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Elevated peripheral benzodiazepine receptor expression in simian immunodeficiency virus encephalitis

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Measurement of central nervous system (CNS) expression of the peripheral benzodiazepine receptor (PBR), a microglia and macrophage activation marker, by positron emission tomography (PET) would aid clinical management of human immunodeficiency virus (HIV)-infected patients. To evaluate the utility of examining PBR expression in the CNS as a cellular activation marker in HIV CNS disease, PBR levels were measured in frontal cortex of simian immunodeficiency virus (SIV)-infected macaques with encephalitis and uninfected animals via PK11195 ligand autoradiography. [^{3}H]-(*R*)-PK11195 binding to both grey matter ($P = .017$) and white matter ($P = .038$) was significantly higher in animals with SIV encephalitis ($n = 10$) versus control animals ($n = 3$). When PK11195 binding was compared with other microglial/macrophage activation markers (obtained via quantitative image analysis), a strong, significant association was found for both HAM56 ($P = .004$) and KP-1 (anti-CD68; $P = .006$) immunostaining in white matter. In contrast, grey matter PK11195 binding did not correlate with HAM56 ($P = .46$), KP-1 ($P = .06$), or glial fibrillary acidic protein (GFAP) immunostaining for astrocytic activation ($P = .09$). The regional nature of these increases in activation within the brain illustrates the crucial need to focus functional neuroimaging analyses of HIV-infected individuals on subcortical white matter to assess activation of microglia and macrophages. *Journal of NeuroVirology* (2003) **9,** 94–100.

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Introduction

Microglial activation has been detected in various chronic central nervous system (CNS) diseases, ranging from multiple sclerosis to spongiform encephalopathies (Aloisi, 2001; Walsh *et al*, 2000). Activation of microglia and macrophages has also been reported widely in human immunodeficiency virus (HIV)-infected individuals, particularly during late stages of disease associated with viral replication in microglia as well as the production of proinflammatory cytokines in the CNS, including tumor necrosis factor alpha (TNFμ) and interleukin (IL)-6 (Sopper *et al*, 1996). Studies demonstrating a close correlation between microglial and macrophage activation and the severity of HIV-induced neurologic disease illustrate the central role microglial/macrophage activation plays in acquired immunodeficiency syndrome (AIDS) dementia (Glass *et al*, 1995). Thus, the ability to measure progressive alterations in

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microglial/macrophage activation by functional neuroimaging would be of great value in the clinical management of neuroAIDS patients.

An emerging marker of microglial/macrophage activation in the CNS is expression of the peripheral benzodiazepine receptor (PBR). The PBR plays a role in steroidogenesis by mediating mitochondrial cholesterol transport (Brown and Papadopoulos, 2001; Gavish and Weizman, 1997). In the CNS, PBR up-regulation, reflecting activation of microglia and brain macrophages, has been reported in both acute and chronic CNS diseases, including trimethyltin toxicity, multiple sclerosis/experimental autoimmune encephalomyelitis (EAE), Rasmussen's encephalitis, and Alzheimer's disease (Banati *et al*, 1999; Cagnin *et al*, 2001; Kuhlmann and Guilarte, 1999; Vowinckel *et al*, 1997). The PBR ligand PK11195 has been radiolabeled for use in both autoradiography on frozen brain tissue sections and *in vivo* for positron emission tomography (PET) imaging studies. This potential use for detecting microglial activation via functional neuroimaging makes assessments of PBR expression particularly valuable. This study demonstrates the utility of measuring PBR expression as a microglial activation marker in a well-characterized simian immunodeficiency virus (SIV)/macaque model of HIV CNS disease, and suggests that functional PET imaging studies to measure PBR expression will serve as valuable means of assessing activation of microglia and macrophages.

Results

PBR expression is increased in animals with SIV encephalitis

To measure expression levels of the peripheral benzodiazepine receptor, the levels of receptor binding by the PBR ligand (*R*)-PK11195 were measured in frontal cortex of SIV-infected macaques and uninfected animals via PK11195 ligand autoradiography (Figure 1). [^{3}H]-(*R*)-PK11195 binding to cortical grey matter was significantly higher in the group of animals with SIV encephalitis, with a median value of 123.5 femtomoles (fmol) PK11195 bound/mg tissue ($n = 10$ animals), versus the group of control animals, with a median value of 85 fmol PK11195 bound/mg tissue ($n = 3$ animals), when compared by the Mann-Whitney test ($P = .017$; Figure 2**A**). Cortical grey matter PK11195-binding values ranged from 82 to 175 fmol PK11195/mg tissue in animals with encephalitis, compared to a range of 71 to 86 fmol PK11195/mg tissue in control animals. Similarly, [^{3}H]-(*R*)-PK11195 binding to subjacent white matter was significantly higher in the group of animals with SIV encephalitis (median = 62 fmol PK11195 bound/mg tissue) versus control animals (median = 37 fmol PK11195 bound/mg tissue, $P = .038$, Figure 2**B**). In white matter, PK11195 binding ranged from 34 to 74 fmol/mg tissue in animals with encephalitis versus a range of 30 to 51 fmol/mg tissue in control animals. In contrast, the amount of [^{3}H]-(*R*)-PK11195 binding in cortical grey matter (47 fmol/mg tissue) and subcortical white matter (33 fmol/mg tissue) in the only SIV-inoculated animal that did not develop encephalitis approximated levels found in control animals (Figure 2). All infected animals were examined 3 months post

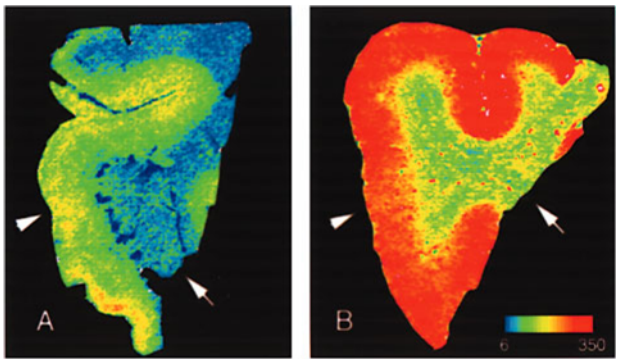

Figure 1 Representative pseudocolored autoradiographs of frontal cortices from a control animal (**A**) and an animal with SIV encephalitis (**B**) demonstrate increased binding of radiolabeled PBR ligand PK11195 to both grey matter (*arrowheads*) and white matter (*arrows*) of animal with SIV encephalitis. The color scale bar (*inset, lower right*) denotes the correspondence between image colors and the amount of bound PK11195, ranging from 6 to 350 fmol PK11195 bound/mg tissue.

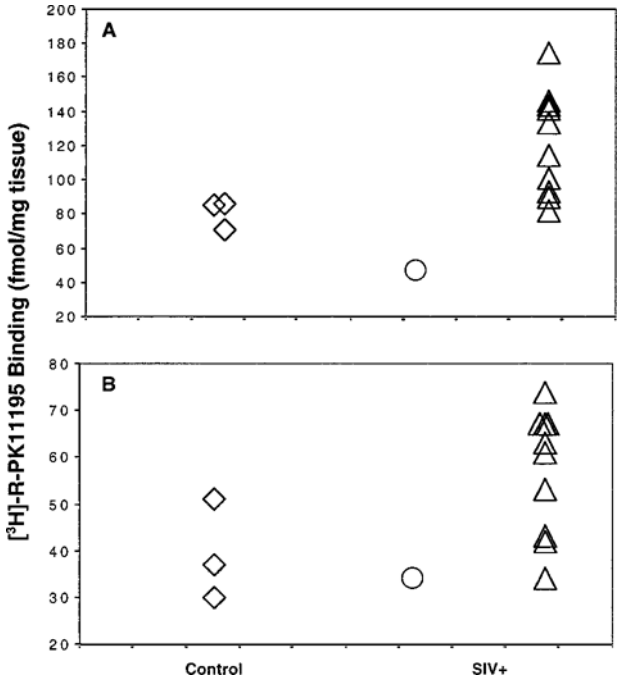

Figure 2 Scattergrams of PK11195 binding values in cortical grey matter (**A**) and subjacent white matter (**B**) in control animals (●) and SIV-infected animals with encephalitis (△). A single animal that was infected with SIV but did not develop encephalitis is represented by a circle (●).

Table 1 Median values of mean total area of immunostaining (pixels) for CNS cellular activation

	Grey matter			*White matter*		
Antibody	*Control*	*SIV*	*P values*	*Control*	*SIV*	*P values*
KP-1	1007	9138	.03	213	6607	<.01
HAM 56	146	4137	<.01	137	1982	.01
Anti-GFAP	203	91257	<.01	Not done	Not done	Not done

Note. Statistically significant increases in microglial/macrophage activation were present in both cortical grey matter and white matter of SIV-infected animals with encephalitis compared to uninfected control animals. The antibodies KP-1 (anti-CD68) and HAM56 both detect activation of microglia and macrophages. Statistically significant increases in astrocytic activation were also present in cortical grey matter, demonstrated by increased immunostaining for GFAP; *P* values were established by the two-tailed Mann-Whitney test.

inoculation with SIV. In this autoradiography binding assay, the use of (*R*)-PK11195 improved the specificity for binding to PBR sites over the racemic isoform of PK11195.

Development of encephalitis and extent of CNS cellular activation

To identify concurrent morphologic alterations associated with elevated PBR expression, including activation of microglia and macrophages as well as astrocytes, brain tissue sections from the frontal cortex were evaluated by histologic examination and immunohistochemical staining. The severity of encephalitis ranged from mild to severe in the 10 animals with SIV CNS disease. SIV-induced CNS lesions generally were most severe in subcortical white matter in basal ganglia and thalamus, consisting of multifocal, perivascular aggregates of macrophages and multinucleated giant cells, giant cells scattered in the neuropil, and multifocal glial nodules. A previous study using this SIV/macaque model has demonstrated a significant correlation between the level of CD68 expression and the severity of CNS lesions as determined by histopathologic assessment of hematoxylin and eosin–stained brain sections (Zink *et al*, 1999). In this study, to measure the extent of microglial/macrophage activation objectively in each individual animal with encephalitis, quantitative measurements of macrophage and microglial activation in grey matter and white matter were obtained for each animal employing quantitative image analysis. After immunostaining with either the antibody HAM56 or KP-1 (anti-CD68), the mean total area of immunostaining per 200≈ field was calculated for each animal. In animals with SIV CNS disease, the antibodies HAM 56 and KP-1 both bind to activated microglia as well as infiltrating, activated macrophages that likely have entered the brain from the blood, predominating in perivascular areas (Figure 3**A–D**).

Statistically significant increases in microglial/macrophage activation were present in both cortical grey matter and white matter of SIV-infected animals with encephalitis compared to uninfected control animals (Table 1, Figure 3**A–D**). To independently confirm measurements of activation of microglia and macrophages, two different antibodies, KP-1 and HAM 56, were used for immunostaining. Statistically significant increases in astrocytic activation also were present in cortical grey matter of macaques with encephalitis versus uninfected animals, demonstrated by increased immunostaining for glial fibrillary acidic protein (GFAP) (Figure 3**E–F**). Astrocyte activation was not measured by image analysis in white matter because the relatively high level of constitutive immunostaining for GFAP in uninfected animals limits the dynamic range of this technique for measuring increases in astrocyte activation in white matter. *P* values were established by the two-tailed Mann-Whitney test (Table 1).

Microglial/macrophage activation correlates with increased PK11195 binding in white matter

To evaluate the correlation between microglial/macrophage activation and PBR ligand binding, measurements of microglial/macrophage activation in grey matter and white matter were compared with levels of PK11195 binding by the Pearson's correlation coefficient. A strong, significant association was found between the amount of PK11195 binding and both HAM56 ($P = .004$) and KP-1 (anti-CD68; $P = .006$) immunostaining in white matter (Table 2). In contrast, grey matter PK11195 binding did not correlate with either HAM56 ($P = .46$), or KP-1 ($P = .06$) immunostaining, although the correlation between PBR ligand binding and KP-1 immunostaining approached statistical significance.

Table 2 Correlation between PK11195 binding and CNS cellular activation

Antibody	*Grey matter*	*White matter*
KP-1	$r = .532$ ($P = .06$)	$r = .715$ (≈$P = .006$)
HAM 56	$r = .223$ ($P = .46$)	$r = .736$ (≈$P = .004$)
Anti-GFAP	$r = .493$ ($P = .09$)	Not done

Note. The correlations between the amount of PK11195 binding in grey and white matter and cellular activation in the CNS were quantified by Pearson's correlation coefficient. Asterisks represent statistically significant correlations ($P < .05$).

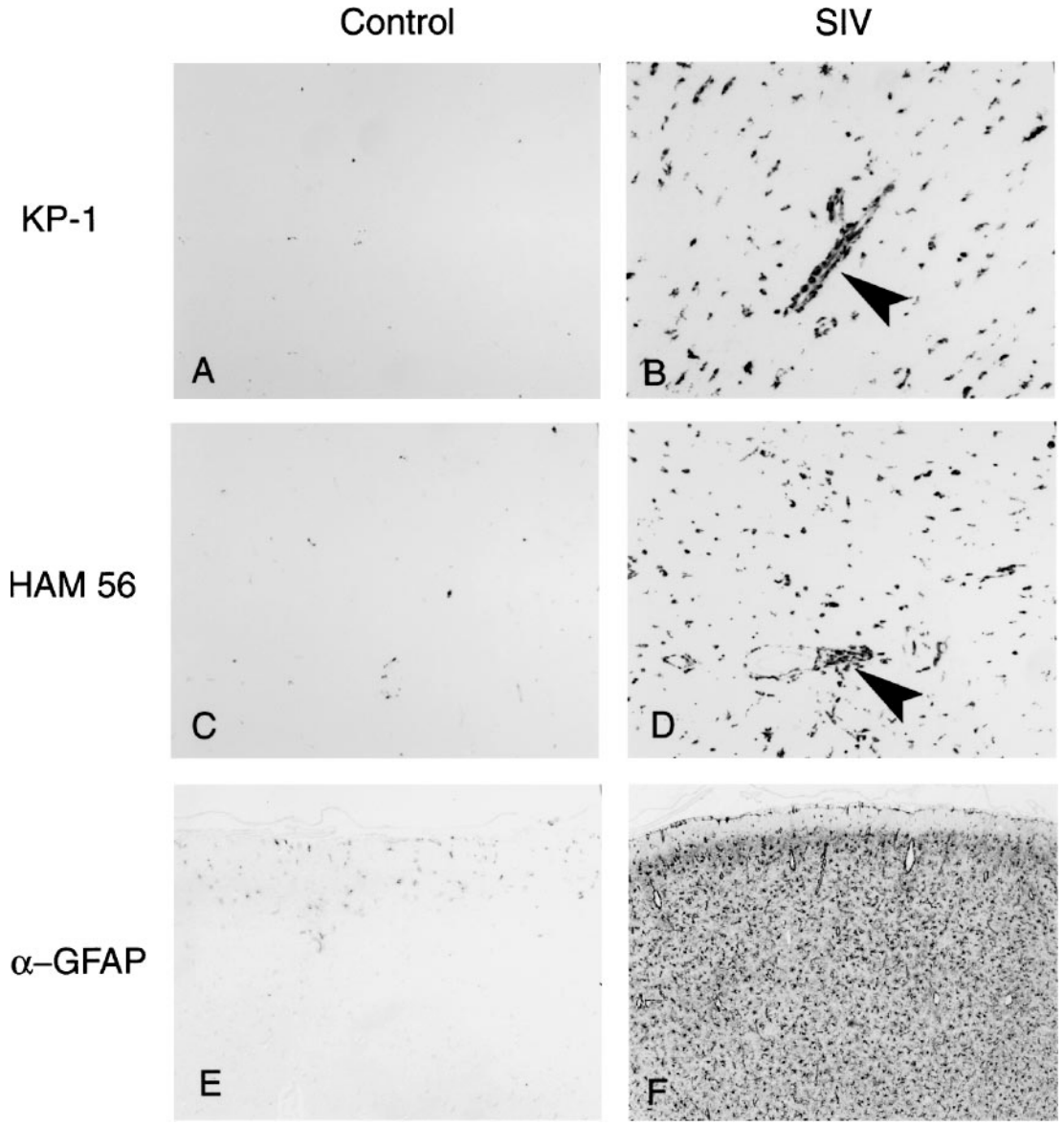

Figure 3 Immunohistochemical staining using the antibodies KP-1 (**A** and **B**) and HAM 56 (**C** and **D**) demonstrates that both antibodies stain activated parenchymal microglia throughout the neuropil as well as infiltrating perivascular macrophages (*arrows*) in the white matter of SIV-infected animals with encephalitis (**B** and **D**). In contrast, staining in control animals is limited to occasional perivascular cells (**A** and **C**). In cortical grey matter, GFAP immunoreactivity denoting astrocytic activation is markedly up-regulated in animals with SIV encephalitis (**F**) versus control animals (**E**). **A–D**, 100≈ original magnification; **E–F**, 40≈ original magnification.

Cortical astrocytosis is a common finding accompanying HIV/SIV infection (Seilhean *et al*, 1997; Weihe *et al*, 1993; Weis *et al*, 1993; Zink *et al*, 2001). To determine whether astrocytic activation was associated with increases in PK11195 binding in cortical grey matter, brain sections were immunostained with anti-GFAP antibody and GFAP expression was measured by quantitative image analaysis. Astrocytic activation did not correlate with the level of grey matter PK11195 binding by the two-tailed Mann-Whitney test ($P = .09$), indicating that the extent of astrocyte activation does not correlate with PBR expression levels in cortical grey matter (Table 2).

Discussion

Activation of microglia and macrophages is a common morphologic feature of many CNS disease processes. The ability to measure microglial and macrophage activation *in vivo* would contribute to both diagnostic evaluation and clinical management of patients with varied diseases, guiding selection of appropriate therapy, and assessing responses to treatment. Several studies have demonstrated the utility of evaluating PBR levels in the brain as an indicator of microglial/macrophage activation using quantitative PET employing the PBR ligand PK11195. Increased

PK11195 binding reflecting activated microglia and brain macrophages has been shown in the brains of patients with Rasmussen's encephalitis by PK11195 PET (Banati *et al*, 1999). In patients with multiple sclerosis, PK11195 binding has been correlated with CD68 immunoreactivity denoting both activated microglia and infiltrating mononuclear cells in perivascular cuffs (Banati *et al*, 2000; Vowinckel *et al*, 1997). Increased PK11195 binding detected by PET also has been demonstrated in patients with Alzheimer's disease, including those with mild, early forms of dementia (Cagnin *et al*, 2001). These observations suggest that PK11195 PET may be a sensitive neuroimaging technique for quantifying activation of microglia and brain macrophages in many diverse neurologic diseases, including HIV CNS disease.

Despite observations of a close correlation between microglial and macrophage activation and the severity of HIV-induced neurologic disease (Glass *et al*, 1995), a reliable clinical method for measuring activation of macrophages and microglia in the CNS of HIV-infected individuals has not been identified. In the SIV/macaque model of HIV CNS disease, increases in microglial activation also have been associated with development of cognitive and motor deficits (Berman *et al*, 1999). To evaluate the utility of measuring CNS PK11195 binding as an indicator of PBR expression levels in HIV CNS disease, this study characterized PK11195 binding in cortical grey matter and white matter of SIV-infected macaques with encephalitis.

SIV-inoculated macaques that developed encephalitis had elevated levels of PK11195 binding in frontal cortex consistent with increased expression of the PBR. In contrast, the amount of PK11195 binding to frontal cortex in an SIV-infected macaque without encephalitis was not elevated over control animal values. In macaques with encephalitis, increases in PK11195 binding corresponded with activation of microglia and macrophages in white matter. However, in cortical grey matter, increases in PBR expression were not clearly associated with either microglial/macrophage activation or astrocyte activation. The lack of a relationship between PBR ligand binding and microglial/macrophage activation in grey matter (as opposed to the correlation observed in white matter) may reflect the presence of different subpopulations of macrophages and microglia in grey matter versus white matter, including infiltrating macrophages that predominate in white matter in SIV/HIV encephalitis. The regional nature of these increases in cellular activation within the brain illustrates the importance of targeting specific regions in the brain, including subcortical white matter, for examination by functional neuroimaging to assess microglial/macrophage activation. Future studies addressing the relationship between PBR expression and microglial/macrophage activation in basal ganglia and thalamus may be particularly valuable as these regions, like centrum semiovale, are profoundly affected by HIV infection. This study demonstrates that measurement of activated microglia and macrophages by PET employing PK11195 as the injected PBR ligand may be of great value in the clinical assessment and management of HIV-infected individuals.

The physiologic role of the PBR in the CNS includes mediating transport of cholesterol into mitochondria during steroid synthesis, as well as stabilizing mitochondrial membranes to prevent apoptosis (Brown and Papadopoulos, 2001; Papadopoulos *et al*, 2001). The specific role that the PBR plays in CNS diseases including neuroAIDS remains to be defined but may involve mediating CNS immune responses. One study has demonstrated that HIV Tat-induced migration of microglia may be inhibited by PBR ligands, implying that microglial chemotaxis may be regulated by PBR expression during HIV infection (Lokensgard *et al*, 2001). PBR regulation of CNS steroid production also may modulate CNS inflammatory responses. As PBR agonists have been shown to have antiapoptotic activity *in vitro*, CNS PBR expression also may serve to limit programmed cell death *in vivo* (Bono *et al*, 1999). A recent study has reported that overexpression of the PBR in mice infected with the neurovirulent Sinbis virus may be a protective mechanism to prevent neuronal apoptosis (Johnston *et al*, 2001).

Although elevated PBR levels in injured brain generally are believed to be specific to activated microglia and infiltrating macrophages, a rodent study of the neurotoxicant trimethyltin has suggested that activated astrocytes also up-regulate PBR expression (Kuhlmann and Guilarte, 2000). Thus, increases in PBR expression may be indicative of reactive gliosis rather than specific microglial/macrophage activation. As cortical astrocytosis develops with both SIV and HIV infection, to determine whether astrocytic activation correlated with elevated cortical grey matter PBR expression in SIV-infected macaques, this study examined the association between increased PK11195 ligand binding and GFAP expression as a marker of astrocytic activation (Weihe *et al*, 1993). We were unable to demonstrate a statistically significant correlation between PBR expression and alterations in GFAP immunostaining ($P = .09$). As the relationship between PBR expression and the amount of both CD68 and GFAP measured by immunostaining approached statistical significance, increases in PBR expression in cortical grey matter may reflect contemporaneous activation of both microglia and astrocytes. Although it would be ideal to directly examine the cellular basis of PBR expression in grey matter by double-labeling studies, appropriate reagents to detect PBR expression in conjunction with microglial and astrocyte activation are currently unavailable. Future studies examining larger groups of animals with SIV CNS disease, including analyses at serial

time points by PET, will clarify our understanding of the relationship between peripheral benzodiazepine receptor expression and the CNS immune responses that develop during HIV infection.

Materials and methods

Animals
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*, 1999). Infected animals were sacrificed at 3 months post inoculation (11 animals). Three additional macaques were mock-inoculated with medium alone and served as virus-negative controls. At sacrifice, all animals were perfused with sterile saline to remove blood-borne leukocytes from the CNS vasculature. The animal procedures in this study were reviewed and approved by the Johns Hopkins University Institutional Animal Care and Use Committee, in accordance with Animal Welfare Act regulations and the USPHS Policy on Humane Care and Use of Laboratory Animals.

PK11195 ligand binding
Eight-micron-thick, frozen frontal cortex sections were prepared on a cryostat microtome, mounted onto poly-L-lysine–coated slides, and frozen at ≈80℃ until used. Prior to study, the slides were thawed at 37℃ for 30 min, then preincubated in 50 mM Tris-HCl (pH 7.4) for 5 min. Slides were then incubated with 1 nM [^{3}H]-(*R*)-PK11195 or 10 μM PK11195 for 30 min at room temperature. The use of the (*R*) enantiomer of PK11195 was used to improve the specificity of PK11195 binding (Guilarte *et al*, 1995; Kuhlmann and Guilarte, 1997; Kuhlmann and Guilarte, 1999). Slides were washed twice with 50 mM Tris-HCl for 3 min on ice, followed by two washes with ddH_2O for 3 min on ice, and dried under vacuum and opposed to Amersham hyperfilm-^{3}H for 14 days. Plastic standards of known ^{3}H radioactivity were included with the sections to determine radioactive density. Following exposure, film was developed and [^{3}H]-PK11195 binding measured using an image analysis program (Inquiry, Loats Associates, Westminister, MD) (Guilarte *et al*, 1995; Kuhlmann and Guilarte, 1999).

Histopathology and immunohistochemical staining
Hematoxylin and eosin–stained sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum from each animal were examined microscopically. SIV-infected macaques with encephalitis developed typical lesions consisting of multifocal perivascular accumulations of macrophages and multinucleated giant cells that predominated in white matter, basal ganglia, and thalamus. Primary antibodies used to assess microglial and macrophage activation included KP-1, an antibody directed against CD68 (diluted 1:2,000; DAKO, Carpinteria, CA), and HAM-56 (diluted 1:50; DAKO), an antibody that detects a poorly characterized cytoplasmic protein present in activated microglia and macrophages (Lane *et al*, 1996). An antibody directed against GFAP was used for evaluation of astrocyte activation (diluted 1:4,000; DAKO). All brain tissue sections were immunohistochemically stained by an automated immunostainer (Optimax Plus, BioGenex, San Ramon, CA) for uniformity. Streck-fixed, paraffin-embedded brain tissue sections were deparaffinized, rehydrated, and then postfixed in Streck tissue fixative (Streck Laboratories, Omaha, NE) for 20 min. After rinsing in water, tissues were heated in a microwave in sodium citrate buffer (0.01 M, pH 6.0) for 8 min to retrieve antigen. Endogenous peroxidase was quenched with 3% H_2O_2 for 10 min and then sections were blocked with buffered casein for 10 min. Primary antibody was applied to tissue sections for 60 min at room temperature, the tissues were washed in buffer, and then secondary biotinylated multilink antibody (Biogenex, San Ramon, CA) was added for 20 min. After washing, streptavidin–horseradish peroxidase was applied for 20 min, followed by diaminobenzidene tetrahydrochloride in buffer containing H_2O_2 for 10 min. Sections were then washed, dehydrated, and mounted.

Quantitative image analysis
To standardize sampling from animal to animal, coronal brain tissue sections from all animals were obtained from the same location, 5 mm posterior to the head of the caudate nucleus. For each animal, 20 adjacent fields in cortical grey matter of the cingulate gyrus or subjacent white matter from sections immunostained with the antibodies HAM 56, KP-1, or anti-GFAP were captured at 200≈ magnification (an area of 2.8 mm^2) using a Sensys 2 digital camera (Photometrics, Tucson, AZ), then analyzed by IP Lab imaging software (Scanalytics, Vienna, VA). Images were binarized and the total area occupied by immunopositive pixels calculated to measure the total area of immunostaining. This approach provides objective measurements of amounts of antigen in tissue sections and thus facilitates statistical analyses.

Statistical analysis
To test the hypothesis that two samples are from populations with the same distribution, the two-tailed Mann-Whitney test was used. This nonparametric analog to the two-sample *t* test allows for comparisons of samples without the assumption of normally distributed data. The Mann-Whitney test was used to determine statistical significance of differences in measurements of microglial/macrophage activation and PK11195 binding in the group of

SIV-infected animals with encephalitis versus the group of control animals. Pearson's correlation coefficient was calculated to measure the magnitude of the linear association between the markers of microglial/macrophage activation (and astrocyte activation) and PK11195 binding. The statistical significance of the correlation coefficient was determined by the Wald statistic (i.e., *z*-score).

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