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Alterations in blood-brain barrier glucose transport in SIV-infected macaques

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The neurological manifestations of HIV infection may be in part due to alterations in the blood-brain barrier. These may be caused by structural changes in the barrier or may consist of subtle metabolic or biochemical disturbances in barrier function. In the CNS, the family of glucose transporter proteins plays a key role in controlling movement of glucose across cell membranes. The 55 kDa isoform of glucose transporter 1 (GLUT1) regulates import of glucose from blood to brain across the endothelial cells of the blood-brain barrier (BBB), whereas the 45 kDa form of GLUT1 predominantly regulates nonvascular glial glucose uptake. In this study, expression of 55 and 45 kDa forms of GLUT1 in different regions of the brain from 18 SIV-infected macaques was measured by quantitative immunoblot and then compared with the severity of SIV encephalitis to determine whether neurologic disease is related to altered glucose metabolism at the BBB and in brain parenchyma. An inverse relationship was found between severity of SIV encephalitis and expression of the endothelial 55 kDa isoform of GLUT1 at the BBB in cortical grey matter, caudate nucleus, and cerebellum. A similar relationship also was found for the glial 45 kDa GLUT1 isoform in cortical grey matter. In addition, a significant increase in 55 kDa GLUT1 expression was found in caudate nucleus during the early stages of infection. In the brains of macaques with moderate to severe encephalitis, 55 kDa GLUT1 expression had declined to pre-infection levels. These GLUT1 alterations at the BBB and in glial cells may reflect severe disturbances in the CNS microenvironment that contribute to CNS dysfunction.

Keywords: blood-brain barrier; GLUT1; endothelial cells

Introduction

Approximately 20–30% of individuals infected with HIV develop clinical signs of neurologic disease ranging from motor and cognitive deficits to overt dementia. In addition, HIV encephalitis is present in over 50% of people with AIDS (McArthur *et al*, 1993; Spencer and Price, 1992). Despite this high incidence of neurologic disease, the mechanisms responsible for clinical and pathologic manifestations of HIV in the CNS remain poorly defined.

Multiple lines of evidence have suggested that alterations of the blood-brain barrier (BBB) may contribute to HIV neurologic disease. Both morphologic alterations of CNS endothelium and the presence of serum proteins within the CNS may reflect structural BBB compromise (Petito and Cash, 1992; Power *et al*, 1993; Rhodes, 1991; Smith *et al*, 1990). Overt CNS endothelial cell loss by apoptosis has also been reported to occur in HIV-infected individuals and in SIV-infected macaques (Adam-

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son *et al*, 1996; Vallat *et al*, 1998). Subtle biochemical alterations in the BBB, including changes in transport molecule expression, may be difficult to detect by routine histopathologic evaluation, but also may cause profound disturbances in regional brain homeostasis and function.

Glucose transporter molecules play a key role in brain metabolism by regulating the import of glucose from blood into the brain across the endothelial cells forming the blood-brain barrier. The 55 kDa isoform of the glucose transporter GLUT1, a protein expressed on both luminal and abluminal faces of CNS endothelium, facilitates diffusion of glucose from blood into brain (Dick *et al*, 1984). The nonvascular 45 kDa form of GLUT1 mediates transport of glucose into glial cells in the CNS while a related protein, GLUT3, regulates neuronal glucose intake (Leino *et al*, 1997; Yu and Ding, 1998). By quantifying glucose transporter expression at the BBB, metabolic alterations may be detected and then correlated with the presence and severity of HIV or SIV encephalitis to determine whether HIV/SIV causes alterations in BBB function. Similarly, changes in expression of the 45 kDa isoform of GLUT1 may be measured and assessed in the context of the presence and severity of neurologic disease. Alterations in the 55 kDa GLUT1 isoform may reflect primary BBB damage and subsequent disruption of CNS glucose metabolism at the level of both endothelial cells and glia. Changes in GLUT1 expression also may represent altered CNS glucose usage and subsequent compensatory responses in glucose transport both at the BBB and in the CNS parenchyma.

The SIV/macaque model of AIDS dementia is an excellent system in which to study the pathogenesis of neurologic disease. Pig-tailed macaques (*Macaca nemestrina*) co-inoculated with a neurovirulent molecular SIV clone and an immunosuppressive SIV strain develop moderate to severe encephalitis within 6 months, facilitating study of CNS disease (Zink *et al*, 1997). In this study, expression of the two isoforms of GLUT1 was examined in different regions of the brain from 18 SIV-inoculated macaques and three control macaques. The expression of the 55 kDa GLUT1 isoform associated with the blood-brain barrier and the nonvascular 45 kDa GLUT1 isoform in parenchymal glial cells was measured by quantitative immunoblot. These measurements of glucose transporter were then compared with severity of encephalitis to determine whether changes in glucose transport correlated with CNS disease onset and progression. An inverse relationship was found between severity of SIV encephalitis and expression of the endothelial 55 kDa isoform of GLUT1 in cortical grey matter, caudate nucleus, and cerebellum. A similar inverse relationship also was found for the 45 kDa GLUT1 isoform in the caudate nucleus. In addition, a significant increase in 55 kDa GLUT1 expression was found in caudate nucleus during the early stages of infection, followed by a decline to control levels 12 weeks after infection.

Results

Severity of SIV encephalitis

Eighteen pig-tailed macaques co-inoculated with an immunosuppressive SIV strain, SIVDeltaB670, and a neurovirulent clone, SIV/17E-Fr, and sacrificed at three time points post-inoculation were examined to determine whether there were SIV-induced alterations in glucose metabolism at the BBB and in the brain parenchyma. This inoculation protocol reliably and rapidly induces SIV encephalitis in pig-tailed macaques, with over 80% developing moderate to severe encephalitis within 3 months of infection. Groups of six animals each were euthanized at approximately 3 weeks (range 21–24 days PI), 8 weeks (range 49–58 days PI), and 12 weeks PI (range 72–94 days PI) to examine serial alterations in glucose transporter expression and presence and severity of SIV encephalitis. No animals euthanized at 3 weeks post-inoculation had CNS lesions while two of six animals euthanized at 8 weeks PI had mild lesions. In contrast, five of six animals euthanized at 12 weeks PI had moderate to severe encephalitis (Table 1). In all animals with encephalitis, lesions were most commonly found in basal ganglia and subcortical white matter of the frontal lobe but also were present in grey matter of frontal cortex and in cerebellum. Changes included perivascular infiltrates of macrophages, multinucleated giant cells, and lymphocytes, multifocal glial nodules, and isolated multinucleated giant cells scattered throughout the brain parenchyma.

Relationship between GLUT1 expression and encephalitis

In this study, the 45 kDa isoform of GLUT1 representing nonvascular, parenchymal glial glucose transport and the 55 kDa isoform of GLUT1 localized to the CNS endothelial cells comprising the BBB were resolved by PAGE performed on brain homogenates. These homogenates were obtained from grey matter of the frontal lobe (cingulate gyrus), subjacent white matter, caudate nucleus in

Table 1 Development of SIV encephalitis at serial times post-inoculation

Time post-inoculation	*Animals without encephalitis*	*Animals with mild encephalitis*	*Animals with moderate encephalitis*	*Animals with severe encephalitis*
3 weeks	6	0	0	0
8 weeks	4	2	0	0
12 weeks	1	0	2	3
	11	**2**	**2**	**3**

basal ganglia, and cerebellum, all regions with SIV-induced CNS lesions. After transferring protein to membranes, bands representing 45 and 55 kDa GLUT1 isoforms were detected by immunoblotting and then quantified by scanning densitometry (Figure 1). For each sample, GLUT1 protein expression was normalized to levels of actin. A standard curve was established by measuring GLUT1 and actin in serial dilutions of control brain homogenate to ensure that all samples fell within the linear range for detection of both 45 and 55 kDa GLUT1 isoforms.

To examine the relationship between glucose transporter expression and neurologic lesions for each brain region, expression of both 45 and 55 kDa isoforms of GLUT1 was compared between SIV-infected animals with no, mild, moderate, or severe SIV encephalitis. Scatter plots were constructed to graphically represent GLUT1 alterations *versus* lesion severity in different brain regions. The strongest correlation between GLUT1 expression and presence/severity of SIV encephalitis was found in cortical grey matter where there was an inverse relationship between GLUT1 and severity of encephalitis for both the 45 and 55 kDa GLUT1 isoforms (Spearman's Rank Correlation (r^S)=−0.71 ($P<0.01$) and $r^S=-0.52$ (P=0.03), respectively, Figure 2A). These correlations indicate that, in cortical grey matter, macaques with more severe encephalitis have lower glucose transporter expression. Additionally, there was a significant inverse relation in caudate nucleus and cerebellum ($r^S=-0.50$; $P<0.05$) between 55 kDa GLUT1 expression at the BBB and encephalitis severity (Figure 2B,C). In contrast, no correlation was found between glucose transporter expression and encephalitis for either GLUT1 isoform in white matter (Figure 2D and Table 2).

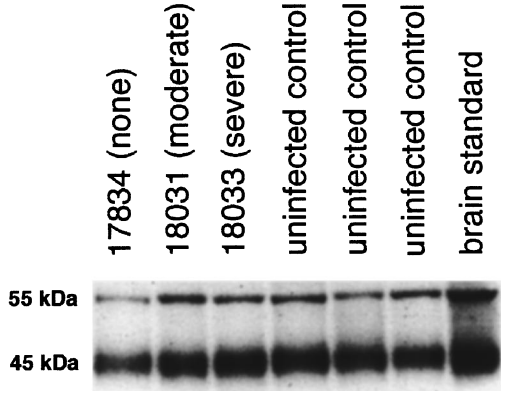

Figure 1 Representative immunoblot demonstrating resolution of both BBB 55 kDa GLUT1 and nonvascular 45 kDa GLUT1 in brain homogenates obtained from the caudate nucleus of macaques including animals infected with SIV and uninfected controls. The presence or severity of SIV encephalitis is denoted above the animal number for three infected animals; other animals served as uninfected controls. Similar immunoblots were obtained from cortical grey matter (cingulate gyrus in frontal cortex) white matter, and cerebellum. Band intensity was measured by scanning densitometry.

Relationship between GLUT1 expression and time after SIV inoculation

To evaluate alterations in GLUT1 expression over time in different brain regions after SIV inoculation, animals were categorized by time of sacrifice (3, 8 or 12 weeks PI). GLUT1 expression also was measured in three control animals to quantify normal expression levels. Immunoblot data were then used to model GLUT1 expression across time for each region of the brain for both GLUT1 isoforms by determining the predicted means of GLUT1 expression and 95% confidence limits (Figure 3).

In caudate nucleus, mean levels of both 45 and 55 kDa GLUT1 isoforms varied significantly across time ($P<0.05$). Given these statistically significant differences in mean GLUT1 levels across time, we were then able to further test for differences between the individual time points. The mean expression of 55 kDa GLUT1 was significantly higher at 8 weeks PI than that of the control group (P=0.02), whereas increases in 45 kDa GLUT1 expression were not significant over this time period (P=0.20). However, there was a significant decrease in expression of both 55 kDa GLUT1 (P=0.01) and 45 kDa GLUT1 ($P<0.01$) from 8−12 weeks. These differences across time in caudate nucleus of basal ganglia imply an increase in glucose transport at weeks 3 and 8 PI followed by a decline to or below control levels at 12 weeks PI. In cortical grey matter, a similar trend over time was observed, with an increase in both GLUT1 isoforms at 3 and 8 weeks PI followed by a decline at 12 weeks PI, although not reaching statistical significance. In contrast, 55 and 45 kDa GLUT1 expression levels in white matter did not change over time PI. In cerebellum, differences in GLUT1 across time were significant only for the 55 kDa isoform ($P<0.05$). Similar to caudate nucleus and cortical grey matter, 55 kDa GLUT1 expression increased (over control expression) at 3 and 8 weeks PI before declining to control levels at 12 weeks PI. The decline in expression of 55 kDa GLUT1 from both 3 weeks PI and 8 weeks PI to 12 weeks PI was statistically significant (P=0.02).

Discussion

In this study, an inverse relationship was discovered between expression of the 55 kDa isoform of GLUT1 on the endothelial cells forming the BBB and the severity of SIV encephalitis in caudate nucleus, cortical grey matter, and in cerebellum. A similar relationship was demonstrated for the nonvascular, glial 45 kDa GLUT1 isoform in cortical

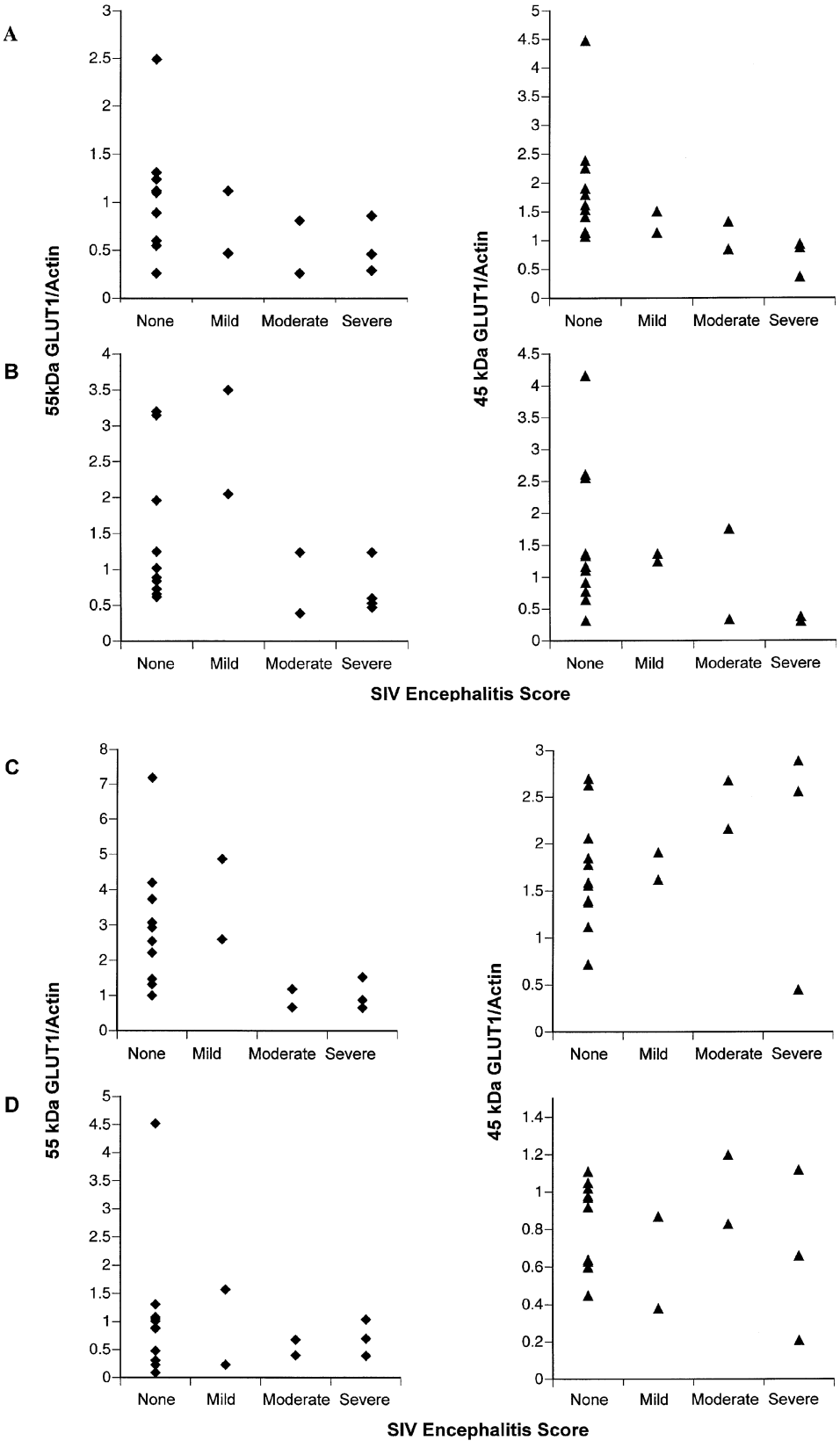

Figure 2 Scatter plots demonstrated a significant decrease in 55 kDa GLUT1 expression that accompanied increasing severity of SIV encephalitis in cortical grey matter (**A**), caudate nucleus (**B**), and in cerebellum (**C**), but not in white matter (**D**). A similar relationship was present for 45 kDa GLUT1 in cortical grey matter as well (**A**).

grey matter, demonstrating metabolic alterations both at the BBB and within the brain parenchyma in SIV-infected macaques. Furthermore, an increase in 55 kDa GLUT1 expression was found in caudate nucleus during the early stages of infection, illustrating that dynamic alterations in GLUT1 expression by the endothelial cells of the BBB occur throughout infection.

The observed inverse association between GLUT1 expression and encephalitis may be indicative of BBB dysfunction that develops with progressive encephalitis. While the CNS endothelial cells are capable of compensating for increased CNS energy demands during early infection, with progression to encephalitis the CNS endothelial cells may be unable to maintain compensatory high level expression of 55 kDa GLUT1. The CNS endothelial cells must also serve other roles beyond glucose transport including regulating leukocyte trafficking via expression of adhesion molecules and thus may be unable to meet these diverse demands. CNS endothelial cells have been reported to be susceptible to infection with HIV and SIV and this also may compromise CNS endothelial cell function (Mankowski *et al*, 1994; Moses *et al*, 1993). Further, both viral protein and cytokines induced by HIV/SIV infection may alter CNS endothelial cell function at the BBB.

The decrease in GLUT1 expression may also reflect decreased CNS metabolic demand for glucose as energy substrate. Loss of dendritic arborization and overt neuronal loss have been documented in people with HIV encephalitis and in SIV-inoculated macaques, suggesting that neuronal glucose requirements may also decline with sever-

Table 2 Spearman's Rank Correlations between GLUT1 expression and SIV encephalitis

	45 kDa		*55 kDa*	
Brain region	$*r^s$	P *value*	r^s	P *value*
Caudate	−0.36	0.14	−0.50	0.04
Cortical grey matter	−0.71	<0.01	−0.52	0.03
White matter	−0.01	0.96	−0.13	0.60
Cerebellum	−0.32	0.20	−0.53	0.02

$*r^s$=Spearman's rank correlation coefficient.

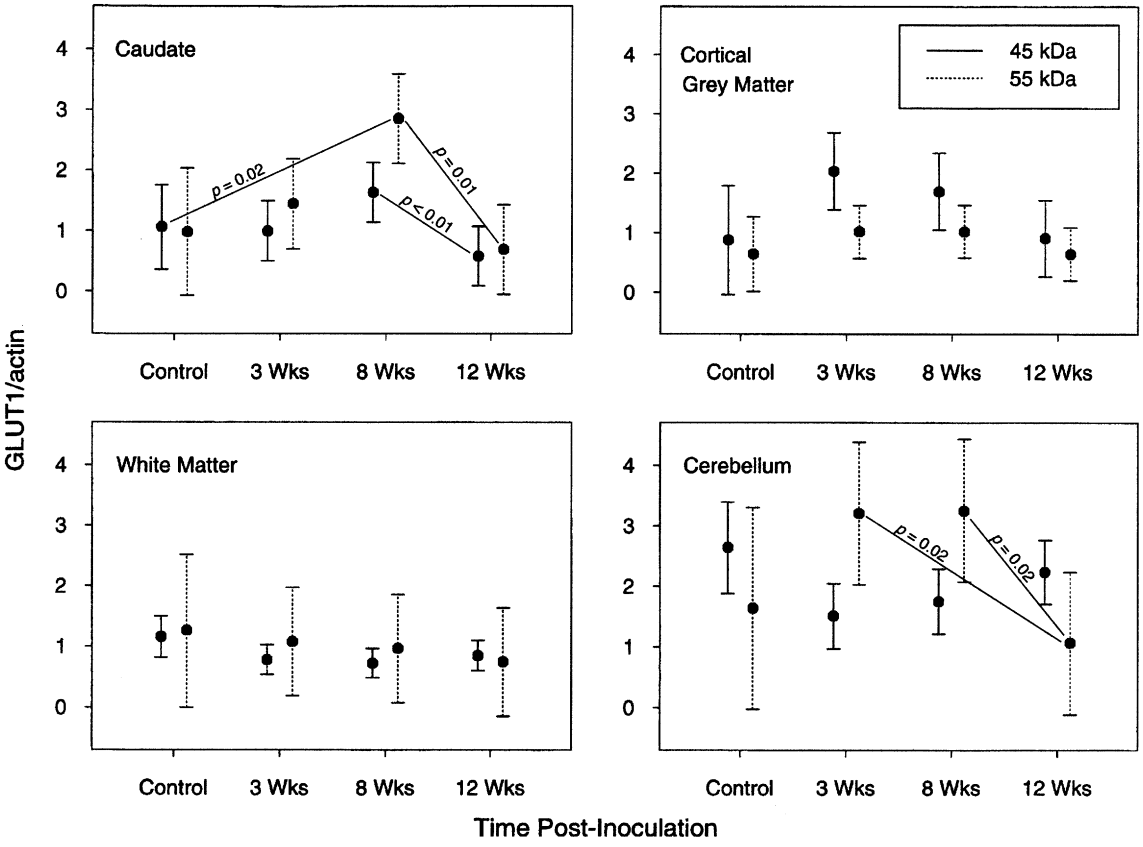

Figure 3 Mean levels and 95% confidence limits of 55 and 45 kDa GLUT1 expression over time in different brain regions showed significant increases in 55 kDa GLUT1 expression in caudate nucleus during early stages of infection followed by a decline to control levels at later stages of infection. A similar trend over time was present in cortical grey matter and cerebellum while white matter GLUT1 values did not change.

ity of encephalitis (Everall *et al*, 1991; Masliah *et al*, 1992b; Montgomery *et al*, 1999; Wiley *et al*, 1991). An additional possibility is that the activation state of astrocytes and microglia changes dynamically throughout infection. Astrogliosis and microglial activation have both been reported in the CNS of HIV-infected individuals (Achim *et al*, 1991; Masliah *et al*, 1992a). In the earlier stages of infection, neuroimmune activation, reflected by elevated CNS metabolic demand, may help to control viral replication in the CNS. With progression to more severe CNS disease, these immune responses in the CNS may decline. As astrogliosis and microglial activation are predominately found in grey matter (including both cortical grey matter and caudate nucleus), this also would explain why changes were found in cortical grey matter from the frontal lobe and the caudate nucleus of the basal ganglia but not in subcortical white matter.

Studies using positive emission tomography (PET) with fluordeoxyglucose (FDG) as tracer have demonstrated that people with various neurologic diseases including Alzheimer's disease, Huntinton's disease, and HIV encephalitis develop cerebral metabolic alterations (Gamberino and Brennan, 1994; Rottenberg *et al*, 1987; Simpson, 1994; van Gorp *et al*, 1992). These PET studies have been extended and complemented by additional studies measuring glucose transporter GLUT1 expression in the CNS. In general, brain regions found to be hypometabolic by PET analysis also exhibit lowered GLUT1 expression (Gamberino and Brennan, 1994). A single report found that people seropositive for HIV without encephalitis had increased expression of GLUT1 while those with HIV encephalitis had expression levels comparable to control brain samples, suggesting a decline from a hypermetabolic state with progression to HIV encephalitis, similar to findings in this study (Kovitz, 1997). In that study, the different isoforms of GLUT1 representing blood-brain barrier (55 kDa) and nonvascular (45 kDa) isoforms were not resolved. Also, the particular regions reported to be hypermetabolic via PET in people infected with HIV, basal ganglia and thalamus, were not examined for GLUT1 expression.

A difficulty of performing these studies in human autopsy material is the potentially confounding presence of the 55 kDa isoform of GLUT1 present at high concentration on erythrocyte membranes within the CNS vessels (Sogin, 1980). An advantage of the SIV-macaque model is that animals may be perfused terminally with saline to remove red blood cells from cerebral blood vessels. As perfusion removes red blood cells expressing high concentrations of the 55 kDa form of GLUT1, measurement of 55 kDa GLUT1 expression in macaque brain homogenates accurately reflects CNS endothelial cell expression. Although a single animal in this study was not perfused, a macaque with severe encephalitis and low 45 and 55 kDa GLUT1 levels, the lack of perfusion in this one animal would bias measurement towards a higher 55 kDa GLUT1 value and thus only strengthens the observed inverse association between encephalitis severity and GLUT1 expression. In this study, findings of increased 55 kDa GLUT1 expression in the caudate nucleus of the basal ganglia were demonstrated in macaques infected with SIV before developing severe encephalitis, like the increases reported previously in people infected with HIV (Kovitz, 1997). Elevated expression was greatest in the caudate nucleus of the basal ganglia, a region commonly involved in HIV encephalitis and the region with the highest expression of 45 and 55 kDa GLUT1 isoforms (Vannucci *et al*, 1998).

This model of rapid, reproducible progression to SIV encephalitis in macaques will facilitate studies examining the dynamic interaction between activation of astrocytes and microglia, alterations of CNS endothelial cells at the BBB, and CNS metabolism throughout the course of infection. Whether compensatory alterations in GLUT1 BBB expression interferes with other biochemical properties of the BBB during HIV infection or whether the ability of the endothelial cells to transport glucose from the blood into the CNS is rate limiting for CNS metabolism remains to be determined.

Materials and methods

Animals

Eighteen pig-tailed macaques (*Macaca nemestrina*) were intravenously inoculated with SIV/DeltaB670 (50 AID_{50}), and SIV/17E-Fr (10 000 AID_{50}) as previously described (Zink *et al*, 1997). Three additional macaques were mock-inoculated and served as virus-negative controls. Six infected animals were sacrificed at each of three different time points post-inoculation: 3, 8 and 12 weeks post-inoculation. Animals were perfused with sterile normal saline to remove peripheral blood cells from the CNS vasculature.

Histopathology

Sections of CNS, including frontal, parietal, temporal and occipital cortex, basal ganglia, thalamus, midbrain, and cerebellum, were examined independently in a blinded fashion by two pathologists (JL Mankowski and MC Zink). To quantify the severity of lesions, sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum were each given numerical scores of 1 (mild), 2 (moderate) or 3 (severe). Sections with more than 30 perivascular macrophage-rich cuffs were given a score of 3, sections with 10 to 30 perivascular cuffs were given a score of 2, and those with less than 10 perivascular cuffs were given a score of 1. The scores for all sections were totalled and divided by 6 (six regions were graded for each

brain) to give a mean score (out of a maximum of 3) for severity of CNS lesions.

Quantitative immunoblotting
A 50 mg portion of brain tissue was harvested from each of cingulate gyrus grey matter, subjacent white matter, caudate, and cerebellum using a 5 mm biopsy punch. Samples were homogenized in 0.5% NP-40 containing protease inhibitors (Roche, USA). After protein quantification, 15 μg protein aliquots were solubilized and separated by electrophoresis on 10% Bis-Tris gels in SDS–MOPS buffer (Novex, USA). After transfer, polyvinylidene fluoride membranes (Millipore, USA) were probed with a rabbit polyclonal antibody made against purified human erythrocyte GLUT1 transporter (1 : 4000) and rabbit polyclonal antibody against actin (1 : 500, Sigma Chemical, USA), followed by anti-rabbit HRP-conjugated antibody. Bound antibody was detected by enhanced chemiluminescence (Pierce, USA), and band intensity was measured by scanning densitometry (IPLab Gel Analysis software, Scanalytics, USA). Final values for both 45 and 55 kDa GLUT1 were expressed as the ratio of intensity of GLUT1 bands to actin band to normalize for variation in protein loaded. As expression of endothelial-specific markers have been reported to change with HIV, actin was used for normalization of protein bands (Seigneur *et al*, 1997). To allow comparison of measurements between gels, a brain homogenate standard obtained from a control macaque was included on all gels. Serial dilutions of this brain homogenate sample, ranging from 2–64 μg of protein, were also analyzed under parallel conditions to ensure that all measurements of GLUT1 and actin were within the linear range for detection.

Data analysis
To determine the correlation between glucose transporter expression and SIV encephalitis in the different brain regions, Spearman's rank correlation coefficient (r^s) was used. A one-way analysis of variance (ANOVA) was used to model mean GLUT1 expression by different strata of brain region and isoform across time. To control for multiple comparisons when testing for differences between two specified times within a brain region under a given GLUT1 isoform, we employed Fisher's LSD method.

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