Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons

Fran MAHER, Theresa M. DAVIES-HILL and Ian A. SIMPSON*

Building 10, Room 5N102, NIDDK, National Institutes of Health, 10 Center Drive MSC 1420, Bethesda, MD 20892-1420, U.S.A.

This study examines the apparent affinity, catalytic-centre activity ('turnover number') and stereospecificity of the neuronal glucose transporter GLUT3 in primary cultured cerebellar granule neurons. Using a novel variation of the 3-*O*-[¹⁴C]methylglucose transport assay, by measuring zero-*trans* kinetics at 25 °C, GLUT3 was determined to be a high-apparent-affinity, high-activity, glucose transporter with a K_m of 2.87 ± 0.23 mM (mean $\pm$ S.E.M.) for 3-*O*-methylglucose, a V_{max} of 18.7 ± 0.48 nmol/min per 10^6 cells, and a corresponding catalytic-centre

activity of 853 s^{-1} . Transport of 3-*O*-methylglucose was competed by glucose, mannose, 2-deoxyglucose and galactose, but not by fructose. This methodology is compared with the more common 2-[³H]deoxyglucose methodology and the [U-¹⁴C]-glucose transport method. The high affinity and transport activity of the neuronal glucose transporter GLUT3 appears to be an appropriate adaptation to meet the demands of neuronal metabolism at prevailing interstitial brain glucose concentrations (1–2 mM).

INTRODUCTION

Although glucose is the major fuel for cerebral energy metabolism, little is known about the kinetic and regulatory characteristics of glucose transport into the various cells that comprise the brain, i.e. neurons and glia. Glucose uptake into most mammalian cells is facilitated by a family of glucose transport proteins, named GLUT1 to GLUT7 (for reviews see Bell et al. [1] and Gould and Holman [2]); at least three isoforms, GLUT1, GLUT3 and GLUT5, are known to be expressed in specific cells in the brain (reviewed in Maher et al. [3]). Preliminary reports suggest that GLUT2, GLUT4 and GLUT7 may also be present [1–3].

The kinetic characteristics of the facilitative glucose transporters have been determined most extensively for human erythrocyte GLUT1 [4]. The apparent affinities of different transporter isoforms for glucose or the non-metabolizable glucose analogue 3-*O*-methylglucose (3-*O*-MG) vary considerably depending upon the methodology used. K_m values of 1.5 mM have been determined for GLUT1 when transport was measured by a zero-*trans* methodology (3-*O*-[¹⁴C]MG and unlabelled 3-*O*-MG added to the outside only), compared with 20–30 mM by equilibrium exchange (3-*O*-MG equal on the inside and outside, 3-*O*-[¹⁴C]MG added to the outside). The ranges of K_m values for GLUT2 and GLUT4 are 15–40 mM and 1.5–7.0 mM respectively [1,4–7]. These ranges in values reflect a diversity in both methodology and the cell type employed in their determination. For example, human GLUT3, when overexpressed in *Xenopus* oocytes or CHO cells, appears to have a relatively high apparent affinity, with K_m values for the transport of 3-*O*-MG of 10 mM and 3–4 mM respectively [8,9]. Although heterologous overexpression of glucose transporters is clearly a useful approach for the analysis of transporter characteristics, it places the transporters in a non-physiological membrane environment and, prior to the present study, appropriate kinetic analyses on endogenously expressed neuronal GLUT3 had not been performed.

Three methodologies are currently used to assess glucose transport activity in cultured cells, each of which is based on the uptake of a different hexose: 3-*O*-MG, 2-deoxyglucose (2-DG),

or glucose itself. Inherent within each methodology are certain assumptions and constraints which limit their utility. For example: (i) 3-*O*-MG is not phosphorylated or further metabolized to a significant extent and, consequently, rapidly equilibrates, rendering the assay difficult to perform; (ii) 2-DG is phosphorylated by hexokinase and accumulates in the cell; however, at high concentrations it is this phosphorylation rather than transport which becomes rate-limiting, and thus this is not an appropriate assay for K_m and V_{max} determinations; (iii) when labelled glucose is used at trace, i.e. submicromolar, concentrations the intermediate metabolites accumulate rather than leaving the cell as either lactate or CO₂ [6,10] and thus, although inexpensive and convenient, it cannot be used for kinetic determinations. The 2-DG methodology has been employed in all previous studies of glucose transport in cultured neurons and glia without taking into account the problems of rate-limiting substrate phosphorylation by hexokinase or the existence of two distinct transporters. We have previously characterized GLUT1 and GLUT3 expression in primary rat cerebellar granule neurons, a relatively pure neuronal culture system ($\geq 95\%$ granule neurons) [11,12]. Differentiated neurons in this system express about 8–10-fold more GLUT3 than GLUT1 at the cell surface and thus are a good model to study the kinetic characteristics and sugar specificity of GLUT3 in an appropriate neuronal environment. In the present study we compare the various methodologies for transport determination in this neuronal system.

MATERIALS AND METHODS

Cell culture

Primary cerebellar granule neuronal cultures were established from 8-day-old Sprague–Dawley rat pups as described previously [11]. Neurons were plated at 3.75×10^5 cells/cm² on poly(L-lysine)-coated LabTek two-chambered slides and cultured in Eagle's basal medium with Earle's salts (BME), 10% (v/v) fetal calf serum, 25 mM KCl, 2 mM glutamine and 0.1 mg/ml gentamycin. Assays were performed on neurons cultured for 7–9 days.

CHO-G3 cells (CHO cells overexpressing human GLUT3 [13]) were grown to confluence on two-chambered slides. Cell number at the time of assay was determined by counting nuclei of lysed cells, as previously described [11]. Total neuronal membranes were prepared as previously described [12]. Protein content was measured with bicinchoninic acid reagent (Pierce, Rockford, IL, U.S.A.).

3-O-[14 C]MG transport

Primary neurons grown on two-chambered slides were washed three times with Locke's buffer (154 mM NaCl, 8.6 mM Hepes, 5.6 mM KCl, 2.3 mM CaCl_2 and 1 mM MgCl_2 , pH 7.5) and incubated in buffer containing 5 μM (56 $\mu\text{Ci}/\mu\text{mol}$) 3-O-D-[methyl- 14 C]glucose (3-O-[14 C]MG; NEN) and 0.02 μM L-[1- ^3H]glucose (0.4 $\mu\text{Ci}/\text{mmol}$; NEN). Equimolar concentrations of L-glucose were used as markers for non-carrier-mediated uptake and trapping. The time courses for 3-O-MG zero-*trans* transport were performed at 24 °C, as uptake at 37 °C was too rapid for sufficient reproducibility. Under these conditions, maximum uptake was achieved by 30–60 s, and two different time points between 5 and 15 s were generally optimal for determination of initial rates such that the cells were approx. 20–30 % filled which, at the higher 3-O-MG concentrations, may still result in an underestimate of the initial rate [6,14]. Transport was stopped by the addition of 0.1 mM cytochalasin B (CB), followed by four washes with buffer containing CB, L-glucose and 3-O-MG. Zero time points were obtained by preincubation with CB. The cells were solubilized in 0.1 M NaOH, neutralized with an equal volume of 0.7 % acetic acid and the uptake of labelled sugars was determined by liquid scintillation counting. The initial rates of 3-O-MG transport were calculated according to the methodology previously described [15].

2-[^3H]DG uptake

Under the same conditions as described above, neurons were incubated in buffer containing 0.1 mM unlabelled 2-DG and 0.02 μM (25 $\mu\text{Ci}/\mu\text{mol}$) 2-[^3H]DG (2-[1,2- ^3H (N)]deoxy-D-glucose; NEN). Uptake at 37 °C was linear up to 10 min, and a 3 min incubation time was selected for further assays. Uptake was terminated by aspirating the solution and washing the monolayer four times with Locke's buffer at room temperature. The cells were then processed as described above.

[U- 14 C]Glucose uptake

Neurons were incubated in buffer containing 0.08 μM (0.021 $\mu\text{Ci}/\text{ml}$) D-[U- 14 C]glucose (NEN; specific radioactivity 265 $\mu\text{Ci}/\text{mmol}$). Uptake at 37 °C was linear for 20 min, and a 5 min incubation was used for all assays. Cells were processed as described for 3-O-[14 C]MG uptake. The inhibitors CB, cytochalasins D and E, phloretin, phlorizin and forskolin were added 5 min prior to uptake assays. Competing sugars were added for competition assays at the same time as the radioisotope.

RESULTS

GLUT3 apparent affinity, $V_{\max}$ and catalytic-centre activity

Kinetic parameters for apparent affinity (K_m) and maximal transport capacity ($V_{\max}$) were determined in neurons using the 3-O-[14 C]MG transport assay under zero-*trans* conditions over a concentration range of 0–15 mM 3-O-MG. The initial velocities (v) determined as a function of substrate concentration from four separate experiments were fitted (SigmaPlot for Windows) by non-linear regression to the Michaelis–Menten equation $v =$

Figure 1 Determination of K_m and $V_{\max}$ values for 3-O-MG transport in neurons

Four separate zero-*trans* 3-O-MG transport experiments were performed. The concentration of 3-O-MG ranged from 0.05 to 15 mM. Combined data are plotted as v (nmol/min per 10^6 cells) versus $[S]$ (mM) and fitted to the Michaelis–Menten equation by non-linear regression. The K_m was determined to be 2.87 ± 0.23 mM and the $V_{\max}$ was 18.74 ± 0.48 nmol/min per 10^6 cells. The variance (r^2) = 0.998.

$(V_{\max}[S])/(K_m + [S])$, from which the K_m and $V_{\max}$ were calculated [$K_m = 2.87 \pm 0.23$ mM (mean $\pm$ S.E.M.) and $V_{\max} = 18.7 \pm 0.48$ nmol/min per 10^6 cells; Figure 1).

In a previous study, using the impermeant photolabel 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), we determined that the concentrations of GLUT1 and GLUT3 proteins expressed at the plasma membranes of neurons are approx. 2 and 18 pmol/mg of cell membrane respectively, and 20 μg of total membrane protein was obtained per 10^6 cells. Thus the concentrations of GLUT1 and GLUT3 in rat cerebellar granule cells were calculated to be 40 fmol/ 10^6 cells and 360 fmol/ 10^6 cells respectively. The $V_{\max}$ for zero-*trans* entry into human erythrocytes at 24 °C is 56 mmol/min per litre of cell water [16], which corresponds to a k_{cat} or catalytic-centre activity ('turnover number') of 123 s^{-1} [17]. Assuming the same rate of GLUT1-mediated transport in cerebellar granule cells, then the expected uptake mediated by GLUT1 is equal to the number of transporters/cell $\times$ catalytic-centre activity = $40 \times 10^{-15} \times 123 \times 60/\text{min}/10^6 \text{ cells} = 0.32 \text{ nmol/min per } 10^6 \text{ cells}$. Thus the transport mediated by GLUT3 = $18.74 - 0.32 = 18.42 \text{ nmol/min per } 10^6 \text{ cells}$. Finally, the catalytic-centre activity for GLUT3 = $(18.42 \times 10^9)/(360 \times 10^{-15} \times 60) = 853 \text{ s}^{-1}$. These observations suggest that GLUT3 is the most active of the glucose transporters analysed to date.

Substrate specificity

To further determine the specificity of neuronal GLUT3, the effects of various well characterized inhibitors were investigated. The following inhibition constants were determined for 3-O-[14 C]MG transport in neurons: CB ($\text{IC}_{50} \sim 0.35 \mu\text{M}$), forskolin ($K_i \sim 5.5 \mu\text{M}$) and phloretin ($\text{IC}_{50} \sim 9.5 \mu\text{M}$). Transport was not inhibited by cytochalasins D and E, or by phlorizin, an inhibitor of the Na^+ /glucose co-transporter. A representative experiment comparing the ability of various monosaccharides to

Figure 2 Sugar competition of 3-O-[¹⁴C]MG transport (a), 2-[³H]DG uptake (b) and [U-¹⁴C]glucose uptake (c) in primary cerebellar granule neurons

3-O-[¹⁴C]MG transport, 2-[³H]DG uptake (measured for 3 min) and [U-¹⁴C]glucose uptake (measured for 5 min) were assayed as described in the Materials and methods section in the presence of 0–50 mM of the following sugars: D-glucose (■), 2-DG (▲), galactose (△), fructose (□), xylose (○), L-glucose (●) and mannose (▼). Data are expressed as percentage of control. Each curve is representative of two to four independent experiments each performed in quadruplicate. S.E.M. values were within 15% of the mean (excluded from the graph for clarity). A summary of the data is given in Table 1.

inhibit the different transport assays, i.e. the uptake of 3-O-[¹⁴C]MG, 2-[³H]DG and [U-¹⁴C]glucose, is shown in Figure 2. In Figure 2(a), it is apparent that glucose and 2-DG are the most potent inhibitors of 3-O-[¹⁴C]MG transport, with IC₅₀ values of 5 mM and 8–10 mM respectively, compared with galactose (~15 mM) and xylose (~20 mM). These values are in marked contrast to those observed for the 2-[³H]DG assay (Figure 2b) or the tracer [U-¹⁴C]glucose assay (Figure 2c). The IC₅₀ values for inhibition of these assays are compared in Table 1. The apparent enhanced ability of glucose and mannose to inhibit

Table 1 Sugar competition of three glucose uptake assays in cerebellar granule neurons

Values are the means obtained from two to four assays performed on each sugar in quadruplicate wells. Initial concentrations of labelled 3-O-MG, 2-DG and glucose were 5, 100 and 0.3 μ M respectively.

Sugar	IC ₅₀ (mM)		
	3-O-[¹⁴ C]MG	2-[³ H]DG	[U- ¹⁴ C]Glucose
D-Glucose	5	< 0.5	0.1
Mannose	20–25	< 0.5	0.3
3-O-MG	–	8.5	3.5
2-DG	8–12	–	0.6
Fructose	> 50	10	17
Galactose	16	> 50	> 50

the 2-DG assay, and of 2-DG and mannose to inhibit the [U-¹⁴C]glucose assay, is due to the ability of these compounds to serve as substrates of hexokinases; therefore they competitively inhibit hexokinase and thus decrease the accumulation of 2-DG 6-phosphate or glucose metabolites. This is made more apparent by the observation that fructose does not inhibit 3-O-[¹⁴C]MG transport but does appear to gain access to the cells, inhibiting the accumulation of both [U-¹⁴C]glucose (IC₅₀ ~ 12 mM) and 2-[³H]DG (IC₅₀ ~ 9.5 mM). In contrast galactose, which is not a substrate for hexokinase, inhibits 3-O-[¹⁴C]MG transport (IC₅₀ ~ 15 mM) but is essentially without effect on either 2-[³H]DG or [U-¹⁴C]glucose uptake.

Previous studies of GLUT3 have been confined to either *Xenopus* oocytes or CHO cells which overexpress the human GLUT3 isoform. It was therefore important to directly confirm these observations using our assay conditions and compare them with those obtained in cells which endogenously express rat GLUT3. The competitive effects of the same sugars on 3-O-[¹⁴C]MG and 2-[³H]DG transport were investigated in CHO cells overexpressing GLUT3 (Figure 3). The sensitivities to inhibition of the 3-O-[¹⁴C]MG assay by glucose, 2-DG and galactose were identical to those seen in the neurons. As in the neurons, the 2-[³H]DG assay was also hypersensitive to glucose, moderately sensitive to 3-O-MG (IC₅₀ ~ 5 mM) but insensitive to galactose. Similarly, the 3-O-[¹⁴C]MG assay was insensitive to fructose but the 2-[³H]DG assay was clearly inhibited.

DISCUSSION

The present study is the first analysis of the kinetic parameters and stereospecificity of endogenously expressed GLUT3 in an appropriate cellular environment, the rat cerebellar granule neuron. Using a novel methodology which is readily applicable to all cultured cells, the apparent affinity (K_m) for the transport of 3-O-MG by GLUT3 in primary cerebellar granule neurons, computed from the Michaelis–Menten equation as illustrated in Figure 1, is 2.8 mM. This is slightly lower than that measured in CHO cells overexpressing human GLUT3 (K_m = 3–4 mM [9]) and significantly lower than the K_m of 10 mM determined for human GLUT3 expressed in *Xenopus* oocytes [8]. It should, however, be stressed that in the latter study the apparent K_m was determined by equilibrium-exchange 3-O-MG transport. Our attempts to perform similar experiments in the primary neuronal cultures were not successful, as transport rates at room temperature were accelerated (depicted by + + + in Table 2) to such an extent that determinations were inaccurate. Unfortunately the neuron cannot withstand temperatures of less than 18 °C. In

Figure 3 Sugar competition of 3-O-[¹⁴C]MG (a) and 2-[³H]DG (b) transport in CHO-G3 cells

Confluent cultures of CHO-G3 cells were assayed for 3-O-[¹⁴C]MG transport or 2-[³H]DG uptake as described for primary neurons in the legend to Figure 2. Results are expressed as percentage of control (means $\pm$ S.E.M.; $n = 4$), and are representative of four separate experiments. Competing sugars: D-glucose (■), 3-O-MG (▲), galactose (△), fructose (□), L-glucose (●).

human erythrocytes, in which transport can be determined at 4 °C, a similar increase has been observed for GLUT1 when comparing zero-*trans* transport with equilibrium exchange. The K_m for GLUT3 is thus comparable with that observed for GLUT1 determined in erythrocytes by measuring zero-*trans* entry (1.6 mM), but dramatically lower than values obtained for GLUT1 measured by equilibrium exchange, which range from 10 to 26 mM [4,7–9]. The significance of these differences is discussed below. These values should be compared with K_m

values for 3-O-[¹⁴C]MG transport of 42 mM for GLUT2 and 4.3 mM for GLUT4 (for reviews, see [2,4,7]).

All previous studies of glucose transport into neural cells have employed the 2-DG uptake technique to assess both the combined rates and apparent affinities of GLUT1 and GLUT3. However, as discussed above, uptake of metabolizable glucose analogues cannot be used reliably to assess the kinetic parameters of substrate transport because, at higher substrate concentrations, the hexokinase responsible for the phosphorylation of the hexose (glucose or 2-DG) becomes saturated, as the K_m for the sugars is of the order of 50 μ M [6,10]. Thus the ability of the cell to accumulate the phosphorylated product may be limited by the kinetics of the hexokinase reaction rather than by the ability of the transporters to transport glucose into the cell. This is reflected in the previously reported K_m values for uptake of radiolabelled 2-DG into primary neuronal and glial cells and isolated synaptosomes, which are in the range 0.2–1.5 mM [18–21]. All appear to be lower than values for 3-O-MG transport, and thus probably reflect substrate phosphorylation rather than transport. A similar criticism might be levelled against the data obtained in CHO cells expressing human GLUT3 and GLUT1, where an apparent K_m value for 2-DG of 0.8–0.9 mM was observed for both transporters [9]. Interestingly, the affinity constant (1.4 mM) reported for 2-DG uptake in *Xenopus* oocytes [22] is comparable with that seen in the present study for 3-O-MG. This similarity probably reflects a true affinity for the sugar, as oocytes are essentially devoid of hexokinase and, given that the cells are relatively slow to equilibrate, the measured uptake represents zero-*trans* uptake of non-phosphorylated 2-DG. The shortcomings of the 2-[³H]DG assay and the tracer glucose assay are highlighted by the ability of the ligands to inhibit the complementary assay (Figures 2b and 2c). Thus 2-DG exhibited an IC_{50} of < 0.5 mM in the tracer glucose assay and a similar IC_{50} was observed for glucose in the 2-[³H]DG assay. In both cases, such IC_{50} values clearly reflect the inhibition of hexokinase. However, the uptake of both substrates was inhibited by 3-O-MG with an IC_{50} of approx. 3.5 mM. Fructose, which presumably enters the cell either by diffusion or via a distinct transporter (not GLUT5; F. Maher, T. M. Davies-Hill and I. A. Simpson, unpublished work), blocks the accumulation of both 2-DG and glucose metabolites but was without effect on 3-O-MG transport. Conversely, galactose blocked 3-O-MG transport but not the accumulation of phosphorylated products. This somewhat anomalous finding is currently unexplained but probably reflects the relatively weak inhibitory actions of galactose on transport and the near-saturating concentration of 2-DG for hexokinase. It should also be noted that the apparent K_i for glucose is < 0.5 mM in both

Table 2 Facilitative 3-O-MG transport: kinetic parameters

Data were obtained from *Carruthers [4], †Gould et al. [8] and ‡present study. CCA, catalytic-centre activity ('turnover number').

Transporter	Location
		K_m (mM)	CCA (s ⁻¹)	K_m (mM)	CCA (s ⁻¹)	K_m (mM)	CCA (s ⁻¹)
GLUT1	Human erythrocytes	20–30*	840*	1.6*	123*	5–8*	440*
GLUT3	Oocytes†, neurons‡	10.6†	+++‡	2.8‡	853‡	–	–

the 2-DG and the tracer glucose assays, again reflecting the inhibition of hexokinase activity rather than of transport.

In the present study we were able to compare the competition of 3-O-MG transport in primary cerebellar granule neurons and CHO-G3 cells by various sugars with that described for human GLUT3 expressed in *Xenopus* oocytes [8]. It is clear that most of the characteristics of sugar specificity seen in neurons are retained when the transporter is expressed in oocytes and CHO cells. A distinctive characteristic of human and rodent GLUT3, compared with other isoforms, appears to be the ability to transport xylose and galactose. The relevance of this for neuronal metabolism is unclear as these sugars are not major substrates for brain energy metabolism, but they may provide a mechanism for selective inhibition.

Inhibitors of the glucose transporters, which include CB, forskolin and phloretin, have much higher affinities than the K_m values of the transporters for the sugars. It has also become apparent that the sensitivities to these inhibitors varies among the different glucose transporter isoforms. GLUT1 is extremely sensitive to CB, exhibiting a K_i for inhibition of transport activity and a K_D for binding of the order of 0.1–0.2 μ M, whereas the corresponding values for forskolin are 2.0–2.5 μ M [23–25]. GLUT2 has a much lower affinity for CB, with K_i and K_D values of 1.7 μ M, and is insensitive to forskolin [26]. In the present study of neuronal GLUT3 the sensitivity to both CB and forskolin was less than that of GLUT1, with corresponding IC_{50} values of 0.35 and 5.5 μ M respectively. The latter corresponds fairly closely to the K_D of 4.4 μ M obtained by Hellwig and Joost [24] for rat brain. In contrast, GLUT4 is sensitive to both ligands, exhibiting K_i values for both of 0.1–0.2 μ M [27,28]. These differences in specificities reflect the subtle differences in structure resulting from relatively small changes in glucose transporter sequence (all show 65% sequence similarity), which accord the transporters their kinetic differences and their ultimate cellular expression.

The specific characteristics of different glucose transporter isoforms are important for understanding the mechanics of cerebral glucose utilization. In the present study we have determined the kinetic characteristics of GLUT3 with the ultimate goal of determining what advantages the neuron might accrue by preferentially expressing GLUT3, and what distinguishes this transporter from GLUT1. GLUT1 is highly expressed in the endothelial cells that make up the mammalian blood–brain barrier, and in erythrocytes of primates and dolphins [29]. Both erythrocytes and endothelial cells have relatively low metabolic activities and are therefore essentially in equilibrium with the circulating glucose. Thus the flux of glucose in or out of the cells would approximate to an equilibrium-exchange mechanism. This transport process is characterized by a high rate of transport (439 s^{-1}) [17] which is essentially unsaturable, with a K_m of $\sim$ 20–30 mM (Table 2). In the case of primate and dolphin erythrocytes, this capacity to rapidly mobilize intracellular glucose essentially expands the available glucose space within the capillaries to include that occupied by the erythrocyte. In the endothelial cells, an equilibrium-exchange mechanism clearly increases the capacity to transfer glucose across the blood–brain barrier. It should be noted that values for the rate of zero-*trans* glucose exit lie between those for equilibrium exchange and zero-*trans* entry [4], i.e. 5–8 mM, comparable with those determined experimentally for glucose uptake into rat brain [30]. The interstitial glucose concentration in mammalian brain has been calculated to be of the order of 1–2 mM [31,32]. This con-

centration is comparable with the observed K_m values determined for GLUT1 and GLUT3 for zero-*trans* kinetics in cerebellar granule neurons. At such relatively low glucose concentrations, combined with the high hexokinase capacity of neurons and glia, zero-*trans* transport is the mechanism that would be predicted to prevail. Under normal circumstances the cerebral glucose transport capacity exceeds metabolic demand. However, under conditions of metabolic stress such as hypoglycaemia, seizures or hypoxia when transport capacity may become limiting, and given the equal concentrations of 45 kDa GLUT1 (presumably in glia and neurons) and GLUT3 in rat brain [12], it is the enhanced kinetic capacity of the GLUT3 transporter that provides the neurons with the advantage of glucose utilization and hence potential survival.

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