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Elevated Expression of Glucose Transporter-1 in Hypothalamic Ependymal Cells not Involved in the Formation of the Brain–Cerebrospinal Fluid Barrier

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Abstract Glucose transporters play an essential role in the acquisition of glucose by the brain. Elevated expression of glucose transporter-1 has been detected in endothelial cells of the blood–brain barrier and in choroid plexus cells of the blood–cerebrospinal fluid barrier. On the other hand, there is a paucity of information on the expression of glucose transporters in the ependymal cells that line the walls of the cerebral ventricles. The tanycytes are specialized ependymal cells localized in circumventricular organs such as the median eminence that can be segregated into at least three types, α, β1 and β2. The β2 tanycytes form tight junctions and participate in the formation of the cerebrospinal fluid–median eminence barrier. Using immunocytochemistry and in situ hybridization, we analyzed the expression of hexose transporters in rat and mouse hypothalamic tanycytes. In both species, immunocytochemical analysis revealed elevated expression of glucose transporter-1 in α and β1 tanycytes. Intense anti-glucose transporter-1 staining was observed in cell processes located throughout the arcuate nucleus, in the end-feet reaching the lateral sulcus of the infundibular region, and in cell processes contacting the hypothalamic capillaries. On the other hand, there was very low expression of glucose transporter-1 in β2 tanycytes involved in barrier function. In contrast with the results of the cytochemical analysis, in situ hybridization revealed that tanycytes α, β1, and β2 express similar levels of glucose transporter-1 mRNA. Further analysis using anti-glial fibrillary acidic protein antibodies to identify areas rich in astrocytes revealed that astrocytes were absent from areas containing α and β1 tanycytes, but were abundant in regions containing the barrier-forming β2 tanycytes. Overall, our data reveal a lack of correlation between participation in barrier function and expression of glucose transporter-1 in hypothalamic tanycytes. Given the virtual absence of astrocytes in areas rich in α and β1 tanycytes, we speculate whether the tanycytes might have astrocyte-like functions and participate in the metabolic coupling between glia and neurons in the hypothalamic area. J. Cell. Biochem. 80:491–503, 2001.

Key words: glucose transporter; GLUT1; tanycytes; median eminence; hypothalamus; barrier

Seven facilitative glucose transporters have been molecularly cloned [Birnbaum, 1989; Doege et al., 2000; Kayano et al., 1988, 1990; Mueckler et al., 1985; Phay et al., 2000; Thorens et al., 1988; Waddell et al., 1992]. The glucose transporters transport glucose and other hexoses down a concentration gradient by a saturable and stereospecific non-energy-dependent process. Additionally, the glucose transporters are also efficient transporters of dehydroascorbic acid, the oxidized form of vitamin C [Rumsey et al., 1997; Vera et al., 1993]. Because the metabolism of brain cells depends on a continuous supply of glucose, the regulated expression and function of the glucose transporters are central to maintaining

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normal brain physiology. The glucose transporter-1 (GLUT1) is widely expressed and appears to function in mediating the basal uptake of glucose in most cells and tissues. GLUT1 is highly expressed in erythrocytes and in cells that participate in barrier function. In the brain, GLUT1 is expressed at high levels in endothelial cells that form the blood–brain barrier and in epithelial cells of the choroid plexus that form the cerebrospinal fluid–blood barrier [Farrell and Pardridge, 1991; Gerhart et al., 1995; Harik et al., 1990; Kalaria et al., 1988; Nualart et al., 1999]. GLUT1 is expressed to a much lesser degree in neurons and astrocytes, an observation that led to the concept that elevated expression of GLUT1 may be a property specific to cells that participate in the formation of the different brain barriers [Kalaria et al., 1988]. However, the expression of GLUT1 in other brain cells like oligodendroglia, microglia, and specialized ependymal cells other than choroid plexus cells has not been analyzed in detail, and therefore the use of GLUT1 as a marker of barrier formation needs further analysis [Maher et al., 1994; Morgello et al., 1995]. For example, there is a third barrier in the brain that separates the median eminence in the hypothalamus from the cerebrospinal fluid of the third ventricle [Krish and Leonhardt, 1978]. Although the upper and middle regions of the third ventricle walls are lined by classical ciliated ependymal cells, the lower lateral walls and the floor are lined by tanycytes, cells that differ from classical ependymal cells in many respects (Fig. 1) [Flament-Durand and Brion, 1985; Rodríguez et al., 1979]. The median eminence–cerebrospinal fluid barrier is formed by specialized ependymal cells located in the floor of the third ventricle lining the median eminence, the β2 tanycytes (Fig. 1). The proximal part of the β2 tanycytes is in contact with the cerebrospinal fluid of the third ventricle, while the dorsal part of the cells forms processes where the end-feet reach the pial surface of the brain or the local capillary plexus in the median eminence. The β2 tanycytes develop tight junctions that form the cerebrospinal fluid-median eminence barrier [Chauvet et al., 1995; Flament-Durand and Brion, 1985]. Although the presence of the tanycytes is not restricted to the third ventricle floor, the tanycytes that cover the lower lateral wall of the third ventricle do not develop tight junctions in the ventricular area and therefore do not participate, by this criterion, in the formation of a hypothalamus–CSF barrier. The tanycytes located in the lower lateral wall of the third ventricle are classified as α and β1 (Fig. 1) [Akmayev and Popov, 1977]. The β1 tanycytes are located in the lateral lower part of the third ventricle and develop elongated cell processes that form a bow through the arcuate nucleus and reach the lateral sulcus of the infundibular region; the cell processes also make contact with local capillary walls [Flament-Durand and Brion, 1985]. The α tanycytes line the lower lateral walls of the third ventricle facing the ventromedial and arcuate nucleus of the hypothalamus and their elongated cell processes make contact with blood vessels and neurons [Akmayev and Popov, 1977; Flament-Durand and Brion, 1985; Kobayashi et al., 1972].

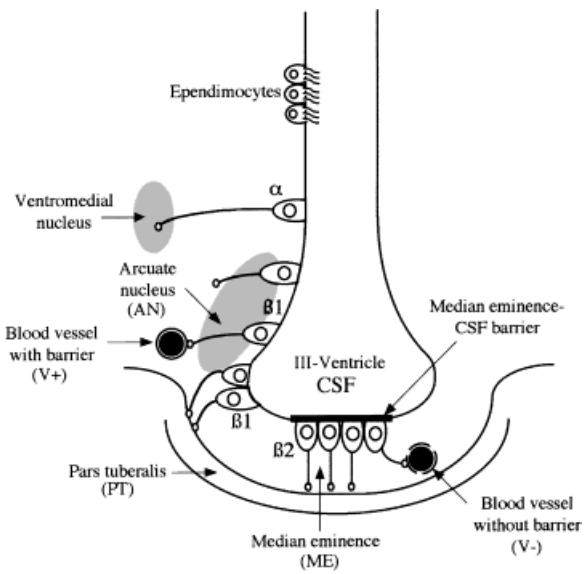

Fig. 1. Schematic representation of rat hypothalamus. The following cellular elements are highlighted in the figure in descending order: (1) The ciliated ependimocytes lining the rostral wall of the third ventricle; (2) the α tanycytes with projections contacting neurons of the ventromedial and arcuate nuclei and blood vessels; (3) the β1 tanycytes located in the lower lateral wall of the third ventricle, with projections contacting neurons of the arcuate nuclei, blood vessels, and the external part of the brain; (4) the β2 tanycytes that cover the floor of the third ventricle with tight junctions that are part of the median eminence–cerebrospinal fluid barrier. The projections of the β2 tanycytes contact the median eminence blood vessels that are characterized by the absence of a blood–brain barrier and the external part of the median eminence. CSF, cerebrospinal fluid.

The function of the tanycytes remains a matter of speculation. Given their special anatomical location, it has been suggested that they could provide a link between the cerebrospinal fluid and the portal vessels, and that they could be involved in regulating neuroendocrine function [Akmayev and Fidelina, 1974; Flament-Durand and Brion, 1985; Kobayashi et al., 1972; Mathew and Singh, 1989; Meister et al., 1988; Nakai and Naito, 1975; Pilgrim, 1978; Rodríguez et al., 1985]. An immunocytochemical analysis of glucose transporter expression in the brain revealed the presence of GLUT1 in hypothalamic tanycytes [Harik et al., 1990]. The elevated expression of GLUT1 in the tanycytes was interpreted as reflecting the participation of these cells in barrier formation, but no detailed localization or cell identification data were provided to support this claim [Farrell and Pardridge, 1991; Harik et al., 1990; Kalaria et al., 1988; Nualart et al., 1999]. In this work, we analyzed the expression of glucose transporters in cerebral tanycytes using immunohistochemistry and in situ hybridization. We determined the precise localization and identified the subpopulation of tanycytes that express high levels of GLUT1 in the hypothalamus of rat and mouse brains. Our results indicate that α and β1 tanycytes express GLUT1, with the highest levels of GLUT1 present in cell processes making contact with local capillary walls. Because α and β1 tanycytes do not appear to have a barrier function and are located in a region of the hypothalamus very poor in astrocytes, we speculate whether these cells might have astrocyte-like functions and facilitate metabolic coupling between glia and neurons in the hypothalamic area. Our data also indicate that β2 tanycytes forming tight junctions at the level of the median eminence express low levels of GLUT1, suggesting that the metabolic and/or transport activity of the ependymal cells participating in the median eminence–cerebrospinal fluid barrier is different from that of endothelial and choroidal plexus that form the blood–brain and the cerebrospinal fluid–blood barriers.

EXPERIMENTAL PROCEDURES

Immunocytochemistry

Rat (Holtzman) and mice (C57BL/J6) brains were dissected and fixed directly by immersion in Bouin's solution, or were fixed in situ using vascular perfusion. Samples were dehydrated in graded alcohol solutions and embedded in paraffin. Frontal sections (5 μm) of the hypothalamic area were obtained and mounted on poly-L-lysine-coated glass slides. Alternatively, thick (40 μm) transverse sections were cut with a cryostat and the sections were processed free-floating. Before immunostaining, the sections were treated with absolute methanol and 3% hydrogen peroxide to inactivate endogenous peroxidase activity.

For immunohistochemical analyses we used a panel of anti-peptide antibodies specific to each isoform of the human facilitative hexose transporters [Nualart et al., 1999]. Sections were incubated with anti-GLUT antibodies (1:200–1:1000) overnight at room temperature in a humid chamber. The antibodies were diluted in buffer Tris–HCl (pH 7.8) containing 8.4 mM sodium phosphate, 3.5 mM potassium phosphate, 120 mM NaCl, and 1% bovine serum albumin. After extensive washing, the sections were incubated for 2 h at room temperature with anti-rabbit IgG (1:50; Sigma Chemical Co, Madison, WI) or with anti-mouse IgG (1:50; Sigma), followed by a 30 minute incubation with PAP complex (1:100; Sigma). The peroxidase activity was developed using a metal-enhanced DAB. Alternatively, some slides were incubated for 1 h at room temperature with secondary antibodies labeled with fluorescein isothiocyanate (1:30; Sigma), and the slides were used for image digitalization using an Axon Instrument image analysis system. As controls, we utilized both primary antibodies pre-absorbed with the respective peptides used to elicit them and preimmune serum. To further characterize the cells displaying immunoreactivity toward the different anti-GLUTs, serial sections were immunostained using an anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:100; Sigma).

In Situ Hybridization

A cDNA of approximately 2.5 kb subcloned in pcDNA3 and encoding the human GLUT1 was used to generate sense and antisense digoxigenin-labeled riboprobes. RNA probes were labeled with digoxigenin-UTP by in vitro transcription [Nualart et al., 1998], and the probe sizes were reduced to approximately 300 nucleotides by alkaline hydrolysis. In situ hybridization was done on serial hypothalamic frontal sections mounted on poly-L-lysine-coated glass

slides. The sections were baked at 60°C for 1 h, deparaffinized in xylene, and rehydrated in graded ethanols. Following proteinase K treatment (5 min at 37°C in PBS, 1 μg/ml proteinase K), the tissue sections were fixed with 4% p-formaldehyde for 5 min at 4°C, washed in cold PBS and then acetylated in 0.1 M triethanol amine-HCl (pH 8.0) at room temperature for 10 min. After a brief wash, the sections were incubated in pre-hybridization solution for 15 min at 37°C, and then 25 μl of the hybridization mix (50% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 × Denhart's solution, 10% PEG 8000, 10 mM DTT, 500 μg yeast tRNA/ml, 50 μg/ml heparin, 500 μg/ml DNA carrier, and 1:20 to 1:100 diluted riboprobe) were added to each slide. The slides were covered with glass coverslips and placed in a humidified chamber at 42°C overnight. After removal of the coverslip, the slides were rinsed in 4 × SSC and then incubated twice for 30 min at 42°C. The slides were washed at 37°C for 30 min each in 2 × SSC, 0.3 × SSC and 0.1 × SSC [Sambrook et al., 1989]. Visualization of digoxigenin was performed by incubation with a monoclonal antibody coupled to alkaline phosphatase (anti-digoxigenin-alkaline phosphatase Fab fragments diluted 1:500) for 2 h at room temperature. Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate were used as substrates for the alkaline phosphatase [García et al., 1998]. Controls included use of the sense riboprobe and omission of the probe.

RESULTS

GLUT1 Immunocytochemistry of Rat Hypothalamus

In initial studies we analyzed the expression of facilitative hexose transporters in the hypothalamus of adult rats using immunocytochemical detection with anti-GLUT polyclonal antibodies. Prominent anti-GLUT1 immunoreactivity was observed in the hypothalamic area, and the immunoreactive material was associated with endothelial cells, neurophil and tanycytes (Fig. 2A). No consistent immunoreactivity for other hexose transporter isoform antibodies was observed in neurons, endothelial or other glial cells of the hypothalamus (data not shown), and therefore we restricted all further analysis to the expression of GLUT1. We examined with special detail the immunoreaction showed by the tanycytes in order to precisely identify the cell types that express GLUT1. The α and β1 tanycytes showed positive anti-GLUT1 immunoreactivity, with the β1 cells showing the greatest immunostaining (Fig. 2A,B). On the other hand, β2 tanycytes that are involved in the formation of the median eminence–CSF barrier were negative for GLUT1 (Fig. 2A). The β1 tanycytes showed a marked immunoreaction with anti-GLUT1 at the level of the proximal part of the cells, in cell processes forming a bow through the arcuate nucleus, and in the end-feet reaching the brain pial surface (Fig. 2B). The greatest immunoreaction was observed in cell processes that contact and coil around the capillaries (Fig. 2D,E). In sagital sections of the hypothalamus we observed β1 tanycyte processes making what appears to be direct contact with neurons of the arcuate nucleus (Fig. 2B, arrows). The α tanycytes showed intense immunostaining in processes located in the arcuate nucleus and projecting to the ventromedial nucleus (Fig. 2A,F).

There was intense anti-GLUT1 immunoreactivity in endothelial cells involved in the formation of the blood–brain barrier (Fig. 2A,B, D, E, labeled V+). In contrast, the endothelial cells of the central and lateral median eminence that do not participate in the formation of the blood–brain barrier were negative for GLUT1 (Fig. 2A, labeled V−). Similarly, there was no positive anti-GLUT1 reaction in the pars tuberalis or in the blood vessels observed in this area (Fig. 2A).

The initial immunohistochemical data are consistent with the absence of GLUT1 expression in the β2 tanycytes, which is in apparent contradiction with the barrier function of these cells. However, one problem associated with immunocytochemical methods is a moderate level of sensitivity, and therefore, lack of immunoreactivity may simply reflect the low sensitivity of the technique rather than absence of antigen expression. To analyze the expression of GLUT1 with increased sensitivity, we used immmunofluorescent detection followed by image digitalization–amplification to increase the signal-to-background ratio. These experiments confirmed that GLUT1 is highly expressed in the α (Fig. 2F) and β1 tanycytes (data not shown). This analysis also revealed a low level of GLUT1 expression in β2 tanycytes, evidenced by a low level of staining that was,

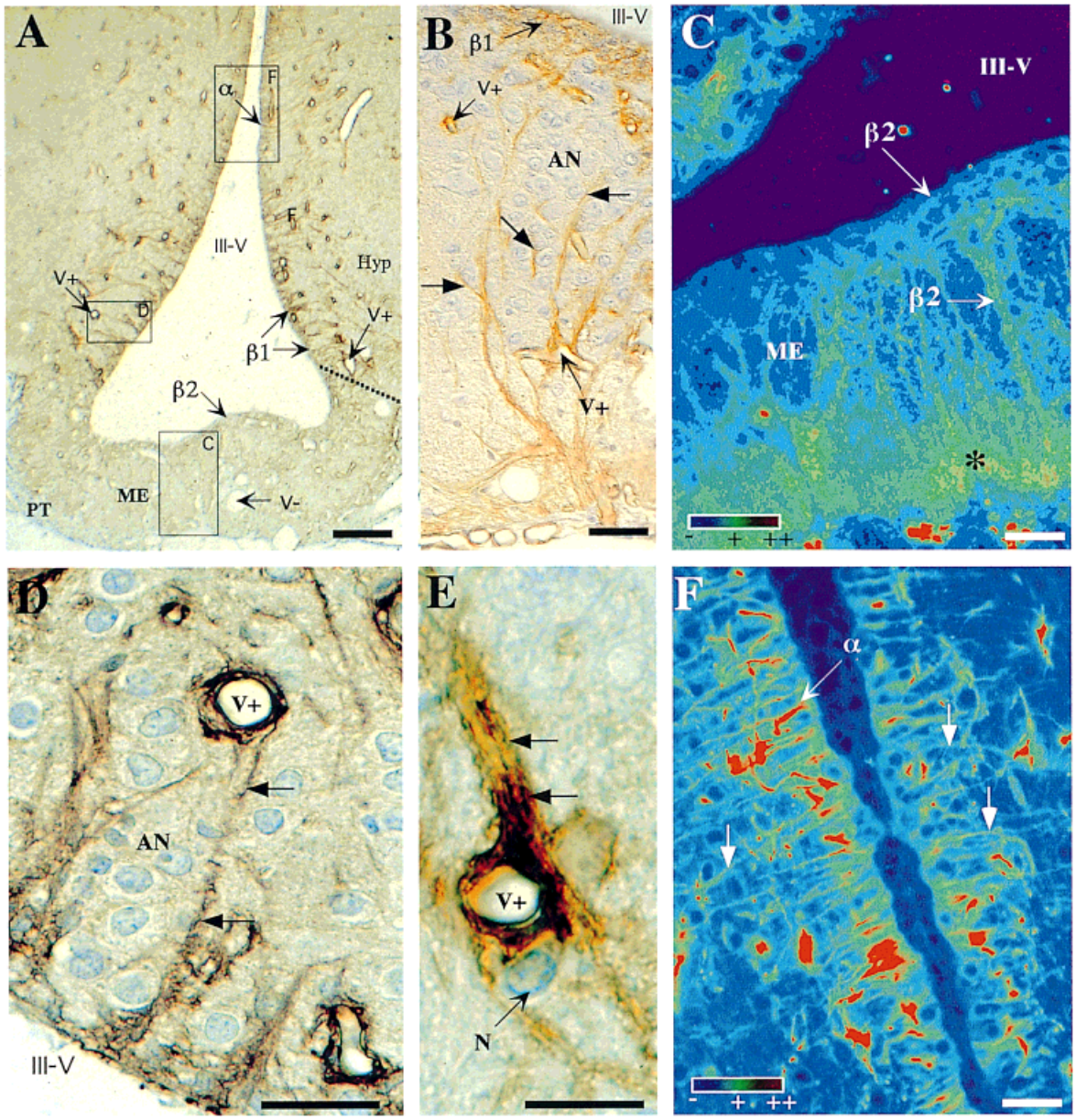

Fig. 2. GLUT1 expression in rat hypothalamus. The sections were incubated with anti-GLUT1 and the immunoreaction was developed using the PAP method (A,B, D and E), or immunofluorescence followed by image digitalization and signal amplification (C, F). **A**: Frontal section of rat hypothalamus. **B**: Sagital section of rat hypothalamus. The approximate orientation of the section is indicated by the dotted line in (A). **D,E**: High magnification of regions containing β1 tanycytes in panel (A). Using the PAP method, the α and β1 tanycytes show a marked immunostaining with anti-GLUT1 in both the frontal and sagital section of the hypothalamus, while the β2 tanycytes are negative. In α and β1 tanycytes, the reaction is intense in the proximal part of the cells that make contact with the cerebrospinal fluid (A,B). The β1 tanycytes show an intense reaction in the processes that form a bow through the arcuate nucleus (filled arrows in B), and in the end-feet that reach the blood vessels (filled arrows in D,E). **C**: Frontal section of rat hypothalamus. The image corresponds to high magnification of an area similar to that contained in box C in panel (A). The β2 tanycytes show a low level of immunoreaction in the anterior region of the cells, with a stronger signal at the level of the cell processes and end-feet (C, asterisk). **F**: Frontal section of the dorsal rat hypothalamus. The image corresponds to a high magnification of an area similar to that contained in box F in panel (A). The α tanycytes show a strong signal in the anterior area of the cells and in the cell processes (filled arrow in F). III-V, third ventricle; N, neuron; V+, blood vessel positive for anti-GLUT1; V−, blood vessel anti-GLUT1 negative. AN, arcuate nucleus; Hyp, hypothalamus; ME, median eminence; PT, Pars tuberalis. Scale bars in A: 250 μm; in B and C: 50 μm; in C and F: 100 μm; in D: 45 μm; in E: 25 μm.

however, clearly different and more intense than the background signal (Fig. 2C). The immunostaining was more intense in cell processes whose end-feet reached the pial surface of the brain or the local capillary plexus in the median eminence (Fig. 2C, asterisk). In conclusion, the lack of anti-GLUT1 immunoreactivity in the β2 tanycytes observed when using conventional immunohistochemistry is due not to absence of but to a very low level of GLUT1 expression in these cells.

GLUT1 Immunocytochemistry of Mouse Hypothalamus

To determine the degree of species-specificity of the above results, we analyzed the expression and distribution of GLUT1 in mouse hypothalamic tanycytes. The pattern of anti-GLUT1 immunoreactivity was similar to that observed in the rat hypothalamus, with the β1 tanycytes showing a strong anti-GLUT1 immunoreactivity (Fig. 3A). The most intense immunostaining in β1 tanycytes was localized at the level of the end-feet that reach the brain pial surface (Fig. 3B). Moreover, and confirming the results observed with rat tanycytes, the mouse β2 tanycytes showed no anti-GLUT1 immunoreactivity, suggesting low-to-absent expression of GLUT1 in these cells (Fig. 3A,B).

To confirm the above findings, we increased the level of immunodetection sensitivity by performing immunostaining in 40 μm thick free-floating sections. This analysis revealed an intense anti-GLUT1 immunoreaction in the proximal part of β1 tanycytes that contact the cerebrospinal fluid, and an even stronger immunoreaction in the end-feet contacting the external membrane of the brain (Fig. 3C,D). However, although the mouse β1 tanycytes end-feet (Fig. 3A) showed a much more intense immunoreaction with anti-GLUT1 than rat β1 tanycytes end-feet (Fig. 2A,B), there was less evidence of mouse β1 tanycyte processes with an intense anti-GLUT1 staining contacting the blood vessels compared to the corresponding rat cells (Fig. 3C,D). Similar to our observation in the rat hypothalamus, the cell processes of β1 tanycytes positive for GLUT1 formed a bow that was in apparent contact with neurons of the arcuate nucleus. The mouse α tanycytes also showed a positive reaction with anti-GLUT1, but the intensity of the immunoreaction at the level of the cell processes was clearly less marked than that observed for rat α tanycytes (Figs. 2A and 3A).

There was also a positive anti-GLUT1 immunoreaction in β2 tanycytes, although to a lesser degree than β1 tanycytes, confirming the low expression of GLUT1 in β2 tanycytes (Fig. 3C). A low level anti-GLUT1 immunoreaction was also detected in the external part of the median eminence where β2 tanycyte end-feet are located (Fig. 3C, arrows).

GLUT1 In Situ Hybridization

Overall, the immunohistochemical data show high-level GLUT1 expression in rat and mouse hypothalamus α and β1 tanycytes, as compared with very low GLUT1 expression in β2 tanycytes. To gain a better understanding of the regulatory processes involved in the preferential expression of GLUT1 in α and β1 tanycytes, we analyzed the expression of GLUT1 at the mRNA level by in situ hybridization using digoxigenin-labeled cRNA probes specific for GLUT1. In mouse brains we detected a positive reaction in the ependymal cells of the choroid plexus and in endothelial cells of the cerebral microcapillaries, although the endothelial cells were not always positive (data not shown). There was also intense labeling of neurons located in different areas of the mouse brain, with the most strongly labeled neuronal areas corresponding to the hyppocampus and the brain cortex, with the neurons of the hypothalamus showing an intense reaction (Fig. 4A,B). Limitations inherent to the in situ hybridization technique precluded us to determine with a minimal degree of certainty whether there was labeling of astrocytes and other glial cells in these experiments. On the other hand, both sets of hypothalamic tanycytes, the α and the β1–β2, showed a positive hybridization signal (Fig. 4A–C). There were no apparent differences in the intensity of labeling of α and β1–β2 tanycytes, suggesting the presence of equivalent levels of GLUT1 mRNA in all the cells, which is different from the immunolocalization experiments indicating a markedly increased expression of GLUT1 in α and β1 tanycytes as compared to β2 tanycytes. Control experiments using a sense riboprobe in serial sections revealed no labeling of the tanycytes, confirming the specificity of the reaction with the GLUT1 antisense riboprobe (Fig. 4D). A similar analysis revealed the presence of mRNA for GLUT1 in rat hypothalamic α, β1, and β2

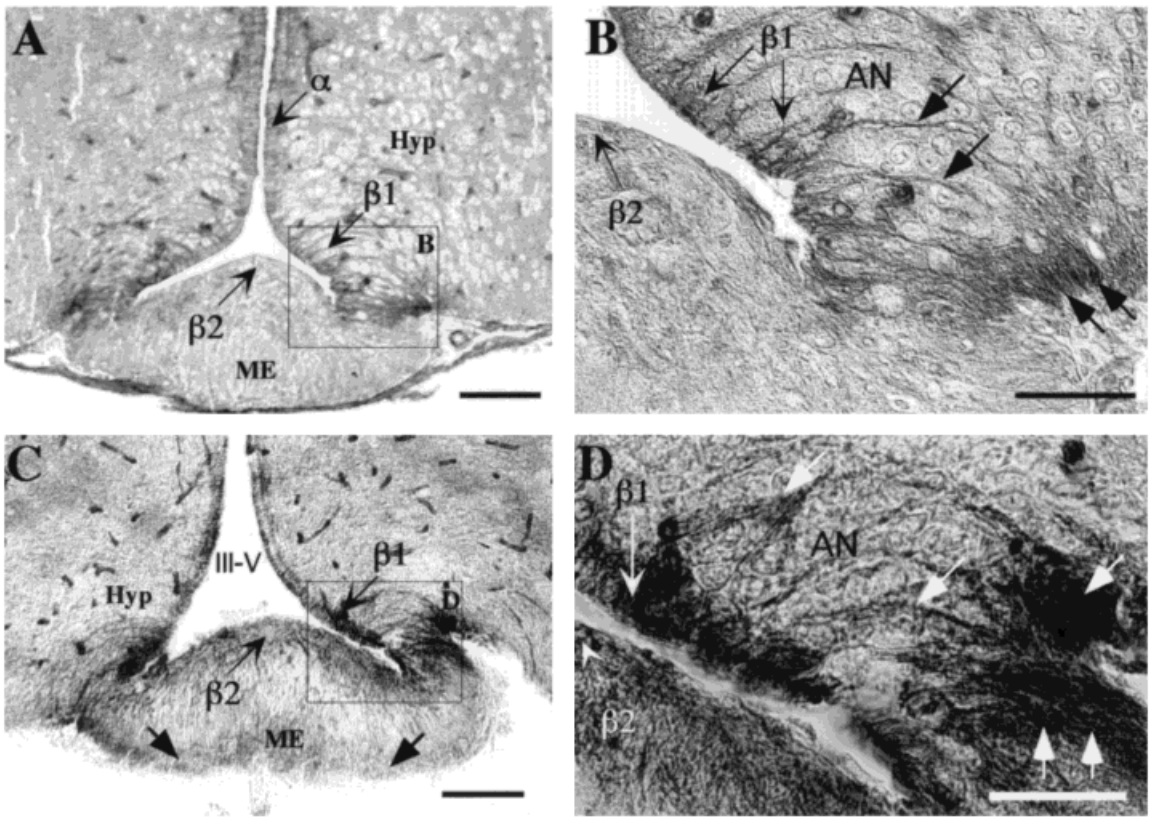

Fig. 3. GLUT1 expression in mouse hypothalamus. The sections were incubated with anti-GLUT1 and developed using the PAP method. **A**: Thin, 7 μm frontal section of mouse hypothalamus. The α and β1 tanycytes show positive immunostaining with anti-GLUT1, while the β2 tanycytes are negative. The greatest intensity of staining is observed at the level of the cell processes and the end-feet. **B**: High magnification of the lateral area of the hypothalamus. The image corresponds to a higher magnification of the box in (A). The filled arrows indicate processes and end-feet of the β1 tanycytes. **C**: Thick, 40 μm frontal section of mouse hypothalamus. The β1 tanycytes show a marked immunostaining with anti-GLUT1, while the β2 tanycytes are positive but to a lesser degree. The filled arrows indicate the β2 end-feet. **D**: High magnification of the lateral area of the hypothalamus. The image corresponds to a higher magnification of the box in (C). The filled arrows indicate the cell processes of the β1 tanycytes. AN, arcuate nucleus; ME, median eminence; III-V, third ventricle; Hyp, hypothalamus. Scale bars in A and C: 200 μm; B: 100 μm; D: 50 μm.

tanycytes, with all the cells showing a similar hybridization signal intensity (Fig. 4E,F).

GFAP Immunocytochemistry

Our data indicate that, in rat and mouse hypothalamus, there is preferential expression of GLUT1 in α and β1 tanycytes as compared to β2 tanycytes. Given the close association of α and β1 tanycytes with the hypothalamic neurons, and in order to better understand the possible metabolic implications of these findings, we analyzed the expression of glial fibrillary acidic protein (GFAP), an astrocyte marker, in the hypothalamus of rat and mouse brains. In rat brain, high expression of GFAP was detected in astrocytes present in the subependymal zone of the third ventricle (data not shown), and also in the median eminence (Fig. 5A), a region that is rich in β2 tanycytes which show low GLUT1 expression. On the other hand, no anti-GFAP immunoreactive material was observed in the periventricular hypothalamic area where α and β1 tanycytes showed the greatest expression of GLUT1 (Fig. 5A and data not shown), suggesting an association between elevated expression of GLUT1 in these tanycytes and absence of astrocytes.

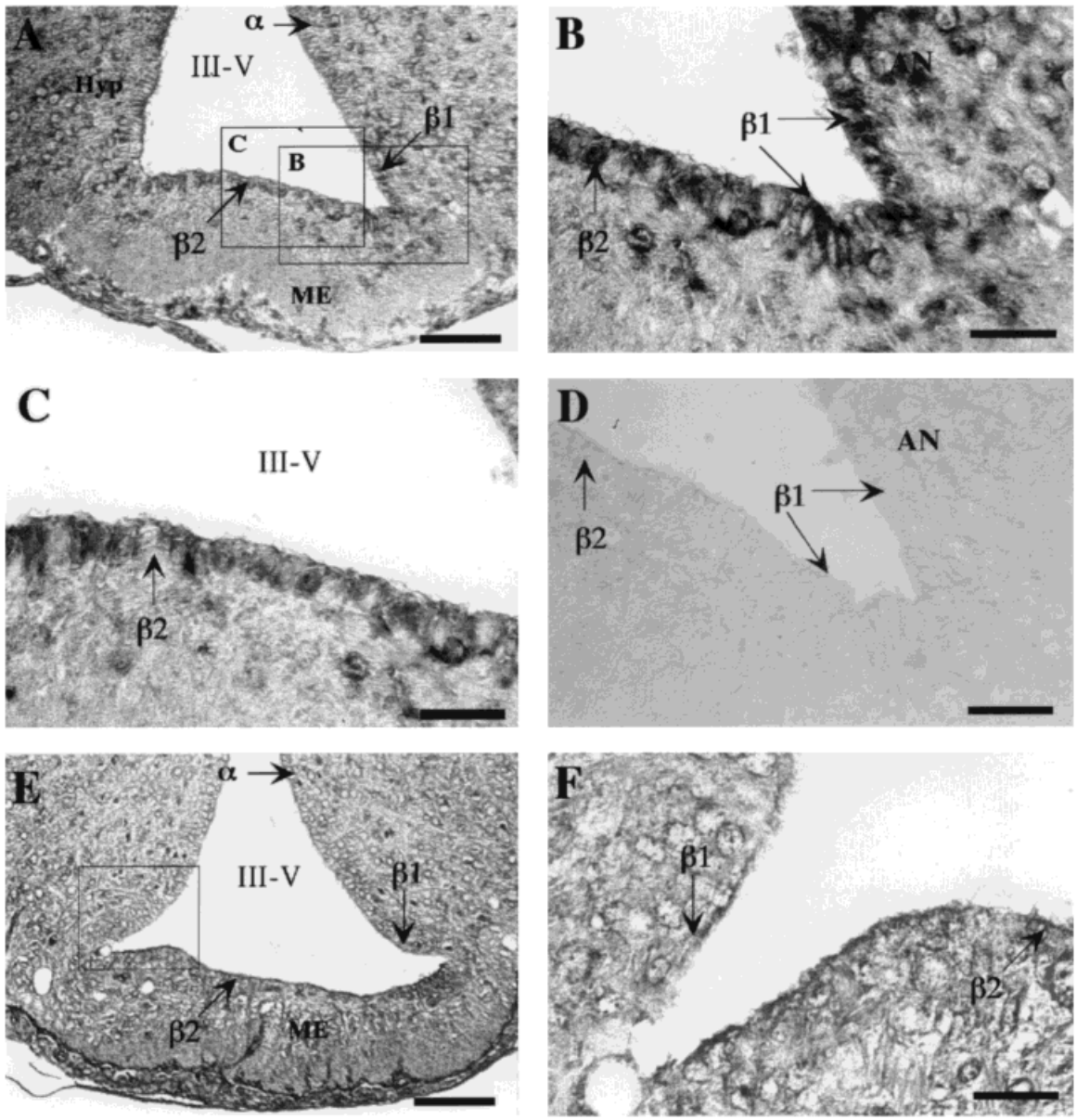

Fig. 4. GLUT1 mRNA detection by in situ hybridization. **A–D**: Frontal section of mouse hypothalamus probed with a GLUT1 riboprobe. The α, β1, and β2 tanycytes show positive hybridization with the antisense GLUT1 riboprobe (A–C), while no hybridization signal is evident when using the GLUT1 sense riboprobe (D). Panels B and C are higher magnifications of boxes labeled B and C in panel (A). **E,F**: Frontal section of rat hypothalamus probed with a GLUT1 riboprobe. The α, β1, and β2 tanycytes show positive hybridization with the antisense GLUT1 riboprobe (E,F). AN, arcuate nucleus; ME, median eminence; III-V, third ventricle; Hyp, hypothalamus. The arrows indicate the approximate location of the different tanycyte types. Scale bars in A: 150 μm; in E: 100 μm; in C, D, and F: 50 μm.

Although analysis is difficult due to the small size of the brain area of interest, a similar pattern of anti-GFAP immunoreactivity was observed in the mouse hypothalamus (Fig. 5B). Elevated anti-GFAP immunoreactivity was observed in the subependimal zone of the third ventricle (data not shown) and the astrocytes of the median eminence (Fig. 5B). Moreover, and confirming the results observed with the rat hypothalamus, a low level of negative anti-

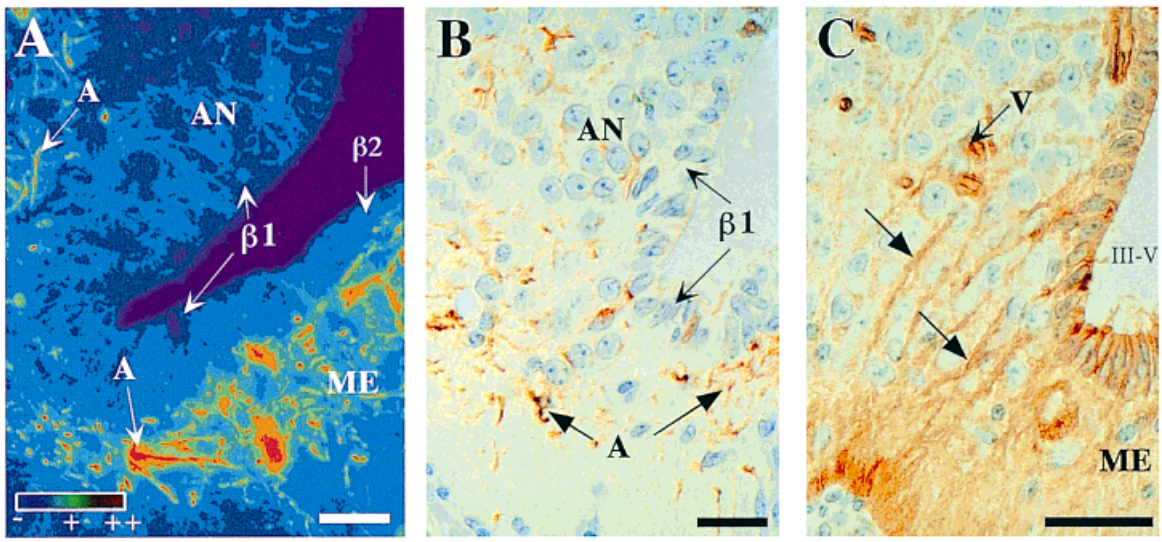

Fig. 5. Glial-fibrillary acidic protein detection in rat hypothalamus. **A**: Frontal section of rat hypothalamus stained with anti-GFAP and developed using immunofluorescence followed by image digitalization and signal amplification. **B**: Frontal section of mouse hypothalamus stained with anti-GFAP and developed using the PAP method. Staining with anti-GFAP is detected in astrocytes of the median eminence. No immunoreaction is detected in hypothalamic areas with high expression of GLUT1. **C**: Frontal section of mouse hypothalamus stained with anti-GLUT1. The filled arrows indicate the cell processes of the β1 tanycytes. A, Astrocytes; AN, arcuate nucleus; V, blood vessel; III-V, third ventricle; ME, median eminence. Scale bars in A: 150 μm; in B and C: 100 μm.

GFAP immunoreactivity was observed in the periventricular hypothalamic area containing α and β1 tanycytes (Fig. 5B) that showed high level expression of GLUT1 (Fig. 5C).

DISCUSSION

We performed a detailed immunohistochemical and in situ hybridization analysis of the expression of glucose transporters in hypothalamic tanycytes. We determined the precise localization and identified the subpopulation of tanycytes that express high levels of glucose transporters in the hypothalamus of rat and mouse brain. Although there are seven different glucose transporter isoforms, all of which have been found to be expressed in different cells of the brain, our data revealed that GLUT1 is the main glucose transporter isoform expressed in the hypothalamic tanycytes. Moreover, the data indicate that the tanycytes can be classified in two groups depending on their level of GLUT1 expression at the protein level:

1. Cells that express high levels of GLUT1 which include tanycytes α and β1, which are cells that do not appear to be involved in barrier function;
2. Cells that express very low levels of GLUT1.

This last group is limited to β2 tanycytes that form tight junctions at the level of the median eminence and are involved in the formation of the median eminence–cerebrospinal fluid barrier. In contrast with the immunohistochemical data indicating preferential expression of GLUT1 by α and β1 tanycytes as compared to β2 tanycytes, the results of the in situ hybridization analysis revealed a similar level of GLUT1 mRNA expression in the different tanycytes.

Data from a number of localization studies have been interpreted as suggesting that, in the brain, GLUT1 is preferentially expressed at high levels in cells involved in brain barrier function [Brant et al., 1993; Farrell and Pardridge, 1991; Harik et al., 1990; Kalaria et al., 1988; Nualart et al., 1999]. There are some functional data supporting this concept. Glucose plays an essential role in the maintenance of normal brain metabolism, which is characterized by an almost absolute dependence on a continuous external glucose supply. Glucose is obtained from the blood and therefore it must be efficiently transported across the barriers that separate the brain from the

bloodstream. The transport of glucose across the blood–brain barrier is mediated by GLUT1, a member of the facilitative glucose transporter family that is characterized by transporting their substrates down a concentration gradient. GLUT1 is abundantly expressed in endothelial cells that are part of the blood–brain barrier and also in epithelial cells of the choroid plexus that form the blood–cerebrospinal barrier. There is also evidence from an immunolocalization study suggesting elevated expression of GLUT1 in tanycytes involved in barrier formation [Harik et al., 1990]. The mentioned study revealed a general positive anti-GLUT1 immunoreactivity in the lower lateral walls of the third ventricle, therefore including α and β1 tanycytes, but the authors failed to identify the cells positive for GLUT1 and provided no direct evidence of the expression of GLUT1 in β2 tanycytes. The results of the more detailed study presented here indicate low-to-non-existent expression of GLUT1 by β2 tanycytes, and therefore our data are in apparent contradiction with the concept that there is a direct correlation between the formation of a brain–fluid barrier and elevated expression of GLUT1. The β2 tanycytes are located in the floor of the third ventricle, where they form tight junctions and are directly involved in the formation of the median eminence–cerebrospinal fluid barrier [Akmayev et al., 1973; Krish and Leonhardt, 1978]. In fact, GLUT1 is expressed at such a low level in β2 tanycytes that its presence was detected only by amplifying the signal using digitalization and computer-assisted methods. We established that the elevated expression of GLUT1 is restricted to α and β1 tanycytes, cells that are located in the lower lateral wall of the third ventricle and do not participate in the formation of the brain–cerebrospinal barrier [Akmayev and Popov, 1977]. It is possible that β2 tanycytes may express a different glucose transporter isoform that could replace GLUT1 in these cells. Our data, however, are consistent with the very low-to-non-existent expression of GLUT2, GLUT3, GLUT4, or GLUT5 in these cells. The results of a previous in situ hybridization analysis suggested the presence of GLUT2 mRNA in ependymal cells that cover the hypothalamic ventricular wall [Alvarez et al., 1996]; however, no identification of the cells was provided and the hybridization analysis was not followed by an immunolocalization study. In conclusion, our data indicate that GLUT1 is the main glucose transporter expressed by ependymal tanycytes and therefore plays a central role in providing these cells with glucose.

When analyzed in combination, the immunohistochemical and the hybridization data are consistent with the concept that the level of GLUT1 expression in tanycytes is probably regulated at a post-transcriptional level. Thus, although the hybridization data failed to reveal any differences in the level of GLUT1 mRNA in α and β1 tanycytes as compared to the β2 tanycytes, the immunohistochemical data revealed elevated expression of GLUT1 in α and β1 tanycytes as compared with absent-to-very-low expression in β2 tanycytes. Whether the differential expression is due to differences in the rate of translation of the mRNA or the stability of the protein, or both, cannot be assessed from our data.

The very low expression of GLUT1 in β2 tanycytes strongly suggests that these cells do not have the capacity to transport glucose efficiently from the cerebrospinal fluid to the median eminence. In other words, β2 tanycytes must have functional and metabolic properties that make them very different from the cells that form the blood–brain and the blood–cerebrospinal barriers, cells that show elevated GLUT1 expression. In this context, the median eminence–cerebrospinal fluid barrier does not fit the classical definition of a fluid–cerebral tissue barrier because it is unable to mediate the transport of glucose from the cerebrospinal fluid into the median eminence with high efficiency. This is probably due to the fact that the endothelial cells of the blood vessels in the median eminence do not form tight junctions and lack a functional blood–brain barrier and therefore soluble components such as glucose diffuse freely from the blood into the brain tissue.

Our data raise the question of the role of GLUT1 in the physiology of α and β1 tanycytes. The expression analysis demonstrated that α and β1 tanycytes express high levels of GLUT1, which endow these cells with the capacity to take up glucose very efficiently. Although no information is currently available on the metabolic activity of the tanycytes and how it may be regulated, ultrastructural analyses have shown the presence of glycogen in these cells [Akmayev and Popov, 1977; Rodríguez et al.,

1979]. This observation suggests that α and β1 tanycytes may have an elevated capacity to take up and metabolize glucose. The uptake of glucose may be facilitated by the fact that these cells are in contact with the cerebrospinal fluid and blood vessels, and both contact sites are rich in GLUT1. The high metabolic capacity of tanycytes is supported by ultrastructural analysis that revealed the presence of a well-developed mitochondrial apparatus [Flament-Durand and Brion, 1985], including the cell processes where we detected the highest signal for GLUT1. It has also been described that the tanycytes have lipid inclusions throughout the full length of the cell [Flament-Durand and Brion, 1985; Rodríguez et al., 1979].

Our data may shed light on the possible functions of tanycytes. The immunohistochemical data indicated that α and β1 tanycytes, all cells showing elevated expression of GLUT1, are present in hypothalamic areas that are rich in neurons but present very few astrocytes. It has been established that astrocytes have an important function in metabolic coupling between the glia and neurons [Tsacopoulos and Magistretti, 1996]. Thus, one intriguing possibility is that tanycytes might have astrocyte-like functions and facilitate metabolic coupling between glia and neurons in the hypothalamus. This proposal is consistent with the very special anatomic localization of the tanycytes, whose processes and end-feet are rich in GLUT1 and make contact with the cerebrospinal fluid, blood vessels, and neurons. There is also evidence from a recent study indicating that tanycytes present in the mediobasal hypothalamus have the remarkable property of supporting neuron survival and axonal regeneration [Chauvet et al., 1995, 1996]. Moreover, deafferentation of the medial basal hypothalamus produced an increase in the metabolic activity of tanycytes [Akmayev and Popov, 1977; Akmayev et al., 1973], an observation that is in line with the reported mobilization of glucose in astrocytes during neuronal activity via receptor-mediated mechanisms [Tsacopoulos and Magistretti, 1996]. It is also possible that β1 tanycytes may be directly involved in the formation of a barrier. In vivo studies utilizing injection of peroxidase and other high molecular weight compounds unable to cross the brain barriers suggest the existence of a barrier at the level of the lateral median eminence, the arcuate nucleus–median eminence barrier [Flament-Durand and Brion, 1985]. There is also some evidence indicating the formation of tight junctions in β1 tanycytes present at the level of the median eminence [Krish and Leonhardt, 1978], an area containing a high density of β1 tanycytes that express high levels of GLUT1.

In conclusion, there is enough evidence regarding the blood–brain and the blood–cerebrospinal barriers to clearly establish the concept of elevated expression of GLUT1 in the cells that form both barriers. However, our current data do not support the concept that elevated expression of GLUT1 can be used as a specific marker of barrier formation in the brain, a concept clearly exemplified by the low-to-absent GLUT1 expression in β2 tanycytes that form the median eminence–cerebrospinal fluid barrier. On the other hand, the elevated expression of GLUT1 in α and β1 tanycytes, although it may be related to the participation of these cells in the formation of a semipermeable barrier, may also have metabolic implications unrelated to direct participation in barrier formation. In fact, a detailed study of glucose transporter expression during the development of the human brain revealed that elevated expression of GLUT1 by endothelial cells of the brain microcapillaries occurs very early during development, and is not directly related to the formation of a functional blood–brain barrier [Nualart et al., 1999]. The elevated expression of GLUT1 in cells that form the blood–brain and blood–cerebrospinal fluid barriers as compared to very low expression in cells that form the median eminence–cerebrospinal fluid barrier probably represents the expression of marked metabolic and functional differences between the different brain regions covered by these barriers.

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