HIV Tat-mediated altered oligodendrocyte maturation involves autophagy-lysosomal dysfunction

Abstract

Objectives: The current study was undertaken to understand the underlying molecular mechanism(s) by which HIV Transactivator of transcription (Tat) alters oligodendrocyte maturation through the generation of reactive oxygen species (ROS), impairment of lysosomal functioning, and dysregulation of autophagy.

Methods: We exposed primary rat immature oligodendrocytes to HIV Tat and utilized various experimental techniques to assess its effects on oligodendrocytes maturation. We measured ROS levels, assessed lysosomal membrane potential, determined cathepsin D activity, and analyzed the expression of autophagy-related markers. Furthermore, we investigated the potential of ROS scavengers and lysosomal protectants to mitigate the damaging effects of HIV Tat on oligodendrocytes maturation.

Results: Exposure of primary rat immature oligodendrocytes to HIV Tat significantly increased ROS levels, indicating the induction of oxidative stress. This oxidative stress impaired lysosomal functioning, as evidenced by a substantial increase in lysosomal membrane potential and a decrease in cathepsin D activity. Compromised lysosomal function resulted in dysregulated autophagy, which was confirmed by increased expression of SQSTM1. However, the administration of ROS scavengers and lysosomal protectants effectively attenuated the detrimental effects of HIV Tat on oligodendrocytes maturation.

Conclusions: Our findings demonstrate that HIV Tat exposure induces oxidative stress, impairs lysosomal functioning, and dysregulates autophagy in oligodendrocytes. These molecular changes likely contribute to the altered maturation of oligodendrocytes observed in HIV-infected individuals. Understanding these underlying mechanisms provides valuable insights into the pathogenesis of HIV-associated neurocognitive disorders and highlights the potential of therapeutic strategies targeting ROS scavenging and lysosomal protection as adjunctive approaches for managing such complications in HIV+ve individuals.

Keywords: heat shock protein 70; HIV Tat; lysosomes; myelin; N-acetylcysteine; oligodendrocytes

Introduction

Although combined antiretroviral therapy (cART) has dramatically increased the lifespan of HIV-1 infected individuals, paradoxically, almost 50% of the infected individuals will go on to develop HIV-1-associated neurocognitive disorders (HAND) ranging from asymptomatic to mild cognitive-motor disorders, which severely impacts the quality of life [1–3]. White matter abnormalities have been linked to neurocognitive dysfunction [4, 5]. Many studies have reported white matter damage in people living with HIV-1, even in the era of cART, and more so with increasing severity in patients with HAND [6–9]. Brain mapping studies using magnetic resonance imaging and diffusion tensor imaging in HIV-1 infected individuals have reported significant white matter damage with disease progression [10–12].

Despite the advances in cART, the ongoing, persistent low-level viral replication in the brain and the simultaneous release and accumulation of viral proteins such as HIV-1 Transactivator of transcription (Tat) is known to contribute to HAND pathogenesis [13–17]. Among the nine HIV-1 encoded proteins, HIV-1 Tat is a crucial component for efficient transcription of the HIV-1 genome. HIV-1 Tat is secreted by the infected cells of the CNS [18] and can also be taken up by the recipient cells leading to cytotoxicity [19–23]. Oligodendrocytes are the myelinating cells of the...
CNS [24–26]. Although HIV-1 Tat-mediated oligodendrocyte and/or myelin injury has been well documented [27–30], the precise molecular mechanism(s) underlying HIV-1 Tat-mediated alterations in oligodendrocyte maturation remain less understood.

Myelination is a complex yet finely regulated process that includes myelin protein synthesis, storage, and transportation [31]. Lysosomes are specialized cellular organelles that play important roles in protein degradation involving various pathways, including autophagy [32, 33]. It has been well reported that lysosomes play a crucial role in myelination, and myelin biogenesis is regulated by transporting one of the major myelin proteins, proteolipid protein (PLP), from the late endosomes/lysosomes to the cell membrane in oligodendrocytes [34–36]. A functional lysosome is necessary for autophagy clearance [37]. Autophagy is essential for oligodendrocyte survival and proper myelination [38, 39]. Dysregulated autophagy in oligodendrocytes has been linked to spinal cord injury [40, 41]. Furthermore, both lysosomal and autophagy dysfunction play critical roles in the pathogenesis of several neurodegenerative diseases [42–47]. However, whether HIV-1 Tat-mediated alterations in oligodendrocyte maturation involve lysosomal dysfunction and defective autophagy is not well explored.

Based on the above, the present study assessed HIV-1 Tat-mediated lysosomal dysfunction and its role in oligodendrocyte maturation. Our findings, for the first time, describe the mechanistic insights underlying HIV-1 Tat-mediated lysosomal dysfunction and possible restoration intervention strategies, such as lysosomal protection, that could be developed as a potential therapeutic target(s) for white matter damage in HIV-infected individuals.

Results
HIV-1 Tat-mediated alteration in primary rat oligodendrocytes maturation

A hallmark of oligodendrocyte maturation is the ability to form elaborated branched processes and myelin protein (PLP and MBP) [48–50]. First, we sought to determine whether exposure of immature oligodendrocytes to HIV-1 Tat could alter their myelin protein. For this, immature oligodendrocytes were exposed to varying doses of HIV-1 Tat (12, 25, 50, and 100 ng/mL) for 24 h, following

Figure 1: Exposure of immature oligodendrocytes to HIV-1 Tat resulted in decreased myelin proteins and impaired cell process. For this, immature oligodendrocytes were exposed to varying doses of HIV-1 Tat (12, 25, 50, and 100 ng/mL) for 24 h, following which the expression levels of myelin proteins such as PLP and MBP were determined using western blotting. (A and B) HIV-1 Tat dose-dependently downregulated the expression of myelin proteins – PLP (A) and MBP (B) in oligodendrocytes. According to the manufacturer’s instructions, two bands observed near 20 kDa were analyzed for PLP. For MBP, bands between 14 and 21 kDa were analyzed. (C–E) HIV-1 Tat (50 ng/mL)-mediated decreased cellular morphology of oligodendrocytes. (C) Representative fluorescent-microscopic image showing HIV-1 Tat-mediated decreased actin cytoskeleton staining in oligodendrocytes. (D and E) Representative bar graph showing HIV-1 Tat-mediated decreased cell process length and secondary branches in oligodendrocytes. (F–H) HIV-1 Tat (50 ng/mL)-mediated decreased myelin PLP in the cellular process. (F) Representative fluorescent-microscopic image showing HIV-1 Tat-mediated decreased myelin PLP staining in oligodendrocytes. (G and H) The representative bar graph shows myelin PLP in the oligodendrocytes cell soma and cell process. Data are from three independent experiments. Actin served as a protein loading control for western blots. Data are expressed as means ± SEM and were analyzed using student t-test or one-way ANOVA. *p<0.05 vs. control.
which the expression levels of myelin proteins such as PLP and MBP were determined using western blotting. As shown in Figure 1(A and B), in the presence of 50 ng/mL of HIV-1 Tat, there was a significant decrease (p < 0.05) in the expression of myelin proteins (PLP and MBP) in oligodendrocytes. This concentration (50 ng/mL) of HIV-1 Tat was thus chosen for the subsequent experiments. The rationale for choosing this concentration of HIV-1 Tat was based on the premise that the Tat concentration in the serum and the cerebrospinal fluid of HIV-1-infected individuals ranges from 1 to 40 ng/mL [51, 52] and that this could be higher in the CNS in the vicinity of HIV-1 positive perivascular cells [53]. As expected, exposure of immature oligodendrocytes to heat-inactivated HIV-1 Tat (50 ng/mL) did not significantly affect the expression of myelin proteins (Figure 1(A and B)). Next, we assessed the morphology of the oligodendrocytes exposed to HIV-1 Tat. Cultured oligodendrocytes were stained for actin (Figure 1(C)), which stains the cytoskeleton of the cell. Data showed that cells exposed to HIV-1 Tat exhibited diminished cellular processes (Figure 1(D)) and decreased secondary branches (Figure 1(E)). Next, we sought to assess the distribution of myelin PLP in HIV-1 Tat-exposed oligodendrocytes (Figure 1(F)). Quantification data showed no change in myelin PLP expression in the cell soma (Figure 1(G)); however, there was decreased myelin PLP expression in the cell processes (Figure 1(H)) of HIV-1 Tat exposed oligodendrocytes.

**HIV-1 Tat-mediated lysosome dysfunction in primary rat oligodendrocytes**

Lysosomes are important for myelin protein secretion and sorting in oligodendrocytes [54]. Next, we sought to explore the impact of HIV-1 Tat on lysosomal function in the oligodendrocytes. Immature oligodendrocytes were exposed to HIV-1 Tat (50 ng/mL) for 3–24 h, then the expression of the lysosomal marker, LAMP2 (lysosomal-associated membrane protein 2), was assessed. As shown in Figure 2(A), in oligodendrocytes exposed to HIV-1 Tat, there was significant downregulation of LAMP2 expression starting at 12 h with a continued trend of downregulation up to 24 h. Interestingly, HIV-1 Tat exposure also decreased the expression of mature cathepsin D (mCTSD) in oligodendrocytes (Figure 2(B)). We next examined lysosomal functioning by assessing lysosomal pH and the enzyme activity in HIV-1 Tat exposed oligodendrocytes. As shown in Figure 2(C–E), we found an increase in lysosomal pH (Figure 2(C)) and increased lysosome membrane permeability (LMP) (Figure 2(D)) that was accompanied by a significant decrease in CTSD activity (Figure 2(E)) in oligodendrocytes exposed to HIV-1 Tat.

![Figure 2](image-url)
HIV-1 Tat-mediated autophagy dysregulation in rat primary oligodendrocytes

During the autophagy process, lysosomes fuse with autophagosomes and form autolysosomes. The formation of autolysosomes ensures complete clearance of misfolded proteins. Also, studies have shown that autophagy plays a key role in oligodendrocyte maturation [38, 55]. We thus rationalized that impaired lysosomal functioning could block the autophagic flux. We thus sought to assess the effects of HIV-1 Tat on autophagy mediators. We assessed the expression of multiple autophagy-related proteins, including BECN1/Beclin1 (beclin 1), MAP1LC3B/LC3B (microtubule-associated protein 1 light chain 3 beta), and SQSTM1/p62 (sequestosome 1) in oligodendrocytes treated with HIV-1 Tat (50 ng/mL) for varying times ranging from 3 to 24 h. As shown in Figure 3, expression of BECN1 (Figure 3(A)), MAP1LC3B (Figure 3(B)), and SQSTM1 (Figure 3(C)) were significantly upregulated in a time-dependent manner in the oligodendrocytes treated with HIV-1 Tat (50 ng/mL). To confirm whether the increased expression of MAP1LC3B was a result of enhanced autophagosome synthesis or reduced autophagosome turnover (due to delayed trafficking or reduced fusion with the lysosomes), MAP1LC3B turnover and SQSTM1 degradation assays in the presence of bafilomycin A1 (BAF - a known fusion inhibitor of autophagosome and lysosome) alone, HIV-1 Tat alone and combination of BAF (400 nM) with HIV-1 Tat (50 ng/mL) were performed. As shown in Figure 3(D) and (E), western blot analysis showed no significant difference in the accumulation of MAP1LC3B-II and SQSTM1 in oligodendrocytes exposed to HIV-1 Tat in the presence or absence of BAF, further indicating the involvement of defective autophagy flux in HIV-1 Tat exposed oligodendrocytes. MAP1LC3B turnover and the SQSTM1 degradation assays determine the autophagy flux. Accumulation of MAP1LC3B-II and SQSTM1 in HIV-1 Tat exposed oligodendrocytes indicated blockade of the autophagic flux. Adding BAF (autophagosome-lysosome fusion inhibitor) to HIV-1 Tat-treated oligodendrocytes failed to cause a further increase in the expression of MAP1LC3B-II and SQSTM1, thereby confirming maximal accumulation of MAP1LC3B-II and SQSTM1 in HIV-1 Tat exposed oligodendrocytes. Studies have shown that the accumulation of MAP1LC3B-II and SQSTM1 protein levels

Figure 3: Exposure of immature oligodendrocytes to HIV-1 Tat resulted in autophagy dysregulation in oligodendrocytes. We assessed the expression of multiple autophagy-related proteins, including BECN1/Beclin1 (beclin 1), MAP1LC3B/LC3B (microtubule-associated protein 1 light chain 3 beta), and SQSTM1/p62 (sequestosome 1) in oligodendrocytes treated with HIV-1 Tat (50 ng/mL) for varying times ranging from 3 to 24 h. (A–C) HIV-1 Tat (50 ng/mL) time-dependently increased the expression of autophagy markers – BECN1 (A), MAP1LC3BII (B), and (C) SQSTM1 in oligodendrocytes. Two bands were observed for LC3B; LC3BI and LC3BII. For analysis, LC3BII was evaluated and compared to a loading control to assess autophagy in all the experiments. (D and E) HIV-1 Tat (50 ng/mL)-mediated decreased autophagic flux in oligodendrocytes. Representative western blots showing the expression of MAP1LC3B-II (D) and SQSTM1 (E) in oligodendrocytes exposed to HIV-1 Tat (50 ng/mL) for 24 h followed by treatment with 400 nM BAF, added in the last 4 h of the 24 h treatment period. Data is from three independent experiments. Actin served as a protein loading control for western blots. Data are expressed as means ± SEM and were analyzed using student t-test or one-way ANOVA. * p<0.05 vs. control; N.S., non-significant.
directly correlates with the number of autophagosomes and impaired autophagic vesicle degradation [56, 57].

**HIV-1 Tat-mediated ROS generation in primary rat oligodendrocytes**

Having established that HIV-1 Tat dysregulated both lysosomal function and autophagy flux while blocking oligodendrocyte maturation, we next sought to explore the mechanism(s) underlying these processes. It is well-known that oxidative stress is one of the critical mediators responsible for the dysregulation of antioxidant enzymes leading, in turn, to cognitive impairment in HIV-1 patients [58]. Intriguingly, exacerbated generation of ROS via dysregulated antioxidant enzymes has also been demonstrated in the monocytes and CSF of HIV-1 infected individuals [59]. We next sought to determine the possible involvement of ROS in HIV-1 Tat-mediated lysosomal dysfunction and autophagy dysregulation. Immature oligodendrocytes exposed to HIV-1 Tat (50 ng/mL) for various time points (0–4 h) were monitored for ROS production using the DCFH-DA assay. As shown in Figure 4(A), exposure of oligodendrocytes to HIV-1 Tat resulted in significant induction of ROS within 1 h (Figure 4(A)).

Next, we inhibited ROS generation by pretreating the oligodendrocytes with various ROS scavengers such as TEMPOL (20 μM) and N-acetyl cysteine (NAC) (5 mM) for 1 h followed by HIV-1 Tat (50 ng/mL). As shown in Figure 4(B), pretreatment of the cells with these ROS scavengers significantly abrogated HIV-1 Tat-mediated induction of ROS.

**NAC abrogated HIV-1 Tat-mediated defects in primary rat oligodendrocytes**

Next, we examined whether HIV-1 Tat-mediated induction of ROS played a role in lysosomal dysfunction, autophagy dysregulation, and decreased oligodendrocyte maturation. To validate this, immature oligodendrocytes were pretreated with NAC (5 mM) for 1 h followed by exposure of cells to HIV-1 Tat (50 ng/mL) for 24 h, following which the protein expression of lysosome markers (LAMP2 and mCTSD), autophagy markers (MAP1LC3B and SQSTM1) and myelin markers (PLP and MBP) was assessed by western blotting. Interestingly, pretreatment of oligodendrocytes with NAC abrogated HIV-1 Tat-mediated downregulation of LAMP2 (Figure 5(A)) and mCTSD (Figure 5(B)). We next examined the protective effects of NAC on HIV-1 Tat-mediated dysregulation of autophagy. As expected, and as shown in Figure 5(C and D), pretreatment of oligodendrocytes with NAC notably blocked HIV-1 Tat-mediated upregulation of MAP1LC3B and SQSTM1, thereby implying increased autophagosome-lysosome fusion. Next, we examined whether pretreatment with NAC could also block HIV-1 Tat-mediated altered oligodendrocyte maturation. As shown in Figure 5(E and F), pretreatment of oligodendrocytes to NAC significantly blocked HIV-1 Tat-mediated downregulation of myelin proteins such as MBP and PLP, thereby indicating the protective role of NAC in HIV-1 Tat-exposed oligodendrocytes.
Figure 5: NAC reversed HIV-1 Tat-mediated defects in oligodendrocytes. Immature oligodendrocytes were pretreated with NAC (5 mM) for 1 h, followed by exposure to HIV-1 Tat (50 ng/mL) for 24 h, following which the protein expression of lysosome markers (LAMP2 and mCTSD), autophagy markers (MAP1LC3B and SQSTM1) and myelin markers (PLP and MBP) were assessed by western blotting. (A and B) Representative western blots showing pretreatment of oligodendrocytes to NAC reversed HIV-1 Tat-mediated downregulation of LAMP2 (A) and mCTSD expression levels (B). (C and D) Representative western blots showing pretreatment of oligodendrocytes to NAC reversed HIV-1 Tat-mediated upregulation of SQSTM1 (C) and MAP1LC3BII expression levels (D). (E and F) Representative western blots showing pretreatment of oligodendrocytes to NAC reversed HIV-1 Tat-mediated downregulation of myelin MBP (E) and myelin PLP expression levels (F). Data is from three independent experiments and is represented as means ± SEM using one-way ANOVA. *p<0.05 vs. control; #p<0.05 vs. HIV-1 Tat.

**Hsp70 overexpression abrogated HIV-1 Tat-mediated defects in primary rat oligodendrocytes**

Having confirmed the importance of LMP in HIV-1 Tat-mediated dysfunctioning of oligodendrocytes, we next sought to determine the protective role of Heat shock protein 70 (Hsp70) in blocking LMP. Hsp70 has been shown to stabilize lysosomes by blocking LMP [60, 61]. Overexpression of Hsp70 protected immature oligodendrocytes against HIV-1 Tat-mediated defects. As shown in Figure 6, Hsp70 overexpression reversed HIV-1 Tat-mediated downregulation of LAMP2 (Figure 6(A)), upregulation of SQSTM1 (Figure 6(B)), and downregulation of myelin protein, MBP (Figure 6(C)). Additionally, Hsp70 overexpression abrogated HIV-1 Tat-mediated increased lysosomal pH (Figure 6(D)) and decreased CTSD activity (Figure 6(E)). To further validate the findings on HIV-1 Tat-mediated lysosome and myelin defects, we performed immunostaining to demonstrate the LAMP2 puncta and myelin PLP intensity in oligodendrocytes (Figure 6(F)). Quantification data showed that Hsp70 overexpression significantly blocked HIV-1 Tat-mediated downregulation of LAMP2 puncta (Figure 6(G)) and PLP intensity (Figure 6(H)).

**Discussion**

In the cART era, white matter abnormalities are frequently observed in HIV + individuals and with increased severity in those afflicted with HAND [6–9]. HIV-1 Tat continues to be detectable in the CSF of HIV-1-infected patients with well-controlled viremia [62]. HIV-1 Tat protein has been implicated in the pathophysiology of the neurocognitive deficits associated with HIV infection [20]. HIV +ve individuals suffer from cognitive, behavioral, or motor abnormalities, currently classified as HIV-associated neurocognitive disorders (HAND) [63]. Oligodendrocyte damage can cause axonal demyelination and neuronal injury, leading to neurological disorders, and demyelination also cause cognitive impairment in a variety of neurological disorders, including HAND [64]. While HIV-1 Tat-mediated oligodendrocyte and/or myelin injury has been well documented [27–30], the precise molecular mechanism(s) underlying
Figure 6: Hsp70 overexpression reversed HIV-1 Tat-mediated defects in oligodendrocytes. (A–C) Representative western blots and bar graphs showing overexpressing Hsp70 in oligodendrocytes reversed HIV-1 Tat-mediated downregulation of LAMP2 (A), upregulation of SQSTM1 (B), and downregulation of MBP (C) expression levels. (D) Representative bar graph showing Hsp70 overexpression in oligodendrocytes reversed HIV-1 Tat-mediated increased lysosome pH. (E) Representative bar graph showing Hsp70 overexpression in oligodendrocytes reversed HIV-1 Tat-mediated decreased cathepsin D activity. (F) Representative fluorescent-microscopic image showing Hsp70 overexpression reversed HIV-1 Tat-mediated downregulation of myelin PLP and LAMP2 in oligodendrocytes. (G) Representative bar graph showing Hsp70 overexpression in oligodendrocytes reversed HIV-1 Tat-mediated downregulation of LAMP2 puncta. (H) Representative bar graph showing Hsp70 overexpression in oligodendrocytes reversed HIV-1 Tat-mediated downregulation of PLP intensity. Data is from three independent experiments. ACTB served as a protein loading control for western blots. Data is from three independent experiments and is represented as means ± SEM using one-way ANOVA. *p<0.05 vs. control; #p<0.05 vs. HIV-1 Tat.
these processes remain less understood. Our findings outline a molecular pathway underlying HIV-1 Tat-mediated impaired oligodendrocyte maturation that involves lysosomal dysfunction and autophagy dysregulation. While the current study focused on investigating the effects of a specific dose of HIV Tat, future research will explore the impact of HIV Tat at different doses and its relevance to other diseases or conditions.

Lysosomes are specialized cellular organelles crucial for the degradation of misfolded proteins and macromolecules/damaged organelles. Myelin abnormalities are a common feature in lysosomal disorders [65]. Lysosomes are well-known targets of cytotoxic HIV proteins, with dysfunction of lysosomes being inherently implicated in pathogenesis [66]. Reports indicate HIV-1 Tat-mediated disruption of endolysosomal function, leading to the accumulation of amyloid beta in rat hippocampal neurons, ultimately culminating in neurotoxicity [67]. Lysosomes contain several proteases, including cathepsins. Inactive pro-cathepsins (pCTSD) can be proteolytically converted to active mature cathepsins (mCTSD) in an acidic environment (low pH) of the lysosomes [68–70]. Our findings suggest increased lysosomal pH, decreased mCTSD expression, and decreased CTSD activity in the oligodendrocytes exposed to HIV-1 Tat.

LMP is the primary cause of elevated lysosomal pH. Protons leak through the destabilized membrane, resulting in a loss of the pH gradient [71, 72]. LAMP, a glycosylated transmembrane protein, protects the lysosomal membrane from its enzymes. Paraquat-induced oxidative stress has been shown to permeabilize the lysosomal membrane and causes lysosomal alkalization in astrocytes [73]. Our results showed HIV-1 Tat-mediated downregulation of LAMP2 expression with increased LMP. Overall, HIV-1 Tat-mediated LMP resulted in elevated lysosomal pH and decreased CTSD activity.

Lysosomes are critical for the maturation of the autophagy process. Lysosomes fuse with autolysosomes to form autophagosomes, which is critical for protein degradation [74, 75]. Autophagy dysregulation has been implicated as one of the hallmark features of HIV neuropathogenesis [76, 77]. Our findings demonstrated HIV-1 Tat-mediated dysregulation of autophagy, assessed by increased expression of autophagy markers such as BECN1, MAP1LC3B, and SQSTM1. MAP1LC3B turnover and the SQSTM1 degradation assays determine the autophagy flux. Accumulation of MAP1LC3B-II and SQSTM1 in HIV-1 Tat exposed oligodendrocytes indicated blockade of the autophagic flux. Adding BAF (autophagosome-lysosome fusion inhibitor) to HIV-1 Tat-treated oligodendrocytes failed to cause a further increase in the expression of MAP1LC3B-II and SQSTM1, thereby confirming maximal accumulation of MAP1LC3B-II and SQSTM1 in HIV-1 Tat exposed oligodendrocytes.

HIV-1 Tat-mediated upregulation of ROS has been reported in various CNS cells [78, 79]. Furthermore, it has also been shown that induction of ROS can lead to LMP [72, 80]. ROS can quickly diffuse into lysosomes and interact with free intralysosomal iron to generate highly reactive hydroxyl radicals in a Fenton-type reaction. Such a hydroxyl radical can induce LMP by causing lipid peroxidation of lysosomal membranes and damaging lysosomal membrane proteins [81–83].

We thus explored the role of ROS scavengers, such as NAC and Hsp70, in abrogating HIV-1 Tat-mediated lysosomal dysfunction and autophagy dysregulation. Our findings demonstrated HIV-1 Tat-mediated upregulation of ROS generation within 1 h of exposure in oligodendrocytes. Further, pretreatment of cells with ROS scavenger or lysosomal protector NAC significantly abrogated HIV-1 Tat-mediated lysosomal dysfunction and autophagy dysregulation in oligodendrocytes. Pretreatment with NAC reversed lysosomal pH and CTSD activity while also blocking HIV-1 Tat-mediated upregulated expression of autophagy markers (BECN1, MAP1LC3B-II, and SQSTM1). NAC also abrogated HIV-1 Tat-mediated downregulation of myelin proteins (MBP and PLP) in oligodendrocytes.

Having confirmed the role of LMP in HIV-1 Tat-mediated lysosomal dysfunction in oligodendrocytes, we next sought to understand the role of lysosomal membrane protection in reversing HIV-1 Tat-mediated defects in autophagy and oligodendrocyte maturation. Heat shock protein 70 (Hsp70) has been shown to localize to the luminal side of the endosomal-lysosomal system and stabilizes the lysosomes by blocking LMP [60, 61]. Interestingly, our findings demonstrated that overexpression of Hsp70 in oligodendrocytes significantly abrogated HIV-1 Tat-mediated lysosomal dysfunction (evidenced by analysis of LAMP2 expression, lysosomal pH, and CTSD activity), autophagy dysregulation (evidenced by analysis of LC3BII and SQSTM1 protein expression), and oligodendrocyte maturation (evidenced by analysis of myelin proteins; MBP and PLP).

These observations implicate ROS as an upstream regulator of the lysosomal and autophagic processes. NAC can thus be considered an ideal candidate for scavenging cellular ROS and protecting lysosomal functions, which, in turn, can ameliorate myelin damage in HIV + individuals. Also, lysosome membrane protection by Hsp70 overexpression reversed HIV-Tat-mediated effects. Our findings implicate that lysosomal dysfunction is central in HIV-1 Tat-mediated
Figure 7: Schematic depicting the mechanism(s) involved in HIV-Tat-mediated lysosomal dysfunction in oligodendrocytes. Exposure of immature oligodendrocytes to HIV-Tat increases ROS generation and LMP, leading, in turn, to lysosomal dysfunction and autophagy dysregulation, ultimately leading to decreased oligodendrocyte maturation. The maturation of oligodendrocytes is important for increased myelination. ROS scavenger N-acetylcysteine (NAC) and LMP protector heat shock protein 70 (Hsp70) reversed these deleterious effects of HIV-1 Tat. (CTSD, cathepsin D; LMP, lysosomal membrane permeabilization; MBP, myelin basic protein; PLP, myelin proteolipid protein).

autophagy dysfunction and myelin defects (Figure 7). Lysosomal protective agents could thus be developed as future adjunctive treatment options for dampening white matter damage in HIV-infected individuals.

Materials and methods

Reagents

HIV-1 Tat protein (1032–10, recombinant endotoxin-free; ImmunoDX, MA, USA). N-Acetyl Cysteine (NAC) (A7250), and bafilomycin A1 (B1793) were purchased from Sigma-Aldrich. TEMPOL (sc-200825) was purchased from Santa Cruz. Antibody resources: BECN1 (sc-1427) and were purchased from Santa Cruz Biotechnology. LAMP2 (NB300-591) and MAPLC3B (NB100-2220) were purchased from Novus Biological Company. CTSD (ab75852), myelin PLP (ab9311) and myelin MBP (ab62631) were purchased from Abcam. SQSTM1 (PM045) was purchased from MBL International. Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) [111035-003]; Goat Anti-Mouse IgG (H+L) [115-035-003] used at 1:1000 dilution; from Jackson ImmunoResearch Inc.

Primary oligodendrocyte isolation

Oligodendrocyte progenitor cells (OPCs) were cultured by a previously published method [84, 85]. Briefly, OPCs were isolated from postnatal day 0–2 rat cortices. Dissociated cells were plated on poly-L-lysine (P4707, Millipore Sigma)-coated T75 cm² flasks with high-glucose Dulbecco’s modified Eagle’s medium (11995-065, Thermo Fisher) containing 10% fetal bovine serum (16000-044, Thermo Fisher) and with penicillin-streptomycin (P0781, Millipore Sigma) then incubated at 37 °C with 5% CO₂. The culture medium was changed every third day for 10–12 days to obtain mixed glial cultures containing OPCs on the astrocytic monolayer bed. Purification of OPCs was done by shaking (18–20 h) on an orbital shaker at 37 °C, followed by differential adhesion for 1 h on the non-tissue culture plastic petri dish (351006, Fisher Scientific). Isolated OPCs were plated in Sato medium [DMEM, 1X Insulin-Transferrin-Selenium (ITS-G, 41400045, Thermo Fisher) 30 nM triiodothyronine (T6397, Millipore Sigma), penicillin-streptomycin (P0781, Millipore Sigma), 1X GlutaMAX™ Supplement (A1286001, Thermo Fisher)) for 2 days to reach immature status before further experiments [29, 84]. The purity of oligodendrocyte cultures were consistently >95% oligodendrocytes with <1% astrocyte contamination [84, 86, 87].

Treatments

At day 2, immature oligodendrocytes [27, 29] were treated with HIV-1 Tat protein with varying doses (12, 25, 50, and 100 ng/mL) for 24 h. The rationale for choosing HIV-1 Tat concentration was based on the premise that the HIV-1 Tat concentration in the serum and the cerebrospinal fluid of HIV-1 infected individuals ranges from 1 to 40 ng/mL [51, 52] and that this could be higher in the CNS in the vicinity of HIV-1 positive perivascular cells [53]. Immature oligodendrocytes were pretreated with ROS scavengers such as TEMPOL (20 μM) and N-acetyl cysteine (NAC) (5 mM) for 1 h followed by HIV-1 Tat.

Western blotting

Cells were lysed using a Lysis kit (Sigma, MCL1-1KT). After protein quantification, samples were electrophoresed in a sodium dodecyl
sulfate-polyacrylamide gel under reducing conditions and then transferred to PVDF membranes (Millipore, IPVH00010). After the transfer, PVDF membranes were blocked with 5% nonfat dry milk in 1× TBS buffer (0.124 g Tris [Fisher Scientific, BP52-5], 8.77 g NaCl [Fisher Scientific, BP358-212], 500 μL Tween-20 [Fisher Scientific, BP357-500], pH 7.6 for 1 h). Membranes were then probed with antibodies targeting proteins of interest. Beta-actin (ACTB; AS4421 Sigma-Aldrich) was used to normalize the loading protein. Goat anti-mouse/rabbit IgG secondary antibodies were HRP conjugated.

**Immunocytochemistry**

After the treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.1% Triton X-100 (Fisher Scientific, BP151-500) in phosphate-buffered saline (PBS; HyClone Laboratories, SH3025801). 10% normal goat serum (31872, Thermo Fisher) in PBS was used as blocking for 1 h at room temperature. Primary antibodies against the protein of interest were added and incubated overnight at 4°C. After 3 washes with buffer, the secondary antibody was added (2 h at room temperature). Cells were washed 3 times in buffer and mounted with Prolong gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, P36935). Fluorescence images were taken with a Zeiss Observer using a Z1 inverted microscope, and the acquired images were analyzed using the Axio vs. 40 Version 4.8.0.0 software.

**Morphological analyses**

For the morphological analyses, the visualization of oligodendrocyte cellular process length and branching involved several steps. First, the maximum-intensity confocal images were converted to binary images and then skeletonized using ImageJ software. The tracings of cellular processes were performed using the Neurolucida plugin within ImageJ. The secondary branches of each cellular process were individually traced and counted, with each tracing labeled as primary or secondary. A primary process was defined as any branch emerging from the soma, while a secondary process referred to any branch emerging from a primary cellular process. The number of cell process endpoints and process lengths was measured and normalized with the number of cell somas using the ImageJ software. To establish the pixel-to-μm ratio, the micrometer scale bar from each reconstruction was utilized, enabling the construction of a scale bar in ImageJ. The total length of the cellular process was then summarized for statistical comparisons, employing the Analyze Skeleton plugin within ImageJ software. In addition, for the cell soma analysis, the area around the cell body was delineated using ImageJ selection tools. Subsequently, the region of interest was measured using the ROI Manager in ImageJ, and the values were exported to an Excel file. The percent area and mean fluorescence intensity were multiplied to calculate the total fluorescence intensity for each threshold image [88]. The experiment was repeated three times, with five images per group analyzed. For statistical analysis, a minimum of 25 cells per group were included.

**Quantification of LAMP2 puncta**

Zeiss Observer using a Z1 inverted microscope (Carl Zeiss, Thornwood, NY, USA) was used to take fluorescence images. Image J software was used to analyze the images. The region of interest was chosen using the polygon selection tool. Fluorescence is converted to black pixels over a white background. The regions of interest to be measured were then analyzed by the Measure Particles algorithm to record puncta number, area, and size, and the results were transferred to an excel spreadsheet using the functions of ImageJ.

**ROS detection**

ROS detection was performed as per the manufacturer’s protocol (Life Technologies, D-339) protocol using Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit obtained from Invitrogen (136007). Briefly, cells grown in 96 well-plates were washed with HBSS, supplemented with 25 μM carboxy-H,DCFDA working solution, and incubated for 30 min at 37°C. The change in fluorescence was measured using a spectrofluorometer (485 nm excitation and 530 nm emission).

**CTSD activity determination**

CTSD Activity Assay Kit (Fluorometric) from Abcam (ab65302) was used to analyze CTSD activity. The oligodendrocytes lysates were incubated with reaction buffer for 1 h at 37°C then the fluorescence was measured at Ex/Em=328/460 nm. Protease activities were determined by comparing the relative fluorescence units (RFUs) against the levels of the controls and represented as fold change.

**Lysosomal pH Measurement**

Lysosomal pH was measured using LysoSensor (Yellow/Blue DND-160) from Thermo Fisher Scientific (Waltham, MA, USA). LysoSensor is a dual ratio-metric indicator dye specifically designed for measuring the pH of acidic organelles such as lysosomes. In our experimental procedure, cells were incubated with 2 μM LysoSensor for 5 min at 37°C. The fluorescence intensity was recorded at wavelengths of 340 and 380 nm. The obtained 340/380 nm ratios were then converted to pH units using a calibration curve established with 20 mM MES (+120 mM KCl, 20 mM NaCl, 10 μM Monensin, 20 μM Nigericin). The pH was adjusted using either HCl or NaOH to cover a pH range of 3.0–7.0.

**Lysosomal membrane permeability assay**

Acridine orange, a fluorescent dye, reversibly accumulates into the acidified membrane-bound compartments of the cell. Acridine orange shows fluorescence emission in a concentration-dependent manner. At high concentrations (e.g., in lysosomes), it shows red fluorescence; at low concentrations (e.g., in the cytosol) shows green fluorescence and yellow fluorescence as intermediate (e.g., upon trapping in nucleiol), thus monitoring lysosomal leakage or change in lysosomal pH. Cells were cultured in 96-well culture plates and firstly exposed with acri-dine orange (5 μg/mL) at 37°C for 15 min, then rinsed and incubated in HBSS with or without HIV-1 Tat and NAC for the different time points as indicated. Fluorescence of the cells was examined at 1 h intervals using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments) with excitation at 485 nm and emission at 530 and 620 nm.

**MAP1LC3B turnover and SQSTM1 degradation assays**

MAP1LC3B turnover and SQSTM1 degradation assays were already published [89]. Briefly, oligodendrocytes were seeded at a density of
5 × 10^5 cells/well in a 6-well plate at 5% CO₂ incubator at 37°C for attachment. Cells were starved overnight in the serum-free culture medium. Oligodendrocytes were treated with HIV-1 Tat (50 ng/mL) for 24 h alone, or the cells were exposed to 400 nM BAF in the last 4 h of the 24 h HIV-1 Tat treatment. At the end of the experiment, cells were harvested, and protein samples were prepared for western blotting analysis.

**Plasmids transfection**

For plasmid transfection, immature oligodendrocytes were maintained in the SATO medium. Upon 70% confluence, the culture medium was replaced with 250 μl of Opti-MEM® 1 Reduced Serum Medium. Using Lipofectamine® 3000 Reagent (manufacturer’s protocol), cells were transfected with pEGFP Hsp70 plasmid (a gift from Lois Greene; Addgene, 15215) for 12 h. After that medium was replaced with the fresh medium for another 24 h. The transfection efficiency of the immature oligodendrocytes was 60–65%. Hsp70 plasmid (a gift from Lois Greene; Addgene, 15215) is tagged with EGFP, a green fluorescent protein that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. After transfection, green fluorescent cells were counted, taking the ratio with the total cell by counting DAPI staining. Then, cells were treated with various reagents for the indicated time and processed for further analysis.

**Statistical analysis**

The results are presented as means ± SEM and were evaluated using a one-way analysis of variance followed by a Bonferroni (Dunn) comparison of groups using least-squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

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**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical Approval:** Animals were housed and maintained in accordance with the National Institutes of Health institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center.

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