

Spectrin Cleavage Induced by LLP-1 Lentivirus Lytic Peptide Domain in the Intracytoplasmic Tail of Human Immunodeficiency Virus Type 1 GP41 in Rat Organotypic Hippocampal Slice Cultures

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We previously demonstrated that the lentivirus lytic peptide 1 (LLP-1) corresponding to the carboxyl terminus of HIV-1 gp41 induced cell death in human neuronal cells. Present study was conducted to further elucidate the pathogenic mechanisms involved in HIV-1 gp41-induced neurodegeneration in AIDS patients with cognitive deficits. The effect of LLP-1 on activation of calpain-1, a calcium-activated cysteine protease, which has been implicated in neuronal degeneration and death, was monitored by the proteolysis of spectrin in rat organotypic hippocampal slice cultures. Protease specific spectrin breakdown products revealed that LLP-1 generated ~150/145-kDa fragments characteristic of calpain-1 activation in hippocampus undergoing cell death as evidenced by LDH release. This spectrin cleavage pattern was further confirmed by *in vitro* calpain-1 proteolysis. Furthermore, calpeptin and MDL28170, inhibitors of calpain activity, blocked calpain-1-mediated spectrin cleavage. Spectrin cleavage likely occurred in the absence of overt synaptic loss, as suggested by the preserved levels of synaptophysin. Among pharmacological agents tested, apocynin, NADPH oxidase inhibitor, ameliorated the LLP-1-induced spectrin. Given the role of spectrin essential for synapse stabilization, LLP-1-induced spectrin cleavage as occurs with the activation of calpain-1 may be an important effector in LLP-1-mediated cell injury in hippocampus, which is primarily linked to cognitive dysfunction.

Key Words: HIV-1 associated dementia, LLP-1, gp41, Calpain-1, Spectrin

Abbreviations: Human immunodeficiency virus-1, HIV-1; HIV-1 associated dementia, HAD; lentivirus lytic peptide 1, LLP-1; AIDS dementia complex, ADC.

INTRODUCTION

During the progression of AIDS, a significant proportion

of Human immunodeficiency virus-1 (HIV-1) infected individuals develops neurological complications characterized by cognitive, motor, and behavioral change termed HIV-1 associated dementia (HAD) or AIDS dementia complex (ADC) (11,15). Although there have been substantial improvements in the control of viral infection in the periphery, the exact injurious mechanisms potentially contributing to neuronal death in association with HAD and an effective therapy for HIV-1 associated dementia (HAD) is still not in sight. It is widely believed that the underlying mechanism for neurological injury does not involve a direct viral infection of the brain. Rather, indirect mechanisms involving the

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neuronal interaction with HIV-1 encoded proteins, damaging inflammatory related agents and other neurotoxic host factors which are released or expressed by HIV-1 infected brain macrophages and microglia as well as reactive astrocytes are primarily responsible for the severity of neurological impairment in HAD (8,10,11,15). Several lines of evidence suggest that elevated brain levels of Ca^{++} and glutamate and subsequent calpain activation are associated with neuronal dendritic damage, apoptosis, and necrosis (7,14,29). However, less information is available on the viral or cellular factors capable of inducing glutamate receptor-mediated excitotoxicity and downstream signaling pathways, which are associated with HIV-1-related neuropathology and their underlying molecular mechanism has not been fully identified.

Earlier report demonstrated that the extent of dendritic and neuronal damage in HIV encephalitis may be more closely correlated with the amount of HIV-1 transmembrane envelope protein gp41 in the brain (4). Furthermore, there is a strong association of highly increased HIV-1 gp41 levels in HIV-1 infected brains with the severe and rapidly progressive cognitive dysfunction in HAD (1,23). Recent studies implicated gp41 as one of many viral proteins that may contribute to neurodegenerative cascade seen in AIDS patients with dementia (8,15). However, potential role of gp41 involved in the development of HAD have not been extensively explored.

A strongly amphipathic and arginine-rich helical lytic peptide, designated lentivirus lytic peptide (LLP-1) present in the carboxy terminal cytoplasmic domain of HIV-1 gp41 (amino acids 828~855) has been demonstrated to be a potent eukaryotic and/or prokaryotic cytolytic agent and have a calmodulin binding property (16,17). Recently, LLP-1 has been characterized to perform critical functions in cell to cell fusion and virion envelope incorporation (9). In addition to the channel-inducing and cytotoxic effects of LLP-1, its role specific to the central nervous system (CNS) in excitotoxic damage to neurons in HIV-1 infection through impairment of excitatory amino acids transport in glial cells was reported (13). Our previous study clearly demonstrated HIV-1 gp41 derived LLP-1 induced toxic effect of on human neuronal (26).

The aim of this study, therefore, was to further elucidate the molecular mechanisms underlying LLP-1 mediated neurotoxicity in hippocampus, which is primarily associated

with cognitive deficits in AIDS patients. We specifically determined the effect of LLP-1 on activation of calpain-1, downstream target of glutamate-induced Ca^{++} influx, in the hippocampus. Activation of calpain-1 was monitored by the proteolysis of spectrin. Protease specific spectrin breakdown products revealed that LLP-1 generated ~150/145-kDa fragments characteristic of calpain-1 activation in rat organotypic hippocampal slice cultures. Furthermore, this spectrin cleavage pattern was further confirmed by *in vitro* calpain-1 proteolysis. Among pharmacological agents tested, apocynin, NADPH oxidase inhibitor, reduced the LLP-1-induced spectrin cleavage, which suggests the involvement of superoxide production in LLP-1-induced spectrin cleavage. Our study provide the evidence for one injurious mechanism potentially contributing to neuronal death in association with HAD.

MATERIALS AND METHODS

1. Materials

Synthetic peptides representing the cytolytic LLP-1 (RVIEVVQGACRAIRHPRRIRQGLERIL) and the non-cytolytic LLP-1 analog 2: (EVIEVVQGACRAIRHIPREIRQGLERIL) were generous gift from Dr. Montelaro of University of Pittsburgh School of Medicine and peptide production and characterization have been described in detail previously (26). Apocynin, the NADPH inhibitor, and the calpain inhibitors, calpeptin and MDL28170 were obtained from Calbiochem. Anti- β -actin antibody and other chemicals were obtained from the Sigma (St. Louis, MO).

2. Hippocampal slice cultures

Hippocampal slice cultures were prepared from 10-day-old Sprague-Dawley rat pups slices as described (18). Slices were cut at 400 μm on a McILWAIN tissue chopper and transferred to Millipore (Millipore Corp., Bedford, MA) membrane inserts (0.4 μm), and placed in six-well culture plates. The upper surfaces of the slices were exposed to a humidified 37°C atmosphere containing 5% CO_2 . Slice culture media consisted of Basal Eagle Medium with Earle's balanced salt solution, 20% heat-inactivated horse serum and the following supplements: 20 mM NaCl, 5 mM NaHCO_3 , 1.7 mM MgSO_4 , 0.2 mM CaCl_2 , 26.7 mM HEPES, 26.6 mM L-glutamine, 48 mM L-(+)-glucose, 100 U/ml peni-

cillin, and 100 mg/L streptomycin, pH 7.25. The medium was changed every other day. Slices were examined periodically for viability, and any dark or abnormal slices were discarded.

3. Experimental treatment

On Days 10~11 postdissection, treatment of the slices was started. All reagents were added to serum-free medium (no horse serum), which was equilibrated at 37°C, 5% CO₂ before their addition to the slices as described (3). Slices were pretreated with various pharmacological agents, as described in the text. All concentrations were selected on the basis of the maximal effects of these drugs on their specified targets. The LLP-1 or LLP-1 analog 2 Peptide was then added to cultures in serum-free medium at various concentrations for 30 h as noted in the text. Vehicles were treated the same way except with no peptide. Slices were then rinsed twice in 1x PBS then harvested by removing the Millicell-CM membrane insert after freezing samples on dry ice, and processed for immunoblotting as described below.

4. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) enzymatic activity in the culture medium was used to evaluate the extent of cellular damage produced in cultured slices subjected to the different treatments as previously described (3, 26). Briefly, culture medium was collected after incubation of slices as indicated in the figures, and from the vehicle treated control cultures. 100 µl aliquots of culture media were taken for determination of LDH activities using Tox-7 (Sigma) according to the manufacturer's directions. The activity was expressed as the relative percentage of neuronal death using respective values for vehicle- or LLP-1 treated slice cultures as 100%.

5. Polyacrylamide gel electrophoresis and western blotting

Immunoblotting was carried out as recently described in detail (3). Briefly, slices were homogenized in ice-cold extraction buffer (10 mM triethanolamine, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, 0.15 M NaCl, 0.3% NP-40) supplemented with the protease inhibitors pepstatin (2 µg/ml), leupeptin (10 µg/ml), aprotinin (10 µg/ml), and PMSF (1 mM), and the phosphatase inhibitor sodium orthovanadate

(1 mM). The protein concentration was determined by the method of BCA (Pierce, Rockford, IL). Equal amounts (20 µg) of sample proteins were separated according to their molecular weight on 10% SDS polyacrylamide gels and transferred onto a polyvinylidene fluoride Immobilon-P (Millipore, Bedford, MA). After transfer, membranes were blocked with 3% milk in TBS-T for 0.5 h. Membranes were probed with the primary antibody diluted with 1% milk and incubated overnight at 4°C. The following antibodies were used: anti-spectrin (1:1000; Chemicon Int., Temecula, CA), anti-synaptophysin (1:3000; DAKO, Carpinteria, CA), and anti-β-actin (1:5000; Sigma). The signal was obtained using ECL system after incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson). Densitometric values were normalized using β-actin as internal controls as indicated.

6. *In vitro* cleavage assay of spectrin and synaptophysin and protease inhibitor treatment

For experiments using purified calpain-1 (Calbiochem), total lysates were prepared from hippocampal slices and then centrifuged for 10 min at 16,000 x g. The supernatants (20 µl) were then incubated with calpain-1 for 1 h at 37°C, as described previously (12). To inhibit calpain activation, the supernatants of hippocampal slices were cotreated with 5 µM ALLN (Santa Cruz Biotechnology) or 10 µM MDL 28,170 (Calbiochem) for 1 h). The reactions were terminated by adding 1 vol of 2x SDS sample buffer and boiled for 5 min. Samples were subjected to Western blot analysis using anti-spectrin body or anti-synaptophysin antibody.

RESULTS

1. Treatment of cultured hippocampal slices with LLP-1 increases LDH release

The organotypic hippocampal slice culture is a good model system to study the mechanisms of neurodegeneration. This is because several features of hippocampal circuitry including the maturation of synapses, receptors, and intrinsic fiber pathways are preserved as *in vivo*, and the preparation is well suited for prolonged pharmacological treatment and recovery; which would be difficult to perform in an intact animal system (6,18). In order to investigate the direct toxic effect of LLP-1 on neuronal viability, in the

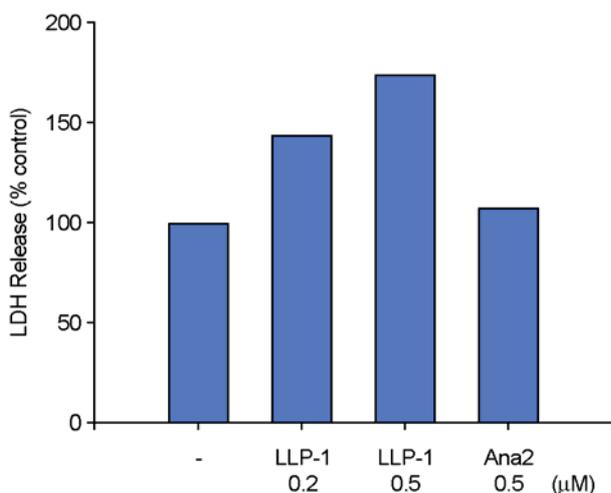


Figure 1. LLP-1 increases LDH release in cultured media of organotypic hippocampal slice cultures. Dose response of LDH release by LLP-1 was examined by incubation with increasing concentrations of peptides as indicated for 30 h. LDH activity in cultured media from hippocampal slices was assayed after exposing rat hippocampal slice cultures to gp41 derived LLP-1 or LLP-1 analog 2 (Ana2). Values are expressed as a percentage of the vehicle treated controls, considering the values obtained in the hippocampal slices as 100%.

organotypic hippocampal slices, we examined the degree of cell death by measuring LDH release. As shown in Fig. 1, the amount of LDH in the media containing hippocampal slices treated with LLP-1 was significantly increased above control level, indicating that cell death had occurred. Exposure to low micromolar concentration of LLP-1 (0.5 μM) for 30 h increased the percentage of LDH release to approximately a 1.5 fold over the vehicle treated control (Fig. 1). In parallel, slices were also exposed to the control peptide LLP-1 analog 2 under the same experimental conditions, but there was no observed increase in cell death.

2. LLP-1-induced cell death was accompanied by calpain-1 activation

To further clarify the mechanism by which LLP-1 causes cell death in the hippocampal slices, we evaluated the activation of calpain-1, calcium-activated proteases that cleave cytoskeletal proteins such as spectrin, neurofilament, cytoskeletal elements, such as spectrin, neurofilament, and MAP-2, and functional elements, such as NMDAR-2B (5,25). Activation of calpain-1 was monitored by the proteolysis of spectrin. Protease specific spectrin breakdown products (SBP) revealed that LLP-1 generated ~150/145-kDa frag-

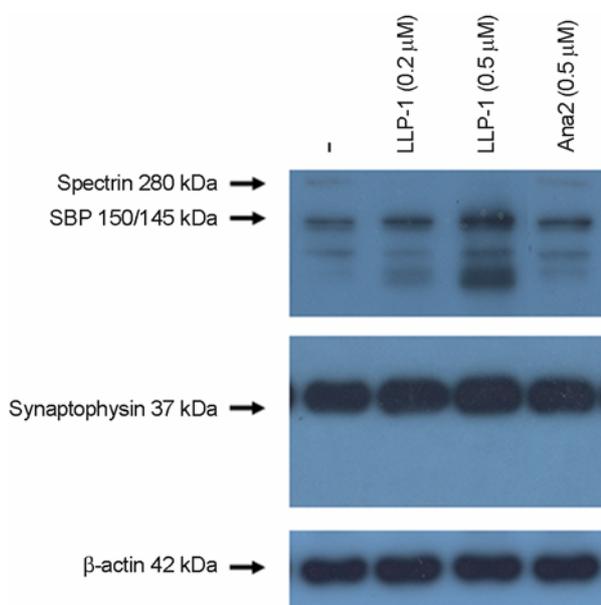


Figure 2. LLP-1-induced cell death was accompanied by calpain-1 activation as analyzed by spectrin cleavage. Western blot analysis of spectrin and synaptophysin content in whole cell extracts prepared from hippocampal slices as described in Fig. 1 was performed as detailed in Materials and Methods section. LLP-1 treatment for 30 h clearly generated ~150/145-kDa spectrin breakdown products (SBP) characteristic of calpain-1 activation. Representative blots from two to three experiments with similar results are shown. Uniformity of gel loading was confirmed with β-actin as the standard.

ments characteristic of calpain-1 activation in rat organotypic hippocampal slice cultures, whereas LLP-1 analog 2 had no appreciable effect (Fig. 2). By contrast, synaptophysin levels, which correlate closely with synapse number and hence are commonly used to assay for loss of synapses, were not significantly altered and cleavage of synaptophysin was not clearly seen under lower concentration of LLP-1 used. This activation pattern of calpain-1 was closely correlated with the pattern of cell death obtained by the LDH assay (Fig. 1).

3. This spectrin cleavage pattern was confirmed by *in vitro* calpain-1 proteolysis

To compare LLP-1-mediated spectrin cleavage pattern in hippocampal slices to that generated by calpain-1, we subjected the total lysate of hippocampal slices to *in vitro* calpain-1 digestion. Indeed, *in vitro* calpain-1 digestion prominently generated the spectrin cleavage pattern observed in the hippocampal slices exposed to LLP-1 (Fig. 3). Furthermore, specific inhibitors of calpain-1, calpeptin and

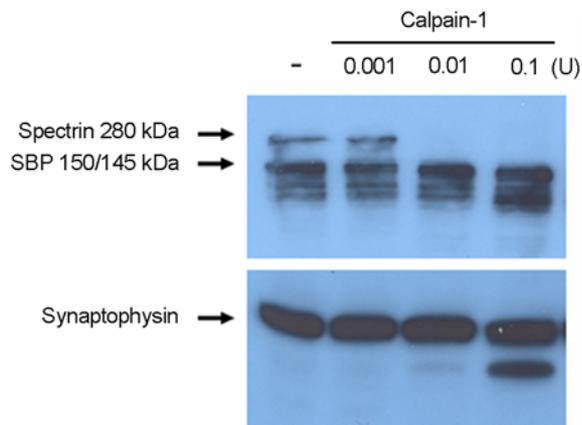


Figure 3. *In vitro* calpain-1 proteolysis confirms the spectrin cleavage pattern induced by LLP-1. Representative immunoblots of *in vitro* spectrin proteolysis by calpain-1. Spectrin or synaptophysin present in total lysate of hippocampal slices treated with vehicle only for 30 h are digested with increasing concentrations of calpain-1 for 1 h as indicated. Western blot analysis of spectrin and synaptophysin contents was performed as described in Fig. 2. Representative blots from two to three experiments with similar results are shown.

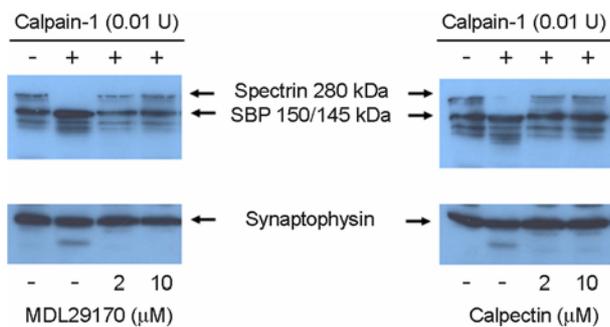


Figure 4. Calpain-1 inhibitors blocked the spectrin cleavage pattern. Effects of specific calpain-1 inhibitors, calpeptin and MDL-28170 on spectrin proteolysis *in vitro* by calpain-1 were analyzed. Whole cell extracts prepared from hippocampal slices treated with vehicle only were digested with calpain-1 in the presence of either calpeptin or MDL28170 as indicated for 1 h. Western blot analysis of spectrin and synaptophysin contents was performed as described in Fig. 2. Representative blots from two to three experiments with similar results are shown.

MDL28170, blocked calpain-1-mediated spectrin cleavage *in vitro* (Fig. 4). These results clearly showed that calpain-1 activation mediated cell death events downstream of LLP-1 exposure to hippocampal slices. Higher concentration of calpain-1 (≥ 0.01 U) also cleaved synaptophysin, generating a breakdown product with MW ~ 30 kDa.

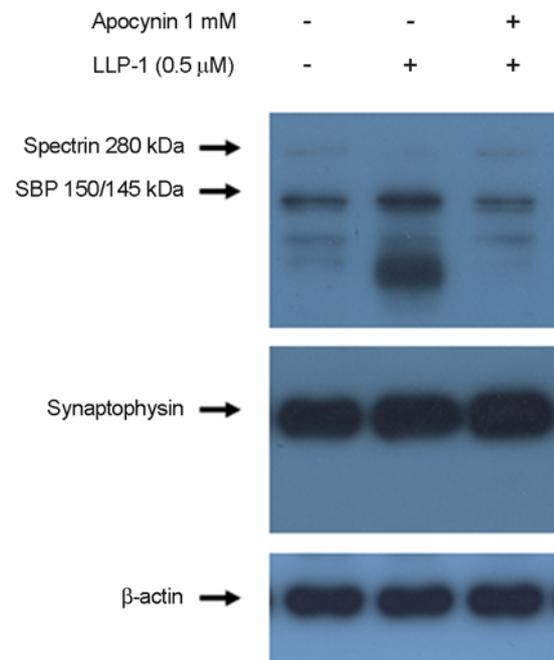


Figure 5. Apocynin reduced the LLP-1-induced spectrin cleavage. Effects of apocynin, NADPH inhibitor, on spectrin proteolysis triggered by LLP-1 in hippocampal slices were analyzed by 1 h pretreatment with apocynin before exposure of hippocampal slices to LLP-1 for 30 h. Western blot analysis of spectrin content in whole cell extracts prepared from hippocampal slices was conducted as described in Fig. 1 and Fig. 2.

4. Apocynin ameliorated the LLP-1-induced spectrin cleavage

Finally, in order to search for the inhibitors to reduce calpain-1 activation triggered by LLP-1 treatment in hippocampal slices, we treated them with various pharmacological inhibitors prior to LLP-1 treatment. Among pharmacological agents tested, apocynin, NADPH oxidase inhibitor, which was effective in inhibiting LDH release induced by LLP-1 (data not shown), significantly reduced LLP-1-mediated spectrin cleavage (Fig. 5). This finding suggests the involvement of superoxide production in LLP-1-induced events. and Thus this study is in part consistent with our previous study showed that LLP-1 may cause a substantial loss of redox poise, which could be a strong contributory factor leading to neuronal death (26).

DISCUSSION

The biological function of HIV-1 gp41 remains critical to unraveling the neuropathogenic mechanisms of HIV-1

associated dementia (HAD) with regard to the development of new therapeutic modalities for the treatment and/or prevention of HIV-1 associated neuropathology. Present study showed that HIV-gp41 derived LLP-1 can induce proteolytic cleavage of spectrin, a downstream target for calpain-1 that is likely activated in hippocampus undergoing LLP-1-induced neuronal cell death. Given the role of LLP-1 in cell death and spectrin essential for synapse stabilization, LLP-1-induced spectrin cleavage as occurs with the activation of calpain-1 may be an important effector in HIV-1 gp41 derived LLP-1-mediated neuronal cell injury in hippocampus, which is primarily linked to cognitive dysfunction associated with HAD. Furthermore, LLP-1 induced cell death and spectrin cleavage were prevented by preincubation of hippocampus with apocynin, suggesting the involvement of superoxide production in LLP-1-induced events. Thus, our study identified one injurious mechanism potentially contributing to neuronal death in hippocampus, which is in association with the development of HAD. This study is also in consistent with earlier studies demonstrating that the levels of gp41 are closely correlated well with the severity and rapid progression of AIDS associated dementia (1,23).

Although HIV infection of macrophages within the brain is thought to be a critical factor in triggering events leading to neuronal damage and death, some use application of recombinant HIV-1 proteins to neuronal cultures to model HIV-1 associated neurotoxicity. In all, these studies have implicated a number of HIV- and macrophage-associated neurotoxins that directly or indirectly activate neuronal NMDA receptors, including glutamate, quinolinic acid, platelet activating factor, reactive oxygen species, NTox, Tat, and gp120 (7,10,14,19,27,29). Importantly, our study confirms that gp41-derived LLP-1 peptide was directly neurotoxic in hippocampus, which provides *in vivo* relevance because HIV-associated neuronal damage is particularly severe in hippocampus, frontal cortex, but not in cerebellum (2,20,24,29). Therefore, it is interesting to speculate that high levels of HIV-1 gp41 expression could at least partially account for selective neuronal damage in hippocampus particularly associated with the development of HAD.

Calpains are calcium-activated proteases that cleave cytoskeletal elements, such as spectrin, neurofilament, and

MAP-2, and functional elements, such as NMDAR-2B (5, 25). Calpains are known to be activated in a number of excitotoxic insults and activity of calpains is often associated with cell death models of ischemia, spinal cord injury, and a range of neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease (5,28). Inhibition of calpain activation has been demonstrated to protect neurons against such insults (22). Our study clearly demonstrated that LLP-1 activated neuronal calpain-1 activity as evidenced by detection of calpain-specific spectrin cleavage products in the hippocampus. We also confirmed that spectrin cleavage was the result of calpain activation by blocking the production of the cleavage product by the calpain inhibitors calpeptin and MDL28170. Furthermore, present study showed that LLP-1 induced cell death and calpain activation was prevented by preincubation of neurons with apocynin, suggesting the involvement of superoxide production in LLP-1-induced events. This finding is consistent with our previous study showed that LLP-1 may cause a substantial loss of redox poise, which could be a strong contributory factor leading to neuronal death (26).

One consequence of gp41-derived LLP-1 treatment in our hippocampal cultures is the observed cleavage of the structural protein spectrin, as a target for calpain, in the hippocampus undergoing neuronal cell death. Although the functional consequences of spectrin cleavage by LLP-1 are not yet defined, calpain-cleaved spectrin remained in the hippocampal neuron and might increase the susceptibility of the cell to excitotoxic injury. Currently, little is known about the molecular mechanisms that specify synapse stability versus disassembly. Loss of presynaptic, but not postsynaptic, spectrin leads to the disorganization and elimination of essential synaptic cell-adhesion molecules (21). In addition, altered axonal transport and disrupted synaptic microtubules as events that contribute to synapse retraction in animals lacking presynaptic spectrin. Thus, our data suggest that calpain-1-mediated spectrin cleavage may link loss of presynaptic spectrin functions as an essential presynaptic scaffold. In this regard, precise neural circuitry would be failed due to dysregulated balance of synaptic cell adhesion with the stabilization of underlying microtubule cytoskeleton in the hippocampus undergoing LLP-1-mediated cell death.

Taken together, the present study suggests that high

expression of viral factors such as gp41 derived LLP-1 could lead to activation of calpain-1 and subsequent spectrin cleavage and cell death in hippocampus which is a brain region primarily vulnerable in HIV-1 infected patients. Thus, we speculate that cumulative expression of HIV-1 gp41 would be relevant to understanding the fundamental neuropathology and the progression of AIDS associated dementia. Chronic exposure of hippocampus to gp41-derived LLP-1 in concentrations correlating with the high level of HIV-1 envelope expression and virus replication in the CNS of AIDS patients could mediate calpain-activation and subsequent spectrin cleavage that culminate in the neuronal cell death, ultimately contributing to neurodegenerative cascade as seen in AIDS patients with dementia. Accordingly, the down-regulation of calpain-1 activation by the inhibitors of superoxide production and calpain inhibitor implicated may hold promise as a potential adjunctive therapy for the control of HIV-1 associated neurologic damage.

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