Rho-kinase inhibitor hydroxyfasudil protects against HIV-1 Tat-induced dysfunction of tight junction and neprilysin/Aβ transfer receptor expression in mouse brain microvessels

Qiangtang Chen1,2 · Yu Wu1 · Yachun Yu1 · Junxiang Wei1 · Wen Huang1

Received: 17 May 2020 / Accepted: 11 January 2021 / Published online: 6 February 2021
© The Author(s) 2021

Abstract
HIV-1 transactivator protein (Tat) induces tight junction (TJ) dysfunction and amyloid-beta (Aβ) clearance dysfunction, contributing to the development and progression of HIV-1-associated neurocognitive disorder (HAND). The Rho/ROCK signaling pathway has protective effects on neurodegenerative disease. However, the underlying mechanisms of whether Rho/ROCK protects against HIV-1 Tat-caused dysfunction of TJ and neprilysin (NEP)/Aβ transfer receptor expression have not been elucidated. C57BL/6 mice were administered sterile saline (i.p., 100 μL) or Rho-kinase inhibitor hydroxyfasudil (HF) (i.p., 10 mg/kg) or HIV-1 Tat (i.v., 100 μg/kg) or HF 30 min before being exposed to HIV-1 Tat once a day for seven consecutive days. Evans Blue (EB) leakage was detected via spectrophotometer and brain slides in mouse brains. The protein and mRNA levels of zonula occludens-1 (ZO-1), occludin, NEP, receptor for advanced glycation end products (RAGE), and low-density lipoprotein receptor-related protein 1 (LRP1) in mouse brain microvessels were, respectively, analyzed by Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR) analyses. Exposure of the mice to HIV-1 Tat increased the amount of EB leakage, EB fluorescence intensity, blood–brain barrier (BBB) permeability, as well as the RAGE protein and mRNA levels, and decreased the protein and mRNA levels of ZO-1, occludin, NEP, and LRP1 in mouse brain microvessels. However, these effects were weakened by Rho-kinase inhibitor HF. Taken together, these results provide information that the Rho/ROCK signaling pathway is involved in HIV-1 Tat-induced dysfunction of TJ and NEP/Aβ transfer receptor expression in the C57BL/6 mouse brain. These findings shed some light on potentiality of inhibiting Rho/Rock signaling pathway in handling HAND.

Keywords HIV-1 transactivator protein · Rho/ROCK signaling pathway · Tight junction protein · Neprilysin · Receptor for advanced glycation end products · Low-density lipoprotein receptor-related protein 1

Introduction
Highly active antiretroviral therapy (HAART) is not capable of eradicating human immunodeficiency virus (HIV) infection in the central nervous system (CNS), although it succeeds in reducing viral loads in the blood to an undetectable level [1]. Indeed, 50% or more of HIV type 1 (HIV-1)-infected individuals receiving successful HAART suffer some level of HIV-1-associated neurocognitive disorder (HAND). Furthermore, most of the treatments for HAND remain ineffective [2]. The mechanisms of HIV infection causing neurocognitive impairment aren’t elucidated completely. The stained brain slides from acquired immunodeficiency syndrome (AIDS) patients showed a significant increase in amyloid deposition [3], which is closely related to the cognitive impairment of AIDS patients [4]. It has been shown that several mechanisms of vascular pathology caused by HIV-1 can be reproduced by the administration of the HIV-1 transactivator protein (Tat), which is made by HIV-1-infected cells [5]. HIV-1 Tat shows harmful effects on the development and progression of HAND. HIV-1 Tat is an inflammatory factor with neuroexcitability and neurotoxicity [6]; it can facilitate amyloid-beta (Aβ) accumulation...
by reducing Aβ degradation [7] and modulating Aβ transfer in vitro [8]. HIV-1 Tat causes increasing permeability of the blood–brain barrier (BBB) in vitro via downregulation of the expression of ZO-1, ZO-2, and occludin [7–9]. At the structural level, BBB dysfunction is associated with changes in tight junction (TJ) structure and functions [10], which are essential for maintaining low BBB permeability and integrity. TJ proteins include members of the claudin family, occludin, and ZO-1. Occludin is the first TJ protein to be found and is not essential for the formation of TJ chains, but the presence of occludin in the brain endothelial membrane is related to increased transepithelial electrical resistance (TEER) and decreased paracellular permeability [11]. Zonula occludens (ZO)-1 are other primary elements of TJ and they link other TJ proteins such as occludin to the actin cytoskeleton, which is essential for TJ formation [12]. Dysfunction of the BBB is related to neurocognitive deficits sustained in a variety of diseases such as stroke, Alzheimer’s disease (AD), and HIV-1 [13–15]. A functionally damaged BBB with a decreased clearance of Aβ from brain to blood could give rise to brain Aβ accumulation [16]. Therefore, inhibiting HIV-1 Tat-induced BBB dysfunction and Aβ accumulation can be a potential strategy for the treatment of HAND.

Aβ accumulation in the brain results in its gradual oligomerization [17]. Therefore, constant clearance of Aβ from the CNS into the blood is the most significant mechanism to prevent the potential neurotoxic accumulations of Aβ in the brain [18]. Aβ is cleared via enzyme-mediated breakdown or through non-proteolytic pathways. Neprilysin (NEP) is a rate-limiting Aβ-degrading enzyme that is highly expressed in human astrocytes and exhibits the strongest Aβ 1–40 and Aβ1–42 degradative activity [19]. The reduction of NEP levels may result in a significant decline in Aβ degradation, promoting Aβ deposition in the brain [20]. HIV-1 Tat-caused NEP dysfunction is detected in cerebral microvascular endothelial cells [7], neurons [3], and astrocytes [21]. Alternatively, Aβ may be transported across the BBB into the blood by the low-density lipoprotein receptor-related protein 1 (LRP1), while the transport of Aβ from the bloodstream into the brain is achieved by the receptor for advanced glycation end products (RAGE) [22]. Exposing hCMEC/D3 cells to HIV-1 or HIV-1 Tat result in markedly enhanced accumulation of intracellular Aβ due to increased RAGE expression [8, 23] and decreased LRP1 expression [8]. It is suggested that upregulation of NEP and LRP1 expression and downregulation of RAGE expression may become promising targets for the treatment of HAND.

Rho-kinase (ROCK), a serine/threonine kinase, is a key downstream effector of the small GTPase Rho; it alters the cytoskeleton to adjust cell migration and proliferation. It was reported that ROCK mediates BBB destruction [24], and activation of ROCK was observed to coincide with increased BBB permeability in the capillaries of AD mice [25]. ROCK1/ROCK2 inhibition ameliorates cognitive deficits, largely through decreased Aβ deposition, and promotes Aβ internalization in an AD model [26]. Thus, inhibiting ROCK might prevent cognitive decline. Previous studies from our group demonstrated that the Rho-kinase inhibitor hydroxyfasudil (HF) markedly restrained HIV-1 Tat-caused occludin downregulation, HIV-1 Tat-regulated LRP1, and RAGE expression in hCMEC/D3 [8]. Nonetheless, the underlying mechanism of whether HIV-1 Tat-caused BBB damage and Aβ accumulation in the mouse brain can be reversed by the ROCK inhibitor warrants further research. Moreover, ZO-1 and occludin express between adjacent cerebral microvessel endothelial cells [27]. The expression of NEP in the brain is relatively low, and it is mainly expressed in the middle membrane of cerebral cortex vessels and pyramidal neurons [28]. Previous studies had shown that the decrease of NEP expression in cerebral microvessels was negatively related to the deposition of Aβ around cerebral vessels [29]. However, LRP1 and RAGE are expressed in a variety of cells in the brain, while LRP1 and RAGE of cerebral microvessels are related to Aβ transport across the BBB [22]. Therefore, mice cerebral microvessels were used as experimental samples in this study. Our data indicate that ROCK inhibition by HF has protective effects on HIV-1 Tat-caused dysfunction of TJ and NEP/Aβ transfer receptor expression.

Materials and methods

Animals

C57BL/6 mice (22–25 g, 8 weeks old, male) were purchased from Guangxi Medical University Animal Center (Guangxi, China). The animals were housed under constant temperature (23 ± 1 °C), humidity (60 ± 10%) and light-controlled vivarium (12-h light/12-h dark cycle). Food and water were available ad libitum. The mice used in this study have been granted approval from the Committee on the Ethics of Animal Experiments of Guangxi Medical University (Certificate Numbers: SYXK 2014–0003) and the experiment had been proceeded in compliance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Animal experiments

Recombinant HIV-1 Tat clade-B protein (amino acids 1 to 86, product#HIV-129) was obtained from Prospec (Rehovot, Israel). Previous research indicates that the dosage of HIV-1 Tat used in mouse experiments varied widely, especially in BBB damage and Aβ accumulation experiments [30, 31]. To identify what dosage of HIV-1 Tat would have a significant effect on BBB, animals were divided into 4 groups (n = 9...
each) on a random basis and were administered with 0, 25, 50, and 100 μg/kg HIV-1 Tat via tail vein injection. The appropriate HIV-1 Tat concentration was obtained by detecting Evans blue (EB) leakage and EB fluorescence intensity by spectrophotometry and fluorescence microscopy, respectively, meanwhile by measuring the expression of ZO-1 of mouse brain microvessels by Western blotting analysis. The methods will be explained later.

Animals were randomly divided into 4 groups (n = 9 each): the control group, HIV-1 Tat group, HF group and HIV-1 Tat + HF group. In the control group, 1 h after intraperitoneal injection with 100 μl sterile saline, the mice were injected with 100 μl sterile saline via tail vein. In the HIV-1 Tat group, 1 h after intraperitoneal injection with 100 μl sterile saline, the mice were injected with 100 μl of HIV-1 Tat 100 μg/kg and sterile saline through the tail vein. In the HF group, 1 h after intraperitoneal injection with 100 μl of 10 mg/kg HF (product#ab145524, abcam, USA) and sterile saline, the mice were injected with 100 μl of HIV-1 Tat 100 μg/kg and sterile saline through the tail vein. In the HIV-1 Tat + HF group, 1 h after intraperitoneal injection with 100 μl of 10 mg/kg HF and sterile saline, the mice were injected with 100 μl of HIV-1 Tat 100 μg/kg and sterile saline through the tail vein. The above treatment was daily for seven consecutive days. Significant side effects caused by HIV-1 Tat or HF treatment were not observed in the experiments. All mice were sacrificed 24 h after the last treatment and their brains were harvested.

**BBB permeability**

The amount of EB leakage was detected as described earlier [32]. Twenty-four hours after the last treatment, mice of each group were given an EB (product#ab2129, Sigma-Aldrich) solution (2%, 5 mL/kg) through tail vein injection. It was allowed to circulate for 2 h. Mice were anesthetized with sodium pentobarbital (1%, 30 mg/kg, i.p.) and decapitated; then, their brains were removed. Mouse cerebral microvessels were isolated according to the research method of Hrvoje Brzica et al. [33]. The cerebral cortex (300 μg) was used to isolate the brain microvessels. Finally, the brain microvessels were suspended with 50ul brain microvascular buffer and stored at −20 °C for further use. To extract the total protein, the cerebral microvessels (25 ul) were homogenized in 100 ul lysis buffer (product#ab156034, Abcam, USA). Immunoblotting of ZO-1, occludin, NEP, LRP1 and RAGE were performed as described earlier [7, 8]. Rabbit anti-ZO-1 antibodies (product#ab96587, 1:1000, rabbit polyclonal; Abcam, Cambridge, UK), rabbit anti-occludin antibodies (product#ab13409–1-AP, 1:1000, rabbit polyclonal; Proteintech Group, Chicago, IL, USA), rabbit anti-NEP antibodies (product#ab256494, 1:500, rabbit polyclonal; Abcam), rabbit anti-RAGE antibodies (product#ab216329, 1:800, rabbit polyclonal; Abcam, Cambridge, UK), rabbit anti-LRP1 antibodies (product#ab92544, 1:2000, rabbit polyclonal; Abcam, Cambridge, UK), and rabbit anti-GAPDH antibodies (product#ab181602, 1:10000, rabbit polyclonal; Abcam) were used as the primary antibody. GAPDH was used as the loading control. Band density was measured by Image J software.

**Western blotting analysis**

Twenty-four hours after the last treatment, the mice were anesthetized with sodium pentobarbital (1%, 30 mg/kg, i.p.) and decapitated; then, their brains were removed. Mouse cerebral microvessels were isolated according to the research method of Hrvoje Brzica et al. [33]. The cerebral cortex (300 μg) was used to isolate the brain microvessels. Finally, the brain microvessels were suspended with 50ul brain microvascular buffer and stored at −20 °C for further use. To extract the total protein, the cerebral microvessels (25 ul) were homogenized in 100 ul lysis buffer (product#ab156034, Abcam, USA). Immunoblotting of ZO-1, occludin, NEP, LRP1 and RAGE were performed as described earlier [7, 8]. Rabbit anti-ZO-1 antibodies (product#ab96587, 1:1000, rabbit polyclonal; Abcam, Cambridge, UK), rabbit anti-occludin antibodies (product#ab13409–1-AP, 1:1000, rabbit polyclonal; Proteintech Group, Chicago, IL, USA), rabbit anti-NEP antibodies (product#ab256494, 1:500, rabbit polyclonal; Abcam), rabbit anti-RAGE antibodies (product#ab216329, 1:800, rabbit polyclonal; Abcam, Cambridge, UK), rabbit anti-LRP1 antibodies (product#ab92544, 1:2000, rabbit polyclonal; Abcam, Cambridge, UK), and rabbit anti-GAPDH antibodies (product#ab181602, 1:10000, rabbit polyclonal; Abcam) were used as the primary antibody. GAPDH was used as the loading control. Band density was measured by Image J software.

**RNA isolation and qRT-PCR**

Cerebral microvessels were separated as mentioned before [33]. Total RNA was extracted from cerebral microvessels (25 ul). qRT-PCR of ZO-1, occludin, NEP, LRP1 and RAGE were performed as described earlier [7, 8]. The primer sequences were as follows: ZO-1 forward: 5'-GCGCGCT AAGACACAGCAA-3', ZO-1 reverse: 5'-TCCCCACTC TGAAATGAGGA -3', Occludin forward: 5'-TTGAAAT GTCCACCTGCCTACAGA-3', Occludin reverse: 5'-CCGG ATAAAAAGACTAGCTGG-3', NEP forward: 5'-CTCT CTGTTGCTTGCTTGCTC-3', NEP reverse: 5'-GACGTT GCCCTTCAACACGC-3', RAGE forward: 5'- CTTGCT CATAGGGGACGCTGTA-3', RAGE reverse: 5'- GGAGGA
the 2 –ΔΔCt method. The target gene mRNA levels, which were analyzed using TGT AGT TGA GGTCA-3′. The amount of EB leakage and EB fluorescence intensity significantly increased in the HIV-1 Tat group compared to the control group (**P < 0.01). However, the EB fluorescent signal increased significantly in comparison to the control group (***P < 0.001). However, the EB fluorescent signal decreased in comparison to the HIV-1 Tat group (##P < 0.01). Moreover, the mouse brain EB fluorescence intensity of HIV-1 Tat group significantly increased in comparison to the control group (###P < 0.001). The blue staining in the mouse brain and its fluorescence intensity went down in the HF + HIV-1 Tat group compared to the HIV-1 Tat group (###P < 0.001). The blue staining in the mouse brain, the amount of EB leakage, the mouse brain EB fluorescent signals, and fluorescence intensity showed no significant difference between the HF group and the control group.

**Role of the Rho/ROCK signaling pathway in HIV-1 Tat-induced changes in BBB permeability**

The effect of HF on HIV-1 Tat-caused BBB damage was evaluated by EB leakage and frozen slices of brain tissues. When 100 μg/kg of HIV-1 Tat was given to the C57BL/6 mouse once a day for seven consecutive days, the mouse brain of the HIV-1 Tat group was clearly stained in blue in the cerebral cortex surface (Fig. 2a). In addition, the EB leakage of the HIV-1 Tat group (Fig. 2b) showed a significant increase compared to the control group and HF group (**P < 0.01). However, in the HF + HIV-1 Tat group, the blue staining in the mouse brain (Fig. 2a) was not conspicuous and EB leakage (Fig. 2b) significantly decreased in comparison to the HIV-1 Tat group (###P < 0.001). Moreover, the mouse brain EB fluorescent signals and fluorescence intensity of HIV-1 Tat group significantly increased in comparison to the control group (Fig. 2c–d) (**P < 0.01). However, the EB fluorescent signals in the mouse brain and its fluorescence intensity went down in the HF + HIV-1 Tat group compared to the HIV-1 Tat group (###P < 0.001). The blue staining in the mouse brain, the amount of EB leakage, the mouse brain EB fluorescent signals, and fluorescence intensity showed no significant difference between the HF group and the control group.

**Role of the Rho/ROCK signaling pathway in HIV-1 Tat-induced changes in ZO-1 and occludin of mouse brain microvessels**

HIV-1 Tat caused changes in TJ protein expression through the Ras signaling pathway in vitro [9]. To address the effect of ROCK on HIV-1 Tat-caused changes of ZO-1 and occludin protein in vivo, the ROCK-specific inhibitor HF was administered 30 min before being applied to HIV-1 Tat once a day for seven consecutive days. The protein levels and mRNA levels of ZO-1 and occludin in mouse brain microvessels were detected by Western blotting and qRT-PCR, respectively. ZO-1 and occludin protein levels as shown in Fig. 3a and c and their mRNA levels as shown in Fig. 3b and d were downregulated following HIV-1 Tat exposure (**P < 0.01 vs the control group in both Western blotting and qRT-PCR) but were significantly upregulated in the HF + HIV-1 Tat group (##P < 0.01 vs the HIV-1 Tat group in both Western blotting and qRT-PCR).

**Results**

**Dose-dependent effects of HIV-1 Tat on BBB permeability and ZO-1 protein levels**

EB binds to serum albumin, which prevents it from crossing the normal BBB, but dysfunctional BBB allows EB to pass through. Hence, EB leakage is used to evaluate BBB function [34]. HIV-1 Tat has been considered to destroy BBB in vitro [7, 8, 35] and in vivo [31]. To assess the permeability of BBB, EB leakage and EB fluorescence intensity were examined by spectrophotometry and fluorescence microscopy, respectively, and the ZO-1 expression in mouse brain microvessels was assessed with Western blotting. With the dose of HIV-1 Tat increasing, the mouse brain in blue (Fig. 1a), the amount of EB leakage (Fig. 1b), the mouse brain fluorescence signal (Fig. 1c) and the EB fluorescence intensity (Fig. 1d) were gradually enhanced; however, the protein levels of ZO-1 (Fig. 1e) were gradually reduced. HIV-1 Tat at 100 μg/kg body weight significantly increased EB leakage and EB fluorescence intensity compared to the control group (**P < 0.01) and reduced the expression of ZO-1 compared to the control group (##P < 0.01).

**Statistical analysis**

SPSS 17.0 (Chicago, IL, USA) was used for statistical analysis. The data are presented as the means ± standard deviation. One-way Anova was used in conducting comparisons between groups; Student-Newman-Keuls (SNK) was used for the post hoc test. P < 0.05 was regarded to be significant.
Fig. 2 The permeability of the blood–brain barrier to EB was determined by the tissue homogenate method and fluorescence microscopy in the frozen tissue section. (a): Gross specimen: EB staining on the mouse brain surface. (b): The amount of EB was determined by the tissue homogenate method. (c) and (d): The exudation amount of EB was determined by fluorescence microscopy in the frozen tissue section. The arrows represent EB exudation. Compared to the control group, the mouse brains were stained in blue; the exudation amount of EB, EB fluorescence signal, and intensity significantly increased in HIV-1 Tat 100 μg/kg group (***P < 0.001, **P < 0.05). Compared to HIV-1 Tat 100 μg/kg group, the blue staining of the mouse brain surface became shallow; the EB leakage amount, EB fluorescence signal, and intensity decreased significantly in the HIV-1 Tat 100 μg/kg + HF group (### P < 0.001, ##P < 0.01). The data represent the mean ± standard deviation of the three independent experiments.
Role of the Rho/ROCK signaling pathway in HIV-1 Tat-induced changes in NEP, RAGE and LRP1 in mouse brain microvessels

Our previous study had showed that HF significantly inhibited HIV-1 Tat-regulated Aβ transferring protein LRP1 and RAGE expression in hCMEC/D3 cells [36]. To determine the underlying mechanism of the effect of ROCK on HIV-1 Tat-induced dysfunction of NEP/Aβ transfer receptor expression in the mouse brain, HF was administered 30 min before giving HIV-1 Tat once a day for seven consecutive days. The protein and mRNA levels of NEP, LRP1 and RAGE in mouse brain microvessels were measured by Western blotting and qRT-PCR, respectively. NEP and LRP1 protein levels (Fig. 4a and c, respectively), along with RNA levels (Fig. 4b and d) declined after HIV-1 Tat administration in comparison with the control group (** P < 0.01). Compared to the HIV-1 Tat 100 μg/kg group, HF pretreatment significantly upregulated the protein and mRNA levels of ZO-1 and occludin (#P < 0.05, ##P < 0.01). These data represent the mean ± standard deviation of the three independent experiments.


![Fig. 4](image_url) The Rho/ROCK signaling pathway involved in HIV-1 Tat-induced changes of NEP, LRP1, and RAGE of mouse brain microvessels. The protein levels and mRNA levels of NEP, LRP1, and RAGE in the mouse brain microvessels were detected by Western blotting and qRT-PCR, respectively. NEP and LRP1 protein levels (a and c) as well as mRNA levels (b and d) declined following HIV-1 Tat (100 μg/kg) exposure (compared to the control group, **P < 0.01 in both Western blotting and qRT-PCR), but were upregulated in the HIV-1 Tat 100 μg/kg + HF group (##P < 0.01 or #P < 0.05 vs the HIV-1 Tat 100 μg/kg group in both Western blotting and qRT-PCR). RAGE protein and mRNA levels (e, f) were ascended after HIV-1 Tat (100 μg/kg) exposure in comparison with the control group (**P < 0.01 in both Western blotting and RT-PCR), but were downregulated in the HF + HIV-1 Tat group (#P < 0.05 vs the HIV-1 Tat 100 μg/kg group in both Western blotting and qRT-PCR). Data are representative of three independent experiments and shown as the means ± standard error of the mean.

The protein and mRNA levels of RAGE (Fig. 4e–f) were ascended after HIV-1 Tat exposure in comparison with the control group (**P < 0.01 in both Western blotting and RT-PCR), but were downregulated in the HF + HIV-1 Tat group (#P < 0.05 vs the HIV-1 Tat group in both Western blotting and qRT-PCR).

**Discussion**

BBB dysfunction is always accompanied by severe neurological dysfunction and neurodegeneration, including HAND [37]. Our previous data, along with other groups’ data, revealed that exposure of cerebral endothelial cells to HIV-1 Tat caused the expression of ZO-1 [7] and occludin [8] to be downregulated; BBB permeability and Aβ accumulation were upregulated [7]. In this experiment, HIV-1 Tat affected BBB permeability and the expression of ZO-1 in a dosage-dependent manner in the mouse brain; the effect of HIV-1 Tat 100 μg/kg was the most significant (Fig. 1). Therefore, HIV-1 Tat 100 μg/kg was selected for use in the rest of the experiment. The dosage in the current study is different from the findings obtained in previous studies due to the differences in HIV-1 Tat peptide length and purity, as well as differences in method and duration of administration. In the research of William A. Banks et al., it was found that HIV-1 Tat passes through the BBB via a unsaturable mechanism with a unidirectional influx rate of about 0.490 A1/g/min. About 0.126% of an intravenous dose of HIV-1 Tat entered each g of brain [38]. It is consistent with the fact that only a small fraction of intravenously administered Tat can enter the brain of mice [5].

ROCK1/ROCK2 inhibitors rescued the efficacy of BBB damage in several CNS diseases, including experimental autoimmune encephalomyelitis, cerebral ischemia, and intracerebral hemorrhage [39]. Activation of RhoA/ROCK changed TJ and F actin cytoskeleton organization, then upregulated epithelial TJ permeability [26]. HIV-1 Tat caused the disruption of TJ proteins by activating the Ras/mitogen-activated protein kinase (MAPK) pathway in brain endothelial cells [9]. Upstream of the MAPK signaling pathway, the Rho/ROCK signaling pathway may be involved in HIV-1 Tat-induced BBB dysfunction. In this experiment, the levels of Rho-A or ROCK protein were examined by Western blotting, but their total amount did not change. It was also found that the levels of Rho-A or ROCK did not change after exposure to HIV-1 Tat, but their activity was affected in the endothelial cells of the BBB [40]. Our previous studies in vitro showed that the Rho-kinase inhibitor HF markedly restrained HIV-1 Tat-caused occludin down-regulation [8] and HIV-1 Tat inhibited the expression of ZO-1 in brain endothelial cells via the Ras signaling pathway [7]. In this in vivo study, the Rho-kinase inhibitor HF reversed the HIV-1 Tat-caused down-regulation of the protein and mRNA levels of ZO-1 and occludin; it also reversed upregulation of EB leakage and EB fluorescence intensity in the mouse brain (Figs. 2 and 3). In the study of Chen et al., after administrated with HF 12 h, the mice were injected with 25 μg HIV-1 Tat via the internal carotid artery. HIV-1 Tat also reduced the expression of ZO-1 and claudin 5 in the hippocampus of mice; this effect could be reversed by HF [31]. The similar results can be observed in this study, even though the way of interventions and experimental specimens were different. Collectively, these findings indicate that HF protected against HIV-1 Tat-induced BBB dysfunction partly by inhibiting the Rho/ROCK signaling pathway in vivo.

Perivascular Aβ accumulation increased in brains with HIV-1 encephalitis (HIVE)- and HIV-1-associated dementia [4]. In cell-based studies, exposure to HIV-1 Tat significantly enhanced the extracellular levels of Aβ [3, 7]. After HIV-1 infection, several mechanisms contributed to upregulating the levels of Aβ, such as the increase of its production [41], deficient enzymatic degradation [3], and an increased transport mechanism across the BBB [23]. In the brain of an HIV infected patient, Aβ accumulation and deposition drove the pathogenic cascades of neurological disorders; this contributes toward aging or associated dementias [41]. Therefore, targeting Aβ degradation and transport may be a potential strategy for the treatment of HAND. Growing evidence suggests that ROCK influence Aβ production and clearance. In vitro, ROCK2 has been shown to regulate phosphorylation of APP at threonine 654 site, which is critical for b-secretase cleavage [42]. On the other hand, ROCK can also influence Aβ production through regulating APP intracellular transport and Aβ secretion [43]. ROCK1 is also thought to be the promoter of Aβ autophagy; the over-expression of ROCK1 promoted internalization of Aβ and formation of autophagosomes in vitro [44]. ROCK inhibition reduced the brain’s amyloid protein level in a transgenic mouse model of AD [45]. These findings show a strong link between ROCK and Aβ clearance.
NEP is a major Aβ-degrading enzyme, showing the most potent degrading activity of Aβ 1–40 and Aβ 1–42 [19]. A negative correlation between NEP and Aβ plaque load was repeatedly confirmed in post-mortem human brains [46] and in animal models [19]. In an in vitro assay, HIV-1 Tat inhibited 80% of NEP. Exposure of HIV-1 Tat to brain cultures showed an increase of soluble Aβ [3]. Previously in our study [7], HIV-1 Tat diminished NEP expression and weakened NEP immunoreactivity in hCMEC/D3 cells; this effect was reversed by the Ras signaling pathway inhibitor farnesylthiosalicylic acid. In keeping with earlier findings [21], treatment with HIV-1 Tat in the mouse reduced expression of NEP protein and mRNA levels in this study; this effect may directly result in reducing Aβ degradation in the brain which, in turn, added to Aβ accumulation, but inhibition of ROCK by HF effectively restrained HIV-1 Tat-caused NEP reduction in the mouse (Fig. 4a–b). However, cellular NEP expression levels were not downregulated in HIV-1pYu2 infection of human monocyte-derived macrophages [47]. The expression and activity of NEP were reduced by exposing brain vascular endothelial cells or glial cells to HIV-1 Tat [7, 21]. NEP protein expression positively correlates with enzymatic activity [3]. These different results may be attributed to different HIV-1 subtypes and cell types. NEP double bands are shown in Fig. 4a. This is likely to be a phosphorylation or methylation of NEP which is lost upon HIV-1 Tat treatment and is reverted after ROCK inhibition via HF. Similar NEP double bands have been found in other studies [48], but no further studies have been carried out yet.

A functionally impaired BBB with a decreased brain-to-blood clearance of Aβ could facilitate Aβ accumulation [16]. If the BBB is impaired, the circulating Aβ can also enter the brain through pro-inflammatory cytokines, possibly caused by the Aβ–RAGE interaction [49]. Peptides such as Aβ cannot directly cross the BBB, but they can be transferred via the specific transferring receptors LRPI and RAGE. RAGE transports Aβ from the bloodstream into the brain, while LRPI undertakes the opposite. LRPI and RAGE regulate Aβ levels in the brain by transporting Aβ across the BBB. The expression of microvascular LRPI decreased, while the level and immune activity of RAGE increased in the human brain of AD [22]. RAGE is a subtype of Caveolae and is expressed at relatively low levels at the BBB. Our previous data revealed that silencing the Cav-1 gene greatly prevented HIV-1 Tat-caused upregulation of RAGE and downregulation of LRPI [35]. Ibolya E András and colleagues in 2010 reported that simvastatin weaken HIV-1-induced upregulation of RAGE expression; however, HIV-1 did not affect LRPI expression in brain endothelial cells [23]. In this study, treatment with HIV-1 Tat gave rise to downregulation of LRPI protein and mRNA levels, as well as upregulation of RAGE protein and mRNA levels in the mouse brain microvessels. This finding may lead directly to Aβ accumulation in the brain. However, this effect was reversed by HF (Fig. 4c–f). It is consistent with our previous in vitro study [8]. These data indicate that restraining ROCK may weaken HIV-1 Tat-caused dysfunction of Aβ transendothelial transfer and degradation in vivo.

In summary, inhibition of Rho/ROCK signaling by HF in the mouse has a strong protective effect on HIV-1 Tat-induced downregulation of ZO-1, occludin, NEP, and LRPI, as well as upregulation of RAGE and BBB permeability. This may lead to the degradation and transport dysfunction of Aβ in the brain. These findings suggest that Rho/ROCK signaling pathway is a crucial element in the HIV-1 Tat-induced BBB destruction and dysfunction of NEP/Aβ transfer receptor expression. Targeting the Rho/ROCK signaling pathway may be a promising option to treat HAND.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81371333, 81160152 and 82060236) and Natural Science Foundation of Guangxi Province (CN) (2019GXNSFDA245032).

Funding Dr. Wen Huang was funded by National Natural Science Foundation of China (CN) (81371333, 81160152) and Natural Science Foundation of Guangxi Province (CN) (2019GXNSFDA245032).

Data availability All data generated or analyzed during this study are included in this article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, and indicate if changes were made. You may do so, for example by placing a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Cysique LA, Maruff P, Brew BJ (2004) Prevalence and pattern of neuropsychological impairment in human immunodeficiency virus-infected/acquired immunodeficiency syndrome (HIV/AIDS) patients across pre- and post-highly active antiretroviral therapy eras: a combined study of two cohorts. J Neurovirol 10(6):350–357. https://doi.org/10.1080/13550280490521078

2. Heaton RK, Franklin DR Jr, Deutsch R et al (2015) Neurocognitive change in the era of HIV combination antiretroviral therapy: the longitudinal CHARTER study. Clin Infect Dis 60(3):473–480. https://doi.org/10.1093/cid/ciu862
3. Rempel HC, Pulliam L (2005) HIV-1 Tat inhibits neprilysin and elevates amyloid beta. AIDS (London, England) 19(2):127–135. https://doi.org/10.1017/S0269960X0400437X

4. Xu J, Ikezu T (2009) The comborbidty of HIV-associated neurocognitive disorders and Alzheimer’s disease: a foreseeable medical challenge in post-HAART era. J Neuroimmun Pharmacol 4(2):200–212. https://doi.org/10.1007/s11481-008-9136-0

5. Xiao H, Neuvénot C, Tiffany HL et al (2000) Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. Proc Natl Acad Sci U S A 97(21):11466–11471. https://doi.org/10.1073/pnas.97.21.11466

6. Albini A, Ferrini S, Benelli R et al (1998) HIV-1 Tat protein mimics of chemokines. Proc Natl Acad Sci U S A 95(22):13153–13158. https://doi.org/10.1073/pnas.95.22.13153

7. Jiang W, Huang W, Chen Y, Zou M, Peng D, Chen D (2017) HIV-1 transactivator protein induces ZO-1 and Neprilysin dysfunction in brain endothelial cells via the Ras signaling pathway. Oxidative Med Cell Longev 2017:3160360. https://doi.org/10.1155/2017/3160360

8. Chen Y, Huang W, Jiang W, Wu X, Ye B, Zhou X (2016) HIV-1 Tat regulates occludin and αolf-1 transfer receptor expression in brain endothelial cells via Rho/ROCK signaling pathway. Oxidative Med Cell Longev 2016:4196572. https://doi.org/10.1155/2016/4196572

9. Zhong Y, Smart EJ, Weksler B, Couraud PO, Hennig B, Toborek M (2009) PPA-Ralpha and PPARgamma attenuate HIV-induced dysregulation of tight junction proteins by modulations of matrix metalloproteinase and proteasome activities. FASEB J 23(5):1596–1606. https://doi.org/10.1096/fj.08-121624

10. Saitou M, Furuse M, Sasaki H et al (2000) Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 11(12):4311–4312. https://doi.org/10.1091/mbc.11.12.4311

11. Van Itallie CM, Fanning AS, Bridges A, Anderson JM (2009) ZO-1 stabilizes the tight junction solute barrier through coupling to the perijunctional cytoskeleton. Mol Biol Cell 20(17):3930–3940. https://doi.org/10.1091/mbc.e09-04-0320

12. Krizanac-Bengez L, Mayberg MR, Janigro D (2004) The cerebral vasculature as a therapeutic target for neurological disorders and the role of shear stress in vascular homeostasis and pathophysiology. Neurol Res 26(8):846–853. https://doi.org/10.1179/016140404789

13. Logsdon AD, Fuke-Wold BP, Turner RC, Huber JD, Rosen CL, Simpkins JW (2015) Role of microvascular disruption in brain damage from traumatic brain injury. Compr Physiol 5(3):1147–1160. https://doi.org/10.1002/cphy.c140057

14. Salmina AB, Inzhutova AI, Malinovskaya NA, Petrova MM (2010) Endothelial dysfunction and repair in Alzheimer-type neurodegeneration: neuronal and glial control. J Alzheimers Dis JAD 22(1):17–36. https://doi.org/10.3233/jad-2010-19690

15. Zlokovic BV (2005) Neurovascular mechanisms of Alzheimer’s neurodegeneration. Trends Neurosci 28(4):202–208. https://doi.org/10.1016/j.tins.2005.02.001

16. Kayed R, Head E, Thompson JL et al (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science (New York, NY) 300(5618):486–489. https://doi.org/10.1126/science.1079469

17. Mawuenyega KG, Sigurdson W, Ovod V et al (2010) Decreased clearance of CNS beta-amyloid in Alzheimer’s disease. Science (New York, NY) 330(6012):1774. https://doi.org/10.1126/science.1197623

18. Wata N, Tsukuba S, Takai Y et al (2001) Metabolic regulation of brain Abeta by neprilysin. Science (New York, NY) 292(5521):1550–1552. https://doi.org/10.1126/science.1059946

19. Narita Naka V, Fisk LR, Belyaev ND, Turner AJ (2008) Amyloid-degrading enzymes as therapeutic targets in Alzheimer’s disease. Curr Alzheimer Res 5(2):212–224. https://doi.org/10.2174/15672050878395785

20. Martin-Zet-Bone M, Muñoz-Fernández M, Álvarez S (2019) HIV-1 increases extracellular amyloid-beta levels through neprilysin regulation in primary cultures of human astrocytes. J Cell Physiol 234(5):5880–5887. https://doi.org/10.1002/jcp.26462

21. Donahue JE, Flaherty SL, Johanson CE et al (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer’s disease. Acta Neuropathol 112(4):405–415. https://doi.org/10.1007/s00406-006-0115-3

22. András IE, Eum SY, Huang W, Zhong Y, Hennig B, Toborek M (2010) HIV-1-induced amyloid beta accumulation in brain endothelial cells is attenuated by simvastatin. Mol Cell Neurosci 43(2):232–243. https://doi.org/10.1016/j.mcn.2009.11.004

23. Liu K, Li Z, Wu T, Ding S (2011) Role of rho kinase in microvascular damage following cerebral ischemia reperfusion in rats. Int J Mol Sci 12(2):1222–1231. https://doi.org/10.3390/ijms12021222

24. Park JC, Baik SH, Han SH et al (2017) Annexin A1 restores Ap(1-42)-induced blood-brain barrier disruption through the inhibition of RhoA-ROCK signaling pathway. Aging Cell 16(1):149–161. https://doi.org/10.1111/acel.12530

25. Song Y, Chen X, Wang LY, Gao W, Zhu MJ (2013) Rho kinase inhibitor fasudil protects against β-amyloid-induced hippocampal neurodegeneration in rats. CNS Neurosci Ther 19(8):603–610. https://doi.org/10.1111/cns.12116

26. Abbott NJ, Rönnbäck L, Hanson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7(1):41–53. https://doi.org/10.1038/nrn1824

27. Dorfman VB, Pasquini L, Riudavets M et al (2010) Differential cerebral deposition of IDE and NEP in sporadic and familial Alzheimer’s disease. Neurobiol Aging 31(10):1743–1757. https://doi.org/10.1016/j.neurobiolaging.2008.09.016

28. Bourassa P, Tremblay C, Schneider JA, Bennett DA, Calon F (2019) Beta-amyloid pathology in human brain microvessel extracts from the parietal cortex: relation with cerebral amyloid angiopathy and Alzheimer’s disease. Acta Neuropathol 137(5):801–823. https://doi.org/10.1007/s00401-019-01967-4

29. Banerjee A, Zhang X, Manda KR, Banks WA, Ercal N (2010) HIV proteins (gp120 and Tat) and methamphetamine in oxidative stress-induced damage in the brain: potential role of the thiol antioxidant N-acetylcysteine amide. Free Radic Biol Med 48(10):1388–1398. https://doi.org/10.1016/j.freeradbiol.2010.02.023

30. Chen L, Choi JJ, Choi YJ, Hennig B, Toborek M (2012) HIV-1 Tat-induced cerebrovascular toxicity is enhanced in mice with amyloid deposits. Neurobiol Aging 33(8):1579–1590. https://doi.org/10.1016/j.neurobiolaging.2011.06.004

31. Manen Groen A, Chen H, Kammer J, Zhang JH, Tang J (2011) Comparison Evans blue injection routes: intravenous versus intraperitoneal, for measurement of blood-brain barrier in a mice hemorrhage model. J Neurosci Methods 195(2):206–210. https://doi.org/10.1016/j.jneumeth.2010.12.013

32. Brzica H, Abdullahi W, Reilly BG, Ronaldson PT (2018) A simple and reproducible method to prepare membrane samples from freshly isolated rat brain microvessels. J Vis Exp JoVE 135. https://doi.org/10.3791/57698
34. Feng S, Zou L, Wang H, He R, Liu K, Zhu H (2018) RhoA/ROCK-2 pathway inhibition and tight junction protein upregulation by catalpol suppresses lipopolysaccharide-induced disruption of blood-brain barrier permeability. Molecules 23(9). https://doi.org/10.3390/molecules23092371
35. Zou M, Huang W, Jiang W, Wu Y, Chen Q (2019) Role of Cav-1 in HIV-1 Tat-induced dysfunction of tight junctions and AJ-transferring proteins. Oxidative Med Cell Longev 2019:3403206. https://doi.org/10.1155/2019/3403206
36. Chen Y, Huang W, Jiang W, Wu X, Ye B, Zhou X (2016) HIV-1 Tat regulates occludin and αβ transfer receptor expression in brain endothelial cells via Rho/ROCK signaling pathway. Oxidative Med Cell Longev 2016:4196572. https://doi.org/10.1155/2016/4196572
37. Huang W, Mo X, Wu X, Luo W, Chen Y (2015) Rosiglitazone suppresses HIV-1 Tat-induced vascular inflammation via Akt signaling. Mol Cell Biochem 407(1–2):173–179. https://doi.org/10.1007/s11010-015-2467-2
38. Banks WA, Robinson SM, Nath A (2005) Permeability of the blood-brain barrier to HIV-1 Tat. Exp Neurol 193(1):218–227. https://doi.org/10.1016/j.expneurol.2004.11.019
39. Gibson CL, Srivastava K, Sprigg N, Bath PM, Bayraktutan U (2014) Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem 129(5):816–826. https://doi.org/10.1111/jnc.12681
40. Zhong Y, Hennig B, Toborek M (2010) Intact lipid rafts regulate HIV-1 Tat protein-induced activation of the Rho signaling and upregulation of P-glycoprotein in brain endothelial cells. J Cereb Blood Flow Metab 30(3):522–533. https://doi.org/10.1038/jcbfm.2009.214
41. Pulliam L (2009) HIV regulation of amyloid beta production. J Neuroimmune Pharmacol 4(2):213–217. https://doi.org/10.1007/s11481-009-9151-9
42. Herskowitz JH, Feng Y, Mattheyes AL et al (2013) Pharmacologic inhibition of ROCK2 suppresses amyloid-β production in an Alzheimer’s disease mouse model. J Neurosci 33(49):19086–19098. https://doi.org/10.1523/jneurosci.2508-13.2013
43. Herskowitz JH, Seyfried NT, Gearing M et al (2011) Rho kinase II phosphorylation of the lipoprotein receptor LR11/SORLA alters amyloid-beta production. J Biol Chem 286(8):6117–6127. https://doi.org/10.1074/jbc.M110.167239
44. Hu YB, Zou Y, Huang Y et al (2016) ROCK1 is associated with Alzheimer’s disease-specific plaques, as well as enhances autophagosome formation but not Autophagic AJ clearance. Front Cell Neurosci 10:253. https://doi.org/10.3389/fncel.2016.00253
45. Zhou Y, Su Y, Li B et al (2003) Nonsteroidal anti-inflammatory drugs can lower amyloidogenic Abeta42 by inhibiting Rho. Science (New York, NY) 302(5648):1215–1217. https://doi.org/10.1126/science.1090154
46. Akiyama H, Kondo H, Ikeda K, Kato M, McGeer PL (2001) Immunohistochemical localization of neprilysin in the human cerebral cortex: inverse association with vulnerability to amyloid beta-protein (Abeta) deposition. Brain Res 902(2):277–281. https://doi.org/10.1016/s0006-8993(01)02390-3
47. Lan X, Xu J, Kiyota T, Peng H, Zheng JC, Ikezu T (2011) HIV-1 reduces Abeta-degrading enzymatic activities in primary human mononuclear phagocytes. J Immunol (Baltimore, MD) 186(12):6925–6932. https://doi.org/10.4049/jimmunol.1100211
48. Pardossi-Piquard R, Petit A, Kawarai T et al (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP. Neuron 46(4):541–554. https://doi.org/10.1016/j.neuron.2005.04.008
49. Ujiie M, Dickstein DL, Carlow DA, Jefferies WA (2003) Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model. Microcirculation (New York, NY) 10(6):463–470. https://doi.org/10.1080/107385803901780212

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.