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The brain response to 2-deoxy glucose is blocked by a glial drug

John K. Young*, James H. Baker, Melissa I. Montes

Department of Anatomy, Howard University College of Medicine, 520 W. Street NW, Washington DC 20059, USA

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Abstract

Two brain regions — the basomedial hypothalamus and area postrema (AP) — react to changes in circulating glucose levels by altering feeding behavior and the secretion of pituitary and non-pituitary hormones. The precise identity of cells responding to glucose in these regions is uncertain. The recent detection of high-capacity glucose transporter proteins in astrocytes in these areas has suggested that astrocytes may play a role in glucose sensing by the brain. To test this hypothesis, rats were injected with either saline or methionine sulfoximine (MS), a compound that produces alterations in carbohydrate and glutamate metabolism in astrocytes. Eighteen hours later, rats were injected with either saline or 2-deoxy glucose (2-DG) and brain sections were stained to demonstrate 2-DG-activated neurons immunoreactive for Fos protein. MS-treated rats showed a 70% reduction in numbers of Fos+ neurons in the AP region ($p < 0.05$). Also, specialized, Gomori+ astrocytes were particularly abundant in both glucose sensitive regions and showed a distribution identical to that reported for high-capacity glucose transporter proteins. These data suggest that specialized astrocytes influence the glucose-sensing function of the brain.

Keywords: 2-DG; Hypothalamus; Area postrema; Glucose; Methionine sulfoximine; Gomori; c-*fos*

Two brain regions — the basomedial hypothalamus and area postrema (AP) region — show a special sensitivity to circulating levels of blood glucose and react to decreases or elevations in blood glucose to modify feeding behavior and the secretion of pituitary, adrenal, and pancreatic hormones [1,23,24]. Neurons in these regions also react to a so-called glucoprivic agent, 2-deoxy glucose (2-DG), a glucose analogue that competitively inhibits phosphohexose-isomerase and depresses cellular glucose utilization. Administration of 2-DG stimulates neurons in these areas, which synthesize the product of an immediate early gene, c-*fos*. The Fos protein in activated neurons can be detected using immunocytochemistry [3,39]. Neurons near the AP, in the nucleus of the tractus solitarius (NTS), appear mainly responsible for the increases in feeding behavior seen during glucoprivation; these neurons activate other brain regions — the dorsal motor nucleus of the vagus and paraventricular nucleus — that more directly regulate feeding [18,21,34,38,39]. What makes these areas uniquely sensitive to glucose?

One reason for this sensitivity is a high regional permeability of capillaries in the median eminence and AP [25,45,53]. In addition, cells in these brain regions seem to possess some of the features of glucose-sensitive pancreatic beta cells. One such feature is a GLUT2 type glucose transporter that carries glucose into cells only when circulating glucose is elevated and which permits a glucose uptake in beta cells that is 10-fold higher than in other cells [13]. GLUT2 transporters have been detected in a subpopulation of astrocytes bordering the AP and in the hypothalamus [22]. Intraventricular infusions of antisense oligonucleotides that bind GLUT2 mRNA interfere with the neural control of insulin secretion and the feeding responses to 2-DG, suggesting that brain GLUT2 transporters are functionally important [23,46].

Another beta cell feature is an ATP-sensitive potassium channel that adjusts cell membrane potential in proportion to levels of intracellular metabolites. These potassium channels have also been detected in neurons; until recently, neuronal ATP-sensitive channels have been considered an identifying feature of glucose-sensitive neurons. However, a recent

* Corresponding author. Tel.: +1-202-806-6555; fax: +1-202-265-7055.

E-mail address: jyoung@fac.howard.edu (J.K. Young).

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study found ATP-sensitive potassium channels in many neurons in diverse brain regions that are unresponsive to glucose [8]. Thus, ATP-sensitive potassium channels, while conceivably playing a role in matching neuronal firing rate to neuronal metabolism, cannot be considered an identifying feature of a glucose-sensitive brain cell. The presence of GLUT2 transporters in a specialized subpopulation of astrocytes suggests that astrocytes may have a key role in the process of glucose sensing by the brain.

One approach to testing this hypothesis is the use of a compound, methionine sulfoximine (MS), that has metabolic effects largely restricted to astrocytes. MS is a glutamate analogue that inhibits an enzyme, glutamine synthetase, present only in astrocytes [7,15,50]. Blockade of this enzyme prevents the synthesis of glutamine from glutamate and also results in the loading of astrocytes with glycogen [15]. MS can therefore be considered a useful tool to examine whether or not a drug-induced alteration in astrocyte metabolism affects brain glucose sensing.

Another interesting feature of brain glucose-sensitive areas is a high abundance in these regions of a specialized type of astrocyte, the Gomori+ (GP) astrocyte. GP astrocytes are identified by cytoplasmic granules derived from degenerating mitochondria [2,49,51]. The reported anatomical distribution of these cells in the hypothalamus and NTS appears to match that of the subpopulation of astrocytes reported to be immunoreactive for the GLUT2 glucose transporter (Ref. [22] vs. Refs. [19,51]). Another goal of this study was to compare the distribution of GP astrocytes with that of the reported distribution of GLUT2 transporters and with the distribution of neurons activated by glucoprivation to synthesize Fos.

1. Materials and methods

1.1. Study I

Nineteen adult female albino Sprague–Dawley derived rats, weighing 150 g, were divided into four treatment groups in this study. Rats were housed in individual cages and given ad lib access to water and Purina rat chow pellets. Prior to any drug treatment, rats were weighed daily for 2 weeks to accustom them to handling and to minimize any stressful experiences that can also provoke expression of Fos in the brainstem [21,39]. On the first treatment day at 1600 h, rats were injected intraperitoneally with either sterile saline or with MS (100 mg/kg, or 0.55 mmol/kg, at a concentration of 13.5 mg/ml of saline). This relatively low dose of MS was chosen to minimize the probability of MS-induced convulsive effects and because the same dose was previously shown to diminish damaging effects of a toxic form of glucose, goldthioglucose, upon the hypothalamus and AP [50]. On the second treatment day, 18 h later at 1000 h, when the MS-induced accumulation of glycogen in astrocytes is maximal [12], food was removed from all cages and rats were treated with either saline or with 2-DG (300 mg/kg, i.p., 30 mg/ml in sterile saline). This dose of 2-DG was chosen on the basis of a dose–response study by Ritter and Dinh [39]. Three hours later, when induction of Fos by 2-DG is maximal, rats were overdosed with Nembutal. Prior to euthanasia, several drops of blood were collected in capillary tubes from cut tail tips. Blood glucose concentrations in blood samples were measured using an Accu-chek II photometer in combination with ChemStrips impregnated with glucose oxidase and a dye indicator (Boehringer-Mannheim, Indianapolis, IN). Rats were perfused with 10% formalin in 0.2 M phosphate buffer and brains were removed for preparation of 30 μm thick frozen coronal sections. During sectioning, cuts were made into one or more surfaces of each tissue block to provide a feature identifying the brain from which a given section was obtained.

Free-floating sections of all hypothalamic and AP regions were simultaneously stained for Fos using a rabbit polyclonal antibody against Fos diluted 1:200 in phosphate buffered saline (PBS) containing 0.3% Triton-X and 1.0% normal goat serum (Oncogene Research Products, Cambridge, MA). Sections were first all washed in PBS-normal goat serum for 1/2 h in plastic tissue wells and then incubated overnight at 4°C with either the antibody to Fos or with non-immune rabbit serum. Following this, sections were washed in PBS-0.1% Triton-X, incubated for 30 min in biotinylated goat-anti rabbit IgG (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and were washed once more in PBS-0.1% Triton. To nullify endogenous sources of peroxidase such as the cytoplasmic granules of GP astrocytes [19], sections were then incubated for 30 min in PBS-0.3% hydrogen peroxide, followed by washing in PBS and a 60 min incubation in avidin–biotin–peroxidase complex (ABC). Immunoreactivity was then localized via the production of a brown precipitate of diaminobenzidine at the sites of peroxidase-labeled antibody by exposing the sections to a 0.05% solution of diaminobenzidine plus 0.03% hydrogen peroxide for 15 min [51]. Sections were then mounted onto gelatinized slides and coverslipped.

Numbers of Fos+ neurons in three consecutive sections for each brain area were counted under the microscope. For sections of medulla, counting was confined to the NTS regions on each side of the AP. In the hypothalamus, counting was confined to the arcuate nucleus, defined by curved tanycyte processes found at the dorsal margin of the arcuate nucleus that terminate at the ventrolateral border of the arcuate nucleus at the midpoint of the ventral surface of the hypothalamus. Statistical comparisons between groups were performed using analysis of variance and Tukey's protected t-test for comparisons between groups (GB-STAT software for Windows, version 5.0, Dynamic Microsystems, Silver Spring, MD).

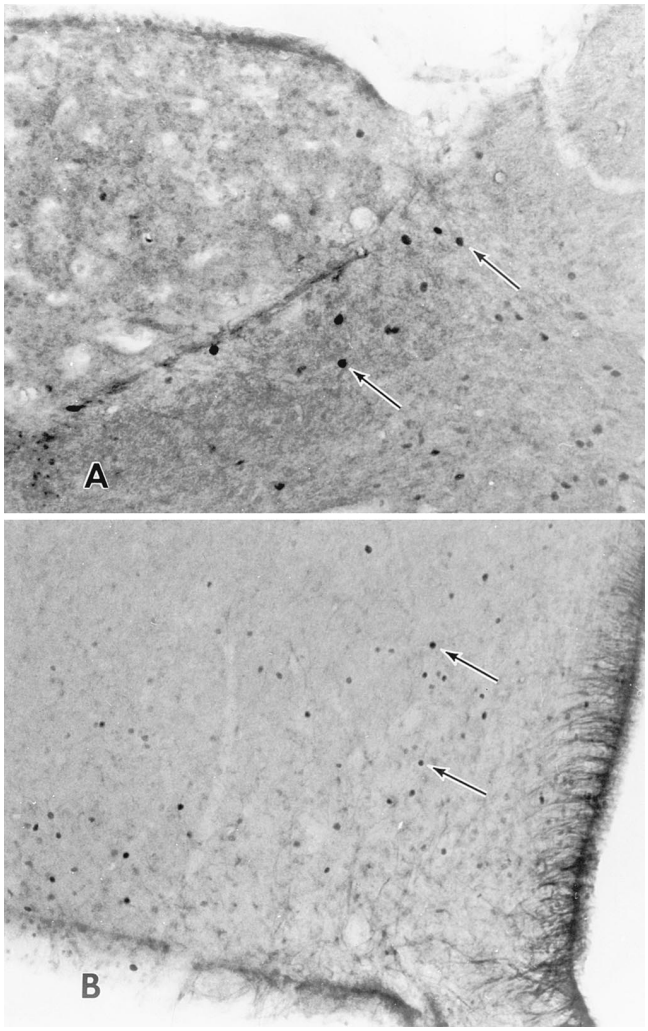

Fig. 1. Neuronal nuclei immunoreactive for Fos (arrows) following administration of 2-deoxy glucose in (A) the nucleus of the tractus solitarius (NTS) in the medulla (magnification = 230 ×), and in (B) the arcuate nucleus of the hypothalamus (magnification = 184 ×).

1.2. Study II

In this study, the anatomical distribution of GP astrocytes relative to the locations of neurons activated by 2-DG was examined. To demonstrate the general distribution of GP astrocytes in several brain areas, two adult female Sprague–Dawley derived albino rats, weighing 300 g, were given lethal overdoses of Nembutal i.p. and were then perfused intracardially with 10% formalin in 0.2 M phosphate buffer. Older female rats (6 months old) were chosen because GP astrocyte granules become larger and more numerous in older animals. Frozen sections (30 μm thick) were mounted onto gelatinized slides and exposed to Janssen IntenSE-M reagents (Amersham, Arlington Heights, IL) that are designed to stain gold particles in tissue sections by forming deposits of silver over them. This procedure was previously found to deposit silver over GP granules [54]. Finally, a hypothalamus from another perfused rat was embedded in methacrylate resin, sectioned at a thickness of 2 μm using dry glass knives, and stained with toluidine blue to provide more detailed illustrations of the morphology of GP astrocytes [51].

2. Results

2.1. Study I

No obvious differences in behavior, posture, or righting reflexes between rats in the different treatment groups were apparent in the 3-h interval prior to euthanasia. Immunocytochemistry of sections of medulla and hypothalamus showed numerous Fos+ neuronal nuclei after incubation in the anti-Fos rabbit serum (Fig. 1); no staining was visible in control sections exposed to only non-immune serum. In the medulla, total counts of Fos+nuclei were significantly higher (3-fold higher) in the 2-DG+saline group than in the 2-DG+MS group or saline+MS or saline + saline groups (F=3.08, df=1,11, p<0.05, ANOVA, Tukey's corrected t-test) (Table 1). MS had no effect on the baseline expression of Fos in the NTS, which appears to reflect responses to stress or to alterations in blood pressure (MS + saline vs. saline + saline groups, p>0.05, NS).

In the hypothalamus, counts of Fos+ nuclei were about 2-fold higher in the 2-DG + saline group compared with the 2-DG + MS group, but this difference did not achieve statistical significance (p>0.05). Rats not treated with 2-DG had very few Fos+ neurons visible in the hypothalamus. Rats treated with 2-DG and either saline or MS showed a significant linear correlation between numbers of Fos+ neurons in the NTS with counts of Fos+ neurons in the arcuate nucleus: low counts in the NTS tended to be accompanied by low counts in the hypothalamus, whereas high counts in the NTS were accompanied by higher counts in the hypothalamus in the same brain (r=0.93, p<0.01).

Table 1
Effects of MS upon the brain response to 2-deoxy glucose

	No. of c-Fos+ cells in medulla (NTS)	No. of c-Fos+ cells in hypothalamus	Plasma concentration of glucose (mg/dl)
Saline + saline, n = 3	54 ± 15.5*	7.5 ± 4.5*	138 ± 3.4*
2-DG + saline, n = 7	249 ± 49	127 ± 33.4	330 ± 34.5
2-DG + MS, n = 6	78.5 ± 37*	64 ± 30.2	201 ± 27.0*
Saline + MS, n = 3	70.5 ± 33*	6.5 ± 0.5*	136 ± 3.5*

* p < 0.05, different from 2-DG + saline, ANOVA, Turkey's protected t-test.

Blood glucose concentrations were also significantly higher in the 2-DG + saline group than in the 2-DG + MS group (F=4.03, df=1,11, p<0.01), showing that the hyperglycemia provoked by 2-DG was substantially attenuated by MS. There were no differences in blood glucose concentrations between the MS + saline and saline + saline groups. In this study, the feeding stimulatory effects of 2-DG were not studied, and food was removed from all cages prior to 2-DG, so that differences in feeding between groups would not complicate analysis of blood glucose levels.

2.2. Study II

Staining of frozen sections for the cytoplasmic granules of GP astrocytes showed that GP astrocytes were abundant along the lateral margins of the AP and within the arcuate nucleus of the hypothalamus, the same regions occupied by neurons that synthesized Fos in response to glucoprivation (Fig. 2). Higher magnification views of methacrylate embedded tissue showed darkly staining GP granules adjacent to astrocyte nuclei in the same regions of the hypothalamus and medulla showing silver deposition over GP granules (Fig. 3).

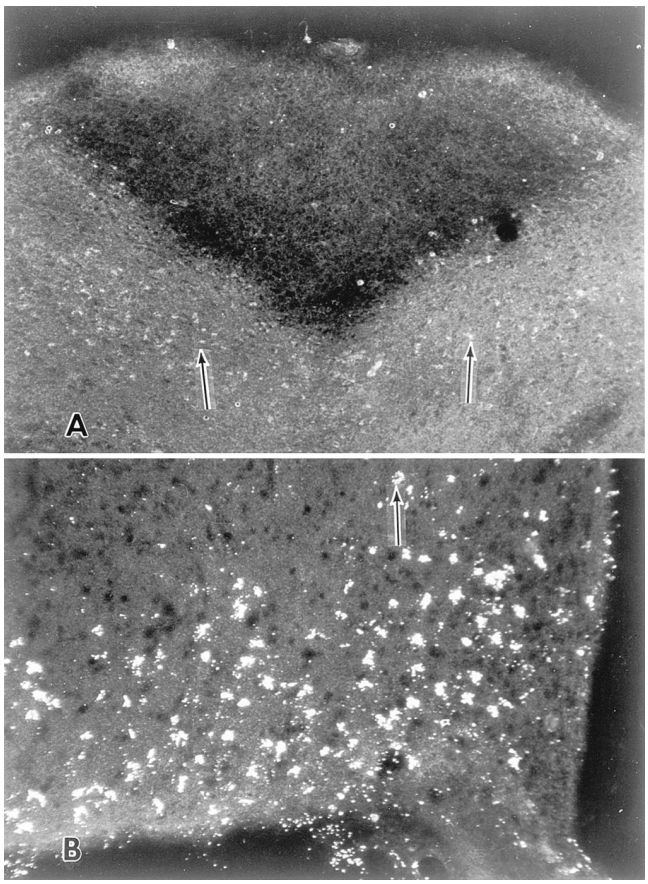

Fig. 2. Distribution of Gomori+ astrocytes (arrows, bright points of light), demonstrated by staining with silver intensification reagents and viewing under dark-field microscopy. (A) Gomori+ astrocytes in the NTS in the medulla (magnification=164 ×). (B) Gomori+ astrocytes in the arcuate nucleus of the hypothalamus (magnification=184 ×).

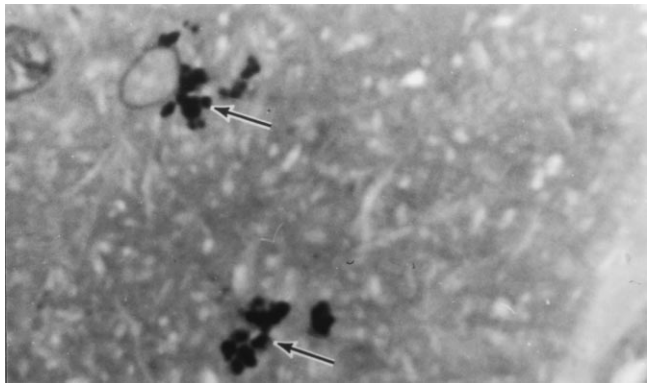

Fig. 3. High magnification view of Gomori+astrocyte (tissue embedded in methacrylate, cut at a thickness of 2 μm, and stained with toluidine blue). Arrows indicate cytoplasmic granules derived from degenerating mitochondria.

3. Discussion

3.1. Study I

This study showed that an MS-induced alteration in astrocyte metabolism significantly attenuated the response to glucoprivation of neurons in the nucleus of the NTS and had a similar, but statistically non-significant, effect upon glucose-sensitive neurons in the hypothalamus. Also, the hyperglycemia provoked by 2-DG, which appears due to a protective reflex release of adrenaline stimulated by glucose-sensing systems of the brain, was also attenuated by MS [1,16,34]. Several explanations for these effects of MS can be proposed.

(1) An MS-induced loading of astrocytes with glycogen could have metabolic consequences for neighboring neurons. In vitro, glycogen-loaded astrocytes exposed to MS become substantially more capable of protecting adjacent neurons from glucose deprivation [43]. This protective effect of astrocytes appears due to transfer of lactate from glia to neurons [27]. An enhanced delivery of nutrients from astrocytes to neurons may allow neurons to "escape" the consequences of a 2-DG-induced impairment in neuronal glucose metabolism that would otherwise lead to neuronal activation and Fos synthesis. Pre-treatment with MS could have enhanced this protective role of astrocytes by increasing astrocyte concentrations of glycogen.

(2) MS-induced alterations in astrocyte uptake of glutamate or in other uncharacterized astrocyte functions could have had functional consequences for adjacent neurons [30]. Also, since neuronal synthesis of GABA is dependent upon transfer of glutamine from astrocytes, an MS-induced change in glial glutamine metabolism could have affected neuronal levels of GABA [7,40]. Possibly,

abnormalities in the neural–glial relationship could affect the neuronal response to many stimuli, as well as the response to glucoprivation; however, baseline production of Fos protein in the AP was not depressed in MS + saline rats relative to controls.

(3) MS could conceivably have had direct effects upon neuronal enzymes. However, available data suggest that direct effects of MS are restricted to astrocytes. After MS, brain astrocytes show ultrastructural abnormalities and glycogen deposition, whereas neurons and oligodendroglia do not [12]. MS increases astrocyte glutamate uptake, but leaves neuronal glutamate uptake and lactate dehydrogenase unaltered [40,43]. Similarly, MS does not affect neuronal levels of choline acetyltransferase or glutamate decarboxylase [42]. Low doses of MS that alter glial glutamine content fail to affect brain levels of neurotransmitters such as dopamine, norepinephrine, serotonin, or their metabolites [4]. Higher doses of MS do appear to reduce brain serotonin, perhaps as a consequence of altered extracellular levels of glutamate [14].

One neuronal enzyme that is affected by MS is γ-glutamylcysteine synthetase, an enzyme required for glutathione synthesis. MS is a potent, irreversible inhibitor of glutamine synthetase and a weak, reversible inhibitor of γ-glutamylcysteine synthetase [36]. Doses of about 1 mmol/kg of MS diminish liver glutathione by 15–50%; however, the same dose has no effect upon brain glutathione [11,32]. High doses of MS and other glutamate analogues that elicit convulsions have no effects upon brain glutathione, whereas drugs that severely deplete brain glutathione but do not affect glutamine synthetase do not cause convulsions like those caused by MS [10]. It thus appears likely that functional effects of MS in the brain are exerted via effects upon astrocyte glutamine synthetase and not upon neuronal glutathione metabolism.

(4) Effects of MS upon peripheral organs such as the liver could have influenced the brain response to 2-DG. However, since severing the neural connection between the brain and the liver by vagotomy has no effect upon the brain response to 2-DG, it is unlikely that alterations in hepatic metabolism could directly affect the brain response to 2-DG [39]. MS conceivably could have altered blood concentrations of 2-DG by affecting hepatic uptake of glucose and 2-DG. However, since blood glucose concentrations are not altered by MS and since 2-DG and glucose are both taken up into peripheral tissues at the same rate and by the same transporters, this explanation is unlikely [15] (Table 1).

3.2. Study II

This study showed that GP astrocytes are unusually abundant in the brain areas occupied by Fos+ neurons activated by 2-DG. These same regions in the hypothalamus and medulla are also uniquely and heavily damaged by a toxic form of glucose, goldthioglucose, that enters these regions via permeable circumventricular capillaries [49,50]. Damaging effects of goldthioglucose are also diminished by MS, perhaps because loading of astrocytes with glycogen diminishes cellular uptake of glucose and/or goldthioglucose [50]. All of these observations suggest that cells in these regions have a special sensitivity to various forms of glucose.

The distribution of GP astrocytes precisely matches the reported distribution of astrocytes immunoreactive for GLUT2 transporters [22]. It would be desirable to see if GP astrocytes are indeed immunoreactive for GLUT2 transporter protein. However, studies using commercially available GLUT2 antibodies, while generally supportive of the results of Leloup et al., do not report a level of staining intensity and specificity needed to conclusively explore this question, which must therefore await the general availability of more suitable antibodies to GLUT2 [17]. Some known features of GP astrocytes, however, are consistent with an unusual glucose metabolism.

The slow, age-related accumulation of mitochondrial damage and GP granules in GP astrocytes appears due to some type of oxidative stress in GP astrocytes [41]. The source of this oxidative stress in vivo is uncertain. Due to their frequent association with dopaminergic neurons, we postulated that oxidative stress in GP astrocytes could be related to dopamine metabolism; however, we were unable to affect the accumulation of GP granules in vivo by chronic administration of a drug that depresses dopamine metabolism [29,52]. The association of GP astrocytes with dopaminergic neurons could instead be related to the fact that many hypothalamic dopaminergic neurons are glucose-sensitive and are activated by 2-DG [3]. Oxidation of glucose and a transfer of glucose metabolites from GP astrocytes to neurons in the hypothalamus could be functional features of GP astrocytes that underlie their distribution and morphological features. Chronic exposure of neural tissue to high levels of glucose damages mitochondria, and a rate-limiting enzyme of glucose metabolism, hexokinase, is bound to astrocyte mitochondria [26,48]. It is therefore possible that high rates of glucose metabolism in circumventricular astrocytes, due to high localized capillary permeability and/or astrocyte GLUT2 glucose transporters, could be responsible for the mitochondrial damage seen in GP astrocytes.

Metabolic characteristics of astrocytes in general are consistent with a role for astrocytes in glucose sensing by the brain. Astrocytes are much more enriched in the enzymes needed to store and break down glycogen than are neurons [33,37]. In culture, the rate of glucose phosphorylation may be 4–5 fold greater in astrocyte-like retinal Müller cells than in neurons [35]. Müller cells are also immunoreactive for GLUT2 glucose transporters [47]. Astrocytes, but not neurons, respond to insulin with increases in glucose uptake [6]. This would seem to indicate a significant role of astrocytes in the production of hyperphagia in the streptozotocin-diabetic rat: in this model of hyperphagia, an impairment of cellular glucose metabolism

due to a lack of insulin disturbs the hypothalamic regulation of feeding [5]. Metabolism of glucose in astrocytes and a subsequent transfer of metabolites to neurons could represent a mechanism by which circumventricular organs respond to changes in circulating glucose.

Other "sensory" functions of the hypothalamus may also involve astrocytes. One example is the ability of supraoptic neurons to respond to changes in blood osmolarity to regulate vasopressin release. Aquaporin-4 type water channels that appear involved in this response to osmolarity are located on astrocyte cell membranes and are absent from neurons [31]. Astrocytes may thus play a regulatory role in the response of neurons to a variety of molecules entering the neuropil from capillaries.

One additional feature of GP astrocytes that may particularly point to an elevated glucose uptake is their elevated immunoreactivity for brain fatty acid binding protein (bFABP) [51]. High levels of bFABP in GP astrocytes may represent a means of sequestering fatty acids. Unbound fatty acids otherwise are known to drastically suppress glucose uptake and the production of GLUT2 glucose transporters [9,20,44].

If, as this study suggests, astrocytes have an important role in brain glucose-sensing, then an age-related astrocyte pathology such as that seen in GP astrocytes could have important implications for age-related neuroendocrine disturbances in humans. Monitoring of blood glucose by the brain may contribute to the regulation of food intake in humans as well as in rats [28]. Accumulation of mitochondrial damage in GP astrocytes could compromise glucose-sensing by the brain and could contribute to age-related changes in hypothalamic function. Thus, more study of the role of astrocytes in brain glucose sensing seems warranted.

Acknowledgments

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