IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Nitrosative Stress Is Associated with Dopaminergic Dysfunction in the HIV-1 Transgenic Rat

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Advances in antiretroviral therapy have resulted in significantly decreased HIV-related mortality. HIV-associated neurocognitive disorders, however, continue to be a major problem in infected patients. The neuropathology underlying HIV-associated neurocognitive disorders has not been well characterized, and evidence suggests different contributing mechanisms. One potential mechanism is the induction of oxidative stress. Using the HIV-1 transgenic (Tg) rat model of HIV, we found increased striatal NADPH oxidase-4 and neuronal nitric oxide synthase expression in the adult (7- to 9-month-old) Tg rat compared with control rats but not in the young (1-month-old) Tg rats. This was accompanied by increased 3-nitrotyrosine (3-NT) immunostaining in the adult Tg rats, which worsened significantly in the old Tg rats (18 to 20 months old). There was, however, no concurrent induction of the antioxidant systems because there was no change in the expression of the nuclear factor-erythroid 2-related factor 2 and its downstream targets (thioredoxin and glutathione antioxidant systems). Colocalization of 3-NT staining with neurofilament proteins and evidence of decreased tyrosine hydroxylase and dopamine transporter expression in the old rats support dopaminergic involvement. We conclude that the HIV-1 Tg rat brain shows evidence of nitrosative stress without appropriate oxidation-reduction adaptation, whereas 3-NT modification of striatal neurofilament proteins likely points to the ensuing dopaminergic neuronal loss and dysfunction in the aging HIV-1 Tg rat. (*Am J Pathol* 2019, 189: 1375–1385; <https://doi.org/10.1016/j.ajpath.2019.03.004>)

Although the introduction of highly active antiretroviral therapy has significantly decreased the incidence of HIV-associated dementia complex, an incapacitating consequence of untreated HIV infection,^{1–3} less severe forms of HIV-associated neurologic disorders have become more common and currently affect approximately 30% to 50% of the HIV-positive patients.^{3,4} The suspected mechanisms underlying HIV-associated neurologic disorders include persistent neuroinflammation,⁵ neurotoxic effects of HIV-1 viral proteins,^{6–8} disruption of the neurotrophic function of astrocytes and oligodendrocytes,^{9,10} as well as the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the underproduction of antioxidants.^{11–17} Related to the latter mechanism, reports indicate that HIV infection

hastens the normal aging-related neurodegenerative process by accelerating neuronal excitotoxicity, atypical Ca⁺ signaling, and dysregulated production of ROS/RNS that contribute to oxidative/nitrosative stress (OS/NS).^{18–20} This imbalance in the oxidation-reduction (redox) state can be cytotoxic to neurons through the inhibition of cellular respiration, dysregulation of mitochondrial electron transport, and

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posttranslational modification of neuronal structural proteins.^{21–24}

Normally, a basal level of ROS/RNS is required to maintain cellular functions.²⁵ An overproduction of these free radicals, however, can cause damage to proteins, lipids, and DNA.²⁶ Superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite^{27,28} are major oxidants implicated in various neurodegenerative disorders. Major sources of ROS that contribute to the pathology of acute brain injury and chronic neurodegenerative disorders are various NADPH oxidases (NOXs).^{29,30} NOX proteins include seven members (NOX1–NOX5 and dual oxidase 1 and 2) that are membrane-bound enzyme complexes playing a critical role in oxidative bursts.^{27,28} NOX4 is unique among the NOX isoforms as it constitutively produces measurable amounts of hydrogen peroxide.³⁰ NOX4 is also ubiquitously expressed in a variety of tissues and has been shown to mediate signal transduction pathways by modulating intracellular Ca⁺ levels.³¹ Depletion of *Nox4* expression was found to reduce oxidative damage to nuclear DNA and delay senescence in endothelial cells.^{32,33}

A major source of RNS is peroxynitrite, produced by the reaction of nitric oxide and superoxide. Peroxynitrite is a destructive oxidant and nitrating agent, capable of oxidizing lipoproteins and causing the nitration of selective tyrosine residues in proteins, thereby altering protein structure, conformation, and function.^{34,35} 3-Nitrotyrosine (3-NT) formation above basal levels indicates nitrosative stress, which correlates with disease progression and severity. On the basis of buffering conditions in the biological milieu, peroxynitrite decomposes and forms highly active free radicals, such as hydroxyl radicals, carbon trioxide, and nitrogen dioxide. These highly reactive products are more efficient nitrating species than peroxynitrite itself.²¹ In fact, nitrogen dioxide is a major contributor to the formation of 3-NT modified proteins.³⁶ Nitric oxide is synthesized by the nitric oxide synthase (NOS) enzyme that exists as three isoforms: neuronal NOS (nNOS), inducible NOS, and endothelial NOS. Neuronal NOS is a major source of NOS in the brain and is regulated transcriptionally and translationally.^{21,37,38}

Under normal conditions, ROS/RNS and subsequent OS/NS are kept under control through the antioxidant system.³⁹ However, under disease conditions, cells are prone to OS/NS damage, likely as a result of inadequate redox adaptation.^{40,41} Nuclear factor–erythroid 2–related factor 2 (Nrf2) is a transcription factor that is induced by OS/NS and binds to the antioxidant response elements of phase 2 antioxidant enzymes. Nrf2 positively regulates the expression of antioxidants that protect cells from oxidative stress.^{39,42} Both the glutathione and thioredoxin (TRX) enzyme systems are phase 2 antioxidants and protect cells from damage caused by ROS/RNS. Glutathione biosynthesis is regulated by Nrf2 in both neurons and astrocytes.^{39,43} The glutathione antioxidant system is responsible for scavenging multiple nitrosative species and reducing lipid hydroperoxides.^{39,44} The TRX system is directly involved in reducing several substrates, including lipid hydroperoxides

and hydrogen peroxide.^{45–48} Curiously, not only does Nrf2 positively regulate expression of phase 2 antioxidants but it also is a positive regulator of enzymes regulating NADPH, NADH, FADH₂, mitochondrial membrane potential, and respiration.²² Therefore, a dysregulation in Nrf2 activity likely alters both cellular respiration and redox homeostasis.

The HIV-1 transgenic (Tg) rat is a well-characterized small animal model of HIV that has been proposed as a model for treated HIV-positive patients.^{16,49–52} This noninfectious rat model expresses seven of the nine HIV viral proteins (including gp120 and Tat), has been reported to develop neuropathology and cognitive deficits,^{52–56} and demonstrates regional variations in redox adaptation, mitochondrial dysfunction, and neurotoxicity.^{16,57–60} Our previous research using this animal model, which includes imaging as well as behavioral and histologic assessments, supports dopaminergic dysfunction in the Tg rat.^{54,61–63} The goal of the current study is to investigate a possible link between this dopaminergic dysfunction and OS/NS. Therefore, striatal NOX4 and nNOS expression was measured, and the extent of nitration of tyrosine residues in neurofilament proteins of the Tg rats compared with age-matched controls was evaluated. The dopaminergic system of old Tg and control rats was also evaluated using immunohistochemistry by looking at tyrosine hydroxylase (TH), dopamine transporter (DAT) in the striatum, and NeuN (a neuronal marker) in the substantia nigra. Finally, the induction of Nrf2 and members of the phase 2 glutathione and TRX antioxidant pathways was evaluated to assess the integrity of the expected secondary antioxidant response.

Materials and Methods

Animals

Male HIV-1 Tg (F344/Hsd, Tg) and age-matched wild-type control (F344/Hsd) rats were purchased from Envigo Inc. (Indianapolis, IN). All rats were housed in a temperature-controlled environment with a 12-hour light/dark cycle and free access to food and water. All studies were approved by the Animal Care and Use Committee of the NIH/Clinical Center (Bethesda, MD). Three animal cohorts of different ages were used for various experiments: 1-month-old (young), 7- to 9-month-old (adult), and 18- to 20-month-old (old) rats.

Oxidative Stress RT² Profiler PCR Array Assay

Total cellular RNA from young ($n = 7$ each) and adult ($n = 6$ each) Tg animals and controls was extracted from striatal tissue using an RNeasy Lipid Tissue minikit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Synthesis of cDNA was performed using RT² First Strand Kit and the cDNA was then used on the Rat Oxidative Stress Real-Time RT² Profiler PCR Array (Qiagen; catalog number PARN-065Z) in combination with RT² SYBR Green qPCR Mastermix (Qiagen). Relative changes in expression levels of

specific mRNA were quantified using the CFX96 Real-Time qPCR System (Bio-Rad, Hercules, CA). C_t values were normalized to a selection of housekeeping gene panels, and fold changes were calculated using the $\Delta\Delta C_t$ method. The RT² RNA Quality Control PCR Array was performed on each sample before RT² Profiler PCR Array Assay, according to the manufacturer's instructions (PARN-999Z). Each gene identified with at least a 1.5-fold change in expression by the PCR Array was further validated using individual real-time quantitative PCR (qPCR) assays. The qPCR primer sets used for further gene validation were as follows: *Nox4* (PPR45975A), hemoglobin α adult chain 2 (*Hba1*; PPR56540A), uncoupling protein 3 (*Ucp3*; PPR44548A), and albumin (*Alb*; PPR48689A). Separately, an individual qPCR assay was also performed for *Nrf2* (PPR45094A).

Western Blot Analysis

Aliquots (20 μ g) of striatal lysates from young (control, $n = 3$; Tg, $n = 4$) and adult (control, $n = 3$; Tg, $n = 4$) animals were run on Any KD TGX SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes, as previously described.⁶⁴ The primary antibodies used were NOX4 (Abcam, Cambridge, UK; catalog number ab133303; dilution 1:3000), nNOS (Abcam; catalog number ab95436; 1 μ g/mL), Nrf2 (Abcam; catalog number ab89443; 1 μ g/mL), and actin (CST, Danvers, MA; catalog number 8457S; dilution 1:1000). The secondary antibodies used were goat anti-rabbit (Jackson Laboratory, West Grove, PA; catalog number 111-035-003; dilution 1:50,000) and goat anti-mouse (Jackson Laboratory; catalog number 115-035-174; dilution 1:50,000). All antibodies, except actin, were diluted in 5% milk with Tris-buffered saline containing Tween 20. The actin antibody was diluted in 5% bovine serum albumin with Tris-buffered saline containing Tween 20.

Immunofluorescence

HIV-1 Tg brain sections were evaluated using immunofluorescence and compared with age-matched control rat brains in the adult (control, $n = 4$; Tg, $n = 6$) and old (control, $n = 5$; Tg, $n = 4$) animal cohorts, as previously described.⁶¹ The animals were anesthetized with isoflurane (3% with 700 mL/minute O₂), followed by transcardial perfusion with saline and 4% paraformaldehyde. The brains were removed, and the striatal and substantia nigra sections (bregma, 0.48 to 0.12 mm and -2.92 to -3.24 mm, respectively) were then selected for immunofluorescence staining, as per the atlas of Paxinos and Watson.⁶⁵ Staining protocols were used to identify microglia using ionized calcium binding adaptor molecule 1 (IBA-1; Wako Chemicals, Richmond, VA; catalog number 019-19741), tyrosine hydroxylase (Novus Biologicals, Centennial, CO; catalog number NB300-110), dopamine transporter (DAT extracellular; Santa Cruz Biotechnology, Dallas, TX; catalog number sc-32259), neuronal soma (NeuN; EMD Millipore, Burlington, MA; catalog number ABN90P), reactive

astrocytes [glial fibrillary acidic protein (GFAP); US Biological, Salem, MA; catalog number G2032-27Q], and nitrosative stress (3-nitrotyrosine; Abcam; catalog number ab110282). Multiepitope labeling protocols were then applied to identify the above targets and imaged, as previously described.⁶³ After postprocessing, the data sets were imported into Adobe Photoshop CS6 (Adobe Inc., San Jose, CA) to produce pseudo-colored composite images.

Image quantification was then performed using the FIJI implementation of ImageJ version 1.6.0 (NIH; <https://imagej.nih.gov/ij>). The locations of the striatal and substantia nigra regions of interest were identical for all of the animals, and the fluorescence intensity was calculated as described previously.⁶³ The data are expressed as percentage fluorescence intensity relative to the area of the region of interest for all stains.

Confocal Microscopy

Frozen striatal brain sections from old male Tg ($n = 3$) and control ($n = 3$) rats (30 μ m thick) were evaluated using immunofluorescence labeling for colocalization of 3-NT to a neurofilament cocktail consisting of light, medium, and heavy chains, using Zeiss LSM 780 with a 63 $\times$ Zeiss plan-apochromat oil, 1.4 numerical aperture, differential interference contrast objective (Zeiss, Jena, Germany). Striatal sections were stained with cocktails of neurofilament antibodies (Abcam; catalog numbers ab9035, ab9034, and ab8135) and anti-3-NT (Abcam; catalog numbers ab61392 and ab110282). The neurofilaments and 3-NT were visualized using goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA; catalog number A11008) and goat anti-mouse IgG2b-Alexa Fluor 647 (Invitrogen; catalog number A21242) secondary antibodies, respectively. The cell nuclei were counterstained using 1 μ g/mL DAPI. All confocal images were acquired using ZEN software 2011 version (Zeiss), in which 20 nonoverlapping fields per section (two sections per slide) were analyzed for colocalization; these data were converted to percentage colocalization per animal for statistical analysis.

Statistical Analysis

Statistical significance was determined by unpaired two-tailed *t*-test or two-way analysis of variance with Sidak's multiple comparisons test using Prism GraphPad version 7 (GraphPad Software, San Diego, CA). All data are represented as means $\pm$ SEM. Correlations and simple linear regression analysis were determined by Pearson correlation coefficient. $P < 0.05$ was considered significant.

Results

Dysregulated Expression of Oxidative Stress Markers in Adult Tg Rats

Our previous data supported dopaminergic dysfunction as the Tg rat ages.^{54,61–63} We hypothesized that chronic oxidative

stress in the striatum is one of the causes for those findings. Using a PCR array, only 4 of 84 genes were found to be differentially expressed above or below the 1.5-fold cutoff: *Nox4*, *Hba1*, *Ucp3*, and *Alb*. Specifically, *Nox4* expression was found to be increased by 1.6- and 2-fold in the young and adult Tg rats, respectively (Table 1). Further evaluation using a specific qPCR assay for *Nox4* showed slightly increased gene transcription in the young Tg compared with age-matched control rats, which became more marked (approximately 2-fold) in the adult Tg compared with age-matched control rats (Figure 1A). However, although those differences were not statistically significant, the differences in the adult group approached significance ($P = 0.06$). On the other hand, no significant differences in the expression of *Hba1*, *Ucp3*, and *Alb* were identified (data not shown).

To further follow up on the dysregulation in *Nox4* mRNA levels, protein expression of these genes was quantified using Western blot analysis in the same animals. NOX4 protein expression in the young cohort was not significantly different from the control rats (Figure 1C). A significant elevation in NOX4 protein expression, however, was detected in the adult Tg rats ($P = 0.02$) compared with age-matched control rats (Figure 1C). Using a two-way analysis of variance analysis of NOX4 expression in the young and adult cohorts, a significant age $\times$ transgene effect interaction was found [$F(1,10) = 5.6$, $P = 0.04$].

Table 1 Striatal Oxidative Stress Response: RT² Profiler PCR Array

Gene symbol	Fold change for transgenic versus control	
	Young	Adult
<i>Nox4</i>	1.6	2.0
<i>Alb</i>	3.0	1.5
<i>Gclc</i>	1.2	1.4
<i>Gclm</i>	1.1	1.2
<i>Gpx1</i>	1.1	1.2
<i>Gpx2</i>	1.2	1.4
<i>Gpx3</i>	1.1	1.3
<i>Gpx4</i>	1.1	1.3
<i>Gpx7</i>	1.1	1.1
<i>Gsr</i>	1.1	1.3
<i>Gstk1</i>	1.0	1.1
<i>Gstp1</i>	1.0	−1.1
<i>Hba1</i>	−2.3	7.8
<i>Prdx1</i>	1.0	1.1
<i>Prdx2</i>	−1.0	1.2
<i>Prdx3</i>	−1.0	1.2
<i>Prdx4</i>	1.0	1.1
<i>Prdx5</i>	1.1	1.3
<i>Prdx6</i>	1.0	1.3
<i>Srxn1</i>	−1.1	1.2
<i>Txn1</i>	1.1	1.1
<i>Txnrd1</i>	1.1	1.3
<i>Txnrd2</i>	−1.1	1.2
<i>Ucp3</i>	−1.3	2.9

Dysregulated Antioxidant Response in the Adult Tg Rats

In view of the above findings, *Nrf2* expression was assessed in the adult animals using qPCR as a measure of the antioxidant response because *Nrf2* is the master regulator of *Gsh* and *Trx* antioxidant gene transcription, and its expression is expected to increase in response to NOX4 up-regulation. Unexpectedly, the relative change in *Nrf2* mRNA expression in the adult Tg and control rats was not significantly different (Figure 1B). Consistent with *Nrf2* mRNA expression, no significant difference in *Nrf2* protein levels was detected between adult Tg and control rats (Figure 1D). Similarly, no increase in the expression of *Gsh* and *Trx* antioxidants was seen in the Tg rats by the PCR array (Table 1).

Nitrative Stress in the Basal Ganglia of Tg Rats Increases with Age

To assess for NS in the striatum in the young and adult Tg rats, levels of nNOS protein were measured by Western blot analysis. In the young cohort, no significant difference was identified between Tg and age-matched control rats; in the adult Tg rats, on the other hand, a significant 3.4-fold increase in striatal nNOS protein expression was identified relative to age-matched controls ($P = 0.0005$) (Figure 2). A two-way analysis of variance analysis showed a highly significant age $\times$ transgene effect interaction [$F(1,10) = 18$; $P = 0.002$].

Immunohistochemical Evidence of Dopaminergic Dysfunction

Immunohistochemical analysis of striatal brain tissues showed increased 3-NT staining in adult and old Tg rats compared with their corresponding age-matched controls. This was highly significant in the old Tg compared with control rats ($P = 0.0003$) but not significant in the adult cohort (Figure 3A). Again, a two-way analysis of variance analysis found a significant age $\times$ transgene effect interaction [$F(1,15) = 8.2$; $P = 0.01$]. This suggests that the normal age-related buildup of nitration is exacerbated in the Tg rats. In view of the pattern of 3-NT staining, which seemed to follow the axonal tracts, we hypothesized that neurofilament proteins would be a likely target for nitration. Using confocal microscopy, an approximately 40% increase in tyrosine nitration of striatal neurofilament proteins ($P = 0.01$) was identified in old Tg rats relative to controls (Figure 3B). These data suggest that the dysregulation of neuronal function in the aging Tg rat might be related to NS and that the posttranslational modification of neurofilament proteins could subsequently lead to neuronal loss.

Presynaptic Dopaminergic Dysfunction and Astrocytic Loss in the Tg Rats

To further assess potential effects of NS on the presynaptic dopaminergic neurons in the old animals, multiplex immunohistochemical analysis of various targets in the brains of old

Figure 1 Characterization of striatal oxidative stress response in HIV-1 transgenic (Tg) rats. Real-time quantitative PCR assessment of *Nox4* mRNA expression in young and adult HIV-1 Tg rats relative to controls (**A**) and nuclear factor-erythroid 2-related factor 2 (*Nrf2*) mRNA expression in adult HIV-1 Tg rats relative to control (**B**). Western blot analysis for the determination of protein expression of NADPH oxidase-4 (NOX4) in young and adult HIV-1 Tg rats relative to controls (**C**) and Nrf2 in adult HIV-1 Tg rats relative to controls (**D**). Western blot analyses were quantified using FIJI (ImageJ version 1.6.0) and are shown below the respective blots. Statistical analysis of NOX4 expression in young and adult cohorts was performed using two-way analysis of variance with Sidak multiple comparisons test. Nrf2 expression data were analyzed by *t*-test. * $P < 0.05$.

control and Tg rats was performed. As expected, a significantly lower expression of DAT ($P = 0.02$), TH ($P = 0.0003$), and GFAP ($P = 0.03$) was identified in old Tg rats, indicating dopaminergic and astrocytic dysfunction (Figure 4, A–C). A significant negative correlation was observed between the percentage fluorescence intensities of striatal 3-NT versus DAT, TH, and GFAP fluorescence intensities (Figure 4, D–F).

In addition to the striatal defects, a significant increase in 3-NT staining ($P = 0.03$) and a concomitant decrease in NeuN-positive cells ($P = 0.03$) were also identified in the substantia nigra of the old Tg rats relative to age-matched controls (Figure 5, A and B), indicating neuronal loss. However, no significant correlation was observed between 3-NT and NeuN staining (Figure 5C).

Discussion

There has always been an interest in the role of oxidative/nitrosative stress in the pathophysiology of HIV-associated

dementia complex and HIV-associated neurologic disorders,^{15,17,66} its association with viral protein expression,^{67–79} and how it affects the dopaminergic system.^{7,67,80} Herein, the HIV-1 Tg rat, a known model of viral protein toxicity, was used to assess the relationship between OS/NS markers and the end result of dopaminergic dysfunction and damage. We were mainly motivated by our previous findings in the HIV-1 Tg rat of decreased brain volumes, lower striatal TH, GFAP, and DAT expression, decreased striatal DAT and dopaminergic receptor (D2/D3) binding on positron emission tomography, and motor and behavioral deficits consistent with dysregulation in dopaminergic function.^{61–63,81,82} Those markers of dopaminergic loss/dysfunction were generally exacerbated with age in this animal model, with progressive loss of DAT, TH, and D2/D3 receptors in the basal ganglia of Tg compared with control rats shown on either positron emission tomography imaging or immunohistochemistry.^{54,61,63} As expected, evidence of OS/NS was found in this study with increased 3-NT staining in

Figure 2 Characterization of neuronal nitric oxide synthase (nNOS) response in the HIV-1 transgenic (Tg) rats. nNOS protein expression in HIV-1 Tg rats relative to controls, as shown by Western blot analysis with quantification in young and adult rats. Quantification data were analyzed using two-way analysis of variance with Sidak multiple comparisons test. *** $P < 0.001$.

the striatum of adult and old Tg rats associated with aging and transgene status (Figure 3A). Concurrently, decreased presynaptic TH and DAT staining was found in the striatum of old Tg rats with significant negative

correlation between the fluorescence intensities of 3-NT and TH (Figure 4, A and D) and 3-NT and DAT (Figure 4, B and E). At the same time, a decrease in striatal GFAP staining, which also negatively correlated

Figure 3 Characterization of nitrative stress response in the HIV-1 transgenic (Tg) rats. **A:** 3-Nitrotyrosine (3-NT) staining of striatal tissue sections from adult and old animal cohorts. Quantification data are represented as percentage fluorescence intensity (%FI) and analyzed using two-way analysis of variance with Sidak's multiple comparisons. **B:** Confocal microscopy was performed to assess percentage colocalization between 3-NT and neurofilament proteins in the striatal region of old HIV-1 Tg and control animals. Data were analyzed using *t*-test. ** $P < 0.01$, *** $P < 0.001$. Scale bars: 100 μ m (A); 5 μ m (B).

Figure 4 Cellular damage in HIV-1 transgenic (Tg) rats. Immunostaining of striatal regions of old HIV-1 Tg and control rats with tyrosine hydroxylase (TH; **A**), dopamine transporter (DAT; **B**), and glial fibrillary acidic protein (GFAP; **C**). All quantification data are represented as percentage fluorescence intensity (%FI) and analyzed using *t*-test. Correlation of 3-nitrotyrosine [3-NT (%FI)] in striatal regions of the old rat cohort with TH (%FI; **D**), DAT (%FI; **E**), and GFAP (%FI; **F**). Pearson correlation coefficient (*r*) and the *P* values are indicated in the graphs. Open circles indicate control rats; closed circles, HIV-1 Tg rats. The *P* values for control versus HIV-1 Tg animals are indicated. *n* = 9 old rats (**D–F**). **P* < 0.05, ****P* < 0.001. Scale bars = 100 μm (**A–C**).

with 3-NT staining, indicating concurrent astrocytic loss, was observed (Figure 4, C and F).

At the molecular level, borderline increases were found in Nox4 (mRNA) and significant increases were found in NOX4 and nNOS protein expression in the striatum of the adult but not young Tg rats compared with age-matched controls (Figures 1, A and C, and Figure 2). Nox4 induction can be mediated by gp120 and Tat HIV-1 proteins^{32,83}; and the expression of gp120 and tat mRNA in striatal brain lysates as well as gp120 in the cerebrospinal fluid of the Tg rat has been shown, suggesting local viral protein production in the brain.⁶² Consistent with published *in vitro* data, these findings may reflect a pattern of OS/NS induced by chronic exposure to HIV-1 proteins in the Tg rat. Downstream effects would include NOX4-mediated production of oxidants, such as hydrogen peroxide, superoxide, and nNOS-dependent production of peroxynitrite. The latter would go on to react with other molecules and form additional types of RNS, including nitrogen dioxide and dinitrogen trioxide, causing further mitochondrial electron

transport chain dysregulation, protein nitration, and neuronal injury.^{21,49,50,84} Alternatively, Villeneuve et al⁶⁰ have demonstrated that, in the HIV-1 Tg rat, viral proteins directly enhance neuronal mitochondrial energy demand and also reduce expression of mitochondrial electron transport chain subunit proteins, likely increasing superoxide production. Some other studies have reported that increases in mitochondrial metabolism can direct the release of hydrogen peroxide and nitric oxide to the cytosol, whereas electrons prematurely exit the electron transport chain to further augment superoxide levels and associated cellular nitrosative stress.^{85–89} Interestingly, gp120 and Tat are also reported to increase intracellular Ca²⁺ levels, which can subsequently activate nNOS.^{31,90–92} Although Cho et al⁵⁷ described the induction of inducible NOS as the molecular source of nitric oxide and nitration of heat shock protein 90 from hippocampal lysates in the HIV-1 Tg rat, the presence of inducible NOS (or endothelial NOS) in the striatal lysates of either the control or the HIV-1 Tg rats was not detected.

Interestingly, the increase in markers of nitrosative stress in these animals occurred with no corresponding increase in Nrf2 expression or the Nrf2-regulated *Gsh* and *Trx* antioxidant enzyme systems (Figure 1, B and D, and Table 1). Nrf2 is the major transcription factor regulating the biosynthesis, use, and regeneration of glutathione, TRX, and NADPH, thereby controlling the cellular redox activity.²² A previous report in the Tg rat describes a reduction, rather than an increase, in Nrf2 and heme oxygenase-1 expression in the whole-brain, cerebellum, and hippocampus lysates.⁴⁹ No induction in Nrf2 expression or its dependent phase 2 antioxidants was found within the striatum. Thus, it is possible that the nigrostriatal dopaminergic dysfunction in aging HIV-1 rats is also a consequence of dysregulation in Nrf2 and redox adaptation, eventually failing to counteract the nitrosative stress affected by NOX4 and nNOS expression. This could, in turn, lead to the nitration of several targets, such as heat shock protein 90,⁵⁷ and the striatal neurofilament proteins, as we demonstrated, albeit only in a small sample size. An additional direct effect of Nrf2 expression imbalance could be neuronal cytotoxicity through the inhibition of cellular respiration and dysregulation of mitochondrial electron transport chain.^{21–24}

These results support a model in which gp120 and/or Tat induce NOX4 expression and nNOS activation, which subsequently leads to ROS/RNS generation and nitrosative stress, protein tyrosine nitration, and neuronal injury; this neuronal pathology is influenced by aging and transgene status. The lack of effective antioxidant activity could further exacerbate the negative effects of OS/NS. This could eventually lead to the dopaminergic dysfunction that we and other groups have previously described.^{7,54,56,61–63,67,80–82} However, questions concerning the mechanism(s) by which Nrf2 activation is suppressed still need to be addressed, and our future work will concentrate on better understanding how Nrf2 is regulated in the HIV-1 Tg rat.

Ultimately, free radical damage to the brain is a process that is found to be common to the pathogenesis of HIV-associated neurologic disorders and many neurodegenerative diseases. Therefore, the identification of increased 3-NT modification of neurofilaments in dopaminergic neurons may be both a biological indicator and/or a contributor to the dopaminergic dysfunction in the HIV-1 Tg rat. We, therefore, propose the HIV-1 Tg rat as an appropriate model of OS/NS in the setting of exposure to HIV viral proteins.

Figure 5 Neuronal loss in the substantia nigra. **A** and **B**: Immunostaining in the substantia nigra of the old animal cohort for 3-nitrotyrosine (3-NT; $P = 0.03$; **A**) and NeuN ($P = 0.03$; **B**). Quantification data are represented as percentage fluorescence intensity (%FI) and analyzed by *t*-test. The *P* values for control versus HIV-1 transgenic (Tg) animals are indicated. **C**: Correlation of 3-NT (%FI) with NeuN (%FI) is shown. Pearson correlation coefficient (*r*) and the *P* values are indicated in the graph. Open circles indicate control rats; closed circles, HIV-1 Tg rats. * $P < 0.05$. Scale bars = 100 μ m (**A** and **B**).

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W.R. and D.A.H. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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