The GLUT3 glucose transporter is the predominant isoform in primary cultured neurons: assessment by biosynthetic and photoaffinity labelling

Fran MAHER* and Ian A. SIMPSON

EDMNS, NIDDK, Building 10, Room 5N102, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Cerebellar granule neurons in primary culture express increasing levels of two glucose transporter isoforms, GLUT1 and GLUT3, as they differentiate *in vitro*. We have determined the relative abundance of GLUT1 and GLUT3 in these neurons by three different labelling methods. (1) Photoaffinity cell surface labelling of neurons with an impermeant bis-mannose photolabel revealed 6–10-fold more GLUT3 than GLUT1 and dissociation constants (K_d) for the photolabel of 55–68 μ M (GLUT3) and 146–169 μ M (GLUT1). Binding to both transporters was inhibited by cyto-

chalasin B. (2) Photoaffinity labelling of neuronal membranes with a permeant forskolin derivative showed 5.5–8-fold more GLUT3 than GLUT1, whereas in rat brain membranes containing both neuronal and glial membranes, GLUT3 and GLUT1 were detected in similar proportions. (3) Biosynthetic labelling of neurons with [35 S]methionine and [35 S]cysteine showed GLUT3 to be 6–10-fold more abundant than GLUT1. Thus GLUT3 is quantitatively the predominant glucose-transport isoform in cultured cerebellar granule neurons.

INTRODUCTION

Glucose is a major fuel for the brain. Its transport into mammalian cells is mediated by a family of facilitative glucose-transporter proteins, designated GLUT1–GLUT7 (Bell et al., 1993). Two isoforms, GLUT1 and GLUT3, are known to be expressed in brain. GLUT1 is present as two forms: 55 kDa GLUT1 is associated with cerebral microvessels (Gerhart et al., 1989; Pardridge et al., 1990; Maher et al., 1993) and 45 kDa GLUT1 is found in the neuronal/glial fraction of the brain (Maher et al., 1993).

There is now compelling evidence that GLUT3 is a major neuronal glucose-transporter isoform (Yano et al., 1991; Maher et al., 1991, 1992; Bondy et al., 1992; Nagamatsu et al., 1992). However, little is known about its activity or regulation. Studies of the expression of the human GLUT3 gene in *Xenopus* oocytes have reported a lower K_m for 3-*O*-methylglucose transport (10.6 mM) than that of GLUT1 (17 mM) and GLUT2 (42.3 mM) measured in the same studies (Gould et al., 1991). The implications of a low- K_m neuronal glucose transporter may be important for understanding brain glucose uptake and utilization *in vivo*. Apart from the putative difference in K_m , there is currently no strong evidence for functional or regulatory characteristics specific to GLUT3 for which this isoform would be selectively expressed in neurons.

We recently reported the expression of the GLUT3 isoform in primary cultured cerebellar granule neurons and neuronal cell lines (Maher et al., 1991, 1992). In order to use these cells as models to study the characteristics and regulation of GLUT3 it is necessary to determine whether it is the major isoform. To date there has been no direct demonstration of quantitative or functional predominance of GLUT3 in neurons.

A number of photoactive compounds have previously been employed to assess the abundance, cellular localization and regulation of glucose transporters. The membrane-impermeant tritiated bis-mannose photolabel 2-*N*-4-[3 H](1-azi-2,2,2-trifluoro-

ethyl)benzoyl-1,3-bis-(*D*-mannose-4-yloxy)-2-propylamine ([3 H]ATB-BMPA) (Holman et al., 1990) is a relatively specific ligand for glucose transporters and has comparable affinity constants (150–200 μ M) for GLUT1, GLUT2 and GLUT4 (Clark and Holman, 1990; Holman et al., 1990; Jordan and Holman, 1992; Palfreyman et al., 1992). Forskolin, a competitive inhibitor of glucose transport (Shanahan, 1982), and forskolin derivatives selective for glucose transporters have been used for ligand-binding and photolabelling studies in different tissues (Wadzinski et al., 1987; Zorzano et al., 1989; Morris et al., 1991). Recently a 7-position derivative of forskolin has been used to map the location of glucose-transporter sites in brain sections (Appel et al., 1992; Robbins et al., 1992). We have used a membrane-permeant iodinated photoactive derivative of forskolin, 7-azido[125 I]iodophenylpropionylaminoethylcarbamyl-7-desacetylforskolin ([125 I]AIPP-Fsk), to photolabel glucose transporters in membranes prepared from cultured neurons and rat brain. In addition primary cultured neurons were biosynthetically labelled with [35 S]methionine and [35 S]cysteine to determine the relative proportions of GLUT3 and GLUT1 by a method not dependent on ligand-binding characteristics. All three labelling methods demonstrate the predominance of GLUT3 relative to GLUT1 in cultured cerebellar granule neurons, in contrast with whole brain in which the proportions of GLUT3 and 45 kDa GLUT1 are similar.

EXPERIMENTAL

Materials

Sprague–Dawley rat pups were obtained from Taconic Farms. All culture media were purchased from Gibco, Grand Island, NY, U.S.A. Hormones and media supplements were cell-culture-grade reagents purchased from Sigma, St. Louis, MO, U.S.A. ATP-BMPA and [3 H]ATB-BMPA (specific radioactivity 10 Ci/mmol) were provided by G. Holman, University of Bath, Bath, Avon, U.K. [125 I]AIPP-Fsk (specific radioactivity 2200 Ci/mmol)

Abbreviations used: [3 H]ATB-BMPA, 2-*N*-4-[3 H](1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannose-4-yloxy)-2-propylamine; [125 I]AIPP-Fks, 7-azido[125 I]iodophenylpropionylaminoethylcarbamyl-7-desacetylforskolin; CB, cytochalasin B.

* To whom all correspondence should be addressed.

was provided by K. Seamon and D. Morris, NIH, Bethesda, MD., U.S.A. L-[³⁵S]Methionine (> 1000 Ci/mmol; stabilized with pyridine-3,4-dicarboxylic acid) and ¹⁴C-labelled molecular-mass markers were purchased from Amersham, Arlington Heights, IL, U.S.A. L-[³⁵S]Methionine and L-[³⁵S]cysteine (> 1000 Ci/mmol; in 50 mM Tricine) were purchased from New England Nuclear-Dupont, Wilmington, DE, U.S.A. Rabbit polyclonal antisera specific for the C-terminal peptide sequences of rat GLUT1 (Birnbaum et al., 1986) and mouse GLUT3 (Nagamatsu et al., 1992) were kindly produced by Hoffman La Roche, Nutley, NJ, U.S.A., using synthetic peptides conjugated to keyhole limpet haemocyanin. Antiserum specificity was characterized as described previously (Maher et al., 1992). Protein A-Sepharose CL4B was purchased from Pharmacia, Piscataway, NJ, U.S.A. All other reagents were analytical grade.

Neuron culture

Primary cerebellar granule neurons were isolated from postnatal day 8 rats and cultured on poly(L-lysine)-coated 100 mm NUNC or 60 mm Costar culture dishes in serum-free medium as previously described (Maher et al., 1991). Under these conditions, non-neuronal cells (primarily astroglia) comprised less than 5% of total cell number after 9 days in culture. Photolabelling experiments were all performed on neurons cultured for 7–9 days, when GLUT1 and GLUT3 expression, as determined by immunoblot analysis, is maximal (Maher et al., 1991).

[³H]Cytochalasin B (CB) binding

[³H]CB binding to neuron and total rat brain membranes was performed as previously described (Weber et al., 1988).

[³H]ATP-BMPA photolabelling

Neurons were washed twice with Dulbecco's PBS (D-PBS). For surface labelling, [³H]ATP-BMPA (0.5–1 mCi/ml per dish) was added and the open dish of cells exposed for 2 min to u.v. light in a Rayonet photochemical reactor with 300 nm lamps. After being washed with D-PBS, cells were scraped from the dish and total membranes were prepared essentially as previously described (Maher et al., 1991) but with disruption by sonication and without a low-speed centrifugation. Average membrane protein yield was 100–150 µg per 100 mm dish and 60–70 µg per 60 mm dish. The membrane pellet was resuspended in 2% Thesit (Boehringer) buffer for immunoprecipitation. Alternatively, cells were directly solubilized in 2% Thesit before immunoprecipitation.

Determination of the binding constants K_d and B_{max} of ATB-BMPA for GLUT3 and GLUT1 in neurons

Neurons in 100 mm and 60 mm dishes (one dish for each concentration) were photolabelled in the presence of 500 µCi/ml (50 µM) [³H]ATB-BMPA and unlabelled ATB-BMPA to final concentrations of 150, 250 and 500 µM. The ³H-labelled and non-radioactive ATB-BMPA were mixed together in 1 ml of D-PBS, then 0.75 ml and 0.25 ml added to the 100 mm and 60 mm dishes respectively, immediately before exposure to u.v. for 2 min. After immunoprecipitation and gel electrophoresis of GLUT1 and GLUT3 (see below) the size of each peak was

calculated after subtraction of the background determined for each sample. Values for the binding constant (K_d) and the total number of binding sites (B_{max}) per dish were calculated as previously described (Jordan and Holman, 1992).

The yield of total neuronal membranes typically represents 50–70% recovery of membranes, based on the different recoveries of labelled transporter from isolated membranes compared with solubilized whole cells. The data are expressed per dish and, for estimates of B_{max} , values of 200 µg of membrane protein/100 mm dish and 80 µg/60 mm dish were used.

[³⁵S]Methionine and [³⁵S]cysteine biosynthetic labelling

Neurons were grown in 100 mm dishes for 4–8 days. On the day of labelling, the medium was changed to low-methionine (1.5 µg/ml) minimal essential medium plus 100–150 µCi/ml L-[³⁵S]methionine or L-[³⁵S]cysteine. Methionine stabilized with pyridine-3,4-dicarboxylic acid was found to be toxic to neurons over culture periods greater than 12 h, so the isotope in Tricine was used for longer incubations. Biosynthetic labelling was performed with [³⁵S]methionine for 6–24 h during days 4–6 in culture under the above conditions, or from day 1 to 6 of culture in regular medium with [³⁵S]methionine and [³⁵S]cysteine (100 µCi/ml) added on days 1, 2 and 4. Membranes were prepared from labelled cells, solubilized and precleared (see below) before immunoprecipitation. GLUT1 and GLUT3 levels determined by [³⁵S]methionine and [³⁵S]cysteine biosynthetic labelling should be valid because: (1) the rates of synthesis of the two proteins are similar during the first 6 days in culture, as previously determined by immunoblot analysis (Maher et al., 1991); (2) the long labelling periods should avoid minor temporal differences in synthetic rates; (3) according to published sequences, GLUT1 and GLUT3 contain a comparable number of methionine and cysteine residues [GLUT1, 16 methionine and six cysteine residues; mouse GLUT3, 14 methionine and nine cysteine residues (Birnbaum et al., 1986; Nagamatsu et al., 1992)].

[¹²⁵I]AIPP-Fsk photolabelling

The [¹²⁵I]AIPP-Fsk labelling procedure was based on that previously described (McHugh-Sutkowski et al., 1993). Membranes from cultured neurons or from whole rat brain (100–200 µg) in 200 µl of Hepes/EDTA buffer were exposed to u.v. light (Rayonet) for 1 min in the presence of 0.2 µCi of [¹²⁵I]AIPP-Fsk. 2-Mercaptoethanol (12 µl) was added to quench the reaction. The membranes were washed twice by resuspending them in buffer and centrifuging at 150 000 g for 10 min. They were then solubilized in 2% Thesit for immunoprecipitation.

Immunoprecipitation and SDS/PAGE

Membranes were solubilized in PBS/1 mM EDTA/2% (v/v) Thesit (containing protease inhibitors: 0.1 mM phenylmethanesulphonyl fluoride, 10 µg/ml aprotinin, leupeptin and pepstatin) at 4 °C for 60 min. Solubilized membranes from [³⁵S]methionine-labelled cells were precleared by the addition of non-immune rabbit serum (1:20 dilution) for 2 h at 4 °C, followed by two incubations with 100 µl of Protein A-Sepharose (50% slurry) for 1 h at 4 °C. Antisera to GLUT1 and mouse GLUT3 were added at a final dilution of 1:40 and mixed for 12–18 h at 4 °C. Protein A-Sepharose was added (100 µl/ml of lysate) for 1 h at 4 °C and pelleted in a Microfuge at 10 000 g; this step was repeated on the resulting supernatant. Protein A-Sepharose complexes were washed three times with 1 ml of PBS/0.2% Thesit, then 1 ml of PBS, and the bound antigen was eluted with an equal volume of

2% (w/v) SDS/6 M urea/100 mM dithiothreitol. Solubilized immunoprecipitates were recovered by centrifugation through Millipore Durapore 0.45 μ m filter inserts in Microfuge tubes.

Immunoprecipitates were subjected to SDS/PAGE (10% gels) along with prestained or 14 C-labelled molecular-mass markers. Gels containing 3 H-labelled samples were sliced and processed for liquid-scintillation counting as described previously (Vanucci et al., 1992). For 35 S- and 125 I-labelled samples, gels were either dried or transferred to nitrocellulose then exposed to Kodak XAR autoradiographic film or a phosphorimager screen (Molecular Dynamics) for quantification of radioactivity. The immunoprecipitation efficiency, based on immunoblot assay to determine the proportions of labelled transporter immunoprecipitated and remaining in the supernatant, was 85–95% for both isoforms.

RESULTS

3 H]CB binding

The total number of glucose transporters in neuronal and rat brain membranes was measured as the number of glucose-displaceable 3 H]CB-binding sites. Membranes from 7–9-day cultured cerebellar granule neurons contained 16.25 ± 2.3 pmol/mg, $K_d = 77.5 \pm 7$ (mean $\pm$ S.E.M.; $n = 4$). In two separate total crude rat brain membrane preparations, 3 H]CB binding was 10 and 18 pmol/mg.

3 H]ATB-BMPA photolabelling

The specificity and ability of 3 H]ATB-BMPA to label GLUT3 in neurons was determined by labelling cells in the presence and absence of the glucose-transport inhibitor CB (0.1 mM). Labelling of the major peak of radioactivity at 43–45 kDa in both GLUT1 and GLUT3 immunoprecipitates was blocked by CB (results not shown).

Intact neurons were labelled with 3 H]ATB-BMPA to determine the proportions of GLUT1 and GLUT3 accessible to this impermeant ligand at the cell surface. In experiments in which membranes were prepared after photolabelling, GLUT3 was the predominant isoform detected, with 6.6 ± 1.1 -fold higher levels than GLUT1 (mean $\pm$ S.E.M., $n = 6$; after correction for immunoprecipitation efficiency). A representative experiment is shown in Figure 1.

The affinity of ATB-BMPA for GLUT1 and GLUT3 was determined by photolabelling intact neurons with ATB-BMPA concentrations from 50 to 700 μ M. From plots of free/bound versus free photolabel (Jordan and Holman, 1992) the dissociation constants (K_d) for GLUT1 and GLUT3 were determined to be 146–170 μ M and 55–69 μ M respectively (Figure 2). Thus the affinity of bis-mannose is about 2-fold higher for GLUT3 than for GLUT1. Plotting the data as pmol of ATB-BMPA bound (B) against ATB-BMPA concentration ($[S]$ 50–700 μ M), maximal binding was seen at approximately 500 μ M (not shown). The calculated B_{max} values normalized for membrane protein (Figure 2) were 17–19 pmol/mg for GLUT3 and 2–3 pmol/mg for GLUT1, representing a ratio of about 8:1 (GLUT3/GLUT1). Thus when assessed at B_{max} , where the difference in affinity for the two isoforms is no longer relevant, GLUT3 is the more abundant isoform measured with this photolabel in neurons.

125 I]AIPP-Fsk photolabelling

125 I]AIPP-Fsk is freely permeable across membranes and was

Figure 1 3 H]ATB-BMPA photolabelling of neurons

Neurons cultured for 8 days were surface labelled with 500 μ Ci/ml 3 H]ATB-BMPA followed by sequential immunoprecipitation of GLUT1 ($\circ$) and GLUT3 ($\blacksquare$). This is a representative of six experiments; the ratio of GLUT3/GLUT1 in this experiment is 5.5:1. Arrows at the top indicate positions of molecular-mass markers (kDa). 3 H is plotted as c.p.m./gel slice.

Figure 2 Determination of binding constants K_d and B_{max} for ATB-BMPA and GLUT1 and GLUT3 in neurons

Neurons in 100 mm and 60 mm dishes were photolabelled with 500 μ Ci/ml (50 μ M) 3 H]ATB-BMPA plus unlabelled ATB-BMPA to final concentrations of 150, 250 and 500 μ M. The amount of ATB-BMPA (pmol) bound to GLUT1 and GLUT3 per dish (B) was calculated from the known specific radioactivity of 3 H]ATB-BMPA. The two curves on each graph are the results from 100 mm ($\blacktriangle$) and 60 mm ($\bullet$) dishes. Binding constants, K_d and B_{max} , were determined from F/B versus F plots, where B and F are bound and free ATB-BMPA respectively, fitted by least-squares non-linear regression weighted for relative error using the Fig.P program with the equation: $B = (B_{max}/K_d)F/(1 + F/K_d)$. The values calculated for the 100 mm and 60 mm dishes respectively were: for GLUT3, K_d 55.5 ± 1.1 and 68.7 ± 35.4 μ M; B_{max} 17.4 and 18.8 pmol/mg; for GLUT1, K_d 170.0 ± 37 and 146.0 ± 40 μ M; B_{max} 2.0 and 2.6 pmol/mg.

Figure 3 [^{125}I]AIPP-Fsk photolabelling of neurons and rat brain membranes

(a) Membranes from 8–9-day cultured neurons and whole rat brain (100 μg) were photolabelled in the presence (+) and absence (–) of 0.1 mM CB and the total sample subjected to electrophoresis. Lane M, molecular-mass markers. (b) Neuron and rat brain membranes (200 μg) were photolabelled followed by sequential immunoprecipitation of GLUT1 (G1) and GLUT3 (G3). Each immunoprecipitate was subjected to gel electrophoresis and phosphorimager analysis. The arbitrary phosphorimager units for each GLUT immunoprecipitated were: neuron GLUT1, 810; neuron GLUT3, 4121; brain GLUT1, 4720; brain GLUT3, 4058. (c) The same membrane samples (20 μg) analysed by immunoblotting.

used to label total glucose transporters in membrane preparations. When total crude membranes prepared from cultured neurons and whole rat brain were photolabelled and analysed by SDS/PAGE (without immunoprecipitation), proteins of approximately 100, 60, 45 and 20 kDa were detected (Figure 3a), similarly to that seen in bovine brain with the 100 kDa band corresponding to adenylate cyclase (Morris et al., 1991). Only the 45 kDa band was identified as a glucose transporter by the inhibition of labelling by 0.1 mM CB.

Neuron and rat brain membranes were then labelled with [^{125}I]AIPP-Fsk followed by immunoprecipitation of GLUT1 and GLUT3. [^{125}I]AIPP-Fsk-labelled GLUT3 was 5- to 7-fold more abundant than GLUT1 in neuronal membranes ($n = 3$) (Figure 3b). In contrast, in whole brain membranes GLUT3 and 45 kDa GLUT1 were present in similar amounts ($n = 2$) (Figure 3b). The

Figure 4 [^{35}S]Methionine biosynthetic labelling of cultured neurons

Neurons were labelled from days 1 to 6 in culture with 100 $\mu\text{Ci}/\text{ml}$ each of [^{35}S]methionine and [^{35}S]cysteine added on days 1, 2 and 4. GLUT1 and GLUT3 were immunoprecipitated, subjected to SDS/PAGE and transferred to nitrocellulose. (a) Phosphorimager scan of ^{35}S -labelled samples in nitrocellulose. Phosphorimager units of the 45 kDa bands were GLUT1 = 1177 and GLUT3 = 12388. Molecular-mass markers (kDa) are indicated at the side. (b) The same nitrocellulose filter analysed by immunoblot after ^{35}S decay. The bands above the 45 kDa transporters are denatured immunoglobulin detected by ^{125}I -Protein A. Arrows indicate glucose transporters.

result from rat brain membranes confirms that the poor detection of GLUT1 in neuron membranes was not due to methodological problems such as poor labelling or immunoprecipitation of GLUT1.

The microvessel 55 kDa GLUT1 was not clearly labelled, probably in part because of the smaller amount relative to 45 kDa GLUT1 in total brain membranes, as seen on immunoblots of the same samples (Figure 3c) and interference by the 60 kDa band which is present in both the immunoprecipitates.

[^{35}S]Methionine and [^{35}S]cysteine biosynthetic labelling

Biosynthetic labelling was employed to determine the total cellular proportion of GLUT1 and GLUT3 by a method independent of ligand binding. The rates of synthesis of GLUT1 and GLUT3 in primary cultured cerebellar granule neurons are comparable during the first 6 days of culture, with most synthesis occurring between days 4 and 6 (Maher et al., 1991). Biosynthetic labelling was therefore performed for periods of 6–12 h on days 4, 5 and 6 in culture. Labelling with [^{35}S]methionine on each of these days resulted in the detection of GLUT3 but not GLUT1, with more detected on day 6 than days 5 and 4 (results not shown). As insufficient [^{35}S]methionine was incorporated into GLUT1 to allow relative quantification, cells were cultured in the presence of both [^{35}S]methionine and [^{35}S]cysteine (100 $\mu\text{Ci}/\text{ml}$) for days 1–6 in culture. Under these conditions GLUT1 was barely detectable, and quantification by phosphorimager analysis indicated a GLUT3/GLUT1 ratio of 6:1 in [^{35}S]methionine-labelled cells and 10:1 in [^{35}S]methionine-plus-[^{35}S]cysteine-labelled cells (Figure 4a). The ^{35}S -labelled 43–45 kDa protein was identified as GLUT3 by competition of immunoprecipitation with excess GLUT3 C-terminal peptide; no bands in the GLUT1 immunoprecipitate competed with the peptide (results not shown). To confirm the presence of GLUT1 in the ^{35}S -labelled immunoprecipitates, the same samples were assayed by immunoblot analysis (after decay of the ^{35}S radioactivity); GLUT1 and GLUT3 were present at levels similar to those in unlabelled samples immunoblotted at the same time (Figure 4b). Neither

GLUT1 nor GLUT3 was detected in cells pulsed with [35 S]methionine and [35 S]cysteine for 6 h on day 9 in culture.

DISCUSSION

The cerebellar granule neuron culture system involves the development of immature granule cells into cells resembling mature granule neurons, by extension of dendrites and axons and expression of neuron-specific marker proteins, glutamate receptors and synaptic activity (Gallo et al., 1986). We have previously described the increasing expression of both GLUT1 and GLUT3 in cerebellar granule neurons as they undergo differentiation (Maher et al., 1991), which mimics the expression of GLUT3 coincident with neuronal maturation in the developing brain (Vannucci, 1994). However, without analysing the relative proportions of the two isoforms, it is not possible to make any predictions about the contribution of each isoform to glucose transport in neurons *in vitro* or *in vivo*. This point is underscored by the observations that many cells in primary culture lose tissue-specific glucose-transporter isoforms (see below). The present study was therefore designed to determine the major transporter isoform present in neurons. We have also found increasing GLUT3 expression coincident with development of cortical neurons in primary culture (F. Maher, unpublished work), but prefer to use cerebellar granule neurons for quantification of GLUT3 and GLUT1 because they constitute a relatively pure neuronal population. Cortical neurons and other neuronal cultures often require the establishment of an astrocyte monolayer to support neuron survival, and astrocytes in culture express GLUT1 (Walker et al., 1988; Hara et al., 1989; Werner et al., 1989).

The impermeant exofacial photoaffinity label ATB-BMPA has been shown to have comparable binding affinities for GLUT1, GLUT2 and GLUT4 (Clark and Holman, 1990; Holman et al., 1990; Jordan and Holman, 1992; Palfreyman et al., 1992). In this study we show that, whereas the K_d for GLUT1 in neurons (146–170 μ M) is comparable with the most recent assessment in 3T3-L1 cells of 150 μ M (Palfreyman et al., 1992), the K_d for GLUT3 in neurons (55–69 μ M) is lower than for GLUT1. Thus the affinity of bis-mannose is about 2-fold higher for GLUT3 than for GLUT1 and other transporters. Despite this difference in affinity, at maximal binding [3 H]ATB-BMPA labelling revealed an 8-fold predominance of GLUT3 at the neuronal cell surface. Studies of adipocytes indicate that, in intact cells, only the functionally active transporters exposed at the cell surface are accessible to the ATB-BMPA photolabel (Vannucci et al., 1992). Thus the predominance of GLUT3 over GLUT1 accessible to bis-mannose at the neuronal cell surface suggests that GLUT3 is the major contributor to glucose transport into neurons.

Immunofluorescence analysis of GLUT1 and GLUT3 in these cells (Maher et al., 1991) shows a pattern of GLUT3 staining consistent with cell-surface localization, whereas GLUT1 appears to be both in the cytoplasm and at the plasma membrane. A cell-surface localization of GLUT3 is supported by the present results in which the cell-surface ratio of GLUT3/GLUT1 determined with [3 H]ATB-BMPA (about 8:1) is greater than the ratios of total cellular GLUT3/GLUT1 determined by [125 I]AIPP-Fsk photolabelling (about 5:1). Thus it is possible that a specific characteristic of GLUT3 selectively targets the plasma membrane or specific neuronal domains, consistent with the apparent targeting to the cell surface of human GLUT3 expressed in *Xenopus* oocytes (Thomas et al., 1993). However, we cannot make any definitive statement about the proportion of either transporter in an intracellular compartment in neurons

because we have been unable to perform comparable experiments to compare directly surface-labelled and permeabilized neurons, as described in the 3T3-L1 cell by Yang et al. (1992), because of the effects of permeabilizing agents on primary neurons.

[125 I]AIPP-Fsk has previously been shown to photolabel GLUT1 (Morris et al., 1991; McHugh-Sutkowski et al., 1993). Using this photolabel the proportions of GLUT3 and GLUT1 in neuronal membranes (5:1) was markedly different from that in total rat brain membranes (1:1). Total brain membranes contain neurons, glia and brain microvessels. GLUT3 is essentially absent from cultured glia (Maher et al., 1991, 1992) and purified rat cerebral microvessels (Maher et al., 1993). [125 I]AIPP-Fsk labelling clearly shows that the neuron culture enriches for GLUT3, supporting selective localization of GLUT3 in neurons. If cultured neurons are representative of neurons *in vivo*, this result suggests that the majority of the 45 kDa GLUT1 is present in glia and/or other non-neuronal cells (including astrocytes, oligodendrocytes, microglia, choroid plexus, pericytes, ependymal cells). Although both neurons and glia in culture express GLUT1 (Walker et al., 1988; Werner et al., 1989; Maher et al., 1991), there is little clear evidence for neuronal GLUT1 expression *in situ*. GLUT1 has not been detected in neurons under normal physiological conditions, by either *in situ* hybridization or immunohistochemistry. Similarly, the evidence for astroglial expression of GLUT1 *in situ* is scant, as only one immunohistochemical study has detected it in astroglia (Devaskar et al., 1992) while others have detected it (the 55 kDa form) in microvessels only (Gerhart et al., 1989; Pardridge et al., 1990). Our present data, in combination with the evidence cited, suggest that 45 kDa GLUT1 is primarily expressed in glia.

The constants for [125 I]AIPP-Fsk binding to different transporter isoforms have not been determined. A related derivative has been used in ligand-binding studies in rat brain, showing a binding pattern resembling, but not identical with, that of [3 H]CB (Appel et al., 1992; Robbins et al., 1992). Robbins et al. (1992) suggest that this derivative may be selective for GLUT3. If this were the case for [125 I]AIPP-Fsk, then the amount of 45 kDa GLUT1 in rat brain would be underestimated. Nevertheless, the correlation between the [125 I]AIPP-Fsk, [3 H]ATB-BMPA and biosynthetic labelling of neurons implies that any affinity differences are not major.

The proportions of GLUT3 and GLUT1 determined by biosynthetic labelling of cultured cerebellar granule neurons may tend to overestimate the amount of GLUT3, as GLUT1 was so difficult to detect. Nevertheless, the results are comparable with the photolabelling studies and confirm that GLUT3 is the more abundant isoform. The few studies employing biosynthetic labelling of GLUT proteins have determined the half-lives of GLUT1 in rat fibroblasts (13–17 h) (White and Weber, 1988) and GLUT1 and GLUT4 in 3T3-L1 adipocytes (19 h and 50 h respectively) (Sargeant and Paquet, 1993). This suggests that GLUT1, with a shorter half-life, may be more responsive to external stimuli whereas the 'differentiation-specific' transporter (GLUT4 in adipocytes) is more stable. In the present study, GLUT3 could be biosynthetically labelled only during the 'developmental' stage of culture. The inability to detect GLUT3 in neurons pulsed with [35 S]methionine for 6 h on day 9 in culture, a time when GLUT3 levels remain stable and maximal (Maher et al., 1991), implies that GLUT3 protein has a low rate of turnover.

The increase in GLUT3 expression in primary cerebellar granule neurons and primary cortical neurons (F. Maher, unpublished work) is unlike the situation in other cell types in primary culture in which expression of the tissue-specific transporter decreases with or is not induced by differentiation, and

GLUT1 expression increases. For example, cerebral microvascular endothelial cells in culture display a loss of GLUT1, their major transporter (Laterra and Goldstein, 1993); pancreatic β -cells in primary culture lose GLUT2 (exclusive to β -cells and hepatocytes) while GLUT1 is up-regulated (Tal et al., 1992); rat adipocytes in primary culture lose GLUT4 expression while GLUT1 increases (Hajdúch et al., 1992). On the other hand, muscle satellite cells and muscle cell lines express little or no GLUT4 despite other indicators of morphological and biochemical differentiation (Sargeant et al., 1993). The findings that GLUT3 expression increases as neurons differentiate *in vitro* and that GLUT3 is the predominant isoform presents a relatively unique system of *in vitro* glucose-transporter expression.

The specific characteristics of GLUT3 that have evolved for its selective expression in neurons remain unclear. The predominant expression of the GLUT3 glucose-transporter isoform in primary cultured neurons makes this a valid model in which to assess the kinetics, function and regulation of endogenously expressed neuronal GLUT3.

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