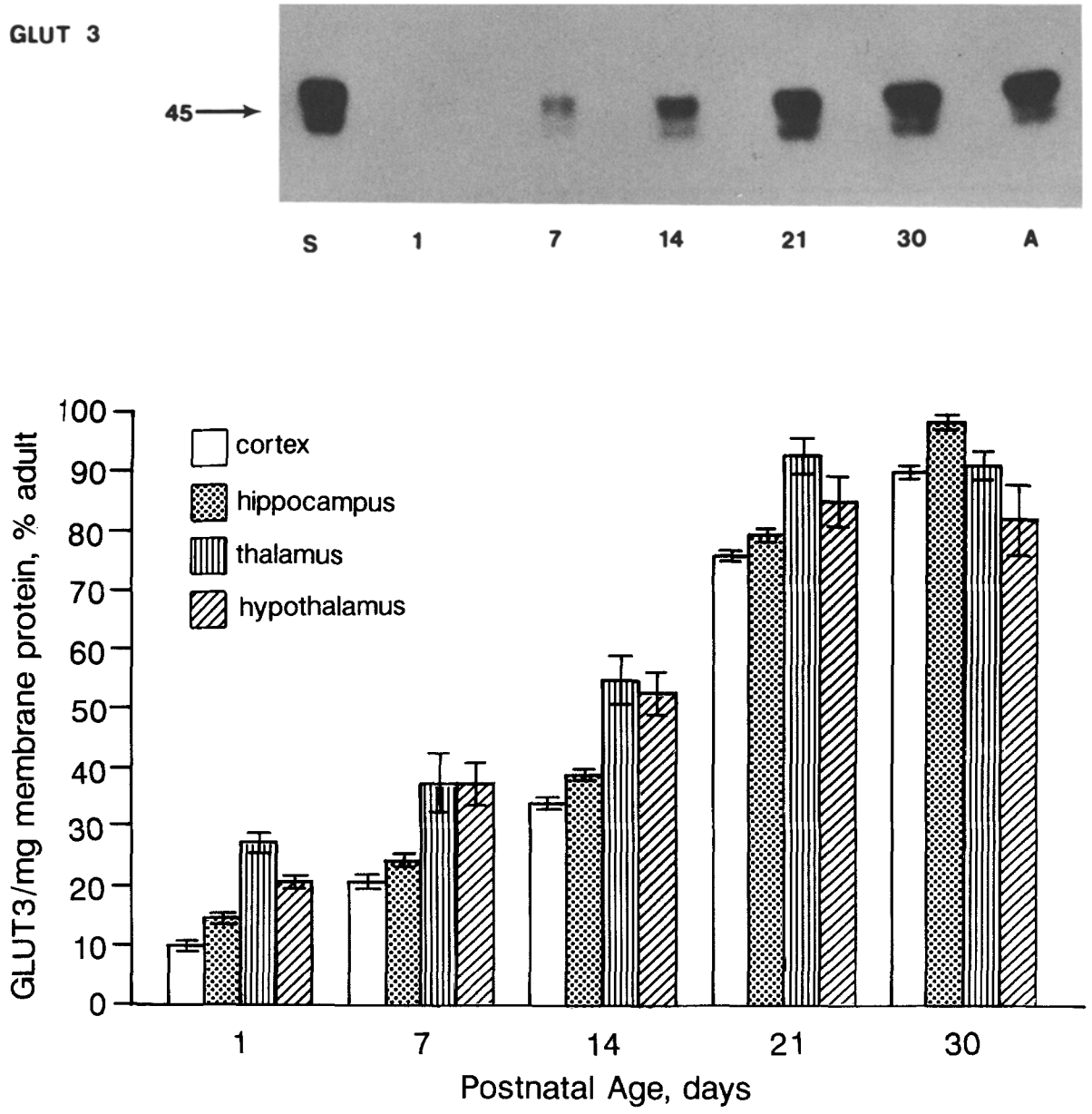


*Fig. 3.* GLUT3 protein expression in immature rat brain. A: Western blot analysis of cerebral cortical membranes from rats of increasing postnatal age (1–30 days), adults (A), and brain 'standard' (S), 15 μg protein/lane. The approximate molecular mass (45 kDa) is indicated in the margin. B: GLUT3 concentration/mg membrane protein, measured in cerebral cortex, hippocampus, thalamus, and hypothalamus, in five rat pups at each age. Concentrations were calculated as described in Fig. 2B and are presented as mean ± SEM (bars). Reprinted with permission from Vannucci, 1994 [12].

both brain activity and metabolism, reflected in rapid increases in the number of synaptic connections [31], increases in bioelectric activity [32], and increased flux of glucose through both synthetic and energetic pathways [33–36]. The increase in GLUT3 expression is coincident with the increased need for the supply of fuel to meet these demands. The association between GLUT3 expression and functional maturity is further seen in the regional variation in the pattern of GLUT3 expression with maturation (Fig. 3B). In this regard, it is interesting that the thalamus, which demonstrates the earliest increases in the level of GLUT3, has also been shown to have a high level of glucose utilization in the infant human [37, 38] and newborn rat [13, 20] brain.

The autoradiographic 2-deoxyglucose method of Sokoloff *et al.*, [14] measures regional rates of cerebral glucose utilization in neurons and glia. This method has been used in

immature rats as young as P10 [13], and a modification of this method for use in younger animals has also been described [19–21]. An initial comparison of previously reported values from all of these studies with the developmental increases in the cellular glucose transporters, 45 kDa GLUT1 and GLUT3, revealed a reasonable agreement (Table 1). Using our modification of the 2-DG methodology, initially reported by us in the 7 day old rat [20], we repeated these measurements in both 7 and 14 day old rats. The results of these studies, together with the data from the other studies, is presented in Table 1. As for the rate constants, all of the CGU measurements are presented as a percent of the adult value for frontal cortex [13, 14]. It is interesting that the measured rates of CGU at all points during the first three weeks are substantially lower than the apparent capacity for blood-brain barrier glucose transport, as described by both the concentration of GLUT1 in the microvessels and the rate constants for glucose transport. A comparison of these values with the relative increases in non-vascular GLUT1 (Fig. 1, bottom panel), and more specifically with GLUT3, demonstrates a much closer relationship. It has been suggested that glucose utilization, as detected with 2-DG is largely synaptic and associated with the energy required to maintain the electrochemical gradients [39, 40]. Thus it is reasonable that CGU and GLUT3 would increase in concert with synaptogenesis and bioelectric activity. These findings would suggest that the ability to transport glucose across the blood-brain barrier may not be the limiting factor to cerebral glucose utilization during development. Rather, it appears that it is the cellular expression of the glucose transporter proteins, and specifically the level of neuronal GLUT3, which is limiting to cerebral glucose utilization during this early postnatal period.

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