14-3-3 Protein in CSF: An Early Predictor of SIV CNS Disease

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Abstract

In neurons, 14-3-3 proteins regulate diverse processes, including signal transduction, neurotransmitter production, and apoptosis by binding to target proteins, but the role 14-3-3 proteins play in the pathogenesis of central nervous system (CNS) disease remains unclear. To examine the relationship between presence of 14-3-3 protein in cerebrospinal fluid (CSF) and encephalitis in the SIV/macaque model of HIV CNS disease, CSF levels of 14-3-3 protein were measured by quantitative immunoblotting throughout infection in 6 SIV-infected pigtailed macaques. Beginning during asymptomatic infection and continuing until death, CSF levels of 14-3-3 were elevated in 4 of 6 SIV-infected animals. Animals with 14-3-3 protein in CSF had the highest viral loads in the CSF after acute infection and the highest levels of both viral RNA and protein in brain (p < 0.001). In contrast, the presence of 14-3-3 protein in CSF was not associated with CNS microglial/macrophage activation measured by quantitative immunohistochemical staining for CD68 (p = 0.13). CSF levels of 14-3-3 protein may be a valuable marker of early neuronal damage, CNS viral replication, and CNS disease progression in HIV-infected individuals.

Key Words: 14-3-3 protein, CSF, HIV, Marker, Neurodegeneration, SIV.

INTRODUCTION

14-3-3 proteins play multiple, varied roles in eukaryotic cells, including regulation of enzyme activity, directing subcellular localization of proteins, and modulating protein-protein interactions by serving as adaptor proteins. 14-3-3 proteins are 28- to 33-kDa acidic proteins consisting of 7 isoforms in mammalian cells designated β, γ, η, σ, ε, τ, and ζ, with the phosphorylated forms of β and γ denoted as α and δ, respectively. As homo- or heterodimers, 14-3-3 proteins bind to conserved phosphoserine or phosphothreonine residues on target cellular proteins. While there are amino acid differences between 14-3-3 isoforms, they share well-conserved binding domains (1–3). 14-3-3 proteins are constitutively expressed in the cytosol of neurons where they play diverse physiologic roles, including regulation of signal transduction and neurotransmitter production (4). Patients with recent ischemic stroke, transverse myelitis, herpes simplex encephalitis, and Creutzfeld-Jakob disease have detectable levels of 14-3-3 in the cerebrospinal fluid (CSF), presumably reflecting neuronal destruction (5, 6).

There are conflicting reports about the utility of measuring CSF 14-3-3 levels in HIV-infected patients. One study suggested that specific 14-3-3 isoforms accumulate in the CSF of patients with HIV dementia, while other studies found that the presence of 14-3-3 in CSF was not associated with HIV dementia (7–9). To determine whether CSF 14-3-3 levels correlated with development of central nervous system (CNS) disease in the SIV/macaque model of HIV dementia, we assayed macaque CSF samples obtained throughout SIV infection for the presence of 14-3-3 by quantitative immunoblotting and then examined associated CNS host inflammatory responses and viral replication.

SIV infection of macaques is an ideal model system to address many of the critical questions about the neuropathogenesis of HIV CNS disease that cannot be answered in humans. The SIV/macaque model recapitulates the key features of HIV-associated CNS disease, including the development of encephalitis with characteristic histopathologic changes and cognitive and motor impairment. Pigtailed macaques inoculated with the neurovirulent clone SIV/17E-Fr and the immunosuppressive strain SIV/DeltaB670 consistently develop CNS disease in an accelerated fashion, with more than 90% of animals developing encephalitis within 3 months postinoculation (p.i.), greatly facilitating pathogenesis studies (10). In this study, 6 pig-tailed macaques inoculated with SIV/17E-Fr and SIVDeltaB670 were examined over a 3-month course of infection to measure the levels of 14-3-3 protein in CSF and then compare with viral and host factors contributing to neurodegeneration.

MATERIALS AND METHODS

Animals and Histopathology

Six pigtailed macaques (Macaca nemestrina) were intravenously inoculated with SIV/DeltaB670 (50 AID50),...
Quantitative Immunoblotting

Duplicate 18-μL CSF samples were boiled for 3 minutes, then separated by electrophoresis on 10% Bis-Tris gels in SDS-MOPS buffer (Invitrogen, Carlsbad, CA). After transfer, polyvinylidene fluoride membranes (Millipore, Bedford, MA) were probed at room temperature with a polyclonal antibody against human 14-3-3 (K-19, 1:2000, Santa Cruz Biotech, Santa Cruz, CA) followed by goat anti-rabbit HRP-conjugated antibody (1:10000, DAKO, Carpinteria, CA). The antibody used to detect 14-3-3 protein was raised against the β isoform of 14-3-3 but cross-reacts with all other 14-3-3 isoforms as well. Bound antibody was detected by enhanced chemiluminescence (Pierce, Rockford, IL), and band intensity was measured by scanning densitometry (IPLab Gel Analysis software, Scanners, Vienna, VA). To allow comparison of measurements between gels, a CSF standard obtained from an SIV-infected macaque with severe encephalitis was included on all gels. Serial dilutions of a CSF sample positive for 14-3-3 were also analyzed under parallel conditions to ensure that all measurements of 14-3-3 were within the linear range for detection.

Immunohistochemical Staining

To identify cellular localization of 14-3-3 in the brain, coronal sections of brain tissue, including basal ganglia and frontal cortex, were immunohistochemically stained with a polyclonal antibody directed against 14-3-3 protein (K-19, 1:2000, Santa Cruz Biotech). For detection of viral protein, kk41, a monoclonal antibody directed against the SIV transmembrane portion of the SIV envelope, was used (diluted 1:400, NIH AIDS Reagent Program, Bethesda, MD). Primary antibody against CD68 was used as a marker of microglial activation and macrophage infiltration (KP-1, diluted 1:2000, DAKO). To identify amyloid precursor protein (APP) accumulation in axons, coronal sections of brain tissue, including basal ganglia, frontal cortex, and corpus callosum, were immunohistochemically stained with the monoclonal antibody anti-β-amyloid precursor protein 695 (Clone LN27, 1:10000, Zymed, South San Francisco, CA). For assessment of astrocytosis, immunohistochemistry using a polyclonal antibody against GFAP was performed (Z0334, diluted 1:4000, DAKO). All brain tissue sections were stained by an automated immunostainer (Optimax Plus, BioGenex, San Ramon, CA) for uniformity. Streak-fixed, paraffin-embedded brain tissue sections were deparaffinized, rehydrated, and then postfixed in 4% paraformaldehyde for 20 minutes. After rinsing in water, tissues were heated in a microwave in sodium citrate buffer (0.01 mol/L, pH 6.0) for 5 minutes to retrieve antigen. Endogenous peroxidase was quenched with 3% H2O2 for 10 minutes and then sections were blocked with buffered casein for 10 minutes. Primary antibody was applied to tissue sections for 60 minutes at room temperature, the tissues were washed in buffer, and then secondary biotinylated multilink antibody (BioGenex) was added for 20 minutes. After washing, streptavidin-horseradish peroxidase was applied for 20 minutes, followed by diaminobenzidine tetrahydrochloride in buffer containing H2O2 for 10 minutes. Sections were then washed, dehydrated, and mounted. To standardize sampling from animal to animal, coronal brain tissue sections from all animals were prepared from the same location in the basal ganglia, 5 mm posterior to the head of the caudate nucleus. For each animal, 20 adjacent fields in subcortical white matter from immunostained sections using a Sensys 2 digital camera (Photometrics, Tucson, AZ) were then analyzed by IPLab imaging software (Scanners, Vienna, VA). Images were binarized and the total area occupied by immunoreactive pixels calculated to measure the total area of immunostaining.

SIV RNA Measurement

To measure SIV RNA in plasma and CSF samples, virus was pelleted from 1 mL of either plasma or CSF, and then viral RNA was extracted and quantitated by real-time RT-PCR. Levels of SIV RNA in brain samples were measured by real-time RT-PCR on RNA isolated from four different sites in brain (basal ganglia, parietal cortex, thalamus, and cerebellum) using primers to quantitate gag as described (12, 13).

Cell Culture

Macaque macrophage, microglia, and astrocyte primary cultures were obtained and infected with neurovirulent SIV/17E-Fr as previously described (14). Western blots for 14-3-3 were performed using 18 μL of culture supernatant obtained day 7 p.i. of macrophages, day 3 p.i. of microglia, and day 20 p.i. of astrocytes, time points when all cell types were positive for SIV p27 by ELISA indicative of productive SIV infection (Beckman Coulter, Miami, FL). As macaque neuronal cultures were not available, human fetal neurons were cultured. These cultures contained approximately 70% MAP-2-positive neurons, 25% to 30% GFAP-positive astrocytes, and 1% to 5% CD68-positive microglia (15). Neuronal culture supernatants were harvested at 6, 24, and 48 hours postexposure...
to a neurotoxic dose of HIV Tat (200 nmol/L) as previously described (15).

Statistical Analysis

For statistical analysis, animals were segregated into one of two groups: animals with persistently elevated CSF 14-3-3 or animals without detectable CSF 14-3-3 postacute infection. The differences in means for CNS viral load (SIV RNA and protein), macrophage/microglial activation, APP accumulation, and GFAP were evaluated by a linear regression model using a generalized estimating equation approach to estimation. The linear model used group as an indicator variable. This method was equivalent to a 2-sample t test assuming equal variances. However, we analyzed all data collected for each macaque (i.e. replicates) not just the subject specific average of the replicates. A simple regression model for this data would violate the assumption of independence, therefore we utilized the generalized estimating equation methodology for estimation to account for the inherent correlation among repeated measurements for each subject (16).

RESULTS

Measurement of 14-3-3 Protein in CSF from SIV-Infected Macaques

To evaluate whether 14-3-3 protein was present in the CSF of SIV-infected macaques, immunoblots were performed on CSF samples obtained from 6 SIV-infected macaques at death time points ranging from 67 to 84 days p.i. with SIV. An ~33-kDa band corresponding to 14-3-3 protein was detected in the CSF of 4 of 5 animals that developed encephalitis, but was not present in CSF samples from an SIV-infected macaque without encephalitis nor in a mock-inoculated macaque (Fig. 1). One SIV-infected macaque with moderate encephalitis (BP41) did not have 14-3-3 protein in CSF at time of death. To determine whether the presence of 14-3-3 in CSF corresponded to either the extent of CNS viral replication or the magnitude of host inflammatory responses, we performed group-based comparisons, segregating animals into a group containing the 4 animals with persistently elevated CSF 14-3-3 protein in CSF at time of death; Fig. 2). Three of the animals with persistent 14-3-3 protein in CSF developed moderate to severe encephalitis (BI55, BM03, and CC33) while 1 animal (BK09) had moderate encephalitis characterized by occasional scattered perivascular cuffs of infiltrating mononuclear cells. In contrast, the 2 animals without 14-3-3 protein in the CSF after 28 days p.i. developed either moderate encephalitis (BP41, comparable to BK09 in character and severity) or no CNS lesions (BP33).

At 10 days p.i., all animals had 14-3-3 in CSF, but these increases during acute infection were not predictive of the presence of 14-3-3 in CSF at later stages of infection. A single animal, BK09, had a small amount of 14-3-3 in CSF prior to infection but 14-3-3 could not be detected in CSF samples from this animal at days 3 and 7 p.i.

SIV RNA Levels and Presence of 14-3-3 in CSF

The levels of SIV RNA in CSF sampled throughout infection were measured by real time RT-PCR and compared with the presence or absence of 14-3-3 in CSF (Fig. 3). Animals with detectable levels of 14-3-3 in CSF had higher CSF viral loads from day 56 p.i. onwards than animals without CSF 14-3-3. In contrast, plasma viral loads measured by real time RT-PCR were not associated with the presence of 14-3-3 in CSF.

To examine temporal trends during earlier stages of SIV infection, 14-3-3 protein levels in the CSF of 6 SIV-infected macaques were then measured by quantitative immunoblotting using samples obtained serially throughout infection. Beginning at either day 28 or day 42 p.i. and continuing until death at 3 months p.i., 14-3-3 protein was continually present in CSF samples from 4 of 6 animals (the same 4 animals with 14-3-3-protein in CSF at time of death; Fig. 2). The levels of SIV RNA in CSF sampled throughout infection were measured by real time RT-PCR and then compared with the presence or absence of 14-3-3 in CSF (Fig. 3). Animals with detectable levels of 14-3-3 in CSF had higher CSF viral loads from day 56 p.i. onwards than animals without CSF 14-3-3. In contrast, plasma viral loads measured by real time RT-PCR were not associated with the presence of 14-3-3 in CSF.

To determine whether the presence of 14-3-3 in CSF corresponded to either the extent of CNS viral replication or the magnitude of host inflammatory responses, we performed group-based comparisons, segregating animals into a group containing the 4 animals with persistently elevated CSF 14-3-3 protein, macrophage/microglial activation, APP accumulation, and GFAP were evaluated by a linear regression model using a generalized estimating equation approach to estimation.
and a group containing the 2 animals without 14-3-3 in CSF postacute infection. The group of animals with 14-3-3 in CSF had significantly higher amounts of viral RNA in all brain regions, including basal ganglia (p = 0.001), thalamus (p < 0.001), parietal cortex (p < 0.001), and cerebellum (p < 0.001), than the group of macaques without detectable CSF 14-3-3 (Table 1; Fig. 4). To extend these findings, we measured SIV transmembrane protein gp41 levels in the caudate nucleus of the basal ganglia by immunostaining followed by quantitation using digitalized image analysis. The group of animals with 14-3-3 in CSF also had significantly higher levels of gp41 expression (p < 0.001) than the group of animals without CSF 14-3-3 protein.

As HIV and SIV predominantly replicate in activated macrophages and microglia in the CNS, we next compared the two groups of animals for extent of macrophage and microglial activation measured by immunohistochemically staining sections of frontal cortex for CD68 followed by quantitative image analysis. Unlike CNS virus replication, CNS macrophage/microglial activation was not tightly associated with the presence of 14-3-3 in CSF (p = 0.128), although CD68 immunostaining was higher in the group of animals with 14-3-3 protein in the CSF. Astrocyte activation in the two groups of animals was measured by immunostaining for GFAP followed by quantitative image analysis to determine whether astrogliosis was associated with the presence of 14-3-3 in the CSF. The amount of GFAP immunostaining was higher in the group of animals with 14-3-3 protein in CSF; however, this difference between groups was not closely associated with presence of 14-3-3 in CSF (p = 0.114). Thus, release of 14-3-3 into the CNS is most closely associated with extent of CNS viral replication rather than macrophage and microglial activation or astrocyte activation, suggesting that viral proteins play major roles in mediating neuronal damage throughout infection prior to the development of overt encephalitis, including asymptomatic stages of infection.

### Relationship between 14-3-3 in CSF and Axonal Dysfunction

Our previous studies have shown a correlation between the amount of gp41 immunostaining and extent of neuronal

![FIGURE 3](image-url) Animals with persistently elevated CSF 14-3-3 protein levels (black symbols) had higher CSF viral loads measured by real-time RT-PCR than the animals that did not have 14-3-3 protein in CSF postacute infection (animals BP33 and BP41 depicted by open symbols). (B) In contrast, CSF 14-3-3 protein did not correspond with plasma viral loads measured by real-time RT-PCR.

![FIGURE 4](image-url) Animals with persistently elevated CSF 14-3-3 protein levels (denoted by black bars in basal ganglia column) uniformly had higher brain viral burdens as measured by real-time RT-PCR at terminal time-points in all examined brain regions, including basal ganglia (BG), thalamus (Thal), parietal cortex (PC), and cerebellum (Cbl), than the animals that did not have 14-3-3 protein in CSF postacute infection (animals BP33 and BP41 depicted by gray bars in basal ganglia; p = 0.001).

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**TABLE 1. Relationships Between Presence of 14-3-3 Protein in CSF and CNS Cellular Activation, Axonal Dysfunction, and SIV Burden (Log Immunopositive Pixels)**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Animals With CSF 14-3-3</th>
<th>Without CSF 14-3-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject mean</td>
<td>3.53</td>
<td>2.32</td>
</tr>
<tr>
<td>Group mean</td>
<td>3.50</td>
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<tr>
<td>GFAP</td>
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<tr>
<td>Subject mean</td>
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<td>4.80</td>
</tr>
<tr>
<td>p value*</td>
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<td></td>
</tr>
<tr>
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</tr>
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<td></td>
</tr>
<tr>
<td>SIV gp41</td>
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<tr>
<td>Subject mean</td>
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<td>1.43</td>
</tr>
<tr>
<td>Group mean</td>
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<td>1.12</td>
</tr>
<tr>
<td>p value*</td>
<td>&lt;0.001</td>
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*Difference in means as estimated by linear regression model using generalized estimating equation (GEE) to account for repeated measurements for each subject.
dysfunction manifest as accumulation of APP within axons in the corpus callosum (17). APP accumulation in the corpus callosum has been correlated with the extent of decline in bimanual motor performance that develops in pigtailed macaques inoculated with SIV/17E-Fr and SIV/DeltaB670 (18). To determine whether CSF 14-3-3 was associated with axonal accumulation of APP, we compared levels of APP accumulation in SIV-infected macaques with or without CSF 14-3-3. Although APP levels were higher in animals with CSF 14-3-3 (group mean = log10 2.61 APP-immunopositive pixels/200× field) than the group without 14-3-3 in CSF (group mean = log10 2.08 APP-immunopositive pixels/200× field), the difference was not statistically significant (p = 0.096, Table 1). Given the small group sizes in this comparison, it is possible that a closer association may be found by examining larger groups of SIV-infected macaques.

**Cellular Source of 14-3-3 in CSF**

To address the possibility that other cells in the brain of SIV-infected macaques in addition to neurons could serve as the chief cellular source of CSF 14-3-3 protein, we examined supernatants obtained from primary cultures of SIV-infected and mock-infected macaque macrophages, microglia, and astrocytes for presence of 14-3-3 protein. 14-3-3 protein was not present in cell culture supernatants from uninfected or SIV-infected macrophages, microglia, or astrocytes by immunoblot, although 14-3-3 protein could be detected by immunoblot in cell lysates prepared from the same cultures (Fig. 5). In contrast, we were able to detect elevated levels of 14-3-3 protein in culture supernatants from human fetal neuronal cultures exposed to a neurotoxic dose of HIV Tat beginning at 6 hours postexposure and increasing at 24 and 48 hours post-Tat treatment (Fig. 6). These in vitro findings were extended by immunohistochemically staining brain sections from SIV-infected macaques for 14-3-3 protein. Immunostaining demonstrated that neurons express high levels of 14-3-3 protein in both uninfected and SIV-infected macaques (Fig. 7). In contrast, we did not detect comparable high levels of 14-3-3 protein in other cell types, including infiltrating macrophages, multinucleate giant cells, parenchymal microglia, or astrocytes in the brains of SIV-infected macaques. Together, these data suggest that neurons are the primary source of CSF 14-3-3 protein in the CNS of SIV-infected macaques.

**DISCUSSION**

In this study, we demonstrated that 14-3-3 protein accumulates in the CSF of SIV-infected macaques after acute infection but prior to development of encephalitis. Four of 6 SIV-infected macaques in this study developed persistently elevated CSF 14-3-3 protein levels, beginning either at day 28 or day 42 p.i. and continuing until death at 3 months p.i. The persistent presence of 14-3-3 protein in the CSF of SIV-infected macaques was predictive for animals that ultimately developed high terminal CNS viral burdens, including high levels of SIV RNA measured by real-time RT-PCR and SIV gp41 measured by quantitative immunostaining. In addition, the persistent presence of 14-3-3 in CSF corresponded with elevated CSF viral loads.

All 3 animals that developed severe encephalitis had detectable 14-3-3 protein in CSF during asymptomatic infection. Interestingly, 1 animal that developed moderate encephalitis (BK09) had lower levels of 14-3-3 protein in CSF than the animals with severe encephalitis. In contrast, the other macaque that developed moderate encephalitis (BP41) did not have detectable levels of 14-3-3 protein in CSF after acute infection. BK09 differed from BP41 in several key ways,
including higher CSF viral load, terminal CNS viral RNA burden, CNS SIV gp141 immunostaining, and CSF MCP-1 and IL-6 levels (unpublished results). In contrast, the amount of immunostaining for the macrophage/microglial activation marker CD68 was lower in BK09 than BP41. As our previous studies have demonstrated that elevated CSF viral load and CSF MCP-1 levels are predictive of progression to SIV CNS disease, these findings suggest that, although BK09 and BP41 had comparable severity of encephalitis, BK09 was likely to progress to severe encephalitis more rapidly than BP41 (10,13).

This study demonstrates that the continued presence of 14-3-3 protein in the CSF was tightly linked with extent of viral replication in the CNS. Our previous studies in this SIV/macaque model examining selection of replicating SIV genotypes in the CNS have demonstrated that the neurovirulent molecular clone SIV/17E-Fr replicates in the brain during asymptomatic infection (Babas T, personal communication). These findings indicate that neuronal damage continues throughout infection in close correspondence with CNS virus replication and suggest that viral proteins such as gp120 and Tat primarily drive neuronal damage during asymptomatic infection rather than toxic factors produced by activated macrophages. In later stages of disease, when encephalitis develops, both viral proteins and neurotoxic macrophage products may contribute to neuronal damage. This relationship between SIV replication and neuronal impairment is consistent with our previous finding in this SIV model that the extent of neuronal dysfunction reflected by axonal accumulation of APP was most closely associated with the amount of viral protein in the brain (17). In this study, APP immunostaining was higher in the group of animals with persistent 14-3-3 protein in the CSF than the group of animals without CSF 14-3-3 protein. Our previous studies have also shown that encephalitis develops predominantly after 56 days p.i. with only 1 of 6 animals examined at 56 days p.i. developing mild encephalitis, whereas more than 90% of animals develop encephalitis at 84 days p.i. As the appearance of 14-3-3 protein in the CSF precedes the 56 day p.i. time point, CSF 14-3-3 protein is not solely a consequence of inflammatory cells entering the brain and thus is not a surrogate marker for encephalitis.

Previous studies have reported varied findings with regard to the relationship between the presence of 14-3-3 protein in the CSF and HIV dementia. While two brief reports did not detect 14-3-3 in small groups of patients with HIV dementia, Wakabayashi et al. reported the detection of specific 14-3-3 isoforms, including 14-3-3 ε, 14-3-3 γ, and 14-3-3 ζ, in the CSF of patients with HIV dementia, whereas 14-3-3 was not detected in individuals seropositive for HIV without dementia (7–9). The relationship between the appearance of 14-3-3 protein in CSF and CNS disease progression was not examined in that study as serial evaluations of CSF obtained from the same patients were not performed (9). Whether specific 14-3-3 isoforms serve key roles in the pathogenesis of various neurologic diseases, including HIV dementia, remains to be determined.

As a marker of neuronal damage, measurement of 14-3-3 protein in the CSF of HIV-infected individuals may be a key prognostic indicator as well as a means of gauging efficacy of antiretroviral treatment. One report has shown that clearance of 14-3-3 protein from the CSF predicted resolution of bacterial meningitis whereas persistence of 14-3-3 protein in CSF was found in those patients who died (19). Similarly, a study of CSF 14-3-3 in multiple sclerosis patients found that the detection of the 14-3-3 protein in the CSF at the initial neurologic event suggestive of multiple sclerosis may be a useful predictor of short-term evolution (20). Examining the correspondence between the appearance of 14-3-3 protein in CSF and contemporaneous alterations in neuroimaging findings in HIV-infected subjects could enable investigators to define the specific neuroimaging abnormalities associated with early neuronal damage. In the SIV/macaque model, the presence of 14-3-3 protein in CSF will enable investigators to pinpoint the earliest stages of neuronal damage.

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