Hippocampal Injury and Alterations in Neuronal Chemokine Co-Receptor Expression in Patients With AIDS

CAROL K. PETITO, MD, BRENDA ROBERTS, JOHN D. CANTANDO, ALEJANDRO RABINSTEIN, MD, AND ROBERT DUNCAN, PhD

Abstract. Hippocampal neurons express high levels of HIV chemokine co-receptors, activation of which causes injury or death in vitro. To determine if their in vivo expression correlates with injury, we evaluated neuronal CXCR4 and CCR5 immunoreactivity and reactive gliosis in autopsy hippocampus of 10 control cases, 11 AIDS cases without HIV encephalitis (HIVnE) or opportunistic infections/lymphomas (O/I/L), and 11 AIDS cases with HIV encephalitis (HIVE). All groups had higher CXCR4 and CCR5 expression in CA3 and CA4 neurons than CA1 neurons (p < 0.05). HIVE cases had increased neuronal CXCR4 and decreased neuronal CCR5 expression as well as increased numbers of hippocampal GFAP-positive astrocytes and LN3-positive microglia. Changes were most severe in CA3 and CA4 and lowest in CA1 regions. These findings also were noted in the 4 HIVE cases with neither hippocampal HIVE nor brain O/I/L and in the HIVnE groups. This study quantitates the regional distribution of hippocampal neuronal CXCR4 and CCR5 and shows their respective increase and decrease in AIDS. It suggests a relationship between neuronal loss and gliosis with intensity of neuronal chemokine expression and raises the possibility of a selective vulnerability of hippocampal neurons to AIDS-related injury.

Key Words: AIDS; Astrocytes; Chemokine co-receptor; Dementia; Hippocampus; HIV; Microglia.

INTRODUCTION

Progressive brain atrophy, neuronal and dendritic loss, white matter gliosis, and blood-brain barrier breakdown are among the pathological abnormalities in AIDS brains. They are commonly associated with encephalitis due to HIV (HIVE) (1–9), and clinically, with a subacute, progressive dementia termed AIDS dementia complex or HIV-associated cognitive/motor dysfunction (HAD) (10–13). Since productive HIV infection in brain is confined to macrophages and monocytes rather than neurons and glia (14, 15), possible mechanisms for brain injury include toxic factors released from activated or infected monocytes and macrophages; the deleterious effects of HIV proteins, such as gp120 and tat; and loss of normal astrocytic functions due to restricted HIV infection of these cells (16–18).

The presence of neuronal chemokine receptors may also be important in neuronal injury or death in HIV-infected patients. Certain of the chemokine receptors, which are divided into 4 subgroups designated as CC, CXC, C, and CX,C on the basis of their chemokine cysteine residues (19), are utilized for HIV cell entry. Once HIV gp120 glycoprotein interacts with host cell CD4 cell surface receptor, a conformational change occurs which allows gp120 to interact with its respective chemokine co-receptor prior to viral entry (20, 21). The V3 loop of viral gp120 is the binding site for both its chemokine co-receptors and CD4 receptor, and its amino acid sequence confers specificity for co-receptor usage (22, 23). T cell-tropic viruses preferentially use CXCR4 chemokine co-receptors and macrophage-tropic viruses preferentially use CCR5 chemokine co-receptors; some viruses are dual-tropic and some chemokine co-receptors are used by different viral strains (24–27). The presence of these HIV chemokine co-receptors on neurons renders them vulnerable to calcium influx and apoptotic cell death when exposed to their chemokine ligand, specific strain of virus, or viral gp120 (28–31).

In the present study, we tested the hypothesis that the expression of neuronal chemokine co-receptors correlates with regional brain damage. We selected the hippocampus as the area of study since its pyramidal neurons express high levels of HIV chemokine co-receptors (32, 33), and since the temporal lobe structures harbor large numbers of the microglial nodules of HIVE (34) and HIV DNA and RNA levels that equal or exceed those in frontal lobe or basal ganglia (35, 36). Additionally, hippocampal damage might contribute to HIV-related cognitive disorders in view of its pivotal role in memory formation (37, 38). We studied this structure in 22 AIDS patients with and without HIVE and in 10 controls without HIV infection, using immunohistochemistry to quantitate changes in neuronal HIV chemokine co-receptor expression, reactive astrocytes, and activated microglia. A preliminary report has been published (39).

MATERIALS AND METHODS

Paraffin-embedded sections of hippocampus were selected from AIDS patients with HIVE (n = 11); AIDS patients without HIVE (HIVnE) (n = 11); and patients with normal brains and...
no evidence of HIV infection (n = 10). The hippocampal sections of all but 3 cases were taken near the level of the lateral geniculate body. Clinical records were reviewed for HIV risk factors, the presence and duration of anti-retroviral therapy, the duration of HIV infection and AIDS, and HAD. The brains were fixed in 10% buffered formalin for 2 wk prior to sectioning as previously described (1). Six-μm-thick, deparaffinized sections were stained with hematoxylin and eosin (H&E) or were incubated in H₂O₂/phosphate buffered saline (PBS) to quench intrinsic peroxidase activity and prepared for immunohistochemistry. We used primary monoclonal antibodies to the following: glial fibrillary acidic protein (GFAP) for astrocytes, dilution 1:100, (Dako Corp, Carpenteria, CA); LN3 for reactive microglia, dilution: 1:500 (Biogenex, San Ramon, CA); HIV gp41 for productive HIV infection, dilution 1:750 (Sanofi, Chaska, MN); CXCR4 and CCR5 chemokine co-receptors, dilutions 1:100 (Pharmingen, San Diego, CA); and amyloid beta precursor protein (ABPP) for injured axons, dilution 1:200 (Zymed Corp, San Francisco, CA). We selected the antibody dilution that gave the strongest immunoreactivity with minimal or no background staining of brain. Antigen retrieval by microwaving in citrate buffer preceded the immunoreactions for ABPP, CXCR4, and CCR5. Following incubation with the primary antibody for 1 to 24 hours (h), we incubated sections with biotinylated secondary antibody for 30 min, freshly prepared avidin-biotin complex for 50 min and exposure to H₂O₂ and 3,3′-diaminobenzidine (DAB) for 5 min. Washes with PBS separated the steps. Since the number of cases (n = 32) was too large to perform the immunoreactions at one time, we randomly incubated the slides in 3 batches and randomly selected 1 of the slides to be repeated in each of the 3 batches to ensure consistency in the immunoreaction. Omission of primary antibodies served as negative controls and known positives and reactive cells in normal brain sections served as positive controls.

We used the presence of the characteristic multinucleated giant cell to diagnose HIV encephalitis (HIVE) (40), confirming the results by immunoreactivity for HIV gp41. ABPP-positive axons were documented as present or absent. Nuclear pyknosis and cytoplasmic eosinophilia identified ischemic neuronal necrosis. We used the wellaccepted terminology of de No to decribe the hippocampal subregions CA1, CA3, and CA4. In human brains, alternative nomenclature for CA4 neurons in-

RESULTS

Table 1 lists the clinical and pathological data for each patient. Ten controls without evidence of HIV infection or immunosuppression had an average age of 48 ± 19 years (yr) and a postmortem interval (PMI) of 18 ± 9 h; 6 of the 10 patients were men. Eleven AIDS cases without HIVE (HIVnE) had an average age of 39 ± 18 yr and PMI of 31 ± 18 h; 6 of the 11 were men. Eleven AIDS cases with HIVE had an average age of 35 ± 9 yr and PMI of 49 ± 30 h; 7 of the 11 were men. According to hospital records, psychoses and changes highly suggestive of AIDS dementia were noted in 1 of 2 HIVE patients with neurological examinations, and mental status changes in the 1 HIVnE case with neurological examination. Information concerning antiretroviral therapy was available in 12 AIDS patients. Three of 5 HIVE patients and 4 of 7 HIVnE patients received anti-retroviral therapy. The average duration of HIV infection and AIDS was 3.4 yr and 0.84 yr in the HIVnE patients, and 5.3 yr and 0.18 yr in the HIVE patients. Risk factor information usually was unavailable but in our hospital population of AIDS patients, intravenous drug abuse accounts for approximately 50% of HIV risk factors for men (personal information). Although the controls were older and the HIVE group had longer PMIs, the differences in age and PMI between groups were not significant.

Eight of the 10 control cases had normal brains, including sections of hippocampus and adjacent temporal cortex and white matter. One control had a small metastatic tumor nodule in the CA3 region and another had hippocampal changes consistent with hyperacute ischemic neuronal necrosis. Mild fibrillar astrocytosis was observed within the periphery of the CA4 pyramidal cell layer adjacent to the granule cell neurons of the dentate fascia. No controls had inflammation, HIV gp41 immunoreactivity, or ABPP-positive axons. Nine of the 11 HIVnE cases had normal brains, 1 had calcific vasculopathy, and 1 had white matter gliosis. Their hippocampal sections revealed fibrillar or gemistiocytic astrocytes in the CA4 region in 3 cases (Fig. 1A). None had hippocampal inflammation, gp41 immunoreactivity, or ABPP-positive axons. Five of the 11 HIVE cases had primary brain lymphoma or opportunistic infection (OI/L) (Cases 1–5). Four of the 11 HIVE patients (Cases 1, 2, 6, 7) had microglial nodules of HIVE in the pyramidal cell layer.
TABLE 1
Patient Demographics and Brain Pathology

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<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Sex</th>
<th>PMI</th>
<th>Group</th>
<th>NPATH Hippocampus</th>
<th>NPATH Temporal white</th>
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<td>53</td>
<td>M</td>
<td>27</td>
<td>Control</td>
<td>INN</td>
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</tr>
</tbody>
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Abbreviations: PMI: postmortem interval (hours); NPATH: neuropathology; HIVE: HIV encephalitis, N loss: CA4 neuronal loss, INN: ischemic neuronal necrosis; chr: chronic; HIVnE: AIDS patients without HIVE; CaV: calcific vasculopathy; Met CA: micronodule of metastatic carcinoma; 0: no lesions seen; NA: not available.

of the hippocampus, which were distributed in all 3 hippocampal regions in 1 case and were solitary, and located in either the CA1, CA3, or CA4 regions in 3 cases. Four of 11 HIVE patients (Cases 8–11) had neither OI/L in brain nor HIVE in the hippocampus. Reactive astrocytes and neuronal dropout were observed in CA4 regions of 5 cases (Fig. 1A–C), and acute diffuse ischemic neuronal necrosis in 1 case. Temporal white matter sampled with the hippocampus contained microglial nodules of HIVE in 6 cases and foci of ABPP-positive axons in 3 cases.

Table 2 summarizes the quantitative analysis of the numbers of reactive astrocytes and microglia per unit area and intensity of neuronal chemokine co-receptor immunoreactivity. Immunoreactivity for hippocampal neuronal chemokine co-receptors, GFAP, or LN3 was not affected by postmortem interval, patient age, or gender (p > 0.05) and did not appear affected by the presence of acute ischemic neuronal necrosis or temporal lobe HIVE and ABPP foci (data not shown). However, when the pyramidal neuronal layer contained HIVE microglial nodules, focal increases in reactive astrocytes and microglia were seen.

Immunoreactivity for CXCR4 and CCR5 neuronal chemokine co-receptors appeared as minute cytoplasmic granules, as previously described by Lavi et al (32), and showed some perinuclear clustering, usually at the dendritic side of the nucleus. For both co-receptors, the immunoreactivity was higher in the pyramidal neurons of CA3 and CA4 when compared to the pyramidal neurons in the CA1 region (Fig. 1D, E). We found an increase in neuronal CXCR4 expression (Fig. 2A) and decrease in neuronal CCR5 expression (Fig. 2B) with AIDS patients and with HIVE. We clustered the results into 2 groups
Fig. 1. A–C: CA4 region of the hippocampus: A: Numerous GFAP-positive fibrillary astrocytes in an AIDS patient without HIVE. (Hematoxylin; original magnification ×400.) B: Normal distribution of pyramidal neurons in a control patient. (H&E; original magnification ×100.) C: An AIDS patient with HIVE shows neuronal loss and gliosis when compared to normal controls. (H&E; original magnification ×100.) D, E: Neuronal CXCR4 chemokine co-receptors AIDS patients with HIVE. D: CA3 neurons exhibit Grade 3 immunoreactivity. E: CA1 neurons shows grade 1 immunoreactivity (see arrow). Hematoxylin; original magnification ×400.

(grades 0–1 and grades 2–3) and used Chi square and Fisher’s exact test for data analysis. In the HIVE group, CCR5 in CA4 neurons was significantly lower than controls (p < 0.011, Chi square; p < 0.023, Fisher’s exact test) and CXCR4 in CA3 neurons was significantly higher than controls (p < 0.019, Chi Square; p < 0.043, Fisher’s exact test). In the HIVnE group, CCR5 expression in CA4 neurons was significantly lower than controls by Chi Square (p < 0.046) but not Fisher’s exact test (p < 0.066).

Figure 2C depicts the numbers per 0.25 mm² of GFAP-positive reactive astrocytes in the pyramidal cell layer of the 3 subregions of the hippocampus; in controls, they were infrequent or absent in CA1 and CA3 regions. They were present in small numbers in the CA4 region, where they congregated at the periphery of the pyramidal cell layer, immediately adjacent to the granule neurons of the dentate gyrus. The number of reactive astrocytes increased in the 2 AIDS groups and was greatest in the HIVE group (p < 0.05). The increase for both HIVnE and HIVE was greatest in the CA4 region where it was 4-fold and 6-fold greater than controls. Due to the small sample size and high standard deviations, statistically significant increases were confined to the HIVE groups.

Figure 2D depicts the numbers of reactive microglia per 0.25 mm² in the hippocampus. LN3-positive microglia were rare or absent in controls and increased in AIDS. Both CA1 and CA3 were similarly increased over controls in HIVnE and HIVE, although the small sample size and high standard deviation confined statistical significance in the HIVE cases (p < 0.05).

The average number of reactive glia and grade of chemokine co-receptor expression also was determined for the 4 HIVE patients (HIVE-4) without brain lymphoma, opportunistic infections, or hippocampal HIVE. The results were similar to the entire group of 11 HIVE cases, although statistical evaluation was not done since the group was too small for meaningful comparisons. One of the 4 (Case 9) had large numbers of reactive astrocytes and microglia in the CA4 region and reactive microglia in CA3. All 4 cases, however, showed the increases in neuronal CXCR4 chemokine receptors and decrease in
TABLE 2

Means \pm Standard Deviations for Glial Cell Counts and HIV Chemokine Co-receptors

<table>
<thead>
<tr>
<th>Group</th>
<th>GFAP-positive Astrocytes</th>
<th>LN3-positive Microglia</th>
<th>CXCR4 Receptors</th>
<th>CCR5 Receptors</th>
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<td>Control</td>
<td>CA1 = 0.24 \pm 0.03</td>
<td>CA3 = 0.62 \pm 0.01</td>
<td>CA1 = 5.33 \pm 1.0</td>
<td>CA1 = 0.49 \pm 0.02</td>
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<tr>
<td>HIVnE</td>
<td>CA1 = 0.15 \pm 0.04</td>
<td>CA3 = 0.41 \pm 0.01</td>
<td>CA1 = 5.44 \pm 1.0</td>
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</tr>
<tr>
<td>HIV (4)</td>
<td>CA1 = 0.24 \pm 0.03</td>
<td>CA3 = 0.62 \pm 0.01</td>
<td>CA1 = 5.33 \pm 1.0</td>
<td>CA1 = 0.49 \pm 0.02</td>
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</tbody>
</table>

Abbrivations: PMI: postmortem interval; HIV: AIDS patients without HIV encephalitis; HIVE: AIDS patients with HIV encephalitis; HIVE: AIDS patients with HIVE. Subgroup of HIVE (4) with neither opportunistic infections, nor lymphoma in brain nor HIVE in hippocampus. Microglial numbers of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes per 0.25 mm²; microglia; numbers of LN3-immunoreactive microglial cells per 0.25 mm². CXCR4 and CCR5 receptors: chemokine co-receptors for HIV. These changes were most severe in those with HIV, many of which had coexisting OI/L and 4 of which had hippocampal HIVE. We found considerable evidence of hippocampal injury in the AIDS cases. This included focal lesions of HIVE, significant increases in reactive astrocytes and activated microglia, and alterations in neuronal chemokine co-receptors for HIV. These changes were most severe in those with HIV, many of which had coexisting OI/L and 4 of which had hippocampal HIVE. However, these changes were also present in the 4 HIV cases, having neither brain OI/L nor hippocampal HIVE, and in the 11 HIVnE patients, none of whom had OI/L. Additionally, adjacent temporal lobe white matter of the HIVE group often contained lesions of HIVE, findings in accord with an early study by De La Monte et al (34), and foci of axonal injury, similar to those described in the centrum semiovale of AIDS patients (42, 43). The actual incidence of these lesions may well be higher since the present study was limited to a single section encompassing hippocampus and that portion of temporal lobe included in the sample.

Evidence of neuronal loss, with accompanying gliosis, was apparent by routine light microscopic observations in the CA4 region in 5 of the 11 HIV cases, although the material available did not allow for quantitative stereological counts of neuronal cell numbers. Such studies have detected neuronal loss in the neocortex, basal ganglia, and cerebellar nuclei in AIDS patients (44), but neuronal loss in hippocampus is controversial. Parvalbumin-containing neurons and somatostatin-containing neurons are decreased in AIDS patients (45, 46), and neuronal loss in CA4 or in all hippocampal subfields occur in familial and simian immunodeficiency virus models of HIV, respectively (47, 48). In contrast, 2 other studies, employing stereological techniques, did not find neuronal loss in hippocampus of AIDS patients (49, 50). This apparent discrepancy concerning neuronal loss in the hippocampus could be due to different methodologies, variability in the rostral-caudal levels from which the hippocampal sections were obtained, or differences in the distribution in HIVE lesions among patients. Our study suggests that hippocampal cell loss does not occur in the absence of HIVE, but further analysis awaits quantitative evaluation.

The present study quantitates Lavi et al’s original observation that CXCR4 expression is high in CA3 and CA4 neurons and absent or low in the CA1 region (32), and documents a similar distribution for hippocampal neuronal CCR5 receptors. Our study also documents an upregulation of neuronal CXCR4 receptors and a corresponding downregulation of neuronal CCR5 receptors with AIDS. This pattern was most pronounced in the
Fig. 2. Quantitative analysis of neuronal chemokine co-receptors (A, B) and number of reactive glia (C, D) in CA1, CA3 and CA4 regions of hippocampus. Controls, n = 10; AIDS patients without HIVE (HIVnE), n = 11; AIDS patients with HIVE (HIVE), n = 11. Significant differences were determined in (A) and (B) with ANOVA and Student t-test, and in (C) and (D) by Chi square and Fisher’s exact test. HIVE (4): Four AIDS cases with HIVE but neither brain opportunistic infections/lymphoma nor hippocampal HIVE; statistical tests were not applied due to small sample size. Hippocampal regions: CA1: dotted bars; CA3: stripped bars; CA4: black bars. A, B: CXCR4 (A) and CCR5 (B) chemokine co-receptor expression, graded from 0 to 3+, was higher in CA3 and CA4 than in CA1 neurons in all 3 patient groups. CXCR4 expression increased and CCR5 decreased in AIDS patients when compared with controls. *: p < 0.05 when compared with controls. C: GFAP-positive reactive astrocytes, expressed as number per 0.25 mm², are higher in the HIVnE and HIVE cases than controls. The greatest differences occurred in the CA4 region. *: p < 0.05 when compared with controls; **: p < 0.05 when compared to controls and HIVnE. D: LN3-positive reactive microglia, expressed as numbers per 0.25 mm², increased throughout the hippocampus in both the HIVnE and HIVE cases. *: p < 0.05 when compared with controls.

HIVE group but was also present in the HIVnE cases with normal brains and in the small subgroup of 4 HIVE patients with neither OI/L nor hippocampal HIVE. Lavi et al (32) and McManus et al (51) briefly describe increased neuronal CXCR4 immunoreactivity in hippocampus and cerebral and cerebellar cortex of AIDS patients with HIVE, findings consistent with the present quantitative study. Other conditions with upregulation of neuronal chemokine receptor include transforming growth factor β1 receptor (52) in HIVE, CXCR2 receptors on dystrophic neurites in Alzheimer disease (53, 54), and of CCR5 receptor mRNA in excitotoxic-induced neuronal injury (55). In contrast to HIVE, chemokine co-receptor staining of proximal dendrites was reduced in SIV encephalitis (SIVE) when compared with uninfected controls (56). Both AIDS and SIVE have increased numbers of CXCR4 and CCR5-expressing microglia and perivascular inflammatory cells (56–58).
Potential mechanisms for altered neuronal chemokine receptor expression in HIV infection include latent HIV infection, as Cota et al have shown in cultured astrocytes infected by HIV (59), or alterations in their respective ligands, including HIV gp41 and tat, exposure to which causes upregulation of cultured neuronal CCR5 expression (60). If parallel situations exist in AIDS, changes in their cytokine environment or exposure to virus or viral gp120 could underlie the alterations in neuronal expression of their respective receptors.

There is little evidence that systemic hypoxia-ischemia played a role in the hippocampal abnormalities seen in our AIDS cases. This metabolic insult selectively damages the vulnerable neurons in the CA1 region (61, 62), producing intense astrocystosis in CA1 and only transient increases in GFAP in the resistant CA3 region (63). The reverse was true in our AIDS patients, where the hippocampal gliosis was low in the CA1 region but severe in the CA3 and CA4 regions, and accompanied by neuronal loss in the CA4 regions. Review of the major connections between the different hippocampal fields (the trisynaptic circuit) suggests that we cannot entirely rule out transsynaptic degeneration in the hippocampus due to brain injury in other regions with hippocampal projections. This would, however, require selective damage to the mossy fiber pathway to explain the apparent selective vulnerability of CA4 and CA3 regions (41, 64–66). Whereas the perforant pathway from entorhinal cortex projects to all hippocampal neurons, the mossy fiber pathway arising from dentate gyrus neurons selectively projects to CA4 (CA3h) and CA3 neurons. The Schaffer collaterals from the CA3 to the CA1 neurons, representing the third component of the trisynaptic pathway; the CA1 and subiculum efferents; and diffuse hippocampal afferents from subcortical and brain stem nuclei do not provide appropriate synaptic connections for the selective hippocampal vulnerability found in our AIDS brains.

The selective vulnerability of CA3 and CA4 regions in the hippocampus of AIDS patients may relate to the regional distribution of their neuronal chemokine receptors. The distribution of chemokine receptors is high in CA3 and CA4, paralleling the hippocampal gliosis and neuronal loss that we detected in the current study. Reactive astrocystosis was low or absent in CA1 and high in CA4, and, to a lesser extent in CA3, and neuronal loss was apparent in CA4. This relationship was apparent in the HIVnE and HIVE groups as well as in the subgroup of 4 HIVE cases with neither OI/L nor hippocampal HIVE. Neuronal chemokine co-receptors are functionally active and neuronal signaling, calcium influx, and apoptotic cell death follows exposure to their respective chemokines, virus, or gp120 ligands (28–31, 67). The microglial activation did not correlate well with specific hippocampal subfields, which may reflect the fact that HIV-infected patients, with or without AIDS or HIVE, display a generalized and diffuse microglial activation throughout the CNS (68, 69).

The present demonstration of enhanced neuronal CXCR4 receptors and downregulation of CCR5 receptors suggests that they may be more vulnerable to T-tropic virus or gp120 glycoproteins than macrophage-tropic virus and glycoprotein. This would parallel the in vitro situation in which cultured neurons have higher levels of CXCR4 receptors than CCR5 receptors (58, 70) and are more vulnerable to T-cell tropic viruses than to macrophage-tropic viruses (29, 30). The principal blood-borne viruses in end-stage AIDS patients are T cell-tropic (71, 72); another factor consistent with T-cell-tropic virus-induced neuronal injury in the setting of elevated neuronal expression of CXCR4 receptors. Our preliminary data shows that postmortem brains of AIDS patients contain CD4 T-lymphocytes (73), which are a potential source of virus or viral gp120 that could activate the neuronal CXCR4 receptors. Thus, increased neuronal CXCR4 receptors in AIDS patients may be important mediators of HIV-related brain damage.

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