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HIV-1 Transgenic Female Rat: Synaptodendritic Alterations of Medium Spiny Neurons in the Nucleus Accumbens

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Abstract

HIV-1 associated neurocognitive deficits are increasing in prevalence, although the neuronal basis for these deficits is unclear. HIV-1 Tg rats constitutively express 7 of 9 HIV-associated proteins, and may be useful for studying the neuropathological substrates of HIV-1 associated neurocognitive disorders (HAND). In this study, adult female HIV-1 Tg rats and F344 control rats had similar growth rates, estrous cyclicity and startle reflex inhibition to a visual prepulse stimulus. Medium spiny neurons (MSNs) in the nucleus accumbens (NAcc) were ballistically-labeled utilizing the indocarbocyanine dye DiI. The branching complexity of MSNs in the NAcc was significantly decreased in HIV-1 Tg rats, relative to controls; moreover, the shorter length and decreased volume of dendritic spines, but unchanged head diameter, in HIV-1 Tg rats suggested a reduction of longer spines and an increase in shorter, less projected spines, indicating a population shift to a more immature spine phenotype. Collectively, these results from HIV-1 Tg female rats indicated significant synaptodendritic alterations of MSNs in the NAcc occur as a consequence of chronic, low-level, exposure to HIV-1 associated proteins.

Keywords

HIV-1 associated neurocognitive disorder; Dendritic spines; Diolistic labeling; Indocarbocyanine dye DiI

A large proportion of individuals infected with HIV-1 experience HIV-1 associated neurocognitive disorders (HAND), even in long-standing aviremic patients (Woods et al. 2009; Winston et al. 2013; Alfahad and Nath 2013). Patients with latent HIV-1 infection (i.e., HIV-1+ without HIV-1 viral RNA or p24 present), which display mild to moderate neurocognitive dysfunction, show decreased expression of the presynaptic protein synaptophysin and the dendritic microtubule activation protein 2 (MAP-2), throughout the frontal cortex (Desplats et al. 2013). Thus, in the absence of active infection or viral replication, long-lived, transcriptionally silent, HIV-1 proviruses in the brain may produce synaptodendritic injury in HAND, yet the mechanism(s) of HAND synaptopathy remain unclear. Interestingly, the production of HIV-1 proteins, such as Tat (produced during the early phase of transcription from proviral DNA) is, for the most part, unaffected by antiretroviral drugs. Tat protein is found in the cerebral spinal fluid of aviremic patients (Johnson et al. 2013), suggesting a role for HIV-1 proteins/Tat in producing synaptodendritic injury in HAND.

Indeed, synaptodendritic injury has been observed in response to HIV-1 Tat protein. Tat protein-induced synaptodendritic damage is highly specific and dependent upon the presence of the cysteine region of the Tat protein (aa 21–32) (Bertrand et al. 2013). Moreover, Tat protein-induced synaptodendritic damage occurs prior to cell death, at very low Tat concentrations, and may be reversible (Bertrand et al. 2014). Synaptic alterations in pyramidal cells of the hippocampus (Fitting et al. 2013), as well as decreased spine density in MSNs (Fitting et al. 2010), have also been reported in mice conditionally expressing the HIV-1 Tat protein in astrocytes. Interestingly, gender differences have been suggested in mice conditionally expressing the HIV-1 Tat protein (Hahn et al. 2013). Likewise, gp120 expressing mice demonstrate synaptic dysfunction (Gorantula et al. 2012; Toggas et al. 1994; Kang et al. 2010). Collectively, these single protein (Tat or gp120) transgenic mice suggest that acute exposure to individual HIV-1 proteins affects synaptodendritic processes; however, synaptodendritic processes have not been studied in chronic multi-HIV protein exposures, such as in the HIV-1 Tg rat, which expresses 7 of the 9 HIV-1 proteins including Tat and gp120 (Reid et al. 2001), without viral replication, similar to the state seen in aviremic patients (Peng et al. 2010).

Shifts in dendritic spine density/alterations in spine morphologies often reflect impaired neuronal processing capacity and adverse neurocognitive outcomes (Mancuso et al. 2012; Ovtscharoff et al. 2008; Shen et al. 2009). MSNs are the major inhibitory projection neurons in the core region of the NAcc (Gangarossa et al. 2013). MSNs are characterized by particularly high densities of dendritic spines (Cheng et al. 1997) and play a key role in many motivational and reward-related behaviors (Enoksson et al. 2012; Shen et al. 2009). Dysfunction of the NAcc is associated with apathy (Levy and Dubois 2006), depression (Nestler and Carlezon 2006) and drug addiction (Lesscher and Vanderschuren 2012) which are often co-morbid with HAND (Alfahad and Nath 2013; Weber et al. 2013). Thus, alterations in MSN dendritic spines may play an important role in HAND.

To determine synaptodendritic alterations in HIV-1, we employed the DiOlistic labeling technique, which may have the following advantages over Golgi-Cox silver impregnation: 1) the lipophilic dye DiI, or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine

perchlorate, has higher resolution to distinguish fine spine morphological characteristics (Mancuso et al. 2012; Shen et al. 2009); 2) allows three-dimensional dendritic analysis when using Z-stack confocal imaging (Mancuso et al. 2012; Shen et al. 2009), and 3) is compatible with retrograde tracing (Neely et al. 2009) and immunohistochemistry (Mancuso et al. 2012; Seabold et al. 2010) facilitating identification of the population of labeled cells.

HIV-1+ women have increased neurocognitive impairments relative to HIV-1- women (Manly et al. 2011; Maki et al. 2009), yet few translational studies have focused exclusively on females. Therefore, we used DiOlistic labeling to explore alterations in dendritic arborization and spine morphology of MSNs in the NAcc of HIV-1 Tg female rats. The MSNs in the NAcc represent an important component of the motivational/reward system mediating substance abuse (Ma et al. 2013), which is often co-morbid with HAND in women (Meyer et al. 2013; Maki and Martin-Thormeyer 2009). Thus, identification of altered MSN synaptodendritic structures in HIV-1 Tg female animals is a key step in clarifying possible neuropathological substrates of HAND.

Methods

Subjects

Adult female Fischer F344 rats (HIV-1 Tg, $n=12$; control, $n=12$; approximately 9–10 weeks of age) were purchased from Harlan (Indianapolis, IN). Food (Pro-Lab Rat, Mouse Hamster Chow #3000, NIH diet #31) and water were available *ad libitum*. Rats were maintained according to the National Institute of Health guidelines in AAALAC-accredited facilities. The animal housing room was maintained at 21 ± 2 °C, 50 ± 10 % relative humidity, and had a 12:12 hr light/dark cycle with lights on at 07:00 EST. Animals were handled daily for approximately 2 weeks prior to behavioral testing, and weighed daily. The research protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina, Columbia, SC; animal assurance number A3049-01.

Estrous Cycle Tracking

Vaginal lavage was performed daily at 11:00 AM (10–11 weeks of age) for approximately 3 weeks to examine estrous cyclicity. As previously described (Booze et al. 1999), cellular cytology was examined in vaginal smears using a light microscope (10 $\times$) with the predominant cell type used to determine stage of the estrous cycle (Waynforth and Flecknell 1992; Westwood 2008). Proestrus was identified by flat, circular squamous cells. Predominately cornified cells indicated day of estrus and was confirmed the following day by presence of macrophages and nucleated cells, indicating metestrus. Diestrus state was designated by the presence of thick epithelial cells and absence of stratum granulosum. The criterion for regular cycling was that each rat demonstrated a 4–6 day estrus cycle. Irregular cycling and acyclicity were defined by either persistent estrus, absence of proestrus, failure to progress from proestrus to estrus, or prolonged diestrus.

Prepulse Inhibition Task

Animals were tested in the prepulse inhibition (PPI) task (12–13 weeks of age) similar to our prior publication (Moran et al. 2013a). In brief, the startle apparatus (SR-Lab Startle

Reflex System, San Diego Instruments, San Diego, CA) was enclosed in a sound attenuating chamber (Industrial Acoustic Company, Bronx, NY). All auditory stimuli were delivered via a high frequency loudspeaker (Radio Shack model #40-1278B) mounted inside the chamber 30 cm above the Plexiglas test cylinder. A white LED light was mounted inside the chamber in front of the test cylinder (22 lux; light meter model #840006, Sper Scientific Ltd, Scottsdale, AZ). The whole body startle response to the auditory startle stimulus deflected the test cylinder, which was measured via an accelerometer at the base of the Plexiglas test cylinder. All test sessions were conducted in the dark.

All animals were habituated to the startle response chamber for 1 day prior to PPI testing (Moran et al. 2012). The habituation session consisted of 36 trials beginning with a 5-minute acclimation period (70 dB(A) background white noise), followed by 36 trials of a 100 dB(A) white noise stimulus of 20 msec duration and 10 sec intertrial interval (ITI). The habituation session was administered during the metestrus phase of the estrous cycle.

One day after habituation, rats were tested with 36 PPI trials, i.e. 0, 8, 40, 80, 120, and 4000 msec interstimulus intervals (ISI) between prepulse (single light flash at 22 lux) and the pulse stimulus (100 dB(A) white noise). A variable 20 sec ITI was used (15–25 sec). Six trials for each ISI were conducted using a Latin-square design. Percent PPI was calculated as the difference between average peak amplitude at 0 and 4000 msec ISI and the ISI at which peak inhibition was observed (40 msec) divided by average peak amplitude at 0 and 4000 msec ISI, multiplied by 100. All PPI testing was performed in the diestrus phase of the estrous cycle.

Preparation of Tissue

Animals were sacrificed in the diestrus phase of the estrous cycle (13–15 weeks of age). Animals were deeply anesthetized using sevoflurane (Abbot Laboratories, North Chicago IL) and transcardially perfused with 100 ml of 100 mM PBS wash followed by 100–150 ml of 4 % paraformaldehyde buffered in PBS (Sigma-Aldrich, St. Louis, MO). Brains were dissected and post-fixed in 4 % paraformaldehyde for 30– 60 minutes. After post-fixation, 200 µm thick coronal slices were cut using a rat brain matrix (ASI Instruments, Warren, MI). Serial coronal slices were then washed in PBS 3×, notched for orientation, and placed in tissue cell culture plates (24 well plate; Corning, Tewksbury MA) until further processing.

Preparation of DiOlistic Cartridges

DiOlistic labeling was performed according to published techniques (Seabold et al. 2010). Approximately 300 mg of tungsten beads (Bio-Rad, Hercules, CA) were dissolved in 99.5 % pure methylene chloride (Sigma-Aldrich, St. Louis, MO) and sonicated in a water bath for 30 minutes. Crystallized DiI (14.5 mg; Invitrogen, Carlsbad, CA) was dissolved in methylene chloride and light protected. Following sonication, 100 µl of the bead solution was placed on a glass slide and 150 µl of the DiI solution titrated on top, and slowly mixed using a pipette tip. After air drying, a razor blade was used to collect the dye/bead mixture onto wax-coated weigh paper and the dye/bead mixture transferred to a 15 ml conical tube (BD Falcon, San Jose, California) with 3 ml ddH₂O and subsequently sonicated for 45–60 minutes.

Preparation of Tefzel Tubing

Tefzel tubing (IDEX Health Sciences, Oak Harbor, WA) was cut into three 1.7 M lengths. Polyvinylpyrrolidone (PVP, 100 mg Sigma-Aldrich, St. Louis, MO) was dissolved in 10 ml ddH₂O, briefly vortexed, then passed through each length of the tubing. The 3 ml bead/dye solution was slowly drawn into the tubing and placed in the tubing prep station (Bio-Rad) for 5 minutes. After draining the water from the tube, the dry tubing was spun in the prep station for approximately 10 minutes with nitrogen gas flow of 1.0 LPM. The nitrogen gas flow through the tubing was adjusted to 0.4–0.5 LPM and the tubing was further spun for 50–60 minutes to ensure the tubing was fully dry. Once dry, tubing was cut into 13 mm segments and stored under anhydrous conditions until use.

DiOlistic Labeling using the Helios Gene Gun

The Helios gene gun (Bio-Rad, Hercules, CA) was loaded with the previously prepared cartridges, He gas flow adjusted to 80 PSI, and particles delivered through 3 μ m pore filter paper directly onto the slice with the barrel placed approximately 2.5 cm away from the sample. After washing 3 $\times$ in PBS, sections were stored overnight at 4 °C to allow dye diffusion. Tissue sections were mounted using Pro-Long Gold Antifade (Invitrogen, Carlsbad CA), cover slipped (#1 cover slip; Thermo Fisher Scientific, Waltham, MA), and stored in the dark at 4 °C.

MSN Dendritic Analysis and Spine Quantification

MSNs were analyzed from the NAcc, located approximately 2.28 mm to 0.60 mm anterior to Bregma (Paxinos and Watson 2007). For dendritic branch order analysis, HIV-1 Tg ($n=5$) and Controls ($n=5$), three MSNs per animal were randomly selected. For spine analysis, neurons with continuous dendritic staining extending from the soma, minimal diffusion of the DiI into the extracellular space, and low background/dye clusters were selected. DiI labeling from 4 rats from each group did not meet the selection criteria, yielding HIV-1 Tg $n=10$, Controls $n=10$; one MSN per animal was randomly selected for spine analysis.

For dendritic spine analysis, Z-stack images were obtained with a Nikon TE-2000E confocal microscope utilizing Nikon's EZ-C1 software (version 3.81b). Dendritic spine analysis was performed at 60 $\times$ (n.a.=1.4) with Z-plane intervals of 0.15 μ m (pinhole size 30 μ m; back-projected pinhole radius 167 nm). A green helium-neon (HeNe) laser with an emission of 533 nm was used for DiI fluorophore excitation. The analysis of spine parameters was performed using NeuroLucida version 10.52, utilizing the AutoNeuron and AutoSpine extension modules (MicroBrightField, Williston, VT). Total dendritic length scanned per cell ranged from 500 to 3000 μ m.

Dendritic Spine Parameters

Dendritic spine parameters of length, volume, and head diameter were analyzed. Spine lengths were defined as between .01 to 4 μ m; lengths greater than 4 μ m were considered to be filopodia and excluded from the study (Blanpied and Ehlers 2004; Ruszczycki et al. 2012). Spine volume parameters were defined as those measures between 0.02 and 0.2 μ m³

(Merino-Serrais et al. 2013). Spine head diameters were defined as those measures between 0.3 and 1.2 μm (Bae et al. 2012).

Data Analysis

Data were analyzed using SPSS version 20.0 (IBM). Differences in body weights were compared using a mixed two-way ANOVA with age as the within-subjects factor and rat strain as the between-subjects factor, with a Bonferroni post-hoc correction. Estrous cycle differences were determined using chi-square analysis (Booze et al. 1999). PPI was analyzed using ANOVA with a Greenhouse-Geisser correction to p values (Moran et al. 2013a). Branching order differences were determined using a mixed two-way ANOVA with rat strain as the between-subjects factor and branch order as the within-subjects factor. Spine parameters (length, volume, and head diameter) were analyzed via Student's unpaired t -test with Welch's correction.

Results

HIV-1 Tg and Control Animals Grew at Similar Rates and Had Similar Estrous Cycles

Although HIV-1 Tg animals weighed significantly less than F344 control animals $F(1, 22)=15.4, p=0.001$ (Fig. 1a), there was no significant interaction between rat strain and age; regression analysis further confirmed similar rates of growth for both control and HIV-1 Tg animals (Control $B=0.74\pm 0.09$, HIV-1 Tg $B=0.96\pm 0.10$).

All 12 of the F344 control animals and 10/12 of the HIV-1 Tg animals experienced typical 4–5 day estrous cycles over a series of 4–5 cycles. One HIV-1 Tg animal displayed a persistent diestrus state on earlier tracking days, while another missed one ovulatory cycle; their cycle length was calculated from the cycle data obtained. Means for F344 control and HIV-1 Tg animals were 4.8 and 4.9 days/cycle ($t(22)=0.74, p>0.10$), respective medians were both 5.0, and a Chi-square analysis of cycle length found no significant difference between the two groups, ($\chi^2=4.7$ (df=11), $p>0.10$).

Magnitude of Prepulse Inhibition Did not Differ between Groups Using a 20 Msec Visual Prepulse Stimulus

During visual prepulse inhibition testing, a significant main effect on mean peak amplitude was observed for ISI length, $F(5, 110)=44.2, p_{GG}=0.001$ (Fig. 1b). A significant quadratic trend was observed for each group [Control: $F(1, 11)=26.7, p=0.001$; HIV-1 Tg: $F(1, 11)=43.4, p=0.001$]. There was neither a significant effect of group nor a significant interaction between group and ISI length. Maximum inhibition for the visual stimulus was achieved at the 40 msec ISI for both groups, as shown previously (Moran et al. 2013a), with no significant difference between groups in percent PPI (Controls: $79.6\% \pm 3.3$; HIV-1 Tg: $82.0\% \pm 2.8$).

MSN Dendritic Branching was Reduced in HIV-1 Tg Animals

DiOlistic labeling was found to completely fill the soma and surrounding dendritic fields of NAcc MSNs with little to no background staining (Fig. 2a and b). MSNs from F344 control animals had multiple proximal branches extending from the soma, a complex dendritic

branching pattern, and an extensive fine network (Fig. 2a). Decreased dendritic branching complexity in HIV-1 Tg MSNs was apparent (Fig. 2b). Dendritic sections revealed spines with an increased head-width to neck-width ratio among control slices, indicating advanced spine maturity (Fig. 2c). HIV-1 Tg spines appeared thinner and with a near equal head-diameter to neck-diameter ratio, producing a stubbier appearance (Fig. 2d). Quantities of dendrites differed significantly between groups as a function of both transgene and branch order, $F(1,42)=56.7$, $p=0.0001$ and $F(2,42)=10.4$, $p=0.001$, respectively (Fig. 2e).

Dendritic Spine Length and Volume, but not Head Diameter, was Altered in HIV-1 Tg Animals

Measurement of spine length revealed a significant shift in the distribution from longer to shorter spines for the HIV-1 Tg rats compared to controls (Fig. 3a and b; student's unpaired t -test with Welch's correction, $t(20763)=20.5$, $p=0.0001$). Measurement of spine volume also showed a significant shift in distribution as a function of the HIV-1 transgene, with the HIV-1 Tg rats displaying a reduction in spine volume relative to controls (Fig. 4a and b, student's unpaired t -test with Welch's correction, $t(6078)=5.6$, $p=0.0001$). Measurement of spine head diameter did not reveal any statistically significant reduction in the HIV-1 Tg animals compared to controls (Fig. 5a and b).

Discussion

HIV-1 associated neurocognitive disorders (HAND) are thought to be the result of dendritic injury (Ellis et al. 2007; Moore et al. 2006) and synaptic dysfunction (Desplats et al. 2013; Gelman and Nguyen 2010). In the present study, DiOlistic labeling in HIV-1 Tg female rats was used to identify synaptodendritic injury in the NAcc MSNs via a reduction in dendritic branching. Furthermore, analysis of dendritic spines revealed a shift toward shorter, stubbier spines with an overall decreased spine volume in HIV-1 Tg animals, relative to controls. Collectively, these findings suggest that MSN synaptodendritic injury occurs in response to chronic low-level HIV-1 protein expression in the central nervous system.

The study of female animals is particularly important as, on a global scale, women represent at least 50 % of people living with AIDS (UNAIDS, 2013), yet HIV-1+ women are under-represented in neuropsychological studies (Maki and Martin-Thormeyer 2009). This under-representation may be due to the complexity of studying economically disadvantaged HIV-1+ women with many complicating comorbidities, including substance abuse. However, recent studies suggest that HIV-1+ women are neurocognitively impaired relative to HIV-1-women with similar comorbid conditions (Maki et al. 2009; Manly et al. 2011). Collectively, these studies emphasize the important role that translational work may provide in identifying HIV-1 induced neurocognitive impairments in females and identifying the underlying neuropathological alterations.

MSNs are important in the motivational/reward system and, more generally, the NAcc plays an important role in substance abuse, which is often comorbid with HAND (Bell et al. 2006). Indeed, HIV-1 Tg animals are particularly vulnerable to a variety of drugs of abuse including methamphet-amine (Pang et al. 2013; Moran et al. 2012), nicotine (Vigorito et al. 2013) and ethanol (Sakar and Chang 2013), suggesting a commonality of neuropathological

impairment across many different drug classes. It is possible that MSNs might be a common neuropathological link in altered responses of the HIV-1 Tg animals to drugs of abuse, as alterations in MSN spines have been found following cocaine/amphetamine exposure (Shen et al. 2014; Dumitriu et al. 2012) nicotine exposure (Gipson et al. 2013) and alcohol (Gass and Olive 2012). Although women with HIV-1+ status may be particularly vulnerable to the effects of substance abuse relative to matched HIV-1- women (Meyer et al. 2013), few translational studies have focused exclusively on females.

In general, the HIV-1 Tg young adult female rats were found to be healthy (i.e., normal estrous cycles, similar growth rate as controls), which is consistent with our prior studies (Moran et al. 2012; Moran et al. 2013a; Moran et al. 2013b; Webb et al. 2010). However, it is of note that the health status of our HIV-1 Tg animals differs from the original description of the HIV-1 Tg rat. Reid and colleagues (Reid et al. 2001) described several severe pathological phenotypes associated with the transgene expression (then located on chromosome 2 and 9), including 3 grades of cataracts, accompanied by severe neurological disorders (hind limb paralysis) and wasting at a relatively young age (5–9 months). The HIV-1 Tg animals used in the current studies are a derivation of these originally described phenotypes, on an inbred F344 background, with the transgene (now limited to chromosome 9), and a 100 % penetrant phenotype of light to moderate cataracts. We have found no significant alterations in detection of brief visual stimuli, hearing or growth rates in the present study and have found no general wasting in this contemporary HIV-1 Tg phenotype through 344 days of age (Moran et al. 2013a). Similarly, studies using these animals report alterations in behavioral tasks of spatial learning (Lashomb et al. 2009; Vigorito et al. 2007), without alterations in hearing or locomotor ability. Thus, as suggested by Peng et al. (2010) the moderate phenotype more closely resembles HAND, is suitable for long-term/aging longitudinal studies, and should be considered distinct from the original descriptions of the most severe phenotypes of the initially derived HIV-1 Tg rat.

Although the phenotype of the HIV-1 Tg rat used in the present studies includes the presence of light to moderate corneal cataracts, we found PPI to brief visual stimuli unchanged, compared to control, with the maximum inhibition at 40 msec ISI. Our previous visual PPI studies, comparing light levels of 22 lux (dim light) and 100 lux (bright light), also indicated similar levels of maximum inhibition between control and HIV-1 Tg ovariectomized animals at the 40 msec ISI, indicating detection of the brief (20 msec) dim (22 lux) visual prepulse by the HIV-1 Tg animals (Moran et al. 2013a). Collectively, these results suggest the presence of moderate corneal cataracts does not hinder the ability of HIV-1 Tg rats to detect light cues. It remains that, although visual acuity may be impaired by the presence of light-moderate cataracts in the HIV-1 Tg animals, the ability to respond to visual cues (even brief dim cues) in small behavioral testing chambers is intact in the contemporary HIV-1 Tg phenotype.

The HIV-1 clade B transgenic rat expresses HIV-1 proteins via regulation of the viral long-terminal repeat, with the exception of Gag and Pol proteins (Peng et al. 2010; Reid et al. 2001). Protein expression in this rat is regulated by the viral long terminal repeat (LTR) and uses cyclin-T as a cofactor to regulate Tat; murine cyclin-T is not compatible with this mechanism (Reid et al. 2001). Endogenous rat cyclin-T acts as a modulator of Tat, thereby

establishing proviral DNA without an accompanying state of active peripheral viremia (Reid et al. 2001). This aviremic state may represent individuals infected with HIV-1, but present seronegative viral loads, as seen in individuals on combined antiretroviral therapy (cART). Moreover, in the HIV-1 Tg rats, HIV-1 expression of vif, nef, tat and gp160 proteins, occurs in mononuclear phagocytes/astrocytes, but not in neurons (Royal et al. 2012) – similar to that seen in human HIV-1 brains – accompanied by chronic, low-level, immune activation in the brains of these animals (Royal et al. 2012). Immune activation produces a neuroinflammatory microenvironment (Rao et al. 2011), which may be detrimental to neuronal connectivity and dendritic spines. However, to our knowledge, no studies of synaptodendritic pathology have been reported using the HIV-1 Tg rat.

Previous studies of HIV-1 synaptodendritic and spine alterations have used a variety of methods for spine detection, including MAP-2 staining (Maragos et al. 2003) and Golgi-Kopsch silver impregnation (Fitting et al. 2010). However, Golgi staining is notoriously capricious, and not all Golgi staining techniques are capable of providing the resolution for differentiating fine structures. In particular, the Golgi-Cox method may not have the ability to distinguish thin filopodia, and MAP-2 immunostaining does not identify specific morphological parameters in spines. Also, because white light is reflected from silver stain, silver staining methods may be impractical for 3-D morphometric analysis of confocal Z-stacks (Staffend and Meisel 2011). In one of the few direct comparisons between Golgi staining and DiOlistic labeling, the DiOlistic method was found to produce higher spine counts via 3-D analysis relative to Golgi staining (Shen et al. 2009).

Indocarbocyanine dye labeling via ballistic methods can be done in a fraction of the time it takes to complete the Golgi method (Staffend and Meisel 2011), and is compatible with retrograde tracing (Neely et al. 2009) and immunohistochemistry (Seabold et al. 2010; Staffend and Meisel 2011). DiOlistic labeling can be used to analyze multiple neuronal structures, and is available in multiple fluorescent spectra to be compatible with the majority of immunohistochemical techniques (Gan et al. 2000). DiOlistics may have advantages over microinjection and electroporation as well, since the technique can be modified to allow for minimal tissue damage, many cells can be labeled simultaneously, and no special or toxic media are required (O'Brien and Lummis 2007). However, the DiOlistic method requires careful optimization of the technique to prevent clustering of DiO crystals, excessive tissue damage, and other pressure artifacts (see Seabold et al. 2010, for discussion of DiO artifacts).

The present study measured the length, volume and head width of each spine on the dendritic stalk, providing a population distribution for various spine parameters. Although spine morphological nomenclature suggests a continuum of morphological structure, *a priori* classification of spine morphology is prone to false negatives (Ruszczycki et al. 2012). However, it is commonly thought that dendritic spine morphology is indicative of functionality and capacity for structural change (Lai and Ip 2013). Classic nomenclature divides spine morphology into three categories: thin, stubby, or mushroom. Thin spines are characterized by a relatively short neck length and small head volume, with relatively small quantities of synaptophysin (Brusco et al. 2010; González-Burgos 2012). Stubby spines are quantified by an equal head-to-body volume (Brusco et al. 2010; González-Burgos 2012).

Mushroom spines, indicating stability, contain a high head volume to neck volume ratio, in addition to high synaptophysin and PSD95 protein quantities located at the spine terminal (Blanpied and Ehlers 2004; Golden and Russo 2012; Rusczycki et al. 2012). The size and shape of dendritic spines correlates with their capacity for structural plasticity, leading to the idea that small spines are preferentially involved in learning and attention, whereas the larger, more stable spines, mediate long-term processes, such as memory (Kasai et al. 2010).

The directly measured spine parameters of length and volume in the MSNs of HIV-1 Tg rats suggest a population shift from longer spines with defined head areas to shorter, less projected, spines. The shorter, smaller dendritic spines on the MSNs of HIV-1 transgenic animals indicates alterations in synaptic properties. Shorter, smaller spines have weaker synaptic connectivity, are more prone to potentiation (Matsuzaki et al. 2004) and are transient/dynamic (Kasai et al. 2010; Holtmaat et al. 2005), relative to larger, more stable spines. Given that the HIV-1 animals have a significant shift in the dendritic spine population parameters (i.e., shorter spines), it might be anticipated that cognitive deficits would be present in these animals. Our prior behavioral studies support this idea as we have observed deficits in temporal information processing (Moran et al. 2013a), as well as in attention/inhibition processes (Moran et al. 2014), as does the work of others in which spatial memory deficits were identified (Vigorito et al. 2007). However, direct correlations between dendritic spine parameters and learning/memory deficits remains to be done with HIV-1 Tg animals.

Collectively, this study found synaptodendritic simplification and spine population alterations following long-term exposure to HIV-1 viral proteins, without an active infection. The MSNs of HIV-1 Tg female rats had limited branching patterns, in addition to a population shift to shorter and smaller dendritic spines. In the future, DiOlistic labeling can be used in conjunction with immunostaining to determine the specific MSN subtype(s) and the corresponding cellular mechanisms most affected by chronic HIV-1 protein expression. Identification of altered synaptodendritic structures in the HIV-1 Tg female rat is a key step in identifying neuropathological substrates of HAND and moreover, in determining how such synaptic pathology evolves.

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Abbreviations

HAND	HIV-1 associated neurocognitive disorders
MSNs	Medium spiny neurons
NAcc	Nucleus accumbens
DiI	1-1'-Diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate
HIV-1	Human immunodeficiency virus-1

Fig 1.

Body weight and prepulse inhibition for control and HIV-1 Tg animals. **a:** Body weights of control and HIV-1 Tg animals across weeks (mean±95 % confidence interval). The HIV-1 Tg animals weighed significantly less than controls. However, regression analysis suggested a comparable growth rate of controls and HIV-Tg-1 rats (Control $B=0.74 \pm 0.09$, HIV-1 Tg $B=0.96 \pm 0.10$). **b:** Mean peak auditory startle response (ASR) amplitude is shown as a function of interstimulus interval (ISI). PPI to the brief 20 msec visual prepulse was not significantly different between groups. Furthermore, both control and HIV-1 Tg rats exhibited similar rates of inhibition at the 40 msec ISI (Control: $79.6 \% \pm 3.3$; HIV-1 Tg: $82.0 \% \pm 2.8$)

Fig 2.

DiOlistic labeling of medium spiny neurons (MSN) in the nucleus accumbens core. **a:** MSNs from control animals displayed a complex branching pattern with increased number of dendrites as a function of branch order (60×). **b:** HIV-1 Tg MSNs exhibited stunted branching complexity among primary, secondary, and tertiary branch orders (60×). **c:** Control MSN spines were longer and exhibited a higher head-width to neck-width ratio. **d:** MSN HIV-1 Tg MSN spines exhibited a stubbier appearance and were shorter. **e:** Quantity of MSN dendritic branching differed significantly between HIV-1 Tg and controls F (2,42)=56.7, $p = 0.0001$ * indicates $p < 0.0001$

Fig 3.

Spine length histograms illustrating relative (**a**) and cumulative (**b**) frequencies. The overall reduction in spine length reflects the significant shift in the population of spines observed; HIV-1 Tg rats had predominantly shorter spines relative to controls (Student's unpaired *t*-test with Welch's correction, $t(20763)=20.5$, $p=0.0001$)

Fig 4.

Spine volume histograms illustrating relative (a) and cumulative (b) frequencies. The overall reduction in spine volume reflects the significant shift in the population of spines observed; the population of observed spines of the HIV-1 Tg rats had predominantly less volume relative to controls (Student's unpaired *t* test with Welch's correction, *t* (6078)=5.6, *p* 0.0001)

Fig 5. Spine head diameter histograms illustrating relative (a) and cumulative (b) frequencies. The overall slight reduction in spine head diameter as a function of expression of the HIV-1 transgene was not statistically significant