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Neuronal Apoptosis in HIV-1-Associated Central Nervous Diseases and Neuropathic Pain

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1. Introduction

The human immunodeficiency virus type 1 (HIV-1) pandemic has claimed over 20 million lives, with 38.6 million people worldwide currently infected (2009 AIDS Epidemic Update by UNAIDS/WHO, www.unaids.org), and will continue to contribute to human morbidity and mortality. The number of people living with HIV-1 continues to rise worldwide because of high rates of new infections and the success of antiretroviral therapy (ART, also known as the highly active antiretroviral therapy or HAART). HIV-1 infection results in a variety of syndromes involving both the central and peripheral nervous systems. HIV-1 invades the central nervous system (CNS) early during the course of infection and persists thereafter in the absence of treatment. HIV-1 infection of the CNS is often associated with neurological complications including HIV-1-associated severe and mild neurologic disorders. Prior to HAART, neurologic disorders were the first manifestation of symptomatic HIV-1 infection, affecting roughly 10% – 20% of patients and up to 60% of patients in the advanced stages of acquired immunodeficiency syndrome (AIDS)[1]. HIV-1 infection is also associated with neuropathic pain, which is caused by peripheral nervous system injury. The vast majority (up to 90%) of individuals infected with HIV-1 have pain syndromes that significantly impact their well-being[2-13]. A variety of pain syndromes including peripheral neuropathies, headache, oral and pharyngeal pain, abdominal and chest pain, arthralgias and myalgias as well as pain related to HIV-1/AIDS-associated malignancies such as Kaposi's sarcoma[14,15]. Pain occurs at all stages of HIV-1 infection, although its severity and frequency are correlated with disease progression[2,16]. In the era of HAART, patients infected with HIV-1 live longer, and management of their symptoms including pain has emerged as a top priority for HIV-1 clinical and translational research.

Uniquely, neuronal injury, cell loss and dysfunction during HIV-1 infection appear to occur through soluble neurotoxins rather than productive virus infection, because there is no

evidence that HIV-1 directly infects neurons. Apoptosis, an active process of cell death characterized by cell shrinkage, chromatin aggregation with genomic fragmentation and nuclear pyknosis, appears to be an important feature of HIV-1-associated central neurological dysfunction and peripheral neuropathy. Apoptotic neurons have been observed in the CNS of HIV-1-infected individuals, and are more abundant in HIV-1 patients with peripheral neuropathy. Herein, we review the role of neuronal apoptosis in HIV-1-associated neurological disorders, focusing on HIV-1-associated CNS manifestations and sensory neuropathic pain. This review also summarizes the current data supporting both the direct and indirect mechanisms by which neuronal apoptosis may occur during HIV-1 infection. Finally, we discuss recent and past approaches for the prevention and treatment of HIV-1-associated neurological disorders by targeting specific neurotoxic signaling pathways.

2. Overview of HIV-1-associated neurological disorders

Neurologic disorders are among the most frequent and devastating complications of HIV-1 infection. Prior to HAART, neurologic disorders were the first manifestation of symptomatic HIV-1 infection, affecting roughly 10% – 20% of patients and up to 60% of patients in the advanced stages of HIV-1/AIDS[1]. The incidence of subclinical neurologic disease is much higher. Autopsy studies of AIDS patients have demonstrated pathologic abnormalities of the nervous system in 75 - 90% of cases[17,18]. Neurologic disorders continue to be prevalent in the era of HAART. HAART has reduced the incidence of severe forms of AIDS-associated neurologic disorders such as HIV-1-associated dementia (HAD), but with longer life span, the prevalence of milder forms of neurologic manifestations such as HIV-1-associated neurocognitive disorder (HAND) appears to be increasing[19,20]. In resource-rich settings such as the United States and the European Union, where antiretroviral therapy is relatively available, peripheral neuropathy and HIV-1-associated cognitive dysfunction (including HAD) account for the greatest proportion of neurologic disease burden[21,22]. In resource-poor settings such as developing countries, opportunistic infections of CNS account for most of the reported neurologic morbidity and mortality in AIDS patients[23]. Fulminant bacterial meningitis, cryptococcal meningitis, neurotuberculosis, neurosyphilis, and toxoplasmosis are common among HIV-1-infected individuals in Asia and Africa[23].

The neuropathic syndromes associated with HIV-1 infection are diverse and include both the somatic and autonomic nervous systems. The primary pathological abnormality may be demyelination, both acute and chronic, or axonal degeneration. Either single or multiple nerves may be involved leading to mono- or polyneuropathy. HIV-1 invades the CNS early in the infectious course, and eventually causes mild or severe forms of HIV-1-associated neurologic manifestations[24]. Severe manifestations include HIV-1-assocated dementia complex (HAD, HIV-1-encephalopathy, and subacute encephalitis) and HIV-1-associated myelopathy, whereas mild manifestations include HIV-1-associated neurocognitive/motor disorders and HIV-1-associated neurobehavioral abnormalities (Table 1)[25]. HIV-1 also affects the peripheral nervous system (PNS) and PNS damage can be assessed by electroneurographic examinations quantifying nerve conduction velocity (NCV) and action

potential amplitudes. Defined disorders of the PNS comprise the HIV-1-associated Guillain-Barré syndrome and sensory polyneuropathy (Table 1)[26].

| CNS disorders	PNS disorders

Severe manifestations
  HIV-1-assocated dementia complex
    HIV-1-associated dementia (HAD)
    Subacute encephalitis
    HIV-1-encephalopathy
  HIV-1-associated encephalopathy (HIVE)
  HIV-1-associated myelopathy (HIVM)
	HIV-1-associated Guillain-Barré syndrome
	HIV-1-associated sensory polyneuropathy

Mild manifestations
  HIV-1-associated neurocognitive/motor disorder
  HIV-1-associated neurobehavioral abnormalities	

Table 1. HIV-1-associated CNS and PNS disorders
HAD: HIV-1-associated dementia; HIVE: HIV-1-associated encephalopathy; HIVM: HIV-1-associated myelopathy.

3. Neuronal apoptosis in the central nervous system in HIV-1 infection

Apoptosis of neurons and non-neuronal cells has been demonstrated in the brain of HAD patients[27]. Petito and Roberts reported that neuronal and astrocytic death in HIV-1 infection occurred by apoptosis[27]. They identified apoptotic neurons, astocytes, and multinucleated giant cells in the brain of adults with HIV-1-associated encephalopathy (HIVE) using a combined approach of in situ end labeling (ISEL) and immunohistochemistry[27]. Neuronal apoptosis occurs not only in HIV-1-infected adults but also in infected children. Using an *in situ* technique, Gelbard et al found that newly cleaved 3'-OH ends of DNA, a marker for apoptosis, in the brain of children with HIVE[28]. They demonstrated the presence of apoptotic neurons in cerebral cortex and basal ganglia of children that had HIVE with progressive encephalopathy[28]. In addition, association of the localization of apoptotic neurons with perivascular inflammatory cell infiltrates containing HIV-1 infected macrophages and multinucleated giant cells was observed[28]. Adle-Biassette et al found apoptotic perivascular cells in the brain of adults with HIVE[29]. Neuronal apoptosis was more severe in atrophic brains, and did not directly correlate with productive HIV-1 infection, suggesting that an indirect mechanism of neuronal damage exists. Shi et al demonstrated neuronal apoptosis in brain tissue from AIDS patients and in HIV-1-infected primary human fetal brain cultures[30]. HIV-1 infection of primary brain cultures induced apoptosis in neurons and astrocytes *in vitro* as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and propidium iodide (PI) staining and by electron microscopy[30]. Apoptosis was not significantly induced until 1 - 2 weeks after the time of peak virus production, suggesting that apoptosis of neurons and non-neuronal cells in HIV-1 infection is triggered by soluble factors rather than by direct viral infection[30]. An et al used ISEL technique to examine neuronal apoptosis in the brains of AIDS and pre-

AIDS patients[31,32]. The presence of apoptotic cells in the brains of HIV-1-positive pre-AIDS individuals was observed, although the frequency was lower than that in the brain of AIDS patients. These data suggest that brain damage already occurs during the early stages of HIV-1 infection[31,32].

Apoptotic neurons are found in several regions of the brain of adults with HIVE including the frontal and temporal cortex, basal ganglia and brain stem[27-32]. The basal ganglia apoptotic neurons were found to be more abundant in the vicinity of activated microglia that had higher HIV-1 copies as determined by measuring HIV-1 core proteins[31,32]. Within the cerebral cortex, the extent of neuronal apoptosis correlated with cerebral atrophy patients[31,32]. In addition, apoptotic neurons in the basal ganglia and cerebral cortex of children with HIVE were detected in the vicinity of perivascular inflammatory cell infiltrates containing HIV-1- infected multinucleated giant cells and macrophages[26].
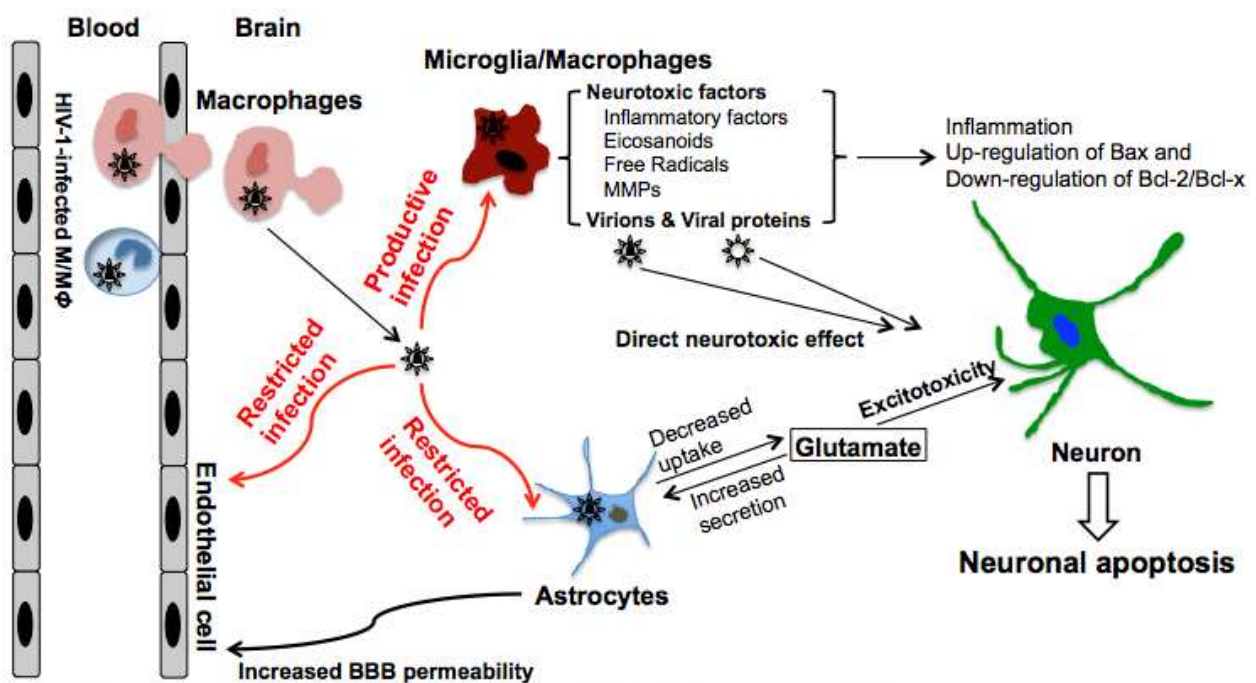
Note: This figure was modified from Jones G. & Power C. Neurobiology of Disease, 2006; 1 – 17
M/MΦ: monocytes/macrophages

Figure 1. Neuronal apoptosis in the central nervous system in HIV-1 infection.
Monocytes/macrophages (M/MΦ) play a crucial role in HIV-1-associated neurologic disorders. They are among the first cells infected by HIV-1, which then forms a reservoir of HIV-1 in infected individuals. HIV-1-infected monocytes/macrophages serve as a means (Trojan horse) of spreading the virus to other tissues such as the brain. HIV-1 gains entry into the brain within macrophages that efficiently produce new virions. These virions infect microglia, astrocytes, endothelial cells, and multinucleated giant cells in the brain. Virions and viral proteins, and the neurotoxic factors released from HIV-1-infected cells induce apoptosis of neurons. The disruption of glutamate homeostasis induces neuronal excitotoxicity, resulting in neuronal cell death.

In addition to apoptotic neurons, apoptosis of other CNS cell types in the brain of HAD patients has also been reported. Apoptotic astrocytes were detected and found to be more

common in the brain of HAD patients compared to non-demented HIV-1/AIDS patients[27-32]. In addition, greater numbers of apoptotic astrocytes were detected in the brains of AIDS patients with rapidly progressing dementia compared to slow progressors[28,29]. Astrocytes are the most abundant cells of the human brain, and perform many functions including biophysical support of endothelial cells that form the blood–brain barrier (BBB), provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord following traumatic injuries. HIV-1-mediated loss of astrocytes impairs maintenance of the BBB and alters the composition of the extracellular environment, resulting in increased BBB permeability. These findings suggest that astrocyte cell loss plays a critical role in the neuropathogenesis of HAD (Figure 1). Table 2 summarizes the studies *in vivo* and *in vitro* on neuronal apoptosis in HIV-1 infection.

In vivo studies	
Petito *et al.*, 1995	Apoptotic neurons, astrocytes, and multinucleated giant cells in the brain of adults with HIVE
Gelbard *et al.*, 1995,	Apoptotic neurons, macrophages, and microglia in the brain of children with HIVE
Adie-Biassette *et al.*, 1995	Apoptotic neurons and perivascular cells in the brain of adults with HIVE
Shi *et al.*, 1996	Apoptotic neurons, astrocytes, and endothelial cells in the brain of adults with HIVE
An *et al.*, 1996	Apoptotic neurons and glial cells in the brain of AIDS patients and pre-AIDS patients
Krajewski *et al.*, 1997	Elevated numbers of Bax-positive microglia and macrophages in the brain of children with HIVE
Vallat *et al.*, 1998	Apoptotic neurons, astrocytes, endothelial cells, pericytes, and macrophages in the brain of AIDS patients
In vitro studies	
Shi *et al.*, 1996	Apoptosis of neurons and astrocytes in primary human brain cultures infected with HIV-1$_{89.6}$
New *et al.*, 1997	Apoptosis of primary human neurons induced by soluble HIV-1 Tat protein
Talley *et al.*, 1995	Apoptosis of differentiated SK-N-MC human neuroblastoma cells induced by TNF-α
Muller *et al.*, 1992	Apoptosis of neurons in rat cortical cultures induced by soluble HIV-1 gp120 protein
Chi *et al.*, 2011	Apoptosis of rat primary dorsal root ganglion neurons directly induced by HIV-1 Tat protein

This table was modified from Shi et al., the Journal of NeuroVirology, 1998; 4, 281 - 290

Table 2. Summary of *in vitro* and *in vivo* studies on neuronal apaoptsois in HIV-1 infection

4. Neuronal apoptosis is associated with pain in HIV-1-infected patients.

The vast majority (up to 90%) of individuals living with HIV-1 have pain syndromes that significantly impact their well-being[2-13]. Pain occurs at all stages of HIV-1 infection, although its severity and frequency are correlated with disease progression[2,16]. In the era of HAART, patients infected with HIV-1 live longer, and management of their symptoms including pain is emerging as a top priority for HIV-1 clinical and translational research. However, pain is often under-assessed and undertreated in people with HIV-1/AIDS, and little progress has been made in understanding the underlying mechanisms that are key for pain management and improvement of quality of life.

The pain in HIV-1-infected patients is believed to result from (1) direct neurotoxic effects of viral components on neurons in both central and peripheral nervous systems, (2) immune dysregulation leading to inflammatory changes, opportunistic infections and/or tumors, and (3) the adverse effects related to antiviral drugs[2,3,33]. Viral proteins are directly involved in neuronal damage and death. HIV-1 encodes a total of nine viral proteins including three structural proteins (Env, Pol, and Gag), two essential regulatory proteins (Tat and Rev), and four accessory proteins (Vif, Vpr, Vpu, and Nef). These viral proteins have been extensively studied for their role in HIV-1-associated CNS neuropathy. To date, five of these proteins including Env, Tat, Vpr, Nef, and Rev have been identified as potent neurotoxic viral

proteins in that they directly induce neuronal cell death (Figure 2). These proteins are implicated in HIV-1-associated CNS pathologies such as mild to severe cognitive impairments and encephalitis[34-43]. Recent studies have shown that Env and Vpr exert neurotoxic activities on peripheral sensory neurons (pain-sensing neurons). Injection of Env into the spinal intrathecal space of rats causes marked pain-like effects[44-48], and Vpr enhances excitability of dorsal root ganglion (DRG) neurons[49]. These results suggest that HIV-1 neurotoxic proteins have direct effects on peripheral nerves, and may be causative factors in the generation of neuropathic pain in HIV-1-infected individuals.
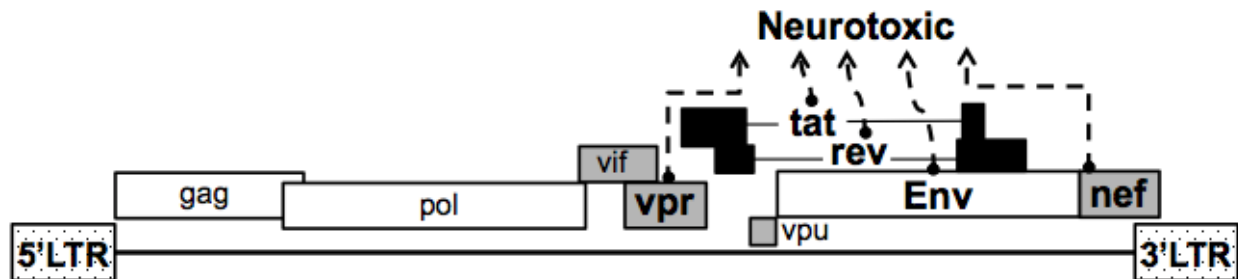
Figure 2. HIV-1 genome and viral proteins. HIV-1 is composed of two copies of single-stranded RNA genome with approximately 9700 bp in length. HIV-1 genome encodes a total of nine viral proteins including three structural proteins (Env, Pol, and Gag in open boxes), two essential regulatory proteins (Tat and Rev in black boxes), and four accessory proteins (Vif, Vpr, Vpu, and Nef in gray boxes). Genes encoding HIV-1 proteins with know neurotoxicity are given in bold.

HIV-1 Env gene encodes a 160-kDa envelope glycoprotein (gp160) precursor, which is proteolytically cleaved into the exterior (gp120) and transmembrane (gp41) glycoproteins. The gp120 remains associated with the mature envelope glycoprotein complex through a non-covalent interaction with the gp41 ectodomain. Three exterior gp120 glycoproteins and three transmembrane gp41 proteins are assembled as a trimer by non-covalent interactions. The gp120 has been shown to directly interact with neurons, leading to neuronal apoptosis. *In vivo* experiments have shown that subcutaneous injection of purified recombinant gp120 in neonatal rats causes dystrophic changes in pyramidal neurons of cerebral cortex accompanied by abnormalities of developmental behaviors[50]. Toggas et al generated transgenic mice expressing gp120 mRNA in astrocytes and found a spectrum of neuronal and glial changes resembling abnormalities in brains of HIV-1-infected individuals[51]. The severity of brain damage correlated positively with the brain level of gp120 expression[51]. These results provide *in vivo* evidence that gp120 plays a key role in HIV-1-associated nervous system impairment. Damage of neurons has also been reported in transgenic mice expressing the entire HIV-1 genome[52], Tat[53], or Vpr[54]. Transgenic mice expressing Nef[55,56] primarily showed severe impairment of thymocyte development and peripheral T-cell function by a CD4-independent mechanism. Nef expression in these mice also indirectly contributes to HIV-1-associated neuropathy by enhancing protein expression of other HIV-1 genes and altering cellular maturation *in vivo*[57]. *In vitro* exposure to HIV-1 proteins including gp160, gp120, gp41, Tat, Nef, Rev, and Vpr has been reported to initiate neuronal damage[58-62].

Dorsal root ganglion (DRG) neurons have central terminals in the spinal cord dorsal horn and peripheral terminals in skin, muscle and other peripheral tissues. These DRG neurons transmit and relay pain-related signals and temperature sensation from peripheral tissues to the spinal cord and brain. Neurotoxins and inflammatory molecules cause hyperexcitability of primary DRG neurons leading to spontaneous or persistent firing. These agents also induce apoptosis of DRG neurons, resulting in permanent neuron damage, death, and nerve lesions. It is well established that peripheral sensitization of primary DRG neurons is the key event in the onset of chronic pain conditions [63]. Peripheral sensitization is defined as the enhancement of a baseline response, such as action potential (AP) firing, after exposure to a defined mediator [63]. For example, exposure to the pro-inflammatory prostaglandin E_2 (PGE_2), increases the number of APs evoked by an excitatory stimulus by about three-fold compared to the control, thus the neuronal output is intensified by PGE_2 [64,65]. This increased firing is believed to be critical in the enhanced perception of pain sensation for both inflammatory and neuropathic conditions [64,65]. Our group has recently explored the direct effects of HIV-1 Tat protein on excitability of rat primary DRG neurons[62]. We demonstrated that HIV-1 Tat triggered a rapid and sustained enhancement of the excitability of small-diameter rat primary DRG neurons, which was accompanied by marked reductions in the rheobase and resting membrane potential (RMP), and an increase in the resistance at threshold (R_{Th}). Such Tat-induced DRG hyperexcitability may be a consequence of the inhibition of cyclin-dependent kinase 5 (Cdk5) activity. Tat rapidly inhibited Cdk5 kinase activity and mRNA production, and roscovitine, a well-known Cdk5 inhibitor, induced a very similar pattern of DRG hyperexcitability. Indeed, pre-application of Tat prevented roscovitine from having additional effects on the RMP and action potentials (APs) of DRGs. However, Tat-mediated actions on the rheobase and R_{Th} were accelerated by roscovitine. These results suggest that Tat-mediated changes in DRG excitability are partly facilitated by Cdk5 inhibition. In addition, Cdk5 is most abundant in DRG neurons and participates in the regulation of pain signaling. We also demonstrated that HIV-1 Tat markedly induced apoptosis of primary DRG neurons after exposure for longer than 48 h. Together, our work indicates that HIV-1 proteins are capable of producing pain signaling through direct actions on excitability and survival of sensory neurons.

However, we still do not know: (1) whether all five HIV-1 neurotoxic proteins induce pain-related signaling, (2) what is their rank order for induction of pain-related signaling, (3) whether these neurotoxic proteins exert synergistic effects on pain-related signaling, and (4) the molecular mechanisms by which HIV-1 neurotoxic proteins exert neuropathogenic effects on sensory neurons. Clarification of these issues will have potentially important implications for developing therapeutic strategies to prevent or treat HIV-1-assciated pain. For example, early initiation of HAART and/or neutralization of HIV-1 protein neurotoxicity may significantly prevent or delay HIV-1-associated pain.

5. Mechanisms of neuronal apoptosis induced by HIV-1 infection

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway[66,67]. There is an additional pathway that involves T-cell

mediated cytotoxicity and perforin-granzyme-dependent killing of the cell by inducing apoptosis via either granzyme B or granzyme A[67]. The extrinsic and intrinsic pathways are initiated by caspase-3 activation induced by proteolytic cleavage and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells, whereas the granzyme pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage. All of these three pathways may be involved in neuronal apoptosis in HIV-1 infection.

5.1. Extrinsic pathway

Several factors including neurotoxins such as tumor necrosis factor alpha (TNF-α) secreted by HIV-1–infected macrophages and microglia, and soluble forms of the HIV-1 gp120 and Tat proteins have been reported to mediate HIV-1-induced neuronal injury[30]. TNF-α is elevated in the serum, cerebrospinal fluid (CSF), and brain of AIDS patients. The elevated TNF-α levels have been shown to correlate with clinical dementia[68]. The binding of TNF-α to TNF-α receptor-1 (TNFR1) on the surface of neurons activates TNFR1-associated death domain protein (TRADD), which in turn interacts with Fas-associated death domain protein (FADD) to induce apoptosis of neurons[69]. However, TNF-α and TNF-beta (TNF-β) have also been shown in certain circumstances to be neuroprotective. TNF-α and TNF-β protect cultured embryonic rat hippocampal, septal, and cortical neurons against glucose deprivation-induced injury and excitatory amino acid toxicity[70]. TNF-α induces the expression of the chemokine CX3CL1 in astrocytes[71], which in turn protects neurons from HIV-1 gp120-mediated toxicity[72]. TNF-α protective or destructive effects on neuronal survival depend on the timing, duration, and concomitant expression of other elements.

The TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is also involved in neuronal apoptosis[73]. TRAIL is a type II integral membrane protein and expressed by multiple cell types[73]. TRAIL protein levels were increased in human monocyte-derived macrophages after HIV-1 infection and immune activation[73]. In the brain of HIVE patients, TRAIL-expressing macrophages were found in association with active caspase-3 positive neurons[73]. *In vitro* studies have shown that TRAIL induces a dose-dependent effect on neuronal apoptosis[73].

Five HIV-1 proteins including Env, Tat, Vpr, Nef, and Rev, are potent neurotoxic viral proteins that cause apoptosis of various neuronal populations in the CNS, resulting in pathologies such as cognitive impairment and encephalitis associated with NeuroAIDS (Figure 2). HIV-1 gp120 binds with high affinity to CXCR4 expressed on human neurons[74], resulting in neuronal apoptosis[40,75]. CXCR4 is a seven transmembrane domain G-protein-coupled receptor and activation of this receptor via binding of gp120 triggers neuronal apoptosis. The exact mechanism underlying gp120-CXCR4-mediated neuronal apoptosis remain unclear, but is not related to increases in intracellular calcium (Ca^{2+}) or to induction of glutamate uptake by functional glutamate receptors[75]. Tat appears to exert its neurotoxic activity via binding to the N-methyl D-aspartate (NMDA) receptor and the low-density

lipoprotein receptor-related protein (LRP)[76-79], whereas evidence of Vpr, Nef, or Rev binding to surface receptor(s) of neurons is lacking. Binding of HIV-1 Tat to LRP triggers formation of a macromolecular complex involving the LRP, NMDA receptors, postsynaptic density protein-95 (PSD-95), and neuronal nitric oxide synthase (nNOS) at the neuronal plasma membrane. This complex leads to neuronal and astrocyte apoptosis. Blockade of LRP-mediated Tat uptake, NMDA receptor activation, or nNOS significantly reduces neuronal apoptosis, suggesting that formation of this complex is an early step in Tat-mediated neuronal apoptosis. CCL2, an inflammatory chemokine, inhibits formation of the complex, resulting in protection against Tat-mediated neuronal apoptosis[80].

During HIV-1 infection, multiple viral proteins co-exist in circulation and tissues, and synergize with one another to accelerate disease progression. For example, Env and Tat act synergistically to cause cell death in CNS neurons[81]. Vpr and Nef each can induce injury of podocytes, and have a synergistic effect on podocyte injury and the subsequent development of glomerulosclerosis[82]. In addition, HIV-1 proteins have synergistic effects with inflammatory factors[83], methamphetamine[84,85], and ethanol[86] in acceleratory HIV-1-associated neurological disorders. Thus, it is possible that the known HIV-1 neurotoxic proteins have synergistic effects on neuronal apoptosis.

5.2. Intrinsic pathway

The intrinsic (or mitochondrial) pathway of apoptosis is intracellularly initiated in response to various types of intracellular signals including growth factor withdrawal, DNA damage, unfolding stresses in the endoplasmic reticulum (ER) and death receptor stimulation. The intrinsic apoptotic pathway is characterized by permeabilisation of the mitochondria and release of cytochrome C (Cyt C) into the cytoplasm. Once it is released, Cyt C binds to the cytosolic protein known as apoptotic protease activating factor 1 (Apaf-1) to facilitate the formation of the apoptosome, a large multi-protein complex formed in the process of apoptosis[87,88]. Subsequently, the apoptosome binds and activates caspase-9 preproprotein. Activated caspase-9 then activates downstream caspases resulting in the activation of caspase cascade[87,88].

Studies have shown that HIV-1 Vpr directly interacts with the mitochondrial membrane to induce neuronal apoptosis through the intrinsic pathway[54]. Vpr directly bind to the adenine nucleotide translocator (ANT), a component of the mitochondria permeability transition pore located on the inner mitochondrial membrane, to trigger a rapid dissipation of the mitochondrial transmembrane potential and the mitochondrial release of apoptogenic proteins such as Cyt C or apoptosis inducing factor[89,90]. Vpr induces neuronal cell death *in vivo* in the absence of both profound microglia activation and pro-inflammatory gene expression[54]. In addition, Vpr causes neuronal apoptosis independent of the presence and function of the p53 tumor suppressor[54,91], a transcription factor which regulates the expression of several pro-apoptotic proteins such as Bax, Bad, Puma, and Bid[92]. These findings further indicate that Vpr-induced apoptosis is effected via the intrinsic pathway.

In addition to Vpr, HIV-1 gp120 and Tat also trigger neuronal apoptosis via intrinsic pathway[93]. Both gp120 and Tat enhance p53 expression and phosphorylation, and promote BCL-2-associated X protein (Bax) insertion into the mitochondrial membrane. Peripheral blood mononuclear cells (PBMCs) and lymph node syncytia from HIV-1 patients show accumulation of phosphorylated p53[94]. Accumulation of p53 protein in neurons of HAD patients and in CNS tissues from monkeys with simian immunodeficiency virus (SIV) encephalitis (SIVE) has been reported[95,96]. It is well established that p53 can mediate the intrinsic apoptosis pathway[94]. HIV-1 can establish a productive infection in perivascular macrophages and a subset of parenchymal microglia in the brain. These infected cells secrete gp120 and other immunomodulatory factors that lead to activation of astrocytes and microglia. Accumulation of p53 protein also occurs in neurons and non-neuronal CNS cells, which is required in both neurons and microglia for gp120-induced neuronal apoptosis[97].

The Bcl-2 family of proteins plays a key role in the regulation of apoptosis[98,99]. This gene family is comprised of antiapoptotic members including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Bfl-1, and Bcl-B, and proapoptotic members including Bax, Bak, Bad, Bok, Bik, Bid, Bim, Hrk, Blk, Bnip3, Noxa, Puma, and Bcl-G[100]. Both Tat and gp120 promote Bax insertion into the mitochondrial membrane and subsequent release of Cyt C[93]. Such effect of HIV-1 gp120 and Tat can be blocked by anti-apoptotic proteins BCL-2/BCL-XL[93]. In addition, Tat also inhibits expression of BCL-2 in neurons, resulting in induction of neuronal apoptosis[101]. *In vitro* studies have shown that over-expression of Bcl-2 in neuronally differentiated human SK-N-MC cells protect neurons against Tat-induced apoptosis[101].

Dysregulation of neuronal cell Ca^{2+} homeostasis plays a central role in both HIV-1 gp120 and Tat-induced neuronal apoptosis through the intrinsic pathway[54,102]. Both gp120 and Tat disrupt neuronal Ca^{2+} homeostasis by perturbing Ca^{2+}-regulating systems in the plasma membrane and ER. By altering voltage-dependent Ca^{2+} channels, glutamate receptor channels, and membrane transporters, HIV-1 gp120 and Tat promote Ca^{2+} overload, oxyradical production, and mitochondrial dysfunction. Exposure to gp120 induces an increased release of arachidonic acid in rat primary neuronal cell culture followed by NMDA receptor-mediated neurotoxicity[103]. The elevated arachidonic acid impairs metabolic balance of glutamate in neurons, resulting in lethal levels of Ca^{2+} influx. As evidence, gp120-induced neurotoxicity is inhibited by dizocilpine and memantine, two blockers of NMDA receptors, and by AP5, a competitive antagonist of the glutamate-binding site on NMDA receptors, but not by antagonists of non-NMDA receptors, which are less permeable to calcium[81,102]. Similar to gp120, Tat activates a wide variety of intracellular signals, some of which play a critical role in neuronal apoptosis. Tat causes activation of c-Jun N-terminal kinases (JNKs), activator protein-1 (AP-1), and phosphatidylinositol 3-kinases (PI3Ks) in a time- and dose-dependent manner[104,105], leading to increases in activity of the protein kinase C (PKC) and mitogen-activated protein (MAP) kinase[104]. Intact molecules of Tat and peptide fragments of Tat, in particular, Tat$_{31-61}$, increased intracellular Ca^{2+} in cultured human fetal neurons[106]. The Ca^{2+} influx contributed to Tat-induced neuron depolarization by activation of glutamate receptors[106]. Tat is also able to increase levels of inositol 1,4,5-triphosphate (IP$_3$) that in turn triggers Ca^{2+} release from IP$_3$-sensitive ER stores in cultured human fetal

astrocytes and neurons[107]. Inhibition of IP$_3$-mediated Ca^{2+} release from the ER has been shown to inhibit Tat-induced neurotoxicity[107]. Notably, HIV-1 gp120 and Tat have a synergistic effect on Ca^{2+} dysregulation in neurons[81]. Subtoxic concentrations of gp120 and Tat causes prolonged increases in levels of intracellular Ca^{2+}, resulting in neuronal cell death. The memantine, a potent NMDA receptor blocker, can completely block the neurotoxicity caused by Tat and gp120 applied in combination[81].

Both HIV-1 gp120 and Tat directly stimulate neurons and non-neuronal cells in the brain to produce inflammatory factors including TNF-α, interleukin (IL)-6 (IL-6), IL-8, and CXCL10 (also known as interferon gamma-induced protein 10 or IP-10)[47,108-110]. These inflammatory factors cause excessive Ca^{2+} influx and oxidative stress, resulting in neuronal apoptosis.

Notably, there is crosstalk between the extrinsic and intrinsic pathways[67]. For example, cleavage of the BCL-2-family member Bid by caspase-8 activates the mitochondrial pathway after apoptosis induction through death receptors, and can be used to amplify the apoptotic signal[111].

6. Future perspectives: Implications for therapy

As discussed, productive infection of macrophages, neurotoxic proteins, inflammatory factors produced by activated non-neuronal cells, in particularly microglia cells and astrocytes, in the brain of HIV-1-infected individuals are involved in neuronal apoptosis in HIV-1 infection. Therefore, prevention or treatment of neuronal injury in HIV-1 infection should include strategies to combat these factors. HAART has reduced the incidence of severe forms of HIV-1-associated neurologic disorders such as HAD, but with longer life span, the prevalence of milder forms of neurologic manifestations such as HAND appears to be increasing. One obstacle to control brain HIV-1 infection is the fact that antiretroviral drugs in the HAART regimens generally have a poor penetration into the CNS[112]. Thus, novel strategies to improve the delivery of these drugs to the CNS are urgently needed. Studies have shown that some host factors may affect the intracellular drug concentration leading to the inability of drug regimens to inhibit HIV-1 replication in cells[113]. For example, the ATP-binding cassette transporter proteins, such as P-glycoprotein and multidrug resistance-associated proteins (MRPs), affect efflux of nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs)[113]. Inhibition of P-glycoprotein or MRPs increases uptake of saquinavir, a protease inhibitor, in the mouse brain[114]. Therefore, targeting these transporter proteins may improve delivery of HAART regimens to the CNS.

Neurotoxic factors released from HIV-1-infected and/or activated macrophages/microglia play a key role in the neuronal apoptosis in HIV-1 infection. Therapies targeting activation of macrophages/microglia can potentially affect neuronal injury. Minocycline, a lipid soluble tetracycline antibiotic that has putative effects on immune system cells, has been proposed as a potential conjunctive therapy for HIV-1 associated neurologic disorders[115]. Minocycline has been shown to effectively cross the BBB into the CNS parenchyma to inhibit activation, proliferation, and viral replication of macrophages/microglia and lymphocytes *in vitro*[116-120],

resulting in reduction of the production of immune activators by these cells and neurons as well[116,121,122]. *In vivo* studies in SIV-infected pigtailed macaques showed that minocycline reduced plasma virus, the pro-inflammatory monocyte chemoattractant protein 1 (MCP-1)/CCL2, and viral DNA in the CNS[115,117]. The mechanisms for these effects include inhibition of immune cell activation, down-regulation of CD16 expression on the surface of monocytes, reduction of monocyte/macrophage and infected cell traffic, and suppression of inflammatory responses[115].

Lexipafant (an antagonist of platelet-activating factor or PAF), prinomastat (an inhibitor of matrix metalloprotease or MMP), inhibitors of TNF-α, and antioxidants have also been proposed as therapeutic drugs for HIV-1 associated neurologic disorders[25]. Some of these inhibitors of neurotoxic factors have been used to prevent or treat the pathogenesis of HAD and HIV-1-related neurodegeneration. One compound that has yielded interesting results is CPI-1189 in the treatment of HAD[123]. CPI-1189 ameliorates TNF-α toxicity by increasing activation of ERK (extracellular signal-regulated kinase)-MAP kinase[124]. CPI-1189 also attenuates toxicity of macrophage culture obtained from HAD patients in the presence of quinolinic acid and gp120[124]. However, a great deal of additional work is still necessary to determine the true effectiveness of any of these therapeutic inhibitors.

Neuronal apoptosis in HIV-1 infection involves activation of NMDA receptors, alterations in Ca^{2+} homeostasis and increase of oxidative stress. MK801 (a NMDA receptor antagonist) or 7-nitroindazole (a NOS specific inhibitor) reduces gp120-induced neuronal apoptosis in the neocortex of rat[125]. Nimodipine, a voltage-dependent Ca^{2+} channel antagonist, significantly decreased the rise in intracellular Ca^{2+} in neurons, but not in astrocytes[123]. A phase I/II trial of nimodipine for HIV-1-related neurologic complications was conducted and the results showed that nimodipine was safe and tolerated by HIV-1-infected subjects with cognitive impairment[126]. Nimodipine has been tested as an adjuvant agent to HAART to improve neuropsychological performance of HIV-1-infected individuals[126]. In addition, a trend toward stabilization in peripheral neuropathy was observed in nimodipine-treated patients[126].

Inhibitors of proapoptotic factors and caspase-3, -8 and -9 have also been tested to treat HIV-1-associated neurologic disorders. Cultured rat cerebrocortical cells exposed to HIV-1 gp120 undergo activation of two upstream caspases including caspase-8 and caspase-9. Pretreatment of these neurons with pan-caspase inhibitor (zVAD-fmk), caspase-3 peptide inhibitor (DEVD-fmk), caspase-8 inhibitor (IETD-fmk), or caspase-9 inhibitor (LEHD-fmk) prevents gp120-induced neuronal apoptosis[127]. Specific inhibitors of both the Fas/TNF-α/death receptor pathway and the mitochondrial caspase pathway also prevent gp120-induced neuronal apoptosis. These data suggest that pharmacologic interventions aimed at the caspase enzyme pathways may be beneficial for the prevention or treatment of HAD or HIV-1-associated pain.

Our research group has recently reported that HIV-1 Tat protein directly causes hyperexcitability and apoptosis of DRG neurons, probably by inhibiting Cdk5 kinase activity and protein expression[62]. Tat-mediated hyperexcitability of DRG neurons may play

a key role in the initiation of HIV-1-associated pain in patients at the early stages of the viral infection. HIV-1 Tat and other proteins induce apoptosis of DRG neurons, which may be involved in HIV-1-associated pain at all stages of viral infection. Our findings have potentially important implications for developing therapeutic strategies to prevent or treat HIV-1-assciated pain. For example, early initiation of HAART and/or neutralization of HIV-1 protein neurotoxicity may significantly prevent or delay HIV-1-associated pain. A recent study using a SIV-macaque model of HAART demonstrates that early initiation of HAART results in a dramatic reduction of viral RNA levels in plasma, CSF and brain[128], suggesting that early initiation of HAART can reduce or delay neurological complications including pain in HIV-1-infected patients. However, pain is often under-assessed and undertreated in people with HIV-1/AIDS illness, and the pain etiologies that are the key for pain management and improvement of the quality of life have been largely unexplored. In addition, like cancer pain, HIV-1/AIDS-associated pain tends to be of more than one type, to involve more than one location, and to increase in intensity as disease progression. Therefore, more comprehensive studies are urgently needed to investigate the current global burden of pain, pain types and origins, and the spectrum of neurological injuries in HIV-1/AIDS patients.

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