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HIV-1 transgenic rats display an increase in [^{3}H]dopamine uptake in the prefrontal cortex and striatum

Jun Zhu[1,*], **Yaxia Yuan**[2,3], **Narasimha M. Midde**[1], **Adrian M. Gomez**[1], **Wei-Lun Sun**[1], **Pamela M. Quizon**[1], and **Chang-Guo Zhan**[2,3]

[1]Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC 29208

[2]Molecular Modeling and Biopharmaceutical Center, University of Kentucky, Lexington, KY 40536

[3]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536

Abstract

HIV viral proteins within the central nervous system are associated with the development of neurocognitive impairments in HIV-infected individuals. Dopamine transporter (DAT)-mediated dopamine transport is critical for normal dopamine homeostasis. Abnormal dopaminergic transmission has been implicated as a risk determinant of HIV-induced neurocognitive impairments. Our published work has demonstrated that Tat-induced inhibition of DAT is mediated by allosteric binding site(s) on DAT, not the interaction with the dopamine uptake site. The present study investigated whether impaired DAT function induced by Tat exposure *in vitro* can be documented in HIV-1 transgenic (HIV-1Tg) rats. We assessed kinetic analyses of [^{3}H]dopamine uptake into prefrontal and striatal synaptosomes of HIV-1Tg and Fisher 344 rats. Compared with Fisher 344 rats, the capacity of dopamine transport in the prefrontal cortex (PFC) and striatum of HIV-1Tg rats was increased by 34% and 32%, respectively. Assessment of surface biotinylation indicated that DAT expression in the plasma membrane was reduced in PFC and enhanced in striatum, respectively, of HIV-1Tg rats. While the maximal binding sites (B_{max}) of [^{3}H]WIN 35,428 was decreased in striatum of HIV-1Tg rats, an increase in DAT turnover proportion was found, relative to Fisher 344 rats. Together, these findings suggest that neuroadaptive changes in DAT function are evidenced in the HIV-1Tg rats, perhaps compensating for viral protein-induced abnormal dopaminergic transmission. Thus, our study provides novel insights into understanding mechanism underlying neurocognitive impairment evident in neuroAIDS.

Keywords

HIV-1 tat; transgenic rat; DA uptake; dopamine transporter; trafficking; striatum

[*]Corresponding author: Jun Zhu, M.D., Ph.D., Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, 715 Sumter Street, Columbia, SC 29208, USA, Tel: +1-803-777-7924; Fax: +1-803-777-8356, zhuj@sccp.sc.edu.

Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

Although the common use of efficacious antiretroviral therapies to control HIV infection and improve the life of HIV patients, HIV-1-associated neurocognitive disorders (HAND) are a highly prevalent and significant health problem (Heaton *et al*, 2010; Mothobi and Brew, 2012; Simioni *et al*, 2010). The incidence (~70%) of HAND is dramatically increased due to substance abuse, such as cocaine (Buch *et al*, 2011; Fiala *et al*, 1998; Larrat and Zierler, 1993; Norman *et al*, 2009; Webber *et al*, 1999). Viral replication and proviral DAT induction within the central nervous system (CNS) in early HIV-1 infection (Nath and Clements, 2011) have been implicated as a risk determinant of HAND (Berger and Arendt, 2000; Purohit *et al*, 2011). Since most antiretroviral therapy medications cannot cross the blood-brain barrier (Buckner *et al*, 2006), these medications have no influence on the production of viral proteins in the CNS. Therefore, viral proteins are associated with the persistence of HIV infection-induced neuropathology and subsequent cognitive deficits (Brack-Werner, 1999; Frankel and Young, 1998; Johnston *et al*, 2001; Power *et al*, 1998). Prolonged exposure to the viral protein impairs the central dopamine (DA) system (Berger and Arendt, 2000; Koutsilieri *et al*, 2002; Nath *et al*, 1987) and the brain pathways controlling motivation (Berridge, 2007; Everitt and Robbins, 2005; Wise and Bozarth, 1987). The HIV regulatory protein, transactivator of transcription (Tat) protein has pivotal effects on the neurotoxicity and cognitive dysfunction evident in neuroAIDS (Rappaport *et al*, 1999). Tat and cocaine exacerbates the development of cognitive impairments in HIV infected individuals (Gannon *et al*, 2011). Viral replication in HIV-1 infected macrophages/microglia within dopaminergic brain regions results in Tat induction (Gaskill *et al*, 2009). The interplay of Tat with cocaine augments synaptic DA levels by inhibiting DA transporter (DAT) activity (Ferris *et al*, 2010). Importantly, the augmented DA levels by Tat and cocaine further stimulates viral replication in human macrophages within DA-rich brain regions, resulting in viral protein release (Gaskill *et al*, 2009), and this contributes to the pathophysiology of HAND (Li *et al*, 2009; Purohit *et al*, 2011). Additionally, recent studies show that other viral proteins, such as gp120 and Nef also influence DAT activity (Acharjee *et al*, 2014; Hu *et al*, 2013). On the other hand, clinical observations have demonstrated that DAT activity is significantly reduced in HIV-infected individuals with cocaine use (Chang *et al*, 2008; Wang *et al*, 2004), which is correlated with impaired learning and memory performance (Hsieh *et al*, 2010; Mozley *et al*, 2001). Reports from the observations made in postmortem brain tissue from HAND patients found significantly increased DAT expression in the striatum (Gelman *et al*, 2006) but no changes in DAT levels in the substantia nigra (Silvers *et al*, 2007). In the early stage of HIV infection, increased levels of DA are found in the cerebrospinal fluid of therapy naïve HIV patients in asymptomatic infection (Scheller *et al*, 2010), which may contribute to decreased levels of DA in DA-rich brain areas in the advanced stages of HIV infection (Kumar *et al*, 2009; Sardar *et al*, 1996). Currently, it is unclear how HIV-1 viral proteins impair the DA system in the CNS of the patients with HAND, thereby producing neurocognitive impairment.

In the last several years, we have studied molecular mechanisms underlying the interplay of HIV-1 Tat with cocaine and DAT. Despite our published data demonstrate that Tat *in vitro* modulates DAT activity allosterically (Midde *et al*, 2012; Midde *et al*, 2013; Zhu *et al*, 2011;

Zhu *et al*, 2009b), it is unclear whether DAT function is altered in a rodent model that are exposed acutely or chronically to low levels of Tat protein. Since HIV does not infect rodents, several approaches have been used for studying the effects of viral proteins on DA system through: 1) rodent brain tissue in the presence of recombinant Tat (Zhu *et al*, 2009b), 2) intra-brain region infusion of recombinant Tat (Ferris *et al*, 2009; Harrod *et al*, 2008), 3) Tat transgenic mouse model (Kim *et al*, 2003), and 4) HIV-1 transgenic (HIV-1Tg) rat model that mimics the conditions of HIV-1-infected patients receiving antiretroviral medications in which viral replication is substantially suppressed, but viral proteins continually expressed throughout the animal life (Ray *et al*, 2003; Reid *et al*, 2001). Tat transgenic mice exhibit a significant increase in DAT levels (Perry *et al*, 2010) and deficits in learning and memory performance (Carey *et al*, 2012). The HIV-1Tg rats carry the *gag-pol* deleted HIV-1 provirus and continually express seven viral proteins: env, tat, rpr, rev, vif, vpu, and nef (Reid *et al*, 2001). Although no differences in DAT mRNA levels (Liu *et al*, 2009) and DAT immunoreactivity (Webb *et al*, 2010) were found in the brains between HIV-1Tg and control Fisher 344 rats, HIV-1Tg rats exhibited a greater affinity for cocaine inhibiting DAT binding site compared to control Fisher 344 rats (McIntosh *et al*, 2015). This study was set to characterize DAT activity and its distribution in the plasma membrane of the prefrontal cortex (PFC) and striatum in HIV-1Tg rats.

Materials and Methods

Subjects

Male HIV-1Tg and Fisher 344 (F344) rats at age of 13 weeks were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). Rats were pair housed in a temperature (21 ± 2 °C)- and humidity (50 ± 10%)-controlled vivarium which was maintained at, on a 12-h light/dark cycle with lights on at 0700 h (EST) as reported previously (Midde *et al*, 2011). F344 rats were used as the controls. Rodent food (Pro-Lab Rat, Mouse Hamster Chow #3000) and water were provided *ad libitum*. All rats were habituated for one week prior to all experiments. Animals were maintained according to the National Institute of Health (NIH) guidelines in AAALAC accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina, Columbia.

Synaptosomal Preparation

Drug-naïve rats were rapidly decapitated and the dissection of PFC and striatum were conducted on an ice-cold plate. The PFC and striatum were chosen because these two regions are critical to HIV infection-mediated neurocognitive and motivation impairments (Chang *et al*, 2008; Coulehan *et al*, 2014). In addition, DAT is relatively expressed in the PFC and striatum (Zhu *et al*, 2005). Synaptosomes were prepared using our published method (Zhu *et al*, 2004). Bovine serum albumin was used as the standard (Bradford, 1976) to measure protein concentration for all samples.

[^{3}H]DA Uptake Assay

To assess the difference in DAT reuptake between HIV-1Tg and F344 rats, the maximal velocity (V_{max}) or Michaelis-Menten constant (K_m) of [^{3}H]DA uptake were conducted in

the PFC or striatum of these animals as described previously (Zhu *et al*, 2004). Notably, DA uptake into prefrontal synaptosomes was performed in the presence of desipramine (1 μM), a norepinephrine transporter inhibitor, and paroxetine (5 μM), a serotonin transporter inhibitor as reported previously (Zhu *et al*, 2004). To determine whether the genetic expression of HIV-1 viral proteins alters the inhibitory effects of substrate and DAT inhibitors on specific [^{3}H]DA uptake into striatal synaptosomes, IC_{50} values for these substrate and inhibitors inhibiting [^{3}H]DA uptake were examined in the presence of various concentrations of DA, cocaine, WIN 35,428 or GBR12909.

Biotinylation and Western Blot Assays

To determine whether viral proteins alter DAT distribution between the plasma membrane and cytoplasmic compartment, we performed cell surface biotinylation assay followed by DAT Western blotting. Synaptosomes from the PFC and striatum were prepared as described above. Biotinylation and DAT Western blotting assays were performed to determine DAT expression in total levels, plasmalemma, and cytoplasm membrane using a previously published method (Zhu *et al*, 2005).

[^{3}H]WIN 35,428 Binding Assay

[^{3}H]WIN 35,428 binding sites are pharmacologically responsible for the characters with DA transport carrier and the cocaine binding domain (Reith and Coffey, 1994). To determine whether viral proteins alter DA uptake sites, we performed kinetic analysis of [^{3}H]WIN 35,428 binding in striatum of HIV-1Tg and F344 rats. Synaptosomes were prepared as described above and [^{3}H]WIN 35,428 binding assay was determined using previously described methods (Zhu *et al*, 2007).

Molecular Modeling

DAT-mediated DA transport involves three typical conformational states of DAT: outward-open state (*i.e.* the extracellular side of substrate-binding site for the transmitter is open, while the intracellular side is blocked); the outward-occluded state (*i.e.* both the extracellular and intracellular sides of binding site are blocked such that the binding site is occluded and no longer accessible for substrate); and the inward-open state (*i.e.* the intracellular side of substrate-binding site is open, while the extracellular side is blocked)(Forrest *et al*, 2007; Penmatsa *et al*, 2013; Singh *et al*, 2007; Wang *et al*, 2012; Yamashita *et al*, 2005).

Based on the reported structures of DAT in three typical conformational states (Huang and Zhan, 2007; Yuan *et al*, 2015), Tat binds most favorably with the outward-open state of DAT in our previous work (Yuan *et al*, 2015), and cocaine also binds to the outward-open state of DAT (Huang *et al*, 2009). The structures of DAT and its complexes with Tat and cocaine all came from the previously reported molecular dynamics (MD) simulation studies (Huang *et al*, 2009; Huang and Zhan, 2007; Yuan *et al*, 2015). The MD-simulated structures were energy-minimized further by using the AMBER 12 (Case *et al*, 2012) program package. Then, the PyMol software (Schrödinger, 2010) was used to visualize the structures (depicted in Figure 6).

Data Statistical Analysis

Data are expressed as mean values ± S.E.M., and n means the number of independent experiments for individual treatment group. IC_{50} values for inhibiting [^{3}H]DA uptake or [^{3}H]WIN 35,428 were examined from inhibition curves by nonlinear regression analysis using a single site model with variable slope. Kinetic parameters (K_m and V_{max} for [^{3}H]DA uptake; K_d and B_{max} for [^{3}H]WIN 35,428 binding) were examined from saturation curves by nonlinear regression analysis using a single site model with variable slope. These kinetic parameters involving comparisons between groups were analyzed using unpaired Student's *t* tests. DAT expression levels were expressed as the ratio of DAT immunoreactivity to control proteins, and analyzed by separate unpaired Student's *t* tests. IBM SPSS Statistics version 20 was used for all statistical analyses, and differences at $p < 0.05$ were determined as significant.

Results

HIV-1Tg rats exhibit an increase in synaptosomal [^{3}H]DA uptake in the PFC and striatum

We performed kinetic analyses of synaptosomal [^{3}H]DA uptake to determine the differences between HIV-1Tg and F344 rats. In the PFC, the V_{max} values for [^{3}H]DA uptake were significantly increased by 34 ± 2.0 % in HIV-1Tg rats (1.2 ± 0.06 pmol/mg/min) compared with F344 rats [0.8 ± 0.04 pmol/mg/min; $t_{(12)} = 2.6$, $p < 0.05$] (Figure 1A). There was no change in the K_m between HIV-1Tg rats (63 ± 2.9 nM) and F344 rats (75 ± 3.9 nM, Figure 1B). Similarly, in the striatum, the V_{max} values were significantly increased by 32 ± 3.6% in HIV-1Tg rats (26.1 ± 2.90 pmol/mg/min) compared with F344 rats [17.8 ± 1.89 pmol/mg/min; $t_{(12)} = 2.4$, $p < 0.05$] (Figure 2A). The K_m values were increased in HIV-1Tg rats (62 ± 1.9 nM) relative to F344 rats [41 ± 3.9 nM; $t(12) = 2.8$, $p < 0.05$, Figure 2B]. Therefore, HIV-1 viral proteins enhanced DAT reuptake function in both PFC and striatum of HIV-1Tg rats. There were no changes in the IC_{50} values for DA, cocaine, WIN 35,428 or GBR12909 inhibiting [^{3}H]DA uptake between HIV-1Tg and F344 rats (Table 1).

The DAT expression was altered in HIV-1Tg rats

We next assessed cell surface biotinylation and immunoblotting assays to determine whether the increased V_{max} in the PFC and striatum of HIV-1Tg rats was result from changes in DAT expression in the plasma membrane. As illustrated in Figure 3, in the PFC, DAT expression in total and non-biotinylated (cytoplasmic pool) fractions were not different between HIV-1Tg and F344 rats, whereas DAT expression in the biotinylated fraction (plasma membrane) from HIV-1Tg rats was lower (37 ± 4.0%) than that in F344 rats [$t_{(6)} = 2.2$, $p < 0.05$]. In the striatum (Figure 4), no difference in total DAT expression between HIV-1Tg and F344 rats was found, whereas DAT expression in non-biotinylated fraction from HIV-1Tg rats was reduced by 22 ± 2.5% relative to F344 rats [$t_{(6)} = 2.2$, $p < 0.05$]. DAT expression in the biotinylated fraction from HIV-1Tg rats was higher (23 ± 1.5%) than that in F344 rats [$t_{(6)} = 2.4$, $p < 0.05$]. These results suggest a region specific correlation between V_{max} and DAT cell surface.

HIV-1Tg rats exhibit a decrease in [^{3}H]WIN 35,428 binding in striatal synaptosomes

We next determined the B_{max} and K_d of [^{3}H]WIN 35,428 binging in striatum from HIV-1Tg and F344 rats. As shown in Figure 5A, the B_{max} value of [^{3}H]WIN 35,428 binding was reduced by 25 ± 0.2% (27.3 ± 0.7 pmol/mg/protein) in HIV-1Tg rats compared with F344 rats (34.0 ± 2.0 pmol/mg/protein; $t_{(6)} = 2.1$, $p < 0.05$]. There were no changes in the K_d between HIV-1Tg (16.4 ± 2.0 nM) and F344 rats [19.3 ± 2.4 nM; $t_{(6)} = 1.8$, $p = 0.0589$]. Thus, taking into account the increase DA uptake (V_{max}) in striatum of HIV-1Tg rats (Figure 2A), a significant increase in uptake turnover rate (V_{max}/B_{max}) in striatum of HIV-1Tg rats (1.5 ± 1.6) was revealed, as compared to in F344 rats [0.78 ± 0.05; $t_{(6)} = 3.1$, $p < 0.05$] (Figure 5B). Note: on Oct 10 (in China), I did online check proof, and found the values for Figure 5B and C not match: so yellow highlighted part was changed to: a significant increase in uptake turnover rate (V_{max}/B_{max}) in striatum of HIV-1Tg rats (1.6 ± 0.19) was revealed, as compared to in F344 rats [0.76 ± 0.07; $t_{(6)} = 3.8$, $p < 0.01$] (Figure 5B). Jun also added new sentence for Figure 5C as “In addition, asignificant decrease in the ratio (K_m/K_d) in HIV-1Tg rats (0.2 ± 0.04) was found, as compared to F344 rats [0.5 ± 0.05; $t_{(6)} = 4.1$, $p < 0.01$] (Fig 5C). There were no changes in the IC_{50} values for substrate and DAT inhibitors inhibiting [^{3}H]WIN 35,428 binding in striatum of HIV-1Tg and F344 rats (Table 1).

Discussion

This study investigated whether the genetic expression of HIV-1 viral proteins alters DAT reuptake function and expression in the rat PFC and striatum. The major finding is that HIV-1Tg rats exhibit increased DAT reuptake in both PFC and striatum, which is opposite to our previous finding showing decreased DAT reuptake *in vitro* in rat synaptosomes and cells expressing hDAT in the presence of Tat protein (Midde *et al*, 2013; Midde *et al*, 2015; Zhu *et al*, 2009b). Moreover, we found that the increased V_{max} in the PFC and striatum is accompanied by distinctly different alterations in DAT expression in the plasma membrane in a brain region-specific manner. Furthermore, we found that HIV-1Tg rats exhibit decreased B_{max} for [^{3}H]WIN 35,428 binding in striatum relative to F344 rats. Considering the increased turnover proportion of DA transport, HIV-1Tg rats may have a neuroadaptive change in DAT function to compensate for viral protein-induced abnormal dopaminergic transmission.

The current findings show that the V_{max} was increased by 34% and 32% in PFC and striatum, respectively, with a slight decrease in the K_m in striatum but not in the PFC. This findings support the previous studies showing Tat-induced increased DAT-DA uptake in primary neurons of Tat-expressing transgenic mice (Perry *et al*, 2010) and the elevated DAT levels in striatum of and HIV-infected individuals (Gelman *et al*, 2006). In contrast, Tat inhibits [^{3}H]DA uptake in rat striatal synaptosomes and cells expressing hDAT *in vitro* (Midde *et al*, 2013; Zhu *et al*, 2009b). Several possibilities may explain the discrepancy between *in vitro* and *in vivo* observations. First, Tat *in vivo* regulates DAT function and expression by disrupting neuronal signaling pathways, such as GSK-3β, thereby elevating DA transport activity and DAT expression in plasma membrane in primary neurons or animals (Perry *et al*, 2010). However, *in vitro* Tat-induced decrease in DA transport and DAT plasma membrane expression in rat synaptosomes could be due to the isolation of

synaptosomes from the neuronal regulatory pathway. Second, the different types of Tat and its concentration may also cause the different Tat effects observed *in vitro* and *in vivo*. For example, a detectable Tat concentration in the frontal cortex of HIV-1 infected individuals is about 140 pmol (Hudson *et al*, 2000), whereas ~500 nM concentration of recombinant $Tat_{1–86}$ was used in the synaptosomal DA uptake (Zhu *et al*, 2009b). Importantly, the native HIV-1 Tat actually detected in the sera (Westendorp *et al*, 1995) or brain (Hudson *et al*, 2000) of HIV-1 infected patients has more biological function and is more neurotoxic to neuronal targets, such as DAT. Third, as documented in the recent reports, the decreased DAT function is caused by not only Tat but also gp120 and Nef (Acharjee *et al*, 2014; Hu *et al*, 2013), implicating that DAT induction could be differentially modulated by the combined or individual of these HIV-1 viral proteins. Nevertheless, results from the present study and another (Perry *et al*, 2010) demonstrate that DAT function can be modulated by viral proteins despite increased V_{max} and increased DAT cell surface expression, which may be a compensatory response to decreased transporting efficiency of individual DAT molecules.

The efficacy of DA uptake largely depends on DAT expression in the plasma membrane, which is dynamically modulated by a trafficking mechanism (Zhu and Reith, 2008). In general, surface biotinylation assay (Zhu *et al*, 2005; Zhu *et al*, 2009a) or sub-fractionation method (Middleton *et al*, 2007) are extensively used to assess the DAT trafficking. In the present observation, HIV-1Tg rats exhibit a 37% increase in striatal DAT expression in plasma membrane, which is comparable to the magnitude (32%) of the increase in V_{max}. However, the magnitude levels of the changes in DAT in plasma membrane were less than (Zhu *et al*, 2009a) or parallel (Zhu *et al*, 2005) to the magnitude of the alterations in V_{max}. This suggests that HIV-1 viral proteins regulate DAT function in striatum via a trafficking-dependent mechanism. Contrasting with the current results, we have demonstrated that *in vitro* Tat exposure decreases DAT expression in plasma membrane and increases DAT level in cytoplasmic compartment without changing the total DAT level in rat striatal synaptosomes (Midde *et al*, 2012). Tat-mediated reduction in the efficiency of DA transport is generally regulated by (1) increasing DAT protein degradation, (2) decreasing DAT turnover efficacy, and/or (3) changing in DAT trafficking on the plasma membrane without changing the DAT protein. Our current results and previous report (Midde *et al*, 2012) demonstrate that total DAT expression in the striatum was not altered after *in vitro* Tat exposure and in HIV-1Tg rats, indicating that Tat-induced changes in DAT reuptake is not caused by DAT degradation. Results from [^{3}H]WIN 35,428 binding experiments show that an reduction in the B_{max} was observed in striatum of HIV-1Tg rats, which is supported by our previous report showing the decreased specific [^{3}H]WIN 35,428 after *in vitro* exposure of rat striatal synaptosomes to Tat protein (Midde *et al*, 2012). DAT turnover proportion reveals the efficacy of DA molecules transported per second per site (Lin *et al*, 2000). Considering the reduced B_{max} along with the increased V_{max} in striatum of HIV-1Tg rats, an increase in DA uptake turnover (V_{max}/B_{max}) was found; however, *in vitro* exposure of synaptosomes to Tat did not affect DA uptake turnover (Midde *et al*, 2012). This may help explain the difference in Tat-induced redistribution of DAT (e.g. upregulation or downregulation of DAT plasma membrane expression) between the present observation and our previous report (Midde *et al*, 2012).

Of interest, our previous report showed that Tat *in vitro* increased IC_{50} value of cocaine inhibiting synaptosomal [^{3}H]DA uptake in striatum, indicating that Tat decreases cocaine's affinity on DAT (Zhu *et al*, 2011). However, the Tat's effect was not documented in HIV-1Tg rats. One possible reason is that Tat expression in HIV-1Tg rats is lower than the concentration *in vitro* study, suggesting that Tat modulates cocaine's binding sites in a concentration-dependent manner. We hypothesize that Tat protein allosterically regulates DAT activity (Zhu *et al*, 2011; Zhu *et al*, 2009b). Indeed, previous study showed that SRI-20041, a novel DAT allosteric modulator, altered cocaine's affinity in a concentration-dependent manner (Pariser *et al*, 2008). Therefore, determining Tat concentration-dependent effect on cocaine's affinity for inhibiting DA uptake will be essential for future study. Notably, viral protein-induced increase of DAT expression is similar to cocaine-induced increase of DAT expression in striatum. For example, chronic administration of cocaine upregulates striatal DAT expression in animals and humans (Schmitt and Reith, 2010), because cocaine can block DA uptake by competitive binding with DA uptake sites in DAT (Huang *et al*, 2009). Mechanistically, as illustrated in Figure 6, HIV-1 Tat can block DA uptake by allosteric modulation of DA transport (Midde *et al*, 2013; Midde *et al*, 2015; Zhu *et al*, 2009b). Specifically, Tat also binds to DAT in the outward-open state; however, the Tat-DAT binding is not to interfere DA binding to DAT rather than prevent the further conformational change of DAT from the outward-open state to the other states (outward-occluded and inward-open states) such that DA cannot be transported by DAT (Yuan *et al*, 2015). Therefore, both cocaine and Tat can independently (and synergistically) disrupt the process of DA transport and, thus, could have the similar role in modulation of the DAT function and expression. Hence, Tat protein likely contributes to the increase of DAT expression in striatum of HIV-1Tg rats observed in the current study.

Assessment of surface biotinylation indicates that the expression of a higher amount of DAT in the striatal plasma membrane, but a relative lower DAT density in the PFC in HIV-1Tg rats, suggesting a region-specific regulatory mechanism underlying DAT function and plasma membrane expression. For example, previous study shows that V_{max} for DA uptake was elevated in both rat striatum and nucleus accumbens; however, the elevated V_{max} was accompanied by increased cell surface DAT in the striatum but not in nucleus accumbens (Samuvel *et al*, 2008). Regulation of DAT function and trafficking involves in DAT distribution, protein-protein interaction, DAT phosphorylation, and presynaptic receptors (Zhu and Reith, 2008), suggesting that DAT expression in the plasma membrane is not always consistent with changes in DAR reuptake. In general, the process of DA uptake reflects a proportion of the DAT expression in the plasma membrane. It is possible that the regulations of DA transport and DAT expression in the PFC of HIV-1Tg rats are mediated via two independent mechanisms. First, the increased V_{max} in the PFC was not supported by elevated DAT in plasma membrane, suggesting a trafficking-independent pathway. Although the current study did not directly determine DAT phosphorylation, our recent report demonstrated that distinct basal phosphorylated levels of cAMP response element binding protein (pCREB) and extracellular regulated kinase 2 (pERK2) were observed among the PFC, nucleus accumbens and ventral tegmental area between HIV-1Tg and F344 rats (Midde *et al*, 2011). Therefore, we speculate that the different phosphorylation levels of signaling pathways may account for the opposite correlations between V_{max} and DAT in the plasma

membrane between the PFC and striatum of HIV-1 Tg rats. Furthermore, the basal level of pERK2 in the PFC is higher in HIV-1Tg rats than that in F344 rats (Midde *et al*, 2011), whereas the inhibition of MEK/ERK activity produces an reduction in DAT expression in plasma membrane (Lin and Uhl, 2003). Second, DA D1 receptor in the PFC has been shown to be highly expressed in HIV-1Tg rats than F344 rats (Liu *et al*, 2009). Given that activation of D1 receptor is associated with an increase in DA transport (Kimmel *et al*, 2001), the increased V_{max} in the PFC of HIV-1Tg rats could be mediated by viral protein-induced increase in D1 expression. Finally, our data showing increased V_{max} along with a lower plasma membrane DAT expression in the PFC suggest a compensatory functional enhancement of efficiency of DAT molecules. Future studies involving determination of the effects of ERK and other signaling proteins, and DA receptors on surface DAT expression in the PFC are necessary to fully understand how viral proteins modulates DAT function and DAT redistribution.

One caveat is that the current study cannot address which viral protein contributes to the alteration of DAT reuptake and DAT plasma membrane expression in HIV-1Tg rats. Although the levels of viral proteins in the PFC and striatum were not measured in this study, previous report has indicated that the mRNA levels of Tat, pg120, nef and vif are detected in these two regions of the HIV-1Tg rats. Particularly, the detected Tat levels are region-specific and age-dependent changes with a higher level in the PFC during 2–3 months and in striatum during 10–11 months (Peng *et al*, 2010). Therefore, determining DAT activity in HIV-1Tg rats across different ages will be essential future work. Several studies have demonstrated that among the viral proteins, Tat plays a critical role in viral protein-induced neurotoxicity (Rappaport *et al*, 1999). For example, our previous report shows that HIV-1Tg rats exhibited attenuated nicotine-mediated behavior sensitization and altered pERK1/2, a protein kinase associated with cell death (Kulich and Chu, 2001; Stanciu *et al*, 2000), in the mesocorticolimbic regions (Midde *et al*, 2011). These behavioral and neurochemical alterations in HIV-1Tg rats can be documented in rats with intra-ventral tegmental area injection of HIV-1 $Tat_{1–86}$ (Zhu *et al*, 2015). Given that gp120 and Nef also inhibit DAT activity (Acharjee *et al*, 2014; Hu *et al*, 2013), the current results may represent a combination effect of viral proteins on DAT activity. Nevertheless, the current findings showing viral protein-mediated alterations in DAT activity provide insights into understanding the role of DA neurotransmission in HIV-1-induced cognitive impairment in HIV-infected individuals.

In conclusion, the present findings suggest that HIV-1Tg animals exhibit neuroadaptive changes in DAT reuptake and DAT plasma membrane expression in the PFC and striatum, which, at least in part, support a compensatory mechanism underlying viral protein-induced inhibition of DAT function observed *in vitro*. We hypothesize that such compensatory response to viral proteins-mediated disruption of DAT reuptake may occur in early HIV-1 infection. Long lasting viral protein-induced dysfunction of DAT activity eventually causes DAT-mediated dysregulation of dopaminergic transmission to accelerate the progression of HAND. Therefore, an intervention for HIV infection-induced dysfunction of DA system has the potential to improve neurocognitive function in patients with the early-stage o HAND. Identifying the binding sites in DAT where Tat interacts with will provide insights into exploring molecular targets for developing potential compound(s) that specifically block Tat

binding site(s) in DAT without affecting DA transport. Ideally, the effectiveness of early intervention for HAND may combine such compounds with anti-retroviral therapy, which will be beneficial to the preservation of neurocognitive function in patients with HAND.

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Abbreviations

AIDS	acquired immunodeficiency syndrome
B_{max}	maximal number of [^{3}H]ligand binding sites
DA	dopamine
DAT	dopamine transporter
EDTA	ethylenediamine tetraacetic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
GBR 12909	1-[2-(*bis*[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine
HBS-TE	HEPES Buffered Saline with Tween 20 and EDTA
hDAT	human dopamine transporter
HIV	human immunodeficiency virus
HAD	HIV-associated dementia
K_d	equilibrium dissociation constant
K_m	Michaelis-Menten constant
Tat	trans-activator of transcription
Tween 20	polyethylene glycol sorbilan monolaurate
V_{max}	maximal velocity
WIN 35	428, 2β-Carbomethoxy-3-β-(4-fluorophenyl)tropane
MD	molecular dynamics

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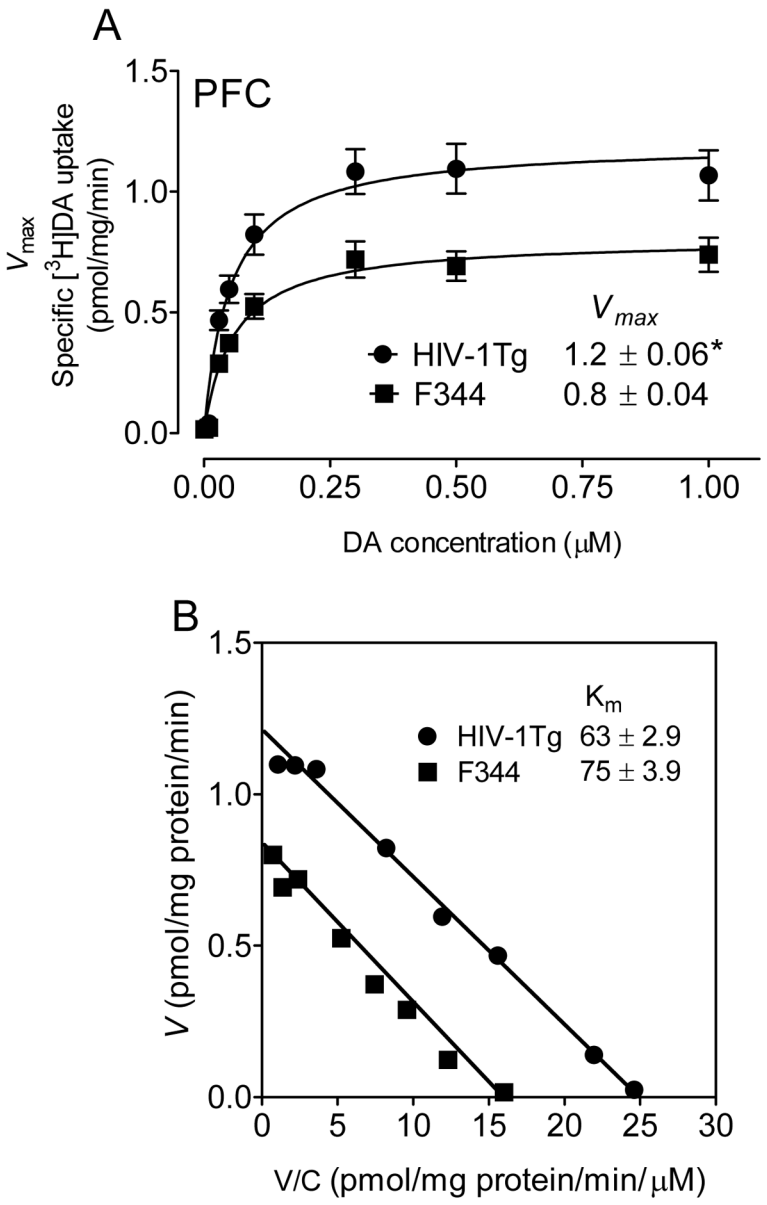

Figure 1. HIV-1Tg rats exhibit an increase in [^{3}H]DA uptake in the prefrontal synaptosomes

Kinetic analysis of the synaptosomal [^{3}H]DA uptake was determined in the prefrontal cortex (PFC) of HIV-1Tg and F344 rats. Synaptosomes were preincubated with a range of mixed DA concentrations (1 – 1000 nM, final concentration). In competition, nonspecific uptake (in the presence of 10 μM nomifensine, 1 μM desipramine, 5 nM paroxetine, final concentration) was determined in the presence of subtracted from total uptake to calculate DAT-mediated uptake. **A.** The V_{max} (pmol/mg/min) and K_m (nM) values were calculated by fitting the data to the Michaelis-Menten equation and represent the means from five independent experiments ± S.E.M. **B.** Eadie-Hofstee transformation of the same kinetic data. *, $p < 0.01$ compared to F344 group.

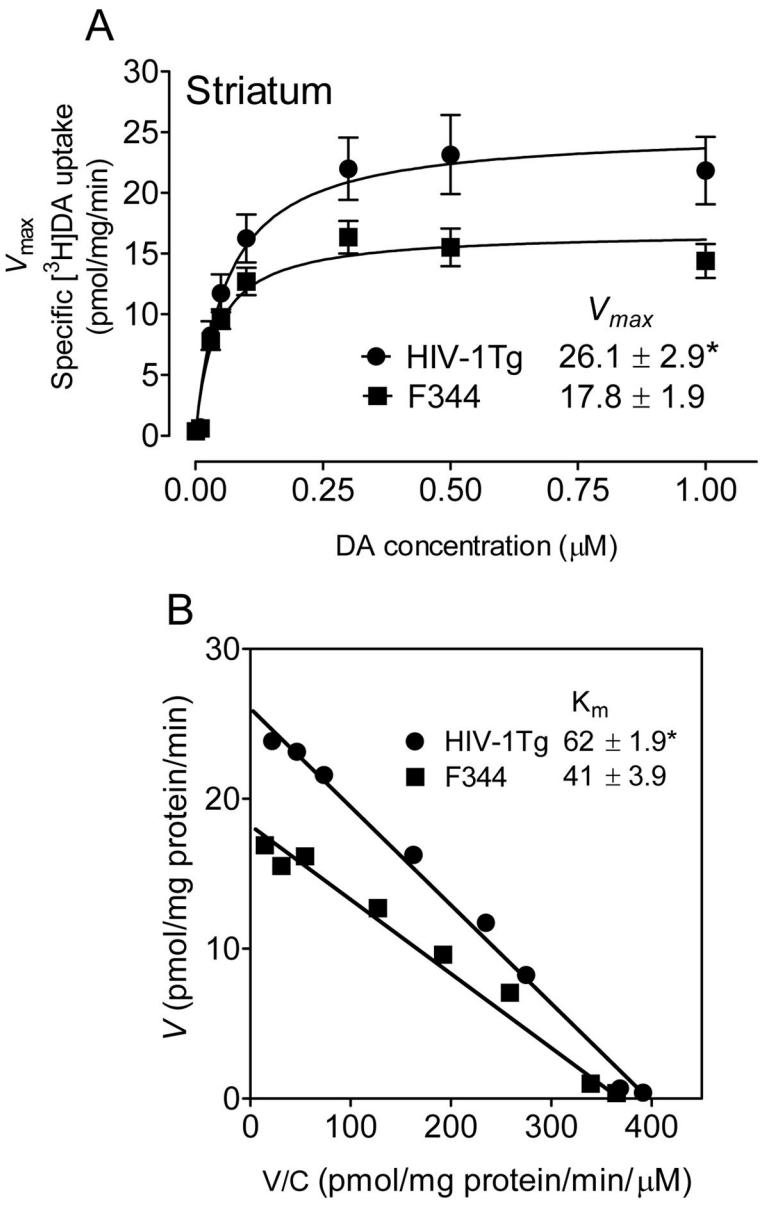

Figure 2. HIV-1Tg rats exhibit an increase in [^{3}H]DA uptake in the striatal synaptosomes

Kinetic analysis of the synaptosomal [^{3}H]DA uptake was determined in the striatum of HIV-1Tg and F344 rats. Striatal synaptosomes were preincubated with one of eight mixed concentrations of the [^{3}H]DA (1 – 1000 nM, final concentration). In competition, nonspecific uptake (in the presence of 10 μM nomifensine, final concentration) was calculated from total uptake to calculate DAT-mediated uptake. **A.** The V_{max} (pmol/mg/min) and K_m (nM) values were analyzed by fitting the data to the Michaelis-Menten equation and represent the means from five independent experiments ± S.E.M. **B.** Eadie-Hofstee transformation of the same kinetic data. *, $p < 0.01$ compared to F344 group.

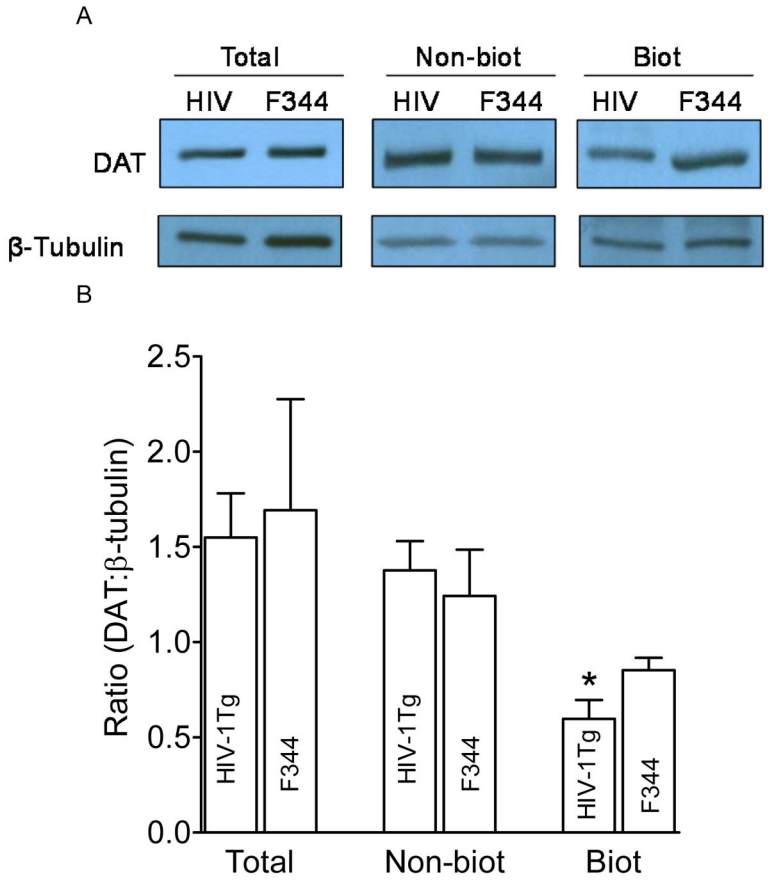

Figure 3. HIV-1Tg rats exhibit an increase in cell surface expression of dopamine transporters in the PFC

A. Representative immunoblots of total synaptosomal fraction (Total), cytoplasmic fraction (non-biotinylated, Non-biot), and plasma membrane fraction (biotinylated, Biot) from PFC of HIV-1Tg (HIV) and F344 rats. β-tubulin was used for monitoring protein loading between HIV-1Tg and F344. **B.** Mean ± S.E.M. ratio of densitometry values of DAT immunoreactivity to β-tubulin immunoreactivity. * $p < 0.05$ compared to F344 group (n=4).

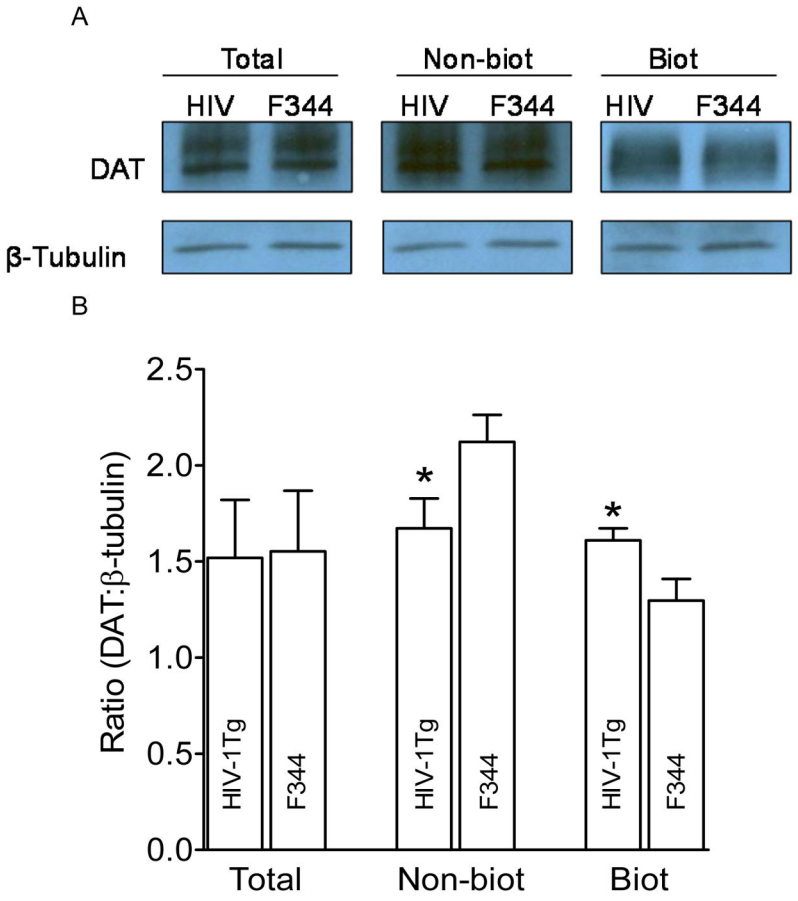

Figure 4. HIV-1Tg rats exhibit an increase in cell surface expression of dopamine transporters in the striatum

A, Representative immunoblots of total synaptosomal fraction (Total), cytoplasmic fraction (non-biotinylated, Non-biot), and plasma membrane fraction (biotinylated, Biot) from striatum of HIV-1Tg (HIV) and F344 rats. β-tubulin was used for monitoring protein loading between HIV-1Tg and F344. **B.** Mean ± S.E.M. ratio of densitometry values of DAT immunoreactivity to β-tubulin immunoreactivity. * $p < 0.05$ compared to F344 group (n=4).

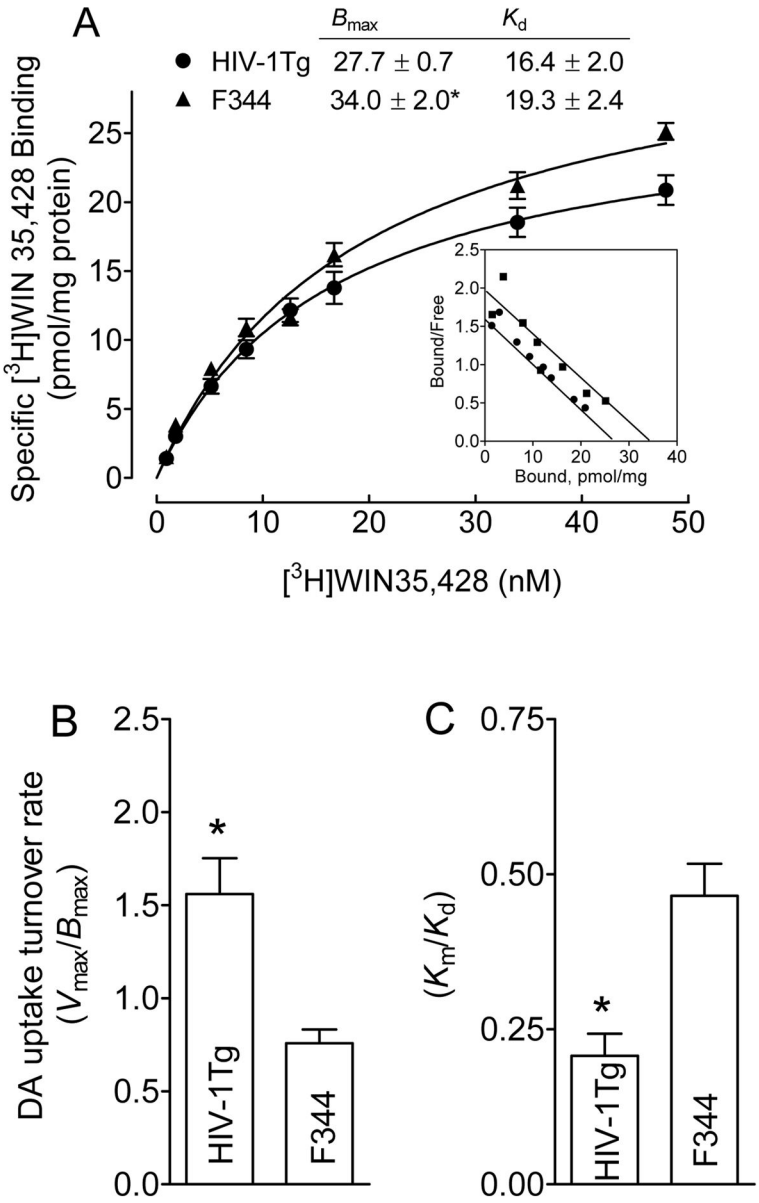

Figure 5. HIV-1Tg rats exhibit a decrease in [^{3}H]WIN 35,428 binding in striatum

For a single experiment, synaptosomes from a single rat were prepared, and half was used for [^{3}H]DA uptake (Figure 2) and the other half was used for saturation isotherm of [^{3}H]WIN 35,428 binding. **A.** B_{max} (pmol/mg protein) and K_d (nM) values of [^{3}H]WIN 35,428 binding in synaptosomes of HIV-1Tg and F344 rats are presented. Scatchard transformations of same data (Insert) are presented. Data were best fit to a single class of binding site and are presented as means ± S.E.M. from four independent experiments. Nonspecific binding for [^{3}H]WIN 35,428 was determined in the presence of 30 μM cocaine. DA uptake turnover rate values are determined from (**B**) V_{max} of [^{3}H]DA uptake/B_{max} of [^{3}H]WIN 35,428 and (**C**) K_m/K_d, respectively. *, $p < 0.05$ compared to F344 group.

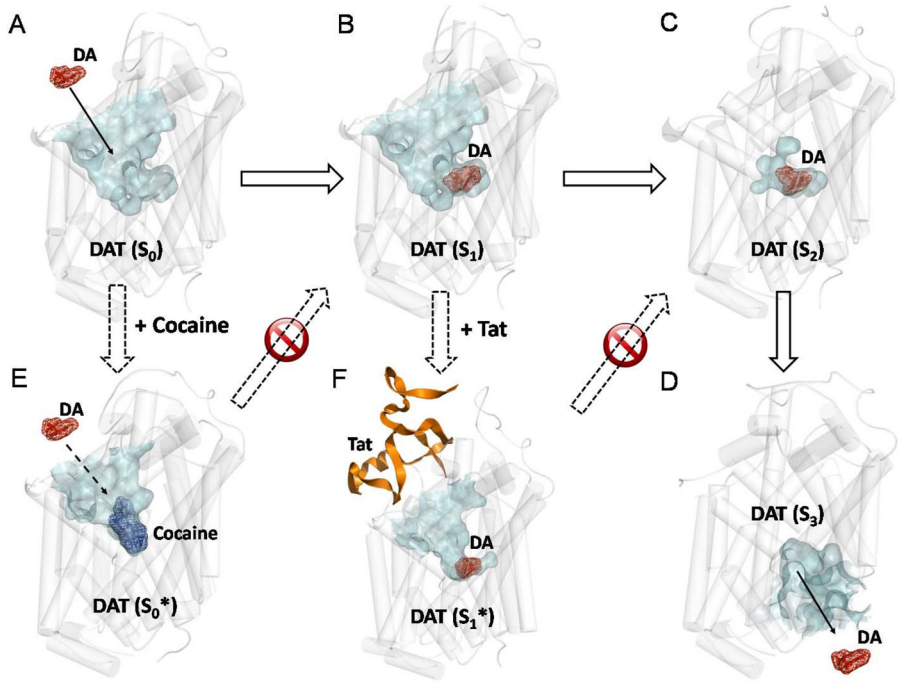

Figure 6. Schematic representation of the mechanisms concerning how cocaine and Tat block the DA transporting cycle

All of the structures were generated with the PyMol software from the energy-minimized structures. All of the initial structures used in the energy minimization were obtained from our previous studies (Huang *et al*, 2009; Midde *et al*, 2013; Midde *et al*, 2015). DAT is represented by white semi-transparent cylinder. The channel for substrate entry or leaving in DAT is represented by semi-transparent surface in cyan. Dopamine (DA) and cocaine are shown as mesh surface and colored in red and blue, respectively. Tat is showed as cartoon and colored in orange. **A.** S_0 indicates the apo DAT in the outward-open state. The substrate binding site is opened to extracellular side of the neuro cell for DA uptake. **B.** S_1 indicates the holo DAT in outward-open state. **C.** S_2 indicates the holo DAT in the outward-occluded state. The substrate binding site is occluded and no longer accessible for DA entry. **D.** S_3 indicates the apo DAT in the inward-open state. The substrate binding site is opened to intracellular cytoplasm and dopamine could leave. The change from S_0 to S_3 represents the process of DAT transporting dopamine from the extracellular side to the intracellular side. **E.** Cocaine can also bind to the S_0 state of DAT with the same binding site of dopamine (S_0*). The competitive inhibition effect of cocaine blocks the $S_0 \rightarrow S_1$ transition step of DAT transporting. (F) Tat could bind to the S_1 state of DAT without occupying the dopamine binding site, which stabilizes the S_1 state (S_1*). The non-competitive inhibition effect of Tat blocks the $S_1 \rightarrow S_2$ transition step of DAT transporting. As a result, although cocaine and Tat adopt slightly different mechanisms in impairing DAT function, they are expected to show similar biological effects on DAT transporting.

Table 1

Effects of substrate and inhibitors on inhibiting [^{3}H]DA uptake and [^{3}H]WIN35,428 binding in the striatal synaptosomes of HIV-1Tg and F344 rats

	[^{3}H]DA uptake		**[^{3}H]WIN35,428**	
	IC_{50}			
	HIV-1Tg	**F344**	**HIV-1Tg**	**F344**
DA (μM)			0.17 ± 0.02	0.59 ± 0.39
Cocaine (μM)	0.90 ± 0.07	0.92 ± 0.13	0.17 ± 0.03	0.20 ± 0.05
WIN 35,428 (nM)	87.2 ± 8.76	93.3 ± 8.93		
GBR 12909 (nM)	3.94 ± 0.36	3.62 ± 0.29	2.77 ± 0.21	2.72 ± 0.28

Data are presented as mean ± S.E.M. of 5 independent experiments performed in duplicate.