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Nicotine mediates expression of genes related to antioxidant capacity and oxidative stress response in HIV-1 transgenic rat brain

Guohua Song^{1,2}, Tanseli Nesil¹, Junran Cao¹, Zhongli Yang³, Sulie L. Chang^{3,4}, and Ming D. Li¹

¹Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, Charlottesville, Virginia, USA

²Shanxi Key Laboratory of Experimental Animal Science and Animal Model of Human Disease, Shanxi Medical University, Taiyuan, China

³Institute of NeuroImmune Pharmacology, Seton Hall University, South Orange, NJ, USA

⁴Department of Biological Sciences, Seton Hall University, South Orange, NJ, USA

Abstract

Oxidative stress plays an important role in the progression of HIV-1 infection. Nicotine can either protect neurons from neurodegeneration or induce oxidative stress, depending on its dose and degree of oxidative stress impairment. However, the relationship between nicotine and oxidative stress in the HIV-1-infected individuals remains largely unknown. The purpose of this study was to determine the effect of nicotine on expression of genes related to the glutathione (GSH)-centered antioxidant system and oxidative stress in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of HIV-1 transgenic (HIV-1Tg) and F344 control rats. Adult HIV-1Tg and F344 rats received nicotine (0.4 mg/kg; base; *s.c.*) or saline injections once per day for 27 days. At the end of treatment, various brain regions including the NAc and VTA were collected from each rat. Following total RNA extraction and cDNA synthesis of each sample, quantitative RT-PCR analysis was performed for 43 oxidative stress-related genes. Compared with F344 control rats, HIV-1Tg rats showed a significant down-regulation of genes involved in ATPase and cytochrome oxidase at the mRNA level in both regions. Further, we found a significant down-regulation of *Gstm5* in the NAc and up-regulation of *Cox1*, *Cox3* and *Gsta6* in the VTA of HIV-1Tg rats. HIV-1Tg rats showed brain region-specific responses to chronic nicotine treatment. This response resulted in a change in the expression of genes involved in antioxidant mechanisms including the down-regulation of genes such as *Atp5h*, *Calml1*, *Gpx7*, *Gstm5*, *Gsr* and *Gsta6* and up-regulation of *Sod1* in the NAc, as well as down-regulation of genes like *Cox5a*, *Gpx4*, *Gpx6*, *Gpx7*, *Gstm5*, and *Sod1* expression in the VTA of HIV-1Tg rats. Together, we conclude that chronic nicotine treatment has a dual effect on the antioxidant defense system and oxidative stress-induced apoptosis signaling in HIV-1Tg rats. These findings suggest that nicotine has a negative

*Correspondence to: Ming D. Li, Ph.D., Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, 450 Ray C Hunt Drive, Suite G-170, Charlottesville, VA, USA 22903, ml2km@virginia.edu.

Conflict of Interest

The authors declare no conflict of interest with the study or preparation of the manuscript.

effect on response to oxidative stress and antioxidant processes in HIV-1 Tg rat brain, especially in the VTA.

Keywords

HIV; nicotine; NAc; VTA; oxidative stress; antioxidants

1. Introduction

The central nervous system (CNS) is one of the major targets of the human immunodeficiency virus (HIV-1) and exhibits high vulnerability to HIV-1-mediated pathogenesis (Churchill *et al*, 2014). HIV-1 proteins can directly cross the blood-brain barrier (BBB) and exert effects on the brain in early stages of HIV-1 infection (Kaul *et al*, 2005; Kimura *et al*, 1993a). HIV-1 proteins affect the activation of monocytic cells (e.g., microglia, macrophages and astrocytes), leading to the increased production of neuroactive and neuroimmune products in the brain (Kimura *et al*, 1993b). Several lines of evidence suggest that an increased inflammatory response to viral proteins can facilitate the activation of oxidative stress and apoptosis signaling cascades (Kaul *et al*, 2005; Kimura *et al*, 1993b). In addition, HIV-1 proteins induce mitochondrial dysfunction by directly interacting with mitochondrial membrane proteins, leading to high levels of oxidative stress in neurons (Ladha *et al*, 2005). Alterations in mitochondrial function and oxidative stress have been suggested as key mediators in developing HIV-1-associated neurocognitive disorders (HAND) during late stage of HIV-1-infected patients (Perez-Matute *et al*, 2013).

Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs) and a major psychoactive component in tobacco smoke (Levin *et al*, 2014). However, research on the neuroprotective and neurotoxic effects of nicotine in the CNS has been inconclusive (Nie *et al*, 2013; Nordberg *et al*, 2002; Picciotto and Zoli, 2008b; Zeng *et al*, 2001). For example, nicotine has been shown to produce significant improvement in the attention span and memory performance in both human and animal studies (Knott *et al*, 2012; Levin *et al*, 2014) and has been implicated in neuroprotective effects against β -amyloid-mediated neurotoxicity as well as the enhanced neuroplasticity in transgenic model for Alzheimer's disease in mice (Nie *et al*, 2013). These effects are likely mediated by up-regulation of nAChR expression and decrease in the accumulation of β -amyloid peptide leading to a decrease in neurotoxicity (Kihara *et al*, 1997; Nordberg *et al*, 2002; Picciotto and Zoli, 2008b; Zeng *et al*, 2001). On the other hand, nicotine has been shown to modulate expression of multiple immune-related pathways in SH-SY5Y cells (Cui *et al*, 2012). The effect of nicotine on immune function was also demonstrated in mitochondrial signaling pathways by inhibiting chemotherapy-induced apoptosis in lung cancer (Zhang *et al*, 2009). There is also considerable evidence from *in vivo* and *in vitro* studies, showing that nicotine increases oxidative stress and consequently neuronal cell death (Guan *et al*, 2002; Quik *et al*, 2007; Soto-Otero *et al*, 2002). Under oxidative stress conditions, neurons increase their antioxidant defense mechanisms by enhancing the production and release of free radical scavengers. This alternation enhances the survival of neurons through the activation of glutathione (GSH) and antioxidant enzymes like catalase and superoxide dismutase (SOD).

Further, nicotine has also been shown to disrupt the antioxidant defense mechanisms by decreasing levels of GSH and SOD in rats and altering the balance between antioxidant capacity and oxidative stress-induced free radicals in the brain (Guan *et al*, 2002; Soto-Otero *et al*, 2002; Wang *et al*, 2009; Yildiz *et al*, 1998). The interaction between nicotine and oxidative stress mechanisms is also detected in the lipid peroxidation mechanism which is initiated by free radical reactions from mitochondria. Nicotine is reported to stimulate lipid peroxidation by increasing the malondialdehyde (MDA) and lactate dehydrogenase in CHO cells (Yildiz *et al*, 1998). The decrease in the antioxidant defense system and increase in the production of reactive oxygen species have been implicated in many forms of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Subramaniam and Chesselet, 2013; Uttara *et al*, 2009).

Epidemiological studies have revealed an inverse correlation between smoking and the incidences of AD and PD (Picciotto and Zoli, 2008a; Zhang *et al*, 2009). Although studies have clearly shown the importance of mitochondria in the biological response to nicotine in these disease states, the relationship between nicotine and oxidative stress in these diseases, especially in HIV-1-infected individuals, remains largely unknown. To better understand the possible neuroprotective and neurotoxic effects of nicotine in HIV-1 patients, we compared the effect of nicotine on the expression of genes related to the (GSH)-centered antioxidant system and oxidative stress in dopaminergic midbrain regions of HIV-1Tg and F344 control rats.

2. Materials and methods

2.1 Animals

Adult male HIV-1Tg and F344 control rats were purchased from Harlan Inc. (Indianapolis, IN). All rats were maintained under controlled lighting (12:12 Light/Dark) and temperature (20°C) with *ad libitum* access to food and water. All animal experiments reported in this study were approved by the Animal Care and Use Committee of University of Virginia.

2.2 Nicotine treatment

Nicotine hydrogen bitartrate (Sigma Aldrich Co., St. Louis, Mo) was dissolved in 0.9% w/v NaCl solution (saline) and neutralized to PH 7.2~7.0 with NaOH. Rats from each strain were divided randomly into two groups (n = 6 per group) Control (i.e., HIV-1Tg_ or F344_saline control) and nicotine-treatment group (i.e., HIV-1Tg_ and F344_nicotine-treated groups). Either nicotine (0.4 mg/kg; base) or saline was administered once a day at 9:00AM by subcutaneous injection for 27 days. This drug treatment paradigm was based on previous reported behavioral studies (Matta *et al*, 2007; Menon, 2001a) and our own reported study where we showed that the 27 days of 0.4 mg/kg dose of nicotine given subcutaneously to HIV-1Tg rats lead to significant changes of gene expression in the brain (Nesil *et al.*, 20015, in press). Furthermore, previous studies have also shown that more than 20 days of nicotine injection leads to oxidative stress and tissue injury (Sener *et al*, 2004; Sener *et al*, 2005).

2.3 Tissue collection

Animals were sacrificed one day after the last injection and brains were immediately removed after decapitation. Using a rat brain matrix, brain slices of approximately 1-mm were taken from each brain. Tissues from the ventral tegmental area (VTA) and nucleus accumbens (NAc) were collected bilaterally from each brain using a 3.00-mm Harris Micro-Punch (GE Healthcare Life Sciences, Piscataway, NJ, USA) according to the rat brain atlas (Paxinos and Watson, 1998). Punched tissues were stored at -80°C until use.

2.4 Quantitative RT-PCR array analysis

Total RNA was isolated from punched brain tissues using the Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. The quantity and purity of the extracted total RNA were measured at the optical densities of 260 and 280 nm with NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). The primers for each gene of interest were designed using Primer Express (v. 3.0) software (Applied Biosystems, Carlsbad, CA, USA) and spanned introns to avoid amplifying genomic DNA, with a melting temperature (T_m) from 59°C to 61°C . The amplicon sequences were subjected to a BLAST search to ensure the specificity of the primers for the target genes of interest. All the primers were tested for their specificity by checking the cycle number and the dissociation curve before they were included in the qRT-PCR array. The primer sequencers of all examined genes in this study are listed in Table 1.

First-strand cDNAs were synthesized from two μg of total RNA using a Reverse Transcription System Kit (Promega, Madison, WI). The qRT-PCR analysis was conducted as described previously (Wei *et al.*, 2011). Briefly, RT product was amplified in a volume of 10 μl containing 5 μl 2 $\times$ Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), equally mixed sense and antisense primers (with a final concentration 250 nM each), and 2.5 μl diluted cDNA in a 384-well plate using the 7900HT Fast Real Time PCR system (Applied Biosystems) (Cui *et al.*, 2012; Wang *et al.*, 2011). The PCR condition was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. A cycle threshold (C_t) was assigned in the beginning of logarithmic phase of amplification and difference in C_t values of the control and treated groups were used to determine the relative expression. Melting curve analysis was applied to characterize the specificity of the amplicons.

2.5 Statistical analysis

Expression levels of all genes of interest were first normalized to the expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then analyzed using a comparative C_t method (Winer *et al.*, 1999; Zhao *et al.*, 2014). The relative gene expression level was compared between F344 and HIV-1Tg rats using the Student's t test. Significant alteration in mRNA expression level was defined as a fold change $>20\%$ with a p value <0.05 .

3. Results

3.1 Strain differences in the expression of genes related to oxidative stress and mitochondrial function

There is a large body of evidence revealing that HIV-1 proteins induce mitochondrial dysfunction as a result of increased oxidative stress which has been implicated in several types of neuropathogenesis of HIV-1 infection in the brain (Churchill *et al*, 2014; Perez-Matute *et al*, 2013). Considering the effects of HIV-1 proteins on the mitochondrial function and oxidative stress, we examined the expression profiles of 43 genes involved in electron transport chain including ubiquinone oxidoreductase (i.e., mitochondrial complex I), succinate dehydrogenase (mitochondrial complex II), cytochrome bc1 complex (mitochondrial complex III), cytochrome c oxidase (mitochondrial complex IV), apoptosis, antioxidant activity, and ATP transporters in the NAc and VTA regions of HIV-1Tg and F344 control rats. Our statistical analysis of mRNA expression data in the HIV-1Tg and F344 rats at the cut-off ratio of > 20% mRNA fold change revealed that 9 genes in the NAc (Table 2) and 12 genes in the VTA (Table 3) showed significant gene expression differences in HIV-1Tg rats compared to F344 rats. In the NAc, expression levels of the genes in mitochondrial complex I (Ndufa2; $P = 0.0004$; Ndubf2; $P = 0.009$), complex IV (Cox5a; $P = 0.018$; Cox6a; $P = 0.007$) and ATPase in complex V (Abcd2, Atp5h and Atp6v1f; $P = 0.001-0.05$) were significantly down-regulated by viral proteins. We also found a significant down-regulation of the expression of antioxidant enzyme glutathione s-transferase subunit Gstm5 in the NAc of HIV-1Tg rats.

The calcium signals and activation of Ca^{+2} /calmodulin-dependent kinase signaling lead to mitochondrial reactive oxygen species (ROS) generation and are critical in oxidative stress-induced neuronal apoptosis. The impact of mitochondrial dysfunction on intracellular calcium levels has been reported in neurodegenerative diseases (Subramaniam and Chesselet, 2013). Our results indicated that expression of calcium binding protein calmodulin 1 (Calm1) in the VTA, which functions as calcium binding protein and activates calmodulin-dependent kinases, was 45.8% higher HIV-1Tg rats than that in F344 rats ($P = 0.042$; Table 2).

In the VTA (Table 3), HIV-1 proteins significantly down-regulated the expression of genes in the mitochondrial complex I (Ndufa2, Ndubf2 and Ndufa8; $P < 0.05$ for all of them) and complex V (Abcd2, Atp5h and Atp6v1f; $P < 0.05$ for all of them). We found a biphasic expression pattern of cytochrome oxidase in mitochondrial complex IV encoding genes where expression of Cox1 and Cox3 mRNA were increased by 28.4% and 35.6% ($P = 0.021$ for both genes), respectively. The expression of Cox8b was decreased by 48% in mRNA expression ($P = 0.021$) in VTA. In addition, genes associated with antioxidant defence (Gsta6; $P = 0.045$) and the p53-dependent apoptosis pathway (Perp; $P = 0.033$) showed a significant increase of mRNA expression in the VTA of HIV-1Tg rats (Table 3).

3.2 The effects of HIV-1 proteins and nicotine on the mRNA expression in the NAc and VTA of HIV-1Tg rats

The role of oxidative stress in midbrain dopaminergic cell loss has been indicated to be one of the major causes of neurodegeneration during HIV-1 infection (Nath *et al*, 2000). Since nicotine has a dual effect on induction of oxidative stress and neuroprotection, we next examined whether nicotine can potentiate mitochondrial dysfunction-mediated oxidative stress caused by viral proteins in the NAc and VTA of HIV-1Tg rats. Chronic nicotine treatment significantly modified the mRNA expression levels in the HIV-1Tg rats; this effect was more pronounced in the GSH-centered antioxidant system-related genes in the NAc and VTA. As shown in Table 4 and Figure 1, nicotine significantly down-regulated the expression of Gsta6, Gpx7, Gstm5 and Gsr by 25%-34% ($P = 0.002-0.049$), but up-regulated the expression of superoxide dismutase encoding gene Sod1 by 69% ($P = 0.02$) in the NAc of HIV-1Tg rats (Table 4; top panel). Further, chronic nicotine treatment decreased the expression of genes involved in mitochondrial complex V (Atp5h; $P = 0.004$) and in Ca^{+2} /calmodulin-dependent kinase signaling (Calm1; $P = 0.005$).

In the VTA, nicotine significantly modulated the expression of genes in the apoptosis signaling, antioxidant defence system, and mitochondrial complex IV in the HIV-1Tg rats. The expression levels of the genes in the GSH-centered antioxidant system (Gpx4, Gpx6, Gpx7, Gstm5) and superoxide dismutase encoding Sod1 were significantly down-regulated by 18.7%-73.3% ($P = 0.02-0.04$) in the nicotine-treated HIV-1Tg rats compared to the saline-treated HIV-1Tg rats (Figure 1). Similarly, expression level of Cox5a in complex IV was significantly down-regulated by nicotine in the VTA of HIV-1Tg rats (27.9%; $P = 0.038$). Different from the observed effect in the NAc, chronic nicotine treatment significantly increased the expression of the genes involved in apoptosis signaling, including pro-apoptotic gene (Casp9; 28.1%; $P = 0.001$) and apoptosis facilitator (Bcl2L11; 26.7%; $P = 0.009$) (Table 4; bottom panel).

3.3 Nicotinic modulation of oxidative stress and mitochondrial function related gene expression in F344 rats

Previous studies have shown that nicotine has neuroprotective effects in the CNS during neurodegeneration (Quik *et al*, 2007). To determine whether nicotine induces oxidative stress or has a positive effect on the antioxidant system under normal physiological conditions, we compared the same set of genes involved in oxidative stress, antioxidant defense mechanism, apoptosis signaling and mitochondrial function between saline and nicotine treatment groups in F344 rats. Nicotine significantly increased the mRNA expression of the gene encoding superoxide dismutase antioxidant enzyme (Sod1; 93.5%; $P = 0.034$), but decreased the glutathione peroxidase 1 (Gpx1) mRNA expression (31.9%; $P = 0.005$) in the NAc (Table 5).

In contrast to the observed effects of nicotine in the HIV-1Tg rats, genes encoding the GSH-centered antioxidant system members (Gpx4, Gpx5, Gpx7, Gstk1 and Gsta6) were significantly up-regulated (27%-150%; $P = 0.02-0.008$) by nicotine in the VTA of F344 rats. However, antioxidant enzyme superoxide dismutase encoding gene Sod2 was significantly down-regulated (23.4%; $P = 0.01$) by nicotine treatment. Our results also

indicated a significant increase in the expression of Casp8 (42.1%; $P = 0.031$) in the apoptosis signaling pathway and of apoptosis facilitator Perp gene (24.2%; $P = 0.035$) by nicotine in the VTA of F344 rats (Table 5).

4. Discussion

We demonstrated the effects of HIV-1 proteins and nicotine on the expression of genes associated with mitochondrial function and the antioxidant defense system in the VTA and NAc of HIV-1Tg and F344 rats. Results showed that HIV-1 proteins disrupted mitochondrial function through mitochondrial complexes I-V and showed a brain region-specific effect on the expression of glutathione-centered antioxidant enzymes in the NAc and VTA. We also found that a moderate dose of nicotine partially attenuated the effects of HIV-1 proteins in the NAc and potentiated the effects of viral proteins on oxidative stress and apoptosis signaling in the VTA. Further, chronic nicotine treatment showed brain region-specific effects on the expression of genes associated with the antioxidant defense system. A significant increase was observed in the expression of genes in the GSH-antioxidant system in the VTA and expression of superoxide dismutase in the NAc of F344 control rats.

Alterations in mitochondrial function play a major role in the development and progression of neurodegenerative disorders, including HAND (Lee *et al.*, 2010; Stack *et al.*, 2008). HIV-1 proteins induce oxidative stress by decreasing the activity of mitochondrial complexes I-V and increasing the production of ROS in the brain (Aksenov *et al.*, 2001; Ladha *et al.*, 2005; Mollace *et al.*, 2001). Previous studies have shown that HIV-1 proteins (e.g., gp120 and Tat) preferentially decrease mitochondrial function and increase the neurotoxicity in the midbrain dopaminergic neurons (Aksenov *et al.*, 2001). The current study showed that HIV-1 proteins induced mitochondrial dysfunction in the NAc and VTA by altering the expression profiles of genes in mitochondrial complexes I-V. The genes involved in complex I showed lower expression levels in the NAc (Ndfun2a) and VTA (Ndfub2, Ndfub8 and Ndfub2a) of HIV-1Tg rats. Impaired complex I activity has been shown in HIV-1-induced apoptosis in T-cells (Tripathy and Mitra, 2010). The decrease in the expression of complex I subunit Ndufa6 has been reported to have a role in the mitochondrial dysfunction during HIV-1 infection (Ladha *et al.*, 2005). Among the mitochondrial respiratory chain complexes, alterations in the activity of complex IV have an important role in mediating the effects of HIV-1 proteins on oxidative stress. Miro *et al.* (Miro *et al.*, 2004) showed that complex IV activity was decreased by 19% in the mononuclear cells of HIV-1-infected patients. In contrast, Tripathy *et al.* (Tripathy and Mitra, 2010) reported that an up-regulation of Cox2, a subunit of complex IV, with a concomitant increase in HIV-1 induced apoptosis in T-cells. In our analysis of the gene expression in HIV-1Tg rats, the complex IV subunits Cox5a and Cox6a in the NAc and Cox8a in the VTA displayed low expression levels in the HIV-1Tg rats compared to F344 rats.

Mitochondrial ATP-synthase (complex V) activity has a pivotal role in energy metabolism of neurons (Chaban *et al.*, 2014). Decreased activity in complexes I-IV and increased oxidative stress are defined as important factors in the impairment of mitochondrial ATP-synthase activity (Mollace *et al.*, 2001; Navarro and Boveris, 2010). There is a strong

relationship between deprivation of energy metabolism and ROS production; however the interaction between excessive ROS production and the decline of mitochondrial ATP synthesis in the midbrain has not been fully investigated during HIV-1 infection. In the current study, our results indicate a possible dysfunction in the complex V due to the reduced expression levels of ATPase subunits in the midbrain regions of HIV-1Tg rats. The expression levels of *Abcd2*, *Atp5h* and *ATP6v1f* genes were significantly down-regulated by HIV-1 proteins in the NAc and VTA. These results indicate that HIV-1 proteins can alter energy metabolism through both complexes I and IV and increase ROS production in the midbrain.

The interaction between ROS and the antioxidant defense system has been shown in the molecular mechanisms of HIV-1-associated neurocognitive disorders (Mollace *et al*, 2001; Price *et al*, 2005). Depletion of the antioxidant defense system leads to an accumulation of ROS in the mitochondria (Mollace *et al*, 2001; Price *et al*, 2005). This alteration increases the formation of apoptosome complex. This formation activates caspase-9-mediated apoptotic signaling cascades during neurodegeneration (Fratiglioni and Wang, 2000). HIV-1 proteins trigger the apoptotic signaling cascades by decreasing the levels of antioxidant enzymes with uncompensated ROS production (Louboutin *et al*, 2012). Our results partially confirm the aforementioned findings and show that HIV-1 proteins decreased the expression of antioxidant enzymes in the NAc (*Gstm5*) and up-regulated the *Gsta6* in the VTA. Our results also showed the effect of HIV-1 proteins on the expression of genes in apoptotic signaling cascades in both VTA and NAc. Compared to F344 rats, HIV-1Tg rats showed higher expression of *Perp*, which is linked to P53-dependent apoptosis signaling, and lower expression levels of pro-apoptotic Caspase-9. The loss of mitochondrial calcium buffering capacity triggers calmodulin-dependent apoptosis signaling pathways in the brain. Also, we showed that HIV-1 proteins increased the calmodulin expression in the NAc, suggesting the interaction of mitochondrial dysfunction and calcium in the loss of dopaminergic neurons.

Previous studies have revealed the dual effects of nicotine on oxidative stress and neurodegenerative disorders (Picciotto and Zoli, 2008a; Wang *et al*, 2009). It has been reported that the dose, route of treatment and the degree of oxidative stress determine the action of nicotine in the brain during neurodegeneration. A low dose of nicotine showed neuroprotective effects depending on activation kinetics of nAChR subunits and localization of the pathogenesis in the brain (Picciotto and Zoli, 2008a). On the other hand, a high dose of nicotine showed neurotoxic effects, which trigger oxidative stress in the CNS resulting in neuronal loss in the midbrain and cortex (Gallo *et al*, 2010; Ravikumar *et al*, 2004). By examining the effect of a moderate dose of nicotine (0.4 mg/kg, base, *s.c*) after 27 days of chronic treatment on the expression of genes in the mitochondrial complexes, antioxidant defense system and apoptosis signaling in the HIV-1Tg rats, we found that nicotine under this moderate dose, had a biphasic effect, i.e., exhibiting antioxidant activities in the NAc and neurotoxic effects in the VTA. Chronic nicotine treatment up-regulated *Sod1* expression and down-regulated the genes in the glutathione-centered antioxidant defense system in the NAc of HIV-1Tg rats. In the VTA, genes encoding the subunits of antioxidant enzymes were down-regulated by nicotine in the HIV-1Tg rats. The balance between oxidant species and removal of oxidants by the antioxidant enzymes determines the ROS-mediated

apoptosis signaling in neurons. Multiple *in vitro* and *in vivo* studies have demonstrated the neuroprotective effect of Sod1 overexpression in neurodegeneration including HAND (Boven *et al*, 1999; Louboutin *et al*, 2012). Results demonstrated that increased levels of Sod1 induced a decrease in ROS levels that lead to an enhanced resistance to caspase-mediated apoptosis in neurons. In contrast to the previous findings (Picciotto and Zoli, 2008b; VanCott *et al*, 1997), our results showed that a moderate dose of chronic nicotine treatment exhibited neurotoxic effects in the VTA of HIV-1Tg rats.

The discrepancies in the protective effects of nicotine might be related to differences in the oxidative stress levels and actions of HIV-1 proteins in the VTA. There is increasing evidence suggesting the involvement of free radicals and iron (Fe)-induced oxidative stress in the pathogenesis of PD, AD and HIV-1 associated neurodegeneration (Tolnay *et al*, 1997). The increased levels of Fe and degeneration of nigrostriatal dopaminergic neurons have been determined to be a future characteristic of PD (Marshall *et al*, 2011). Moreover, the effect of HIV-1 proteins on Fe levels was demonstrated in primary human fetal adrenocortical (HFA) cells (Kaul *et al*, 2005). Results showed that viral proteins increased the concentration of ferritin (important role in the storage of Fe) and oxidative stress. Our results also showed that nicotine could alter the Fe (II) and H₂O₂ levels by increasing the formation of 6-hydroxydopamine (6-OHDA) which is formed through the oxidation of dopamine by Fe (II) and H₂O₂ in Fenton's reaction (Kimura *et al*, 1993b; Linert *et al*, 1999). Given this evidence, the effect of nicotine and HIV-1 proteins in the molecular mechanisms of oxidative stress signaling, we propose that nicotine potentiates the effect of HIV-1 proteins by stimulating dopaminergic neurons in the VTA. This stimulation may increase dopamine production and release from the VTA which contributes to excessive production of Fenton reagents and depletes antioxidant enzyme activity.

Taken together, our results show that a moderate dose of chronic nicotine treatment partially attenuates the effect of HIV-1 proteins in the NAc by up-regulating Sod1 expression levels. However, nicotine potentiates the neurotoxic effects of HIV-1 proteins and stimulates oxidative stress in the VTA of HIV-1 Tg rats.

We also evaluated nicotine's effects on mitochondrial function and oxidative stress induction related genes under normal physiological conditions by exposing F344 rats to chronic nicotine treatment. Our results showed that nicotine altered the expression of antioxidant defense mechanism related genes in a brain region-specific manner. A moderate dose of chronic nicotine treatment decreased the expression of glutathione-centered antioxidant enzyme Gpx1 and increased the Sod1 expression in the NAc of F344 rats. In contrast, nicotine increased the expression levels of glutathione-center antioxidant defense system enzymes (Gsta6, Gpx4, Gpx5, Gpx7 and Gstk1) and decreased Sod1 expression levels in the VTA. In fact, nicotine enhanced the expression of genes involved in p53-apoptosis signaling (Perp) and caspase signaling (Casp8) pathways in the VTA of F344 rats. These results indicate that nicotine showed brain region-specific alterations in the antioxidant enzyme expression and induction of oxidative stress signaling pathways. The antioxidant and neuroprotective properties of nicotine have been demonstrated by reported studies, which showed that the neurotoxic and neuroprotective actions of nicotine in the health state depend on the difference in experimental models and treatment dose of nicotine

(Kimura *et al*, 1993b; Picciotto and Zoli, 2008b). Guan *et al*. (Picciotto and Zoli, 2008b) showed that a low dose of nicotine (10 μ M) provided a protective effect against ROS production, whereas a high concentration of nicotine (10 mM) induced oxidative-stress mediated apoptosis in PC12 cells. In contrast, Linert *et al*. (Linert *et al*, 1999) showed that nicotine had no effect on the formation of ROS or neuronal damage in the neocortex, hippocampus and neostriatum of rats. The same group also showed the beneficial effects of nicotine in neocortical primary cells and indicated the possible effect of nicotine in formation of 6-OHDA through interaction between dopamine and Fenton's reagents. Soto-Otero *et al*. (Dandekar *et al*, 2005) reported that nicotine increased the production of hydrogen peroxide by 6-OHDA autoxidation that resulted in an increase in the neurotoxicity. Chronic nicotine exposure increases the dopamine release from VTA to NAc by regulating nicotinic acetylcholine receptors from VTA. Excessive levels of synaptic dopamine are eliminated by monoamine oxidase (MAO) which is associated with the formation of cellular oxidants (hydrogen peroxide) and oxidative stress induction. Increased production of hydrogen peroxide is scavenged by GSH-centered antioxidant enzymes that protect neurons against oxidative stress (Menon, 2001b). Therefore, brain regions with high concentrations of dopamine are more vulnerable to oxidative stress due to dopamine turnover and subsequent oxidation processes. Consistent with the literature, our results indicate that nicotine has a selective effect on oxidative stress mechanisms in the mesolimbic system. Nicotine increases the antioxidant defense mechanisms by regulating the expression of Sod1 in the NAc and GSHs in the VTA. However, a moderate dose of nicotine decreases the expression of Sod2 that results in an enhancement in the expression of genes related to apoptosis signaling (Casp8 and Perp) in the VTA.

5. Conclusion

Our study focused on the characterization of synergistic effects between HIV-1 proteins and nicotine on mitochondrial function, oxidative stress and apoptosis signaling in the dopaminergic midbrain regions. Our results indicate a novel interaction between nicotine and HIV-1 proteins in the mesolimbic brain regions where a moderate dose of chronic nicotine treatment partially attenuated the HIV-1 proteins, induced oxidative-stress mediated apoptosis by increasing the Sod1 expression, and decreasing the caspase signaling in the NAc. However, nicotine potentiated the effect of HIV-1 proteins in the VTA by decreasing the expression of genes in the antioxidant defense system and by increasing the levels of genes associated with apoptosis signaling. Further, we showed that nicotine exhibits a dual effect on the antioxidant defense system and oxidative stress mediated apoptosis signaling in a brain region-specific manner in F344 rats. These findings suggest that nicotine enhances the detrimental effects of HIV-1 proteins in the VTA and partially attenuates this effect in the NAc. Our findings need further investigation to elucidate the mechanisms of the possible interaction between nicotine and HIV-1 proteins in the dopaminergic pathways in response to psychostimulant drugs of abuse.

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Figure 1.

Brain region-specific effects of nicotine on the expression of genes in the glutathione-centered antioxidant defense system and apoptosis signaling in the NAc (left panel) and VTA (right panel) of HIV-1Tg rats. The relative quantity is presented in a log₁₀ scale. Bars indicate mean $\pm$ SEM. Statistical comparisons were made using two-tailed, unpaired Student's t-test between saline control and nicotine-treated groups. HS = Saline-treated HIV-1Tg rats; HN = Nicotine-treated HIV-1Tg rats (n = 4–6 per group).

Table 1

Primer sequences used for quantitative RT-PCR in this study

Gene Symbol	Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Atp6v1f	V-type proton ATPase subunit F	AGACACTTTCAGGCAGTTTCTAAACA	CCGAACCATCTCTGCGATGT
Abcd2	ATP-binding cassette sub-family D member 2	GATGGCCCTAAGCAGGCTATG	CAGTATAACCCGCTAGTTCAGTGATC
Atp5h	ATP-binding cassette sub-family D member 2	CCATCGATTGGGTATCTTTTGTG	TTATTCAGGACTTCAGAGCATTTC
Abcc8	ATP-binding cassette sub-family C member 8	ATCTCCCCAGGACAGAAGATTG	CGGAAAAAGGCGAGAGAGAA
Ndufa8	NADH dehydrogenase	GGAGGGCAAGCTGGTCAAC	AAAGGCTCCGCACAGTGACT
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	TCTTTGGAGATTTTGGCATGACT	TCATCTGTCCACTTCGATGGAT
Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	CGTGAGATTCGCATTCACTTATG	ACCGTTGCTGGATGAAATCTCT
Ndufb11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11	CCTGCCTGATTACAGGATGCA	TTCTCGGTATTTTCAACAAGTCTCTCA
Ndufs4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	AGGCAAAGGGCAGTAGCTACAG	CAGCTTCCATGTGCATGTGTTC
Cox1	Cytochrome c oxidase subunit I (mitochondrion)	GAGCAGTATTCGCCATCATAGCT	TTTGTCTCATGTGTCATTTAGGTAT
Cox2	Cytochrome c oxidase subunit II (mitochondrion)	CCGTCCCTTCACTAGGGTTAAAA	TGATGTCACTGTAGCTTGGTTTAGG
Cox3	Cytochrome c oxidase subunit 3	ACGAGATATCATCCGTGAAGGAA	TTCCGTATCGGAGGCCTTT
Cox5a	Cytochrome c oxidase subunit 5A, mitochondrial	TGGGCCTTGACAAAGTGTAAGT	TTCCAGGCAACTGTTTCAATCA
Cox6a	Cytochrome c oxidase subunit 6A1, mitochondrial	TGAAGTCGCGACACGAAGAG	GGGAAGGGCTTAGTCCTGATG
Cox8b	Cytochrome c oxidase subunit 8B, mitochondrial	CTCTTCCAAGCCAGCCAAAT	GCCAGCGATTATGACTGACATC
Sod1	Superoxide dismutase [Cu-Zn]	CACTGCAGGACCTCATTTTAATCC	GTCTCCAACATGCCTCTCTTCA
Sod2	Superoxide dismutase [Mn], mitochondrial	CGCTGGCCAAGGGAGAT	CCCCGCCATTGAACTTCA
Sod3	Extracellular superoxide dismutase [Cu-Zn]	GGCAGCTCAGAGGCTCTTTCT	CTGAGGTTCCACACCTGACAAG
Gpx1	Glutathione peroxidase 1	CTCGGTTTCCCGTGCAAT	TCGGACATACTTGAGGGAATTC
Gpx4	Glutathione peroxidase 4	ACGAGTTCCTGGGCTTGTGT	TGCGAATTCGTGCATGGA
Gpx5	Glutathione peroxidase 5	TGGTGAAAAAGAGCAGGAAATCT	CCCAGAATGTATGTTTCTTGTG
Gpx6	Glutathione peroxidase 6	CCCGTACCCAGTAGGATGTTTTA	TTGCAGGCTTTGATCATATGGA
Gpx7	Glutathione peroxidase 7 precursor	GCTGGTGTGCTGGAGAAGT	AGCCACATTGCTAGCTACATT

Gene Symbol	Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Gstk1	Glutathione S-transferase kappa 1	AACACCTCTGGAATATCAAGCTGAA	GTGGTTGGTTTCCACTGTCTTTC
Gstm5	Glutathione S-transferase Mu 5	TCACCCAGAGTAACGCCATCT	CTTCTTCGGTGTCAACACACA
Gsta6	Glutathione S-transferase A6	TCAACTGGAGGACACCTTGGA	CCCCGGGCCTCATCA
Gsr	Glutathione reductase	TGAGCCGCCTGAACAACA	TTGCGTAGCCGTGGATGAC
Perp	P53 apoptosis effector related to PMP-22	TGCCAGAGCCTCATGGAATAC	GAAGCAGATGACCAGGATGATG
Bmf	Bcl-2-modifying factor	ACAGATCGCCAGAAAGCTTCA	CTGCTGGTGTGTTGCATATGA
Bcl-2	Apoptosis regulator Bcl-2	GGAGGCTGGGATGCCTTTG	CTGAGCAGCGTCTTCAGAGACA
Bax	Apoptosis regulator BAX	CTCAAGGCCCTGTGCACTAAA	CCCGGAGGAAGTCCAGTGT
Bad	Bcl2 antagonist of cell death	CGGGACAGGCAGCCAATA	CGACTCCGGGTCTCCATAGTC
Bcl2L1	Bcl-2-like protein 1	GCAGTCAGCCAGAACCTATCT	GTCCAAAACACCTGCTCACTCA
Bcl2L11	Bcl-2-like protein 11	GGATCGGAGACGAGTTCAATG	CCTCTCGGTAATCGTTTGCAA
Casp1	Caspase-1	CCTCAAGTTTTGCCCTTTAGAAAT	TGAAAGTCTGTGCTGCAGATAATG
Casp2	Caspase-2	CCATGCACTCCTGAGTTTACCA	CGAGGCTGAGACTGCAACCT
Casp3	Caspase-3	AGGCCGACTTCCTGTATGCTT	TGACCCGTCCCTTGAATTTTC
Casp4	Caspase 4	TCAGGCAGCCACCATGGT	AGAGTGTCCACTGATTCTTCTGGTT
Casp6	Caspase 6	GAAAACCCAAGATATTCATCATCA	TCCACCACGTCCAGAGGAA
Casp7	Caspase-7	CATCTCATCCCTTCTCTGGAAGA	GCGGTCCCGGGTGGTA
Casp8	Caspase-8	CGAACGATCAAGCACAGAGAGA	CTGGCGAGTCCCACATGTC
Casp9	Caspase-9	GAGAGACATGCAGATATGGCATA	CAGAAGTTCACGTTGTTGATGATG
Camk2b	Calcium/calmodulin-dependent protein kinase type II subunit beta isoform 2	CATCACCAGCCCCAAAGG	TGGCATCTTCGTCTCTATGG

Table 2

Differentially expressed genes in the NAc between HIV-1Tg and F344 rats

Gene Symbol	F344 (Mean $\pm$ SEM)	HIV-1Tg (Mean $\pm$ SEM)	Ratio (HIV-1Tg/F344)	P-value
Calml1	0.972 $\pm$ 0.099	1.417 $\pm$ 0.457	1.458	0.042
Abcd2	0.017 $\pm$ 0.002	0.013 $\pm$ 0.002	0.762	0.017
Atp5h	0.204 $\pm$ 0.028	0.167 $\pm$ 0.010	0.818	0.013
Atp6v1f	0.156 $\pm$ 0.012	0.122 $\pm$ 0.012	0.777	0.0005
Cox5a	0.077 $\pm$ 0.004	0.056 $\pm$ 0.016	0.723	0.018
Cox6a	0.443 $\pm$ 0.060	0.342 $\pm$ 0.026	0.772	0.007
Ndufa2	0.046 $\pm$ 0.002	0.035 $\pm$ 0.004	0.754	0.0004
Ndufb2	0.027 $\pm$ 0.005	0.020 $\pm$ 0.001	0.738	0.009
Gstm5	0.035 $\pm$ 0.004	0.025 $\pm$ 0.003	0.709	0.002

Table 3

Differentially expressed genes in the VTA between HIV-1Tg and F344 Rats

Gene Symbol	F344 (Mean $\pm$ SEM)	HIV-1Tg (Mean $\pm$ SEM)	Ratio (HIV-1Tg/F344)	P-value
Cox1	21.371 $\pm$ 1.912	27.430 $\pm$ 5.076	1.284	0.021
Cox3	14.918 $\pm$ 1.319	20.230 $\pm$ 4.55	1.356	0.021
Gsta6	0.008 $\pm$ 0.0006	0.011 $\pm$ 0.002	1.286	0.045
Perp	0.003 $\pm$ 0.0006	0.005 $\pm$ 0.001	1.545	0.033
Abcd2	0.012 $\pm$ 0.002	0.008 $\pm$ 0.004	0.653	0.044
Atp5h	0.174 $\pm$ 0.028	0.127 $\pm$ 0.026	0.730	0.013
Atp6v1f	0.153 $\pm$ 0.017	0.123 $\pm$ 0.020	0.809	0.022
Ndufa2	0.064 $\pm$ 0.017	0.042 $\pm$ 0.016	0.651	0.042
Ndufa8	0.137 $\pm$ 0.016	0.106 $\pm$ 0.028	0.778	0.044
Ndufb2	0.029 $\pm$ 0.009	0.019 $\pm$ 0.0040	0.640	0.025
Cox8b	0.00002 $\pm$ 0.000005	0.00002 $\pm$ 0.000001	0.524	0.021
Casp9	0.006 $\pm$ 0.001	0.005 $\pm$ 0.0007	0.750	0.002

Table 4 Significantly regulated genes by nicotine in the NAc and VTA of HIV-1T_g rats

Brain Region	Gene symbol	HIV-1T _g Saline (Mean ± SEM)	HIV-1T _g Nicotine (Mean ± SEM)	Nicotine/Saline Ratio	P-value
NAc	Atp5h	0.167 ± 0.004	0.119 ± 0.013	0.715	0.004
	Calml1	1.417 ± 0.457	0.644 ± 0.109	0.454	0.005
	Casp2	0.005 ± 0.0005	0.004 ± 0.0004	0.846	0.007
	Casp7	0.002 ± 0.0003	0.002 ± 0.0001	0.809	0.017
	Gsta6	0.012 ± 0.001	0.007 ± 0.0007	0.609	0.016
	Gpx7	0.004 ± 0.0006	0.003 ± 0.0007	0.757	0.049
	Gsr	0.022 ± 0.002	0.016 ± 0.0008	0.712	0.007
	Gstm5	0.025 ± 0.001	0.015 ± 0.002	0.594	0.002
	Sod1	0.071 ± 0.007	0.119 ± 0.018	1.687	0.028
	Bcl2L11	0.0006 ± 0.00003	0.0008 ± 3.39E-05	1.267	0.009
	Casp9	0.004 ± 0.0002	0.006 ± 0.0001	1.281	0.001
	Casp1	0.0006 ± 5.81E-05	0.0004 ± 4.52E-05	0.661	0.034
VTA	Cox5a	0.067 ± 0.017	0.048 ± 0.004	0.721	0.046
	Gpx4	0.004 ± 0.0004	0.003 ± 0.0002	0.729	0.038
	Gpx6	0.0002 ± 0.0001	0.00005 ± 0.00002	0.267	0.048
	Gpx7	0.004 ± 0.001	0.003 ± 0.0006	0.714	0.046
	Gstm5	0.080 ± 0.003	0.065 ± 0.004	0.813	0.029
	Sod1	0.147 ± 0.050	0.085 ± 0.026	0.576	0.022

Table 5

Significantly regulated genes by nicotine in the NAc and VTA of F344 rats

Brain Region	Gene Symbol	F344_Saline (Mean $\pm$ SEM)	F344_Nicotine (Mean $\pm$ SEM)	Nicotine/Saline Ratio	P-value
NAc	Sod1	0.080 $\pm$ 0.009	0.154 $\pm$ 0.025	1.935	0.034
	Atp6v1f	0.156 $\pm$ 0.005	0.135 $\pm$ 0.005	0.864	0.015
	Gpx1	0.014 $\pm$ 0.0008	0.009 $\pm$ 0.0009	0.681	0.005
VTA	Casp8	0.0004 $\pm$ 3.51E-05	0.0005 $\pm$ 3.78E-05	1.421	0.031
	Cox8b	2.14E-05 $\pm$ 2.13E-06	0.0001 $\pm$ 4.07E-05	5.233	0.023
	Gsta6	0.008 $\pm$ 0.0006	0.013 $\pm$ 0.003	1.595	0.008
	Gpx4	0.003 $\pm$ 0.0003	0.004 $\pm$ 0.0002	1.455	0.004
	Gpx5	0.00005 $\pm$ 0.00001	0.0001 $\pm$ 0.00004	2.5	0.011
	Gpx7	0.004 $\pm$ 0.0009	0.005 $\pm$ 0.0006	1.328	0.021
	Gstk1	0.019 $\pm$ 0.002	0.024 $\pm$ 0.001	1.270	0.022
	Perp	0.003 $\pm$ 0.0006	0.004 $\pm$ 0.0004	1.242	0.035
	Sod2	0.085 $\pm$ 0.005	0.065 $\pm$ 0.004	0.766	0.015