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Chronic Central Nervous System Expression of HIV-1 Tat Leads to Accelerated Rarefaction of Neocortical Capillaries and Loss of Red Blood Cell Velocity Heterogeneity

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Abstract

Objectives—HIV-1 infection of the central nervous system is associated with impairment of cerebral blood flow and neurocognitive function, and accelerated signs of aging. Since normal aging is associated with rarefaction of the cerebral vasculature, we set out to examine chronic viral effects on the cerebral vasculature.

Methods—Doxycycline-inducible HIV-1 Tat transgenic (Tat-tg) and wild-type (WT) control mice were used. Animals were treated with doxycycline for 3 weeks or 5–7 months. Cerebral vessel density and capillary segment length were determined from quantitative image analyses of sectioned cortical tissue. In addition, movement of red blood cells in individual capillaries was imaged *in vivo* using multiphoton microscopy, to determine red blood cell velocity and flux.

Results—Mean RBCV was not different between Tat-tg mice and age matched WT controls. However, cortical capillaries from Tat-tg mice showed a significant loss of RBCV heterogeneity and increased RBCF that was attributed to a marked decrease in total cortical capillary length (35–40%) compared to WT mice.

Conclusions—Cerebrovascular rarefaction is accelerated in HIV-1 Tat transgenic mice, and this is associated with alterations in red cell blood velocity. These changes may have relevance to the pathogenesis of HIV-associated neurocognitive disorders in an aging HIV positive population.

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Authors' contributions

JNS carried out *in vivo* imaging including examination of cortical capillaries and pyramidal cells, and red blood cell velocity acquisition using the two photon imaging line scan technique. He also conceived the study and drafted the manuscript. OP assisted with *in vivo* imaging, and conducted the cytokine analysis; she also contributed to study design. HW and IR provided technical assistance on line scan acquisition and modified existing MATLAB code to process collected line scan data. JC carried out genotyping and breeding of Tat-tg mice and also assisted with conducting the cytokine analysis. CN assisted with thin skull surgeries and collection of physiologic data. CF performed the statistical analysis. SD helped to conceive the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Keywords

HIV-1; Tat-transgenic mice; cerebrovascular; red blood cell velocity; capillary rarefaction

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Introduction

Human Immunodeficiency Virus type 1 (HIV-1) infection of the central nervous system (CNS) can result in HIV-associated neurocognitive disorders (HAND) [6], which are comprised of motor, behavioral and cognitive disorders that are estimated to affect over 50% of persons living with HIV-1, despite use of combined antiretroviral therapy [29]. While the mechanism(s) leading to neurocognitive impairment are incompletely understood, it has been correlated with increased monocyte activation within the CNS, suggesting that neuroinflammation may contribute significantly to HAND [34,44,51]. Neuroinflammation has also been linked to neuronal injury in animal models for HIV infection [15,70] - but additional mechanisms likely contribute to the pathogenesis of HAND, including cerebrovascular dysfunction [3–4,26,59].

Gelman and colleagues recently examined patterns of gene expression in brain tissues from persons with HIV-induced neurocognitive impairment (NCI), and found that subjects with NCI but without severe virally-induced encephalitis had elevated levels of transcripts expressed by vascular and perivascular cells [26]. This suggests the possibility that neurovascular stress may contribute to the pathogenesis of HAND [26], especially among persons with the most common forms of this disease [29]. Consistent with this, other research describes pathological changes to resting cerebral blood flow (CBF) and functional hyperemia [3–4] in infected persons, prior to experiencing neurocognitive decline. Additionally, there has been a reported increase in both hemorrhagic and ischemic stroke in HIV infected people [19], as well as frequent vascular complications that may contribute to HAND [9,17,48,62]. We recently showed that acute, intracranial exposure of mice to HIV-1 Tat resulted in decreased cerebrovascular reactivity (CVR) in response to hypercapnic challenge [59]. Importantly, attenuation of CVR has been associated with increased risk of ischemic stroke, elevated overall cardiovascular risk and cognitive impairment [11,16,45,66].

HIV-1 does not productively infect endothelial and smooth muscle cells, suggesting that the cerebrovascular changes observed in HAND are the result of soluble viral proteins and the inflammatory milieu elicited by viral infection. Viral proteins (including Tat, gp120) that are released from infected cells may be taken up by non-infected vascular cells, inducing pathophysiological changes. This is thought to occur through effects on host cell signaling or transcription, as well as through receptor engagement, leading to an inflammatory phenotype [5,10]. While many other proteins such as gp 120 also induce inflammation, Tat has been most implicated with regard to the changes to vascular homeostatic dysregulation, including angiogenesis and blood brain barrier disruption [30,63,71].

The cerebral microvasculature is encircled by perivascular macrophage and astrocytic endfeet; both have the ability to directly assault vessels with injurious reactive oxygen species (ROS) [42,69], proinflammatory cytokines [18,40,50,53,72] and multiple viral

products [40,42,69,72] in the setting of HIV infection. This is particularly important with respect to CNS restriction observed in the Tat-tg model. Tat production is limited to the CNS and as such, more closely recapitulates the environment blood vessels would be exposed to in actual HIV infection.

New research suggests that premature aging of the vasculature [28] and CNS [17], in HIV-infected individuals, may play a role in the progression of HAND. Microvascular rarefaction within the cerebral cortex is often attributed to pathologies such as neurodegeneration [13,65], hypertension [7,12,49] and normal aging [13,54,60]. With this in mind, we sought to determine the long-term effect of Tat expression on the density of cortical microvasculature and on capillary hemodynamic parameters which may help to explain the witnessed alterations to CBF in humans infected with HIV. However, the quantification of either of these values at the microvascular level in the human cerebral parenchyma is not currently possible. To determine these parameters, we instead used a well-defined mouse model in which viral Tat protein is expressed exclusively within the CNS (doxycycline-inducible, HIV-1 Tat transgenic (Tat-tg) mice [14]). We exposed the animals to either acute (3 weeks) or chronic (5–7 months) regimen of Tat induction (i.e., 5–7 months of doxycycline, DOX), and then assessed capillary structure (segmentation and volume) throughout the neocortex, as well as red blood cell velocity (RBCV), flux (RBCF) and hematocrit (CH) within capillaries of cortical levels I and II. We identified a significant loss of overall capillary length and branching, and an increase in mean capillary segment length throughout all levels of the cortex in mice that were chronically exposed to Tat, compared to age matched WT mice. We also observed a significant change in RBCV in Tat-tg mice compared to WT mice, which was characterized by an overall reduction in the heterogeneity of RBC velocities –with a selective loss of slow-moving RBCs (< 1 mm/s), and an overall increase in median RBCV (0.920 mm/s in Tat-tg animals, versus 0.718 mm/s in WT animals). Taken together, these findings suggest multiple alterations to vascular density and hemodynamic parameters in the context of sustained Tat induction.

Materials and Methods

Chronic and Acute Model of HIV-1 Induced Neuroinflammation

Tat-transgenic (Tat-tg) mice were a generous gift from Drs. Pamela Knapp and Kurt Hauser (Virginia Commonwealth University) [14]. These double transgenic mice express a glial fibrillary-associated protein driven promoter-reverse tet transactivator (GFAP-RTTA) and tetracycline responsive element (TRE)-Tat that effectively create a nervous system restricted, inducible transgenic animal limited to expressing Tat within GFAP producing astrocytes found in the parenchyma of the brain after ingestion of a tetracycline derivative. At 8 weeks old, male mice were fed pellets infused with 6 mg/kg doxycycline (DOX) (Harlan Laboratories, South Easton, MA, USA), *ad libitum*, for 3 weeks (acute regimen) or for 5–7 months (chronic regimen) prior to use in experiments. Non-transgenic animals (WT) receiving DOX-infused and non-infused pellets were used as controls for Tat-tg exposed to DOX. Age-matched WT animal not exposed to DOX were used as a control for the effect of DOX treatment. Animals were housed with a 12-hour light and 12-hour dark photoperiod.

Measuring Tat expression in brain tissue

Cortical tissue was collected at sacrifice from Tat-tg mice that had been exposed to DOX for either 3 or 20 weeks. Specifically, tissue was captured from a 2 mm coronal section adjacent to the section used for imaging. RNA was isolated using NucleoSpin RNA MIDI kit (Macherey Nagel), according to the manufacturer's protocol. cDNA was synthesized from 700 ng RNA using Superscript III (Invitrogen), according to manufacturer's protocol, then qRT-PCR was run using 2 μ l of cDNA and a custom Taqman assay designed to prime/probe the TAT sequence (Life Technologies, Assay ID: AICSWL2) as well as the Taqman assay for mouse GAPDH (Life Technologies, Assay ID: Mm99999915_g1) according to the manufacturer's instructions. Results are expressed as $-Ct$ (calculated by subtracting Ct values for GAPDH from Ct values for Tat). The expression level of Tat was statistically indistinguishable between mice treated with DOX for 3 and 20 weeks.

Examination of Cortical Capillaries and Pyramidal Cells

Mice were injected with Texas Red-dextran as described in the next section. Dye was allowed to circulate for 5 minutes, and anesthetized animals were then sacrificed by cervical dislocation and decapitated. Intact brains were removed, washed with cold artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Starr Life Science Corporation, Holliston, MA, USA) and placed into a brain slicing matrix (Zivic Instruments, Pittsburgh, PA, USA). Tissue was cut into 2 mm coronal slices and placed onto glass slides with a shallow depression containing aCSF. A glass cover slip was placed over the sectioned tissue and a drop of aCSF was added to the cover slip to provide a liquid interface for the water immersion microscope objective. Images were taken from Bregma level -1.0 , interaural 3.10 , approximately 1.5 mm from midline. A $25\times$, $100\text{-}\mu\text{m}$ Z-stack was imaged through the cortex which was then skeletonized and analyzed in three dimensions using AMIRA software (Visage Imaging, San Diego, CA, USA). To assess pyramidal cells per field, a $10\text{-}\mu\text{m}$ stack from a depth of 45 to $55\text{-}\mu\text{m}$ from each of the original $100\text{-}\mu\text{m}$ stacks were analyzed. ImageJ pointpicker analysis tool [52] allowed quantification of pyramidal cells within each of the sections.

Red Blood Cell Velocity Acquisition Using Two Photon Imaging Line Scan Technique

Mice were anesthetized with isoflurane (4L/min induction; 2L/min maintenance) for surgical removal of scalp and overlying membranes. Urethane (1.2 g/kg) via IP injection was then administered in 2 staggered (5 minutes apart) injections on alternating sides. Urethane anesthetized mice were securely placed into a stereotactic frame and a thin skull window was created, as described [46]. Isoflurane was then decreased gradually to $\sim 0.25\text{ L/min}$ for multiphoton imaging.

Urethane anesthetized mice were placed on a water-perfused heating pad (37°C) throughout the duration of the experiment to prevent anesthesia-related hypothermia. Initial baseline temperature measured rectally in mice was $\sim 35^\circ\text{C}$. Before the recording of physiological parameters, arterial blood gas status (see below) was measured to ensure that PaCO_2 , an important regulator of CBF, was in the normal physiological range ($35\text{--}45\text{ mm Hg}$). Arterial blood pressure was monitored via a femoral artery catheter. Blood gas status was measured

with a blood gas analyzer (Rapidlab 248, Bayer) in 40 μ l capillary samples of blood from the femoral artery. Blood samples were obtained prior to recording RBCV data.

To visualize blood vessels, 70 kDa dextran labeled with Texas Red (Invitrogen, Grand Island, NY) was injected intravenously (10 mg/kg, in 0.1 ml of saline) into the right femoral vein. Arterioles were identified by the intrinsic auto-fluorescence of smooth muscle (as visualized using a 480/20 bandpass emission filter), branching patterns and blood flow direction. Arterioles were then followed into capillary beds. Imaging was performed on a Spectra PhysicsMaiTai HP DeepSee/Olympus Fluoview FV1000 multiphoton imaging setup with a 25 $\times$ NA 1.05 water immersion microscope objective, and recorded by FluoView1000 software (Olympus America, Center Valley, PA). The excitation wavelength was 880 nm, with the laser power at the sample set below 10 mW. The Texas Red fluorescence was detected using a 607/36 bandpass emission filter. FV 1000 line scan acquisition was achieved in XT scan mode. Scan speed was set at 10 μ s/pixel and capillaries were scanned along the axis of the vessels; red blood cell velocities were calculated from their distance traveled over time (dx/dt, mm/s) using a particle velocity radon transform algorithm [20] with a window size of 28 and overlap of 0.25. Capillary diameters were extracted from two-photon images of line scanned vessels using ImageJ software [52].

Cytokine Analysis

Brain tissue was collected from WT mice (n=4) and Tat-tg mice (n=5) after acute (3 weeks) or chronic (20 weeks) exposure to DOX. Brain was quickly removed, placed into Zivic sectioning tool, and coronal section at Bregma 0 to 2 was taken. From this section, cortex was dissected and immediately frozen on dry ice. Lysates were prepared from cortex tissue by homogenizing in RIPA buffer containing protease inhibitors, with rotor-stator homogenizer. The lysates were centrifuged for 10 min at 30,000g at 4°C, and supernatants were used to measure IL-1 β and VEGF. Protein levels of IL-1 β and VEGF were measured by MILLIPLEX (MAP assay, following the manufacturer's recommendations (EMD Milliplex Corp., Billerica, MA).

Statistical Analysis

All data are presented as mean $\pm$ SEM. The relationship between RBCV and RBCF was determined using Pearson Correlation. Acute and chronic group means were compared using one-way ANOVA with Tukey's multiple comparison adjustment. Statistical significance between means, for chronic data groups alone, was determined by unpaired, two-sample t-test. For all tests, the level of significance was set at $P < 0.05$.

Results

Chronic Exposure to Tat Caused a Reduction in Overall Capillary Volume, as well as Change in Vessel Length and Branching, Compared to Age Matched Controls

We used multiphoton microscopy in Tat-tg and WT animals exposed to acute or chronic regimen of DOX to determine whether exposure to Tat would affect microvessel morphology and density. Initial results revealed a striking qualitative change in vascular

density after chronic exposure to Tat (Figure 1), and that this change was not a result of variations in Tat production over time (Figure 2A).

To quantitate these data, three-dimensional ($500\mu\text{m} \times 500\mu\text{m} \times 100\mu\text{m}$) image stacks acquired from coronal sections were analyzed. For each image stack (Figure 1) a three-dimensional vascular skeleton was created by manually tracing vessels in three-dimensional space. From this we extracted vessel length, volume and branching parameters (numbers of nodes and segments). Vessel branch was defined as segments between two nodes where a node is either an endpoint of a vessel or point at which multiple segments arise (branching points).

There was a significant decrease in the number of nodes and segments between WT and Tat-tg animals chronically exposed to DOX (Figure 2B; $P = 0.0005$ and $P = 0.0011$, respectively; one-way ANOVA). However, DOX treatment alone had no effect on the numbers of nodes and segments, as revealed by a comparison of data from WT mice that were either chronically treated with DOX or unexposed to DOX throughout the experiment (Fig. 2B). In addition, no difference was observed between WT and Tat-tg animals that were acutely treated with DOX.

Mean segment length (Figure 2C) was also found to be significantly increased between Tat-tg mice and WT mice that were chronically exposed to DOX (Tat, $98.6\mu\text{m} \pm 2.06$ $N=4$, 488 segments total; WT, $76.2\mu\text{m} \pm 2.71$ $N=3$, 758 segments total; $P = 0.038$; two-sample t-test), but not in acutely treated animals. DOX treatment alone had no effect on mean segment length, however (see WT groups $\pm$ DOX in Fig. 2C).

To determine whether vessel density within the cortex was altered in Tat-tg animals, total capillary length (Figure 2D) was extracted from skeletonized sections. Total capillary length was significantly reduced in Tat-tg mice, as compared to WT mice (Tat, $11600\mu\text{m} \pm 683$, $N=4$; WT, $19600\mu\text{m} \pm 2770$, $N=3$; $P = 0.02$, two-sample t-test). This analysis suggests a loss of 35–40 % of total capillary length in the cortical sections from Tat-tg animals. Importantly, DOX treatment alone had no effect on total capillary length (see WT groups $\pm$ DOX in Fig. 2D).

In vivo examination of perfused capillary status revealed an increase in resting diameter in Tat-tg mice as compared to wild-type mice (Tat, 4.54 ± 0.11 , $N=47$; WT, 3.67 ± 0.09 , $n=53$; $P=0.0001$; two-sample t-test; Figure 2E). DOX treatment alone had a moderate effect on this parameter (WT –DOX, 4.00 ± 0.09 , $N=66$; WT, 3.67 ± 0.09 , $n=53$; $P=0.005$; two-sample t-test; Figure 2E). Finally, Figure 2F presents an estimation of total perfused vascular volume (vascular density) within a $500\mu\text{m} \times 500\mu\text{m} \times 100\mu\text{m}$ tissue block from the neocortex. Total volume was calculated from values extracted from *in vivo* resting vessel diameter and total capillary length (Tat, $182000\mu\text{m}^3 \pm 10700$ $N=4$; WT, $208000\mu\text{m}^3 \pm 13300$ $N=3$; $P=0.2$; two-sample t-test). Thus, perfused cortical vessel density was not significantly altered in Tat-tg animals exposed to Tat for 5–7 months compared to age matched control mice.

Chronic Tat Exposure is Not Associated with Loss of Pyramidal Cells

Two photon images used in assessing vascular cortical density were also used to quantify pyramidal cells within a 10 μm stack. The analyzed location was selected midway between the 100 μm stack of the imaged coronal sections, approximately from a 45 to 55 μm depth within the cortical stack. Pyramidal cells quantification data are presented in Figure 3. DOX treatment alone had no effect on pyramidal cell numbers, as revealed by a comparison of data from WT mice that were either untreated with DOX or chronically treated with DOX (Figure 3). In addition, no difference in pyramidal cell numbers was observed between Tat-tg and WT animals chronically treated with DOX (Tat, 147.0 ± 21.7 N=5; WT, 163.0 ± 16.3 N=3; $P=0.63$; two-sample t-test).

Tat-tg Mice Exhibit Altered Capillary Hemodynamics

We examined RBCV, red blood cell flux (RBCF) and capillary hematocrit (CH) to assess hemodynamic regulation within the superficial cortical capillary bed. Mean arterial pressure (MAP) and arterial blood gas (ABG) parameters were not different between groups (Table 1). RBCV data for Tat and WT mice during steady state flow are presented in Figure 4. Chronic doxycycline exposure had no effect on RBCV (see data for WT mice +/- DOX in Fig. 4). Figure 4A also shows that Tat-tg mice did not have significantly different mean RBC velocities compared to WT mice (Tat, $0.93 \text{ mm/s} \pm 0.05$, N=53 in 3 animals; WT, $0.81 \text{ mm/s} \pm 0.07$, N=52 in 3 animals; $P=0.2$, two-sample t-test). However, closer inspection of the data revealed some important differences between Tat-tg and WT mice.

Figures 4B and 4C show a breakdown of velocities into “slower” ($<1.0 \text{ mm/s}$) and “faster” ($>1.0 \text{ mm/s}$) capillary groupings, respectively. Figure 4B shows that very slow-moving capillaries are much less frequent in Tat-tg versus WT mice, and that there is a significant increase in mean RBC velocity within the “slower” capillary grouping as a result (Tat, $0.72 \text{ mm/s} \pm 0.22$, N=32 in 3 animals; WT, $0.47 \text{ mm/s} \pm 0.05$, N=32 in 3 animals, respectively; $P=0.0002$, two-sample t-test; Figure 4B). Analysis of the “faster” capillary grouping revealed no significant difference between Tat-tg and WT mice (Tat, $1.26 \text{ mm/s} \pm 0.06$, N=21 in 3 animals; WT, $1.4 \text{ mm/s} \pm 0.12$, N=21 in 3 animals, respectively; $P=0.16$, two-sample t-test; Figure 4C). Again, chronic doxycycline exposure had no effect on either of these parameters (see data for WT mice +/- DOX in Fig. 4B,C).

RBCF data for Tat-tg and WT mice during steady state flow are presented in Figure 4D. Tat-exposed animals displayed significantly more RBCs per second within cortical capillaries, compared to WT mice (Tat, 86.87 ± 6.08 , N=48 in 3 animals; WT, 67.54 ± 6.151 , N=51 in 3 animals; $P=0.03$, two-sample t-test). Chronic doxycycline exposure had no significant effect on RBCF in WT animals (see data for WT mice +/- DOX in Fig. 4D).

Analysis of the distribution of RBC velocities in Tat-tg and WT mice revealed an increase in the median velocity of RBCs in Tat-tg mice (0.918 mm/s) compared to WT animals (0.751 mm/s) (Figure 4E). There was also a reduction in the heterogeneity of the RBC velocities in Tat-exposed animals, which is reflected by the smaller coefficient of variation (cv) for RBCV in Tat-tg mice (0.38), compared to that seen in WT animals (0.66). Not only was the wide dispersion of velocities observed in normal tissues lost in Tat-tg mice, but so was the

typical skewing toward the lower end of the velocity histogram [21,55–56]. This suggests a loss of the velocity heterogeneity observed in normally functioning capillary beds. The cv in WT (–DOX) (0.64) was similar to WT (+DOX) (0.66) animals reflecting no change in velocity heterogeneity between the groups.

A correlation analysis of RBCV and RBCF is presented in Figure 5. In both WT (Figure 5A, B) and Tat-tg (Figure 5C) mice, there was significant correlation ($P<0.0001$) between RBCV and RBCF. The slope of this relationship dictates the capillary hematocrit (CH), and as such, the shallower slope observed in the Tat-tg mice suggests an increase in CH compared to WT animals.

Tat-tg Mice Exhibit Elevated Levels of IL-1 β and VEGF in Brain Tissue

We collected brain tissue from WT and Tat-tg mice, following both acute (3 weeks) and chronic (20 weeks) exposure to DOX, and prepared brain lysates. These were then analyzed using a MILLIPLEX® MAP assay (EMD Millipore) to quantitate levels of the proinflammatory cytokine, interleukin (IL)-1 β (Figure 6A), as well as vascular endothelial growth factor (VEGF) (Figure 6B). Levels of both VEGF and IL-1 β were significantly elevated in Tat-tg mice, when compared to WT animals.

Discussion

Maintenance of the brain microvasculature is a dynamic process where remodeling often occurs to accommodate deviations in tissue metabolic requirements [8,38], growth factor levels [41,60], and pericyte functionality [22,24,64]. The homeostasis between angiogenesis and rarefaction ensures the optimal level of perfusion for varying parenchymal demands. However, homeostatic disturbance is frequently observed in the context of hypertension [7,12,49], neurodegenerative disease [13,65] and normal aging [13,54,60] resulting in vascular redistribution, density change and hemodynamic alteration.

Typically, changes in vascular morphology or collateral density are not seen in aging mice until 16 to 31 months [23,61]. In agreement with these reports, WT mice (+DOX) did not show decreased capillary density at 7–9 months. In Tat-tg mice exposed to 5–7 months of intracranial Tat induction, there was a significant (~35–40%) decline in overall vessel length (rarefaction) within the neocortex compared to age matched WT controls, without concomitant loss of vascular density (total estimated volume of perfused vessels). This seemingly counterintuitive observation might be best explained by the statistically relevant increase in vessel diameter in Tat-tg animals, as vessel diameter plays a dominant role in estimation of vascular density. Interestingly, there was a significant decrease in vessel diameter in WT animals treated with DOX, relative to untreated (Figure 2E). This suggests that there might be an underestimation of the actual diameter change in Tat exposed animals treated with DOX.

While the underlying mechanism remains to be elucidated, it is likely that Tat-induced brain inflammation contributed to this observation by instigating vascular aging through exposure to chronic inflammation and reactive oxygen species. Vessel rarefaction might also occur as a consequence of decreased tissue metabolic demand (resulting from astrocyte or pyramidal

cell pathology/loss), or via direct dysregulation of microvascular homeostasis/signaling. Although we did not observe any changes in pyramidal cell density between Tat-tg and WT mice (Figure 3), the metabolic status of these cells was not examined - making it difficult to determine pyramidal involvement conclusively. Morphological changes in this cell type were witnessed in the form of increased intracellular inclusions (data not shown) after long term Tat exposure, arguing in favor of metabolic/pathological changes within this cell type compared to WT. It is possible that rarefaction is occurring in the context of normal or increased metabolic demand. In this environment, cells such as neurons and astrocytes experiencing hypoxia would signal for increased capillary density by increased expression of vascular growth factors.

Vascular endothelial growth factor (VEGF) a potent proangiogenic regulator and proinflammatory mediator was significantly upregulated (Figure 6B) in animals chronically exposed to Tat. VEGF, one of the major target genes of hypoxia-inducible factors (HIFs) plays a predominant role in regulation of dynamic vascular remodeling. In an intact pathway, VEGF can function to ameliorate hypoxia. However, if this signaling pathway is dysfunctional, VEGF can actually contribute to ensuing the proinflammatory milieu [47]. Multiple *in vivo* and *in vitro* studies support the deleterious effects of Tat on vascular integrity [30,63,71], and Tat has also been shown to directly interact with and activate the endothelial VEGF receptor (KDR/flk-1), subsequently signaling for angiogenesis [2].

Increased total capillary length in the cortex after 3 week exposure to Tat (Figure 2D) supports the notion that an early, possibly VEGF-driven, angiogenic process may occur in Tat tg mice. However, the balance between angiogenesis and vessel loss is clearly reversed at later time points since chronic (20 week) exposure to Tat was associated with a decrease in total capillary length (Figure 2D).

Levels of IL-1 β were also upregulated in cortical tissue after prolonged exposure to Tat, suggesting that chronic Tat expression is associated with inflammation within the CNS. This finding is consistent with previous studies in which Tat exposure was shown to upregulate IL-1 β expression from microglia and astrocytes [33,36,39]. While inflammation can cause increased adhesion and rolling of immune cells at the post-capillary venules leading to decreased RBCV through some segments of capillaries, this phenomenon is unlikely a significantly contributing factor in the changes we observe in Tat-tg mice due to 1) rarefaction and 2) the resultant increased driving pressure within the vessels. Significant contribution would be represented by an increase in velocity heterogeneity to account for the new slower moving capillaries, rather than the measured decrease.

Figure 2 shows a quantitative loss of total capillary length (Figure 2D) and branching (Figure 2B), with a concomitant increase in mean segment length (Figure 2C) in the capillary bed of chronically induced Tat-tg animals. The resultant loss of vessels to distribute pressure throughout the cerebral vasculature (reduced total capillary length), combined with the increase in the length of parallel resistance vessels (increased mean segment length), and the loss of parallel resistance conduits (reduced branching), can collectively be expected to result in increased vascular resistance within the cerebral parenchyma. If one assumes that total cerebral blood volume through the brain is initially

preserved, as suggested by Figure 2F, pressures driving flow within capillary beds would be expected to increase, resulting in increased RBCV and/or loss of RBCV heterogeneity, RBCF, and CH with possible visible capillary distension [21,55–56]. This is consistent with our experimental findings in Tat-tg mice, which include increased capillary diameter (Figure 2E), loss of RBCV heterogeneity and an increase in median RBCV (Figure 2F), as well as a rise in RBCF and a likely increase in CH compared to WT animals (Figure 2 G,H).

Investigation of literature on rarefactive events in the peripheral microvasculature suggests a prominent role for nitric oxide synthase (NOS) dysregulation - including chronic inhibition of NOS [27,58] or loss of endothelial NOS [25]. Our group recently demonstrated that acute intracranial exposure (3 weeks) to Tat produced a dilatory dysfunction in the pial arterioles in Tat-tg animals, as a consequence of changes in the NO-cGMP signaling axis [59].

In the present study, it is difficult to draw conclusions about dilatory capacity through the examination of changes in relative blood flow rate, because of the loss of vasculature in chronically treated Tat-tg animals compared to WT mice. It is probable that dilatory capacity remains dysfunctional, but has subsequently progressed to capillary rarefaction through further NOS inhibition. Chronic exposure to Tat could result in long-term changes in the NO-cGMP and VEGF pathways, thereby eliciting structural changes in the downstream capillary vasculature.

Increased homogeneity in RBCV has been observed in cerebral capillaries as a response to stimuli such as hypoxia [37], hypercapnia [1,31,67] and functional hyperemia [68] - all states that are expected to produce increased perfusion pressures within the capillary bed. Similarly, the upward shift in velocity observed in slower capillaries has also been reported to occur in physiologic hyperemic states such as hypoxemia [37] and hypercapnia [31] and has been attributed to a functionally distinct group of capillaries [32,43]. Although rarefaction was not reported in any of these studies, the loss of RBCV heterogeneity is likely a direct result of increased flow into parenchyma. Since tissues can only safely allow for limited increases in RBCV in the context of increased perfusion pressure, a capping of the maximum flow rate must exist physiologically before microvessel hemorrhage would be expected to occur. This limitation would likely decrease heterogeneity by causing both the slower and faster moving vessels to move RBCs more quickly, but preferentially affecting the magnitude of these distinct slower moving capillaries to a greater degree in order to prevent capillary rupture.

The RBCV heterogeneity loss we observed due to rarefaction is potentially different than in circumstances where capillary beds contain a normal total length of vessel conduits and are responding to hyperemic change. Rarefaction-induced heterogeneity loss might be better appreciated through a model (Figure 7) that gives plausible interpretation of our data. We speculate that the increased homogeneity in RBCV in Tat-tg mice might be a direct result of the loss of distal microvasculature, through pathological remodeling and subsequent rarefaction of the slowest moving vessels. Additionally, a 35–40% capillary rarefaction will greatly decrease the area available for gas exchange, and may establish a constant state of increased metabolic demand within the parenchyma, analogous to a permanent physiological hyperemic state. Either case would likely result in the significant increase in capillary

diameter to accommodate flow changes observed in Tat-tg animals (Figure 2E), and neither hypothesis is mutually exclusive.

RBCF is defined by the number of cells moving past a given point per unit time where increases in flux are known to enhance O₂ delivery to tissues. Flux and velocity are both factors in the determination of CH, and hyperemic states that increase RBCV generally cause a corresponding increase in CH [35]. Although we did not see a statistically significant increase in mean RBCV between Tat-tg and WT mice, we did observe over a 20% increase in median RBCV (Tat, 0.920 mm/s; WT, 0.718mm/s), due to the selective loss of lower velocity capillaries. This may be sufficient to significantly augment both flux (Figure 4D) and hematocrit (decrease in slope Figure 5C).

Although overall capillary length is decreased in Tat-tg mice, resulting in a decline in the surface area available for gas exchange, the observed changes in hemodynamic parameters suggest a possible compensatory change in the efficiency of O₂ extraction in Tat-tg mice. This is because perfusion is determined in part by RBCF, while diffusion is determined by lineal density, or cells/mm within the tissue (proportional to CH). Increases in RBCF and CH would augment both the perfusive and diffusive O₂ conductances, acting to enhance blood-parenchymal O₂ extraction.

Overall, our findings show that the cortical microvasculature undergoes significant rarefaction, as well as profound hemodynamic changes, in a mouse model for chronic HIV-associated neuroinflammation. It will be important to determine how these changes may affect neuronal function, and whether cerebral microvascular rarefaction continues to progress over time in response to HIV-1 induced neuroinflammation. This may have important implications for persons living with HIV/AIDS, as they age.

Conclusions

Examination of aged Tat-tg mice chronically exposed to Tat showed marked reduction in capillary density with concomitant alterations to capillary blood flow and oxygen extraction parameters (flux and hematocrit) in the somatosensory region of the cortex. RBCV heterogeneity was decreased owing to loss of a subset of “slower moving” capillaries (<1.0mm/s) normally found in tissues [21,55–57], while RBCF and CH were both increased. Changes to flow were attributed to a loss of both vascular branching and overall capillary length within this area.

Perspectives

We have demonstrated that expression of HIV-1 Tat within the CNS causes rarefaction of microvascular networks in the cortex which is associated with concomitant hemodynamic changes. Next, it should be determined whether microvascular rarefaction continues to progress over time in response to HIV-1 induced neuroinflammation, and how these changes affect neuronal function. Future research should look to determine whether similar changes occur in persons living with HIV/AIDS.

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List of abbreviations

ABG	Arterial blood gas
CBF	Cerebral blood flow
CH	capillary hematocrit
CNS	Central nervous system
CVR	Cerebrovascular reactivity
DOX	doxycycline
HAND	HIV-associated neurocognitive disorders
HIV-1	Human immunodeficiency virus, type-1
MAP	Mean arterial pressure
RBCF	Red blood cell flux
RBCV	Red blood cell velocity
SEM	Standard error of the mean
Tat-tg	Transactivator of transcription-transgenic
WT	Wild type

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Figure 1. Chronic exposure to Tat causes a qualitative reduction in cortical capillary density
A, B. Representative images of skeletonized, 3-dimensional ($500\mu\text{m} \times 500\mu\text{m} \times 100\mu\text{m}$) collapsed stacks of cortical capillaries (10x magnification) from WT (panel A) and (panel B) Tat-tg mice, chronically exposed to DOX. Each image in a panel is representative of an individual animal where capillaries were localized to $\sim$ bregma level -1.0 . Scale bars are 50 micrometers.

Figure 2. Chronic exposure to Tat causes a quantitatively significant decrease in cortical capillary density

Data are presented for cortical tissue from WT and Tat-tg mice treated either acutely, for 3 weeks, (WT A, Tat A) or chronically, for 5–7 months (WT C, Tat C) with DOX, or WT mice not treated with DOX [WT C (–DOX)]. **A**. Tat expression in brain of Tat-tg mice after 3 and 20 weeks of DOX treatment. $N = 5$ for each group. Results are expressed as Δ Ct (calculated by subtracting Ct values for GAPDH from Ct values for Tat). **B**. The number of capillary nodes and segments per field of view. **C**. Mean length of individual capillary segments. **D**. Mean aggregate length of all capillaries, per field of view. **E**. Mean capillary diameter. **F**. Mean total volume of all capillaries in one 3-dimensional image stack. **B–F**. Data for mice exposed chronically to DOX (or control age-matched WT mice) were

extracted from a single cohort of animals (4 WT, 4 WT(–DOX) and 3 Tat-tg). **B–D.** Data for mice exposed acutely to DOX were extracted from a single cohort of animals (7 WT and 7 Tat-tg). Statistical significance is denoted as * ($P < 0.05$), ** ($P < 0.001$) *** ($P < 0.0001$) or NS (no significant difference), as calculated by a two-tailed t-test. All data represent mean $\pm$ SEM.

Figure 3. Chronic exposure to Tat does not lead to a reduction in pyramidal cell density
Quantification of pyramidal cells from 10 μ m stacks (from sections used in Fig 2). Numbers of pyramidal cells per field were not significantly different in WT, and Tat-tg mice that were treated chronically with DOX ($p > 0.05$, t-test), or age-matched WT not treated with DOX [WT C(-DOX)]. Data represent mean $\pm$ SEM.

Figure 4. Chronic exposure to Tat is associated with altered capillary hemodynamics, and a selective loss of slow-moving vessels

Data are presented for WT and Tat-tg mice treated for 7 months with DOX (WT C, Tat C) with DOX, or age-matched WT mice that were not treated with DOX [WT C (-DOX)]. **A.** Mean RBC velocities, as measured using the capillary line scan method. Each dot represents an individual capillary. There was no significant statistical difference in mean RBC velocities between the three groups of mice. **B.** The distribution of slower velocity RBCs (<1 mm/s) was different between Tat-tg and WT mice, with the selective loss of slower moving cells in Tat-tg mice, and a significant shift toward higher speeds compared to WT animals. **C.** The distribution of faster velocity RBCs (>1 mm/s) was unchanged between Tat-tg and WT mice. **D.** RBC flux (cells/s) in vessels of Tat-tg animals was increased compared to WT (+/-DOX). **E.** Histogram depicting the distribution of RBC velocities (relative frequency of RBCs traveling at a given velocity). There is a marked shift in the median velocity of RBCs in Tat-tg mice (0.920 mm/s) compared to WT animals (0.718 mm/s). The calculated coefficient of variation (cv) revealed a decrease in the breadth of distribution of data points in Tat-tg (cv=0.38) animals compared to WT animals (cv=0.66). Statistical significance is denoted as * (P<0.05), ** (P<0.001) *** (P<0.0001) or NS (no statistically significant difference), two-sample t-test. Data represent mean ± SEM.

Figure 5. Correlation between red blood cells flow and velocity

A. WT mice not exposed to DOX; **B.** WT mice exposed to DOX; **C.** Tat-tg mice exposed to DOX. A Spearman (non-parametric) correlation analysis revealed a statistically significant correlation in all cases ($P<0.0001$), where slope is representative of CH. All results were generated from a single cohort of mice (3 WT(-DOX), 3 WT, 3 Tat-tg).

Figure 6. IL-1 β and VEGF levels are elevated in brains of Tat-tg mice

Brain tissue was collected from WT and Tat-tg after acute (3 weeks) or chronic (20 weeks) exposure to DOX. Protein levels of IL-1 β (**A**) and VEGF (**B**) in brain lysates were then measured by MILLIPLEX® MAP assay. Results were generated from 4 WT and 5 Tat-tg per group. Statistical significance was determined by two-sample t-test, and is denoted as * ($P < 0.01$ in both cases, when compared to the matched time point for WT animals). Data represent mean $\pm$ SEM.

Figure 7. Proposed mechanism of distal rarefaction in cortical capillaries exposed to Tat

The normal vascular tree (**A**) loses distal capillary branching (**B**) because of ineffective remodeling in the context of pathology or aging. The loss of higher order branch points, such as tertiary branches, would likely increase vascular resistance within the bed. This occurs since each consecutive bifurcation produces drops in perfusion pressure which subsequently leads to concomitant drops in RBCV, in accordance to Ohm's Law ($V=IR$). As total blood volume supplied by arterioles might not initially change due to the gradual nature of the rarefaction, capillary distention may occur from increased luminal pressures as seen in **B**. This increase in driving pressure (heavy black arrow in **B**) would typically cause red blood cells to travel at a higher net velocity. However, in this current scenario, increased perfusion pressure is now tempered by the increased vascular resistance resulting from the lengthening of capillary segments (loss of branch points effectively lengthens segmentation between nodes). This lengthening inadvertently maintains net capillary velocities similar to those witnessed in WT animals. The most striking feature of this pathological remodeling would be the loss of intrinsically slower moving distal vessels which were created from pressure drops across consecutive bifurcations. This phenomenon is reflected as a loss of velocity heterogeneity, where distal vessels with intrinsically slower velocities either become mildly faster to accommodate increased driving pressure or are lost entirely through pathological remodeling.

Table 1

Physiological Parameters in Mice Before Line Scan Data Collection.

	MAP, mm Hg	Arterial blood pH	PaCO ₂ , mm Hg	PaO ₂ , mm Hg
WT (N=3)	74.8 ±1.49	7.37 ±0.01	35.1 ±1.25	104 ±8.12
Tat-tg (N=3)	78.6 ±1.93	7.31 ±0.03	41.7 ±4.71	112 ±12.4
WT (N=4)	79.99±1.74	7.33±0.03	39.15±3.48	110.35±9.38

MAP, mean arterial pressure; PaCO₂, partial arterial pressure of CO₂; PaO₂, partial arterial pressure of O₂; Data shown as mean ± SEM