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Glutamate permeability at the blood-brain barrier in insulinopenic and insulin-resistant rats

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Abstract

The influence of diabetes on brain glutamate (GLU) uptake was studied in insulinopenic (streptozotocin [STZ]) and insulin-resistant (diet-induced obesity [DIO]) rat models of diabetes. In the STZ study, adult male Sprague-Dawley rats were treated with STZ (65 mg/kg intravenously) or vehicle and studied 3 weeks later. The STZ rats had elevated plasma levels of glucose, ketone bodies, and branched-chain amino acids; brain uptake of GLU was very low in both STZ and control rats, examined under conditions of normal and greatly elevated (by intravenous infusion) plasma GLU concentrations. In the DIO study, rats ingested a palatable, high-energy diet for 2 weeks and were then divided into weight tertiles: rats in the heaviest tertile were designated DIO; rats in the lightest tertile, diet-resistant (DR); and rats in the intermediate tertile, controls. The DIO and DR rats continued to consume the high-energy diet for 4 more weeks, whereas the control rats were switched to standard rat chow. All rats were studied at 6 weeks (subgroups were examined under conditions of normal or elevated plasma GLU concentrations). The DIO rats ate more food and were heavier than the DR or control rats and had higher plasma leptin levels and insulin-glucose ratios. In all diet groups, the blood-brain barrier showed very low GLU penetration and was unaffected by plasma GLU concentration. Brain GLU uptake also did not differ among the diet groups. Together, the results indicate that the blood-brain barrier remains intact to the penetration of GLU in 2 models of diabetes under the conditions examined.

1. Introduction

The movement of nutrients into brain from the circulation involves specific transport carriers, located on the membranes of capillary endothelial cells, which form the "blood-brain barrier" (BBB) [1]. For amino acids (AA), a number of such carriers exist that are differentiated by the size and charge of the AA transported [2]. For example, separate carriers are found for large, neutral AA (eg, aromatic and branched-chain AA), basic AA (lysine, arginine), and acidic AA (glutamic [GLU] and aspartic acids). The large neutral and basic AA carriers generally facilitate the uptake of these AA into brain, whereas the acidic AA carriers restrict entry [2]. Acidic AA are excluded from brain because they function directly at neuronal synapses as excitatory neurotransmitters. Indeed, roughly two thirds of all synapses in cerebral cortex use GLU as a neurotransmitter [3], making it the dominant brain transmitter. Glutamate compartmentation in brain is thus an important physiologic issue because the presence of high amounts of GLU in the extracellular fluid (ECF) surrounding neurons can cause them to be overexcited, leading ultimately to their death [3-6]. The brain compartmentalizes GLU by synthesizing all that it needs and keeping it carefully sequestered within neurons and glial cells. It maintains ECF GLU concentrations at very low levels, except during the brief periods when the AA are released from nerve endings to excite GLU receptors on adjacent neurons. And even when GLU concentrations rise in the local ECF milieu of the synapse after its release, powerful transporters on neuronal and glial cells rapidly remove it from the synaptic ECF [3]. The GLU absorbed by glial cells is converted to glutamine and recycled to neurons,

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where it is deaminated to GLU and reused [3]. Such GLU transporters (EAAT-1, EAAT-2, EAAT-3) are also located on the abluminal membranes of capillary endothelial cells (the BBB) and function to remove GLU from ECF, from which it enters the general circulation [7].

Over the past 2 decades, interest has developed in evaluating the possibility that BBB defects are associated with chronic diseases that affect cognition. One example is diabetes; in this case, interest in BBB integrity derives from prior observations that kidney and retinal capillary functions degrade as the disease progresses [8-10]. Indeed, some data suggest that the barrier function of the BBB falters in diabetes [11-13]. However, it is not clear that such changes, if prevalent, compromise the effectiveness of the nutrient transporters of brain capillary endothelial cells. The transporters for the large neutral AA and basic AA may [14] or may not [15,16] be modified, and no data appear to be available for the acidic AA. Because of the importance of the compartmentalization of GLU within brain, which includes normal functioning of GLU transport activities at the BBB, we have examined net GLU uptake into brain in insulinopenic (streptozotocin [STZ] induced) and insulin-resistant (diet-induced obesity [DIO]) animal models of diabetes.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (300-325 g initial weight; Charles River Laboratories, Wilmington, MA) were housed individually at 23°C to 24°C (humidity, 40%-50%), with a daily lighting cycle of 14-hour light/10-hour dark (6:00 AM lights on, 8:00 PM lights off). They had free access to food (Rodent Diet #8640; Harlan Teklad, Madison, WI) and water.

All rats were acquired, cared for, and handled in conformance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (Office of Laboratory Animal Welfare, National Institutes of Health), the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Live Sciences, National Research Council), and the *Guiding Principles for Research Involving Animals and Humans* (recommendations from the Declaration of Helsinki) approved by the Council of the American Physiological Society. The Institutional Animal Care and Use Committee of Rosalind Franklin University approved all experimental protocols.

2.2. STZ-induced diabetes

Rats were anesthetized with halothane and injected with STZ (65 mg/kg) (Sigma-Aldrich, St Louis, MO) or vehicle (154 mmol/L NaCl) through a tail vein (n = 12 per group). This dose of STZ reliably produces diabetes, with rats becoming hyperglycemic and ketotic within 24 hours [17]. After injection, STZ-treated rats had access for 24 hours to water bottles containing 10% glucose; all rats continued to have free access to food and water throughout the study. All rats were weighed 3 times per week, and STZ-treated rats were monitored for the presence of glucose and ketone bodies in the urine using Uriscan 2 Gluketo strips (YD Diagnostics, Seoul, Korea). The BBB GLU transport measurements were conducted 3 weeks after induction of diabetes.

2.3. Diet-induced obesity

A group of 36 rats was given free access to water and a high-energy diet (D12266B: 16.8% protein, 51.4% carbohydrate, 31.8% fat [percentage energy]; 4.41 kcal/g; Research Diets, New Brunswick, NJ). Food intakes and body weights were measured daily. After 2 weeks, the rats were divided into 3 groups (n = 12 per group): those that gained the most weight were designated the DIO group; and those that gained the least weight, the diet-resistant (DR) group. These 2 groups continued to consume the same high-energy diet for an additional 4 weeks. The intermediate-weight group was designated the control group and was switched to standard rodent diet (Rodent Diet #8640: 23.6% protein, 64.1% carbohydrate, 12.3% fat [percentage energy]; 3.82 kcal/g; Harlan Teklad) for the subsequent 4-week period [18]. The BBB GLU transport measurements were conducted at the end of this period, 6 weeks after initiating the study.

2.4. Study subgroups

All rats were studied in the morning. They were not fasted. Each group (STZ and control; DIO, DR, and control) was divided into 2 subgroups. After anesthetization, one subgroup received an infusion of saline, whereas the other received an infusion of L-GLU (100 mmol/L L-GLU infused at 1 mL kg^{-1} min^{-1} for 3 minutes and then at 0.26 mL kg^{-1} min^{-1} until the end of the experiment). The intent in infusing GLU was to test the integrity of the BBB to GLU under conditions of very high circulating GLU concentrations.

2.5. Outline of the basic experimental procedure

The animal model was a rat fitted with an arterial and a venous catheter such that L-[^{14}C]GLU could be introduced into the venous system and samples of arterial blood could be taken continuously throughout the experimental period. At the end of the experiment, the rats were killed and their brains were removed. The measurements necessary for the calculation of GLU clearance are the disintegrations per minute per gram tissue (obtained by autoradiography of brain sections) and the average plasma L-[^{14}C]GLU content over the 1-minute experimental period.

2.6. Surgery

Rats were anesthetized with a mixture of ketamine and xylazine (90 mg ketamine, 10 mg xylazine per kilogram

body weight) (Lloyd Laboratories, Shenandoah, IA). Catheters were then introduced into the left femoral artery and left femoral vein, and lidocaine (Sigma-Aldrich) was applied to all incisions. Infusions (saline or 100 mmol/L L-GLU) were then initiated. Three minutes later, 50 μCi of L-[^{14}C]GLU (GE Healthcare/Amersham, Piscataway, NJ) was injected through the venous catheter over 45 seconds. Blood was withdrawn continuously from the arterial catheter into heparinized syringes and stored in heparinized tubes. This sample was counted to determine the average disintegrations per minute per microliter in plasma for calculating the rate of clearance. Sixty seconds after initiating the injection of L-[^{14}C]GLU, the rats were killed by an intravenous (IV) injection of sodium pentobarbital (150 mg in 1 mL of 0.15 mol/L NaCl, Sigma-Aldrich) and decapitated. This dose of pentobarbital stopped the heart within 3 seconds; the rats did not recover consciousness. Plasma was frozen for later analyses of [^{14}C]GLU, GLU, glucose, leptin, insulin, β-hydroxybutyrate, acetoacetate, and branched-chain AA.

2.7. Autoradiography

Rats were decapitated within 6 to 8 seconds after death. The brains were removed from the skull in less than 3 minutes and frozen between layers of bromobutane and methylbutane chilled to −30°C [19]. Care was taken not to dislodge the pineal or pituitary glands. The brains were sectioned at 50 μm in a precision cryostat (Microm International, Richard Allen Scientific, Kalamazoo, MI) maintained between 8°C and 12°C. Every fourth section was fixed to a slide and packed with Biomax light autoradiography film (Kodak, Rochester, NY) with a set of [^{14}C] standards (GE Healthcare/Amersham) that were calibrated to relate optical density to disintegrations per minute per gram. After 10 days, the films were developed; and the optical density in specific areas was measured using a densitometer (Tobias, Ivyland, PA) with an aperture of 300 μm diameter. The optical densities were converted to disintegrations per minute per gram by comparison with the [^{14}C] standards.

2.8. Plasma measurements

For the assays of β-hydroxybutyrate and acetoacetate, an aliquot of plasma was deproteinized in 0.5 mol/L $HClO_4$, followed by neutralization with 20% KOH (wt/vol). Glutamate, glucose, β-hydroxybutyrate, and acetoacetate were measured in deproteinized extract by specific enzymatic assays [20]. Enzymes and cofactors were purchased from Boehringer Mannheim (Indianapolis, IN).

2.9. Plasma radioactivity

The [^{14}C] activity in plasma samples was measured by liquid scintillation spectroscopy. An aliquot of plasma, 10 μL, was placed in 500 μL of H_2O in a scintillation vial; counting fluid was added (Ready Safe; Beckman Coulter, Fullerton, CA); and the samples were counted in an LS6500 liquid scintillation counter (Beckman Coulter). It has previously been shown [21] that approximately 97% of the radioactivity in plasma was in the form of L-[^{14}C]GLU under the experimental conditions used.

2.9.1. Hormones

Leptin and insulin were measured in untreated plasma by specific radioimmunoassay (rat insulin kit RI-13K and rat leptin kit RL-83K; Millipore, Billerica, MA).

2.9.2. Amino acids

Plasma concentrations of the branched-chain amino acid were determined using high-performance liquid chromatography separation and electrochemical detection, after reaction of samples with *o*-phthaldialdehyde (Sigma-Aldrich) [22].

2.9.3. Calculations

Glutamate transport into brain was calculated according to the following equation:

$$\begin{aligned}&\text{Clearance(microliters per minute per gram)}\\&\quad=\text{brain disintegrations per minute per gram}\\&\quad\div(\text{average plasma disintegrations per minute}\\&\quad\text{per microliter per minute}).\end{aligned}$$

These calculations assume that there was negligible loss of [^{14}C] from brain during the 1-minute experimental period and that virtually all the plasma radioactivity was in L-[^{14}C]GLU [21]. The background contamination due to radioactivity in blood was taken to be homogeneous and equivalent to the amount of radioactivity in 5 μL of plasma [21].

2.9.4. Statistical analysis

Differences between groups were detected by *t* test and analysis of variance (Tukey test) (SigmaPlot 11; Systat Software, San Jose, CA). Values were considered significant at $P < .05$.

3. Results

3.1. STZ-induced diabetes

Of the 12 rats injected with STZ (65 mg/kg IV), 3 lost considerable weight over the subsequent 3-week period and were not used in the study. The remaining 9 maintained stable weight (Table 1); 5 were subsequently tested at normal plasma GLU concentrations (saline infusion), and 4 were tested at elevated plasma GLU concentrations (infused with GLU). Of the 12 control rats, 3 were lost during the surgical protocol; 5 were tested at normal plasma GLU concentrations, and 4 were tested at increased plasma GLU concentrations. All STZ rats became glucosuric and slightly ketonuric (tested with Uriscan 2 Gluketo strips) within 24 hours of STZ injection and remained so throughout the next 3 weeks. At the end of the 3-week period, plasma concentrations of the ketone bodies and the branched-chain AA in STZ-treated rats were substantially elevated over values in control rats (Table 1).

Table 1
Body weights and plasma chemistry in control and STZ-diabetic rats

Variable	Control	STZ-diabetic
Group size	9	9
Initial body weight (g)	300 ± 5	307 ± 2
Final body weight (g)	447 ± 11	305 ± 9*
Plasma glucose (mmol/L)	10.1 ± 0.4	16.9 ± 0.9*
Plasma β-hydroxybutyrate (mmol/L)	ND	2.45 ± 0.36*
Plasma acetoacetate (mmol/L)	ND	0.91 ± 0.14*
Plasma ΣBCAA (nmol/mL)	455 ± 29	1434 ± 92*

Data are means ± SEM. Plasma samples were collected in the *nonfasted* state in the morning at the end of the study. ΣBCAA indicates leucine + isoleucine + valine; ND, below the limit of assay detection.

* $P < .01$, *t* test.

In rats infused with unlabeled GLU before ^{14}C-GLU infusion, plasma GLU concentrations were 30 to 75 times higher than those in rats infused with saline (Table 2). Generally speaking, BBB GLU clearance was very low in all animals (from 2 to 8 μL min^{-1} g^{-1}), except in those areas that have fenestrated capillaries (circumventricular organs [CVOs], Fig. 1). No statistically significant differences in GLU uptake were observed in whole brain or in most of the brain regions examined (striatum, hippocampus, midbrain) when comparing normal with STZ-treated rats or, within each treatment group, animals with normal or extremely high plasma GLU concentrations. A statistically significant main effect (STZ, vehicle) was noted in cerebral cortex and in cerebellum (Table 2).

3.2. Diet-induced obesity

In this study, all rats (N = 36) were initially placed on the high-energy diet. At 2 weeks, they were weighed and separated into 3 groups based on body weight (lowest, highest, intermediate; n = 12 per group) (Table 3). The lowest third was designated DR; and the highest third, DIO. These groups continued to consume the high-energy diet for the remainder of the experiment (4 additional weeks). The group of intermediate weight was designated the control group and switched back to standard rat chow (chow) for the succeeding 4 weeks. All rats continued to gain weight, although at 6 weeks, the chow and DR rats weighed the same, whereas the DIO rats were significantly heavier than the control or DR rats (Table 3). The DIO rats consumed significantly more kilocalories per day than the DR and control rats throughout the study (Table 3).

Plasma glucose, leptin, and insulin were measured in nonfasting samples obtained at the conclusion of the study (6 weeks). No differences in glucose concentrations were noted among the groups, but insulin levels in the DIO group were significantly higher than those in either the DR or chow group. The insulin-glucose (I/G) ratio, an indicator of insulin resistance, was also higher in the DIO group than in either of the other 2 groups (Table 3). Retroperitoneal, mesenteric, and epididymal fat pads were also removed and weighed and used as an index of total fat mass. These weights were greatest in DIO rats, least in chow rats, and intermediate in DR rats (Table 3); and the I/G ratio was correlated with the combined mass of these fat depots (Fig. 2, top panel). Adipose tissue mass and plasma leptin values correlated extremely well (r^2 = 0.92, Fig. 3), consistent with adipose tissue being a major source of circulating leptin [25,26].

In rats infused with unlabeled GLU before ^{14}C-GLU infusion, plasma GLU concentrations were 25 times higher than those in rats infused with saline (Table 4). The BBB GLU clearance was very low (from 0 to 9 μL min^{-1} g^{-1}), except in those areas with CVOs (ie, with fenestrated capillaries; Fig. 4). No statistically significant differences in GLU clearance were observed in any of the brain regions examined, comparing DR, chow, and DIO groups (Table 4). Elevation of circulating GLU concentrations produced a statistically significant effect on GLU clearance in whole brain, hippocampus, and cerebellum (Table 4). Whole-brain

Table 2
Brain GLU clearance in STZ-diabetic and control rats

Variable	Control	Control + GLU	STZ-diabetic	STZ-diabetic + GLU
Group size	5	4	5	4
Plasma GLU (mmol/L)†	0.02 ± 0.03	1.51 ± 0.2‡	0.06 ± 0.02	1.77 ± 0.31‡
GLU clearance (μL·min^{-1}·g^{-1} brain)				
Whole brain[a]	4.4 ± 0.8	2.0 ± 1.0	6.2 ± 2.0	4.3 ± 2.0
Brain region				
Cerebral cortex*	2.3 ± 0.6	0.4 ± 0.2	6.8 ± 2.1	3.7 ± 2.8
Striatum[a]	4.8 ± 1.1	3.0 ± 1.4	5.6 ± 2.4	3.7 ± 2.8
Hippocampus[a]	3.5 ± 1.0	3.2 ± 1.7	5.9 ± 2.6	2.0 ± 1.7
Midbrain[a]	6.6 ± 1.5	1.8 ± 1.4	4.0 ± 2.0	4.8 ± 2.4
Cerebellum*	4.7 ± 2.1	1.8 ± 1.4	8.5 ± 1.9	7.3 ± 1.7

Data are means ± SEM. Within a treatment group, "+GLU" indicates the subgroup with elevated plasma GLU concentrations during the BBB GLU uptake procedure (IV GLU infusion); the other subgroup had normal plasma GLU concentrations (IV saline infusion).

[a] No significant main effect of treatment (STZ, vehicle) or infusion (vehicle, GLU).

* $P < .05$, main effect of treatment (STZ, vehicle), but not infusion (vehicle, GLU).

† $P < .05$, main effect of infusion (vehicle, GLU), but not treatment (STZ, vehicle).

‡ $P < .05$, differs significantly from corresponding noninfusion group; ANOVA (Tukey test).

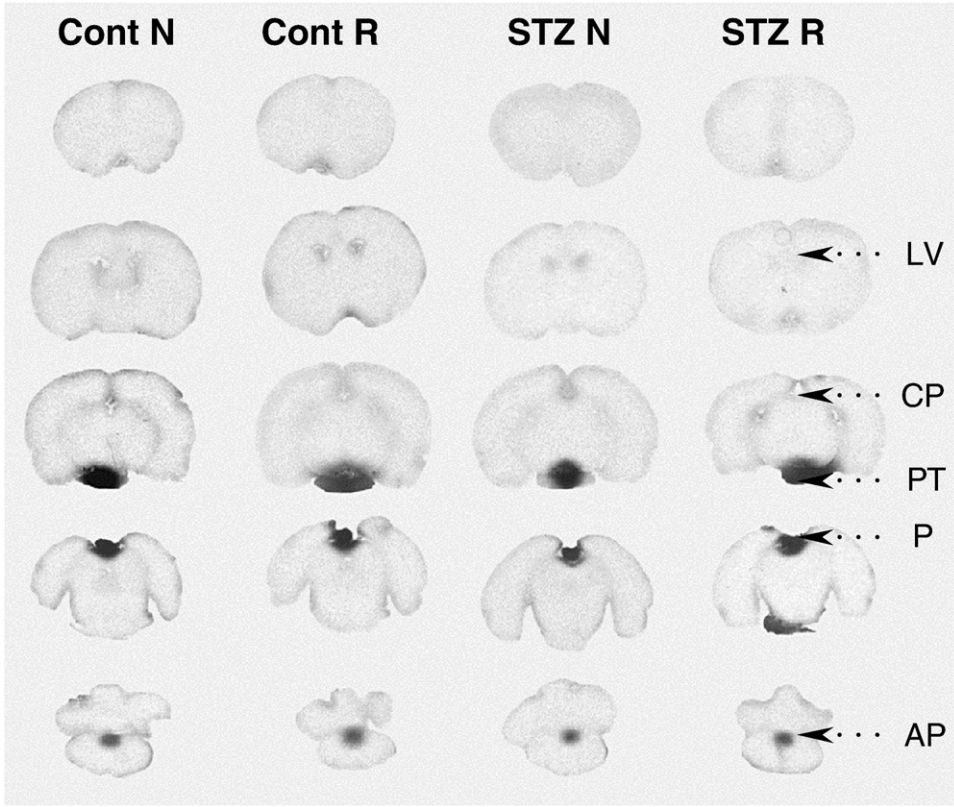

Fig. 1. Autoradiographs of brain from STZ-treated and control rats. Representative coronal sections through the brains of rats infused with [^{14}C]glutamate at normal concentrations and raised glutamate concentrations are shown. The only areas of brain where ^{14}C was notably visible were the CVOs (eg, area postrema, choroid plexus). The CVOs have fenestrated capillaries; however, the epithelial lining of the ventricles surrounding these areas is tightly connected by zonula occludens, thereby preventing the spread of ^{14}C into adjacent parenchyma of brain [23,24]. The pineal and anterior pituitary glands are not part of the brain. AP indicates area postrema; CP, choroid plexus; LV, lateral ventricles; P, pineal gland; PT, pituitary gland; N, normal concentration; R, raised concentration.

GLU clearance showed no relationship to fat mass and was not elevated in animals showing the largest I/G ratios (ie, greatest insulin insensitivity) (Fig. 2, bottom panel).

Table 3
Diet-induced obesity study: body weights, daily food intake, and plasma chemistries

Variable	DR	Control	DIO
Group size	12	11	12
Body weight (g)			
Initial	297 ± 1	300 ± 1	302 ± 2
Final	512 ± 3	510 ± 3	589 ± 4*
Food consumption (kcal/d)			
Weeks 0-2	98 ± 2	103 ± 2	116 ± 2*
Weeks 2-6	97 ± 2	97 ± 1	114 ± 2*
Fat pads (g)†	27.8 ± 1.7	20.3 ± 1.2	36.8 ± 2.3
Plasma glucose (μmol/mL)	10.6 ± 0.6	10.9 ± 0.4	11.2 ± 0.8
Plasma insulin (ng/mL)	7.8 ± 1.9	7.4 ± 2.3	17.3 ± 3.7*
I/G ratio (ng/μmol)	0.73 ± 0.17	0.65 ± .018	1.63 ± 0.38*
Plasma leptin (ng/mL)	4.0 ± 0.6	1.3 ± 0.2‡	5.1 ± 0.5

Data are means ± SEM. Fat pads weighed were epididymal, mesenteric, and retroperitoneal.

* $P < .05$, differs significantly from either DR or control values.

† $P < .05$, any group comparison significantly different.

‡ $P < .05$, differs significantly from either DR or DIO values (ANOVA, Tukey test).

4. Discussion

To pass the BBB, glutamate must cross both membranes of the capillary endothelial cell. The luminal membrane (blood facing) has a facilitative transporter with a very low capacity [27] and a high affinity [28]. This facilitative carrier exists only on the luminal membrane and cannot be found on the abluminal side (brain facing) [29]. Efflux from brain back into plasma appears to be driven in large part by a Na^+-dependent active transport system at the capillary abluminal membrane [7]. Because the Na^+ gradient is about 10 times higher in ECF than in endothelial cells for each GLU molecule that passes into the ECF, it is expected that 10 will be moved back to the endothelial cell whence GLU may diffuse into the plasma [7]. This mechanism thus contributes to the maintenance of the GLU concentration in brain ECF at very low concentrations. As such, it also represents one component of a regulatory system that helps maintain the brain interstitial fluid GLU concentration independent of that in the circulation. [7,28,30]. Consistent with these known relationships, the present results affirm that GLU uptake into the brain is normally very low. They further demonstrate that brain GLU uptake remains low when rats are made

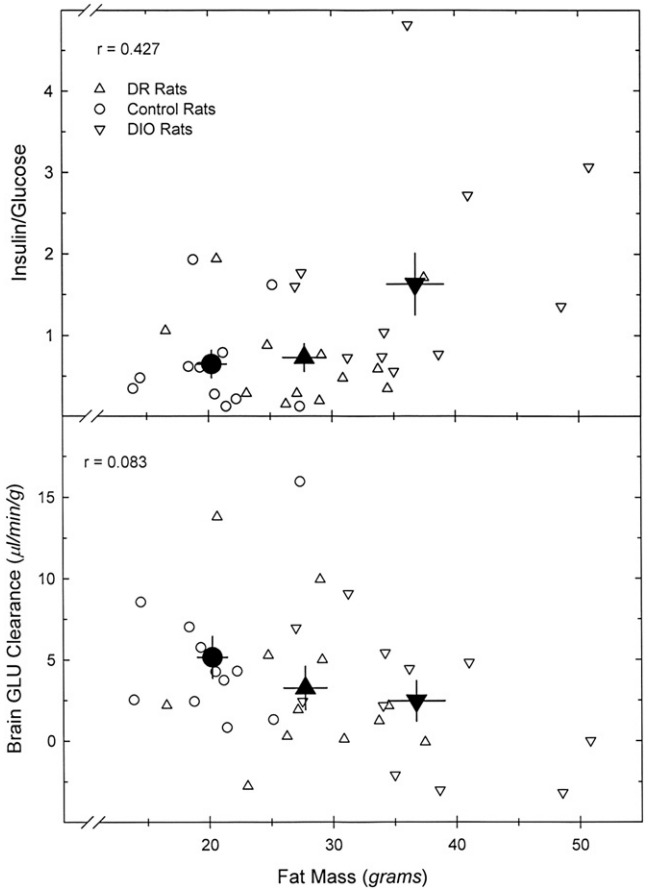

Fig. 2. Correlation between fat mass and the plasma I/G ratio (top panel) and brain GLU clearance (bottom panel). Data points for individual animals are shown as open symbols; filled (black) symbols represent group means; bars are SEM. A linear regression of I/G vs fat mass was statistically significant ($F = 7.127$, $P = .012$), whereas that of whole-brain GLU clearance vs fat mass was not ($F = 2.173$, $P = .150$).

insulinopenic or insulin resistant, even when blood GLU concentrations are very high.

We used the STZ-treated rat as the insulinopenic model of diabetes. First, we observed that GLU clearance in this study was 2 to 8 μL min^{-1} g^{-1} tissue in normal and STZ-treated rats in brain regions having a BBB (Table 2). Such values are normally very low and similar to those obtained previously [21]. In contrast, clearance values for other AA are typically 10- to 30-fold higher. For example, clearance values are 100 to 200 μL min^{-1} g^{-1} tissue for phenylalanine (a large neutral amino acid) and 8 μL min^{-1} g^{-1} tissue for lysine, a basic amino acid [14]. These AA are transported at the BBB by carriers different from that for acidic AA (eg, GLU) [27]. Such differences suggest that the BBB limits the access of circulating GLU to most portions of the brain. In several discrete brain areas—the CVOs—however, where no BBB is found, GLU is not excluded; and indeed, in the present study and a previous study [21], GLU penetration is readily observed (Fig. 1; eg, area postrema). Cellular elements in the CVOs are known not to be sensitive to elevated ECF GLU concentrations and are isolated from adjacent brain regions by tight junctions that exist between the epithelial cells that line the cerebral ventricles [23,30,31]. Second, GLU clearance values were not appreciably different from control values in STZ-treated rats (Table 2). In all cases, clearance values were within the reference range (2-8 μL min^{-1} g^{-1} tissue), although in 2 regions, a statistically significant, but materially insignificant, effect of STZ treatment was observed (cerebral cortex, cerebellum). The latter apparent difference is probably due to chance because, in a previous study [21], cerebral cortex and cerebellum GLU clearance values in normal rats were somewhat higher than those seen in STZ rats in the present study (8-11 μL min^{-1} g^{-1} tissue). And third, GLU clearance values were unaltered in the presence of extremely high circulating GLU concentrations (GLU infusion groups). The GLU infusion group was included based on the hypothesis that if the BBB to GLU appeared intact in diabetic animals at normal blood GLU concentrations, but was actually weakened, this weakness might emerge in the presence of enormously high circulating GLU levels. Such clearly was not the case, further attesting to the intactness of the BBB under the diabetic conditions examined.

By way of comparison, there is but one study that has assessed GLU clearance across the brains of healthy human subjects and subjects having insulin-dependent diabetes of long duration (2 decades) [32]. Clearance was estimated from measurements of the arteriovenous difference of amino acid concentrations and the rate of cerebral blood flow. The GLU clearance was found to be normal in the diabetic subjects. Our findings are consistent with this result. The results in diabetic subjects of long duration are also helpful in balancing the primary limitation of our study in rats, that of time. It could be argued that had the duration of diabetes been longer in our STZ rats, clearance differences might have been observed. The human study

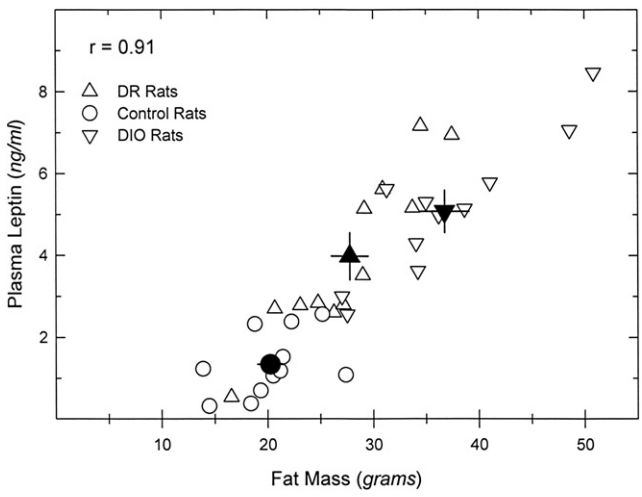

Fig. 3. Correlation between fat mass and plasma leptin concentrations. Data points for individual animals are shown as open symbols; filled (black) symbols represent group means; bars are SEM. A linear regression of plasma leptin vs fat mass was statistically significant ($F = 144.583$, $P < .001$).

Table 4
Brain GLU clearance in diet-induced obesity study

Variable	DR	DR + GLU	Control	Control + GLU	DIO	DIO + GLU
Group size	6	6	6	5	6	6
Plasma GLU (mmol/L)[a,*]	0.10 ± 0.02	2.70 ± 0.10[†]	0.10 ± 0.01	2.40 ± 0.20[†]	0.10 ± 0.02	2.40 ± 0.05[†]
GLU clearance (μL·min^{-1}·g^{-1} brain)						
Whole brain[a,*]	5.7 ± 2.2	0.8 ± 0.8	6.3 ± 2.1	3.7 ± 1.3	3.6 ± 1.7	1.6 ± 1.5
Brain region						
Cerebral cortex[a]	2.1 ± 1.9	1.4 ± 0.5	7.8 ± 2.0	4.6 ± 2.0	3.9 ± 3.6	3.0 ± 1.6
Striatum[a]	3.1 ± 2.4	0.3 ± 0.8	3.4 ± 2.9	2.6 ± 2.1	0.0 ± 2.1	0.0 ± 2.0
Hippocampus[a,*]	8.6 ± 2.9	0.0 ± 1.1	6.3 ± 2.1	3.5 ± 1.4	4.0 ± 1.9	1.5 ± 2.3
Midbrain[a,*]	6.3 ± 2.5	0.6 ± 1.3	6.0 ± 2.3	3.2 ± 1.9	3.1 ± 2.0	0.0 ± 2.0
Cerebellum[a,*]	8.4 ± 3.1	2.2 ± 1.6	8.2 ± 1.8	4.7 ± 1.3	8.7 ± 2.1	3.6 ± 1.4

Data are means ± SEM. Within a treatment group, "+GLU" indicates the subgroup with elevated plasma GLU concentrations during the BBB GLU uptake procedure (IV GLU infusion); the other subgroup had normal plasma GLU concentrations (IV saline infusion).

[a] No significant effect of diet (DR, chow, DIO).

* $P < .05$, significant effect of infusion (vehicle vs GLU).

[†] $P < .05$ vs noninfused value in same diet group (ANOVA, Tukey test).

suggests such might not be the case. Indeed, in rats treated with STZ, although some reports indicate that physical BBB disruption can occur as early as 14 to 28 days after STZ injection for small molecules like sucrose [12,33], other studies offer data providing little indication of BBB disruption (for mannitol or sucrose) in rats treated with STZ as long as 9 or 14 months earlier [34,35]. It seems that diabetes affects the BBB differently for different molecules [36].

We used a dietary paradigm to attempt to create insulin-resistant rats [18]. Rats were fed a highly palatable diet for 2 weeks and then divided into 3 groups of equal size based on body weight gain. The groups gaining the most and the least continued on the same diet, whereas the group of intermediate weight gain was switched to chow for the remaining experimental period. As described by Levin and associates [18], the group that showed the greatest initial weight gain ingesting the highly palatable diet continued to

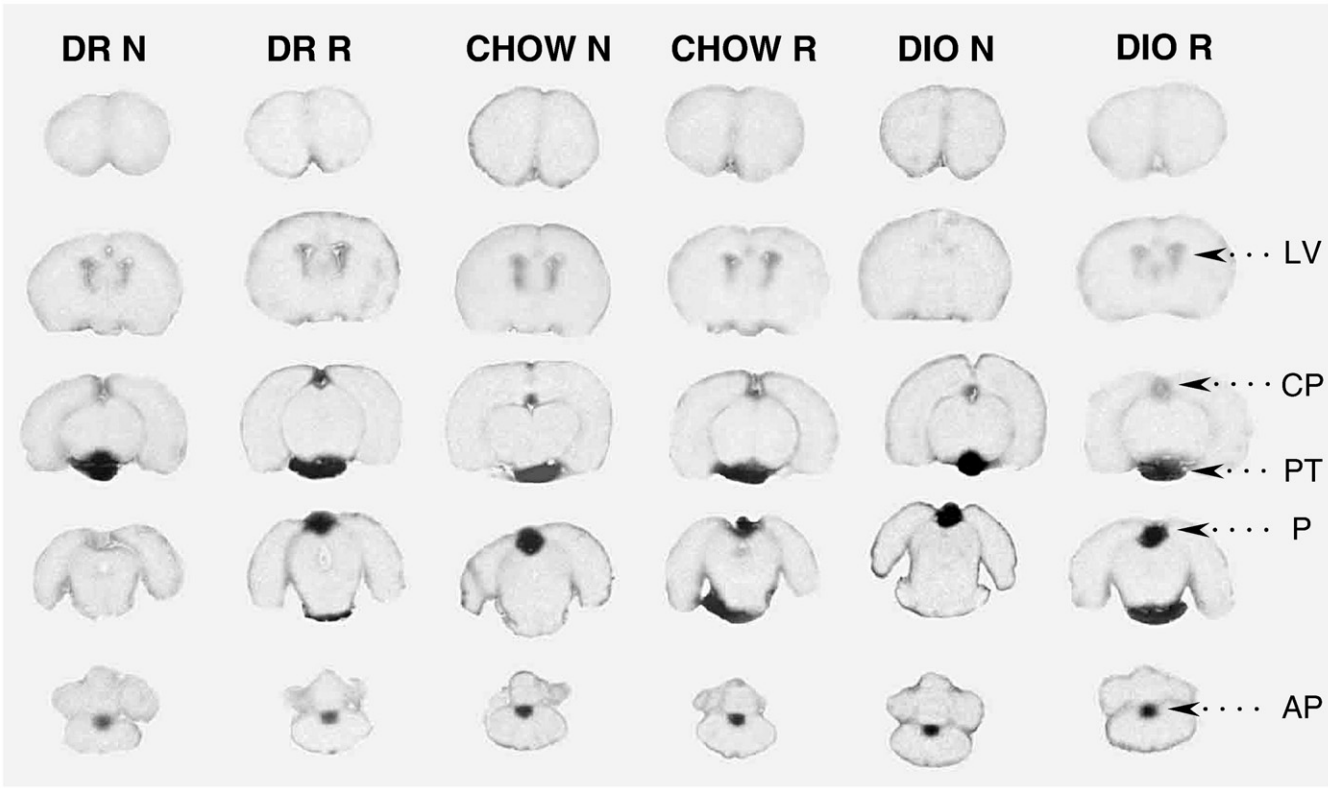

Fig. 4. Autoradiographs of brains from diet-induced obesity study. Representative coronal sections through the brains of rats infused with [^{14}C]glutamate at normal concentrations and raised glutamate concentrations are shown. The only areas of brain where ^{14}C was visible were the CVOs (Fig. 1).

eat more and gained significantly more weight by the end of the experiment than the group initially gaining the least weight on the same diet (DIO vs DR groups, respectively; Table 3). By most measures, DR rats were very similar to the rats that had initially gained an intermediate amount of weight that were switched to the rat chow diet (control group). They differed only in their fat pad mass and serum leptin levels. Indeed, as observed previously in such dietary paradigms, plasma leptin levels were notably different among the groups and correlated remarkably well with fat mass in individual rats across all groups (Fig. 3) [37,38]. In our study, plasma insulin concentrations were also significantly higher in the DIO group compared with the other 2 groups; but the effects were much smaller than those seen for leptin. However, because plasma glucose levels did not differ among groups, a significant difference was evident in the I/G ratio (Table 3), a measure of insulin resistance [39]. To affirm the relationship of fat mass to insulin insensitivity, which should be positive [25,37], data from individual rats were plotted (Fig. 2, top panel) and showed that the I/G ratio tended to rise with increasing fat mass (the effect was statistically significant; linear regression, $P = .01$). In contrast, a plot of brain GLU clearance vs fat mass (Fig. 2, bottom panel) revealed that, if anything, GLU clearance tended to be lower as fat mass increased, although this effect was not significant (linear regression, $P = .15$). A regression analysis of whole-brain GLU clearance vs the I/G ratio was also not statistically significant (not shown; $r = 0.08$, $F = 0.22$, $P = .64$). Hence, rats with higher I/G ratios, indicative of insulin resistance, did *not* show higher brain clearances for GLU. The conclusion, however, that insulin resistance is not associated with increased GLU penetration into brain must be tempered by the length of the study: 6 weeks. This time may be insufficient for the development of the metabolic sequelae that accompany insulin resistance of long duration. In humans with type 2 diabetes mellitus (non–insulin dependent) of long duration, the few data that are available are conflicting regarding the integrity of the BBB: one study indicates that it is intact [40], whereas another indicates that it is not [13]. A third study, quantitating GLU clearance across the brain of long-term diabetic subjects requiring insulin (discussed above), reports no difference relative to nondiabetic subjects, although these subjects had type 1 diabetes mellitus, a more severe form of diabetes [32]. Further work would thus be useful with the DIO model to assess if it does develop more marked insulin insensitivity with time, which is at least suggested by some earlier data [41].

In conclusion, the results with STZ suggest only minor, if any, changes in GLU clearance in whole-brain and brain regions of severely diabetic rats. In addition, no changes in brain GLU clearance were evident in rats made insulin resistant using a dietary paradigm that promotes obesity. Although the findings generally support the opinion, at least in the paradigms used, that diabetes is not associated with changes in GLU transport across the BBB, further studies in an animal model of prolonged insulin resistance would be useful because of the growing prevalence of type 2 diabetes mellitus (non–insulin dependent) in the population, particularly that associated with obesity.

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References

[1] Hawkins RA, Peterson DR, Viña JR. The complementary membranes forming the blood-brain barrier. IUBMB Life 2002;54:101-7.
[2] Hawkins RA, O'Kane RL, Simpson IA, Vina JR. Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr 2006;136:218S-26S.
[3] Meldrum BS. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 2000;130:1007S-15S.
[4] Castillo J, Davalos A, Naveiro J, Noya M. Neuroexcitatory amino acids and their relation to infarct size and neurological deficit in ischemic stroke. Stroke 1996;27:1060-5.
[5] Castillo J, Davalos A, Noya M. Progression of ischaemic stroke and excitotoxic amino acids. Lancet 1997;349:79-83.
[6] Choi DW, Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. J Neurosci 1987;7:357-68.
[7] O'Kane RL, Martinez-Lopez I, DeJoseph MR, Viña JR, Hawkins RA. Na(+)-dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood-brain barrier. A mechanism for glutamate removal. J Biol Chem 1999;274:31891-5.
[8] Edwards MS, Wilson DB, Craven TE, Stafford J, Fried LF, Wong TY, et al. Associations between retinal microvascular abnormalities and declining renal function in the elderly population: the Cardiovascular Health Study. Am J Kidney Dis 2005;46:214-24.
[9] Ward JDE. Abnormal microvasculature in diabetic neuropathy. Eye 1993;7:223-6.
[10] McMillan DE. The microcirculation in diabetes. Microcirc Endothelium Lymphatics 1984;1:3-24.
[11] Bradbury MW, Lightman SL. The blood-brain interface. Eye 1990;4 (Pt 2):249-54.
[12] Huber JD, VanGilder RL, Houser KA. Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats. Am J Physiol Heart Circ Physiol 2006;291:H2660-8.
[13] Starr JM, Wardlaw J, Ferguson K, MacLullich A, Deary IJ, Marshall I. Increased blood-brain barrier permeability in type II diabetes demonstrated by gadolinium magnetic resonance imaging. J Neurol Neurosurg Psychiatry 2003;74:70-6.
[14] Mans AM, DeJoseph MR, Davis DW, Hawkins RA. Regional amino acid transport into brain during diabetes: effect of plasma amino acids. Am J Physiol 1987;253:E575-83.
[15] McCall AL, Millington WR, Wurtman RJ. Metabolic fuel and amino acid transport into the brain in experimental diabetes mellitus. Proc Natl Acad Sci USA 1982;79:5406-10.
[16] Brosnan JT, Forsey RG, Brosnan ME. Uptake of tyrosine and leucine in vivo by brain of diabetic and control rats. Am J Physiol 1984;247: C450-3.
[17] Junod A, Lambert AE, Stauffacher W, Renold AE. Diabetogenic action of streptozotocin: relationship of dose to metabolic response. J Clin Invest 1969;48:2129-39.
[18] Levin BE, Hogan S, Sullivan AC. Initiation and perpetuation of obesity and obesity resistance in rats. Am J Physiol 1989;256:R766-71.

[19] Hawkins RA, Mans AM. Determination of cerebral glucose use in rats using [14C] glucose. In: Boulton AA, Baker GB, Butterworth RF, editors. Neuromethods 11. Carbohydrates and energy metabolism. Clifton (NJ): Humana Press Inc.; 1989. p. 195-230.
[20] Bergmeyer HU, editor. Methods of enzymatic analysis, 2nd ed. New York: Academic Press; 1974. p. 1836-43.
[21] Hawkins RA, DeJoseph MR, Hawkins PA. Regional brain glutamate transport in rats at normal and raised concentrations of circulating glutamate. Cell Tissue Res 1995;281:207-14.
[22] Bongiovanni R, Yamamoto BK, Jaskiw GE. Improved method for the measurement of large neutral amino acids in biological matrices. J Chromatogr B Biomed Sci Appl 2001;754:369-76.
[23] Brightman MW, Reese TW. Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol 1969;40:648-77.
[24] Brightman MW, Reese TS, Feder N. Assessment with the electron microscope of the permeability to peroxidase of cerebral endothelium and epithelium in mice and sharks. In: Crone C, Lassen NA, editors. Capillary permeability. New York: Academic Press; 1970. p. 468-76.
[25] Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1: 1155-61.
[26] Klein S, Coppack SW, Mohamed-Ali V, Landt M. Adipose tissue leptin production and plasma leptin kinetics in humans. Diabetes 1996;45:984-7.
[27] Oldendorf WH, Szabo J. Amino acid assignment to one of three blood-brain barrier amino acid carriers. Am J Physiol 1976;230:94-8.
[28] Smith QR. Transport of glutamate and other amino acids at the blood-brain barrier. J Nutr 2000;130:1016S-22S.
[29] Lee WJ, Hawkins RA, Viña JR, Peterson DR. Glutamine transport by the blood-brain barrier: a possible mechanism for nitrogen removal. Am J Physiol 1998;274:c1101-7.
[30] Hawkins RA. The blood-brain barrier and glutamate. Am J Clin Nutr 2009 [Epub ahead of print] pmid: 19571220.
[31] Reese TS, Feder N, Brightman MW. Electron microscopic study of the blood-brain and blood-cerebrospinal fluid. J Neuropathol Exp Neurol 1971;30:137-138.
[32] Grill V, Bjorkman O, Gutniak M, Lindqvist M. Brain uptake and release of amino acids in nondiabetic and insulin-dependent diabetic subjects: important role of glutamine release for nitrogen balance. Metabolism 1992;41:28-32.
[33] Hawkins BT, Lundeen TF, Norwood KM, Brooks HL, Egleton RD. Increased blood-brain barrier permeability and altered tight junctions in experimental diabetes in the rat: contribution of hyperglycaemia and matrix metalloproteinases. Diabetologia 2007;50:202-11.
[34] Bradbury MW, Lightman SL, Yuen L, Pinter GG. Permeability of blood-brain and blood-nerve barriers in experimental diabetes mellitus in the anaesthetized rat. Exp Physiol 1991;76:887-98.
[35] Rechthand E, Smith QR, Latker CH, Rapoport SI. Altered blood-nerve barrier permeability to small molecules in experimental diabetes mellitus. J Neuropathol Exp Neurol 1987;46:302-14.
[36] Hawkins BT, Ocheltree SM, Norwood KM, Egleton RD. Decreased blood-brain barrier permeability to fluorescein in streptozotocin-treated rats. Neurosci Lett 2007;411:1-5.
[37] Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1995;1:1311-4.
[38] Archer ZA, Corneloup J, Rayner DV, Barrett P, Moar KM, Mercer JG. Solid and liquid obesogenic diets induce obesity and counter-regulatory changes in hypothalamic gene expression in juvenile Sprague-Dawley rats. J Nutr 2007;137:1483-90.
[39] Buse JB, Polansky KS, Burant CF. Insulin resistance and the risk of type 2 diabetes, 10 ed. Philadelphia: Saunders; 2003.
[40] Dai J, Vrensen GF, Schlingemann RO. Blood-brain barrier integrity is unaltered in human brain cortex with diabetes mellitus. Brain Res 2002;954:311-6.
[41] Levin BE, Sullivan AC. Glucose-induced norepinephrine levels and obesity resistance. Am J Physiol 1987;253:R475-81.