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5 α -reduced progestogens ameliorate mood-related behavioral pathology, neurotoxicity, and microgliosis associated with exposure to HIV-1 Tat

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Abstract

Human immunodeficiency virus (HIV) is associated with motor and mood disorders, likely influenced by reactive microgliosis and subsequent neural damage. We have recapitulated aspects of this pathology in mice that conditionally express the neurotoxic HIV-1 regulatory protein, trans-activator of transcription (Tat). Progestogens may attenuate Tat-related behavioral impairments and reduce neurotoxicity *in vitro*, perhaps via progesterone's 5 α -reductase-dependent metabolism to the neuroprotective steroid, allopregnanolone. To test this, ovariectomized female mice that conditionally expressed (or did not express) central HIV-1 Tat were administered vehicle or progesterone (4 mg/kg), with or without pretreatment of a 5 α -reductase inhibitor (finasteride, 50 mg/kg). Tat induction significantly increased anxiety-like behavior in an open field, elevated plus maze and a marble burying task concomitant with elevated protein oxidation in striatum. Progesterone administration attenuated anxiety-like effects in the open field and elevated plus maze, but not in conjunction with finasteride pretreatment. Progesterone also attenuated Tat-promoted protein oxidation in striatum, independent of finasteride pretreatment. Concurrent experiments *in vitro* revealed Tat (50 nM)-mediated reductions in neuronal cell survival over 60 h, as well as increased neuronal and microglial intracellular calcium, as assessed via fura-2 AM fluorescence. Co-treatment with allopregnanolone (100 nM) attenuated neuronal death in time-lapse imaging and blocked the Tat-induced exacerbation of intracellular calcium in neurons and microglia. Lastly, neuron-glia co-cultures were labeled for Iba-1 to reveal that Tat increased

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microglial numbers *in vitro* and co-treatment with allopregnanolone attenuated this effect. Together, these data support the notion that 5 α -reduced pregnane steroids exert protection over the neurotoxic effects of HIV-1 Tat.

Keywords

5 α -reductase; allopregnanolone; finasteride; HIV/AIDS; intracellular calcium; Iba-1; neurosteroid; neurotoxicity; oxidative stress; progesterone

1. Introduction

The advent of highly active antiretroviral therapies (HAART) has made it possible to reduce circulatory viral load of human immunodeficiency virus (HIV) to undetectable levels. However, HAART cannot eradicate HIV, in part due to cellular reservoirs that retain latent infection or low-levels of viral production residing within the central nervous system (CNS) where HAART penetration and/or accumulation are reduced (Iglesias-Ussel & Romero, 2011). As such, neurotoxic HIV proteins continue to be produced within the CNS and are believed to underlie continuing progression of affective, cognitive, and motor dysfunction (a constellation of neurological symptoms referred to as “neuroAIDS”) seen among HIV-infected individuals (Hauser et al., 2007; Hong & Banks, 2015; Nath, 2015).

Within the CNS parenchyma, microglia act as macrophages and are thought to be the primary resident cell type to harbor the virus (Gendelman & Meltzer, 1989; Meltzer & Gendelman, 1992). Given that HIV does not infect neurons, microglial activation and the production of excitotoxic and inflammatory factors likely contribute to the neurodegenerative and behavioral profile observed in neuroAIDS. One viral protein produced by infected microglia (and infected astrocytes to a lesser degree) that may particularly contribute to these effects is the HIV-1 trans-activator of transcription (Tat).

HIV-1 Tat acts independently or in concert with other viral proteins and inflammatory toxins to promote excitotoxic neuronal injury and/or death (Mattson et al., 2005). Among several targets, Tat activates NMDA receptors (Dreyer et al., 1990; Eugenin et al., 2007; Li et al., 2008), interrupts mitochondrial function (Brooke et al., 1998; Perry et al., 2005) and ATP production (Brooke et al., 1998; Norman et al., 2007; Turchan-Cholewo et al., 2006), and can cause focal, transient disruptions to ion homeostasis (Ca²⁺, Na⁺, and potentially K⁺; Fitting et al., 2014; Greenwood & Connolly, 2007; Lee et al., 2003; Perry et al., 2005) resulting in synaptodendritic injury (Greenwood et al., 2007; Park et al., 1996). Microglia are a principal source of Tat-induced cellular toxins including cytokines, reactive oxygen and nitrogen species, and hydrolytic enzymes (King et al., 2006; Sheng et al., 2000; Turchan-Cholewo et al., 2009) as evidenced by central protein oxidation, particularly in striatum (Aksenov et al., 2001, 2003; Turchan-Cholewo et al., 2009). We have utilized a transgenic mouse model wherein HIV-1 Tat₁₋₈₆ is conditionally expressed in a CNS-targeted manner to demonstrate Tat-driven microgliosis within the striatum of male and female mice (Hahn et al., 2015). In a similar transgenic model, conditional Tat exposure was observed to increase microglial activation throughout limbic and extra-limbic brain regions of male mice (Paris et al., 2015). However, in an examination of sex differences, the presence of a reactive

nitrosative marker co-localized with microglia was significantly lower among females, compared to males (Hahn et al., 2015). This occurred concurrent with reduced neuronal cell death, astrogliosis, and reduced motor/anxiety-like pathology among females exposed to central HIV-1 Tat (Hahn et al., 2015). As such, gender may confer protection to some of Tat's neuroinflammatory and neurotoxic effects, but the mechanisms are not known.

One point of convergence between HIV-1 Tat toxicity and sex-specific neuroinflammation occurs with classic steroid hormones and their neuroprotective, non-traditionally-acting metabolites. In particular, concentrations of progesterone and its 5 α -reduced/3 α -hydroxylated metabolite, allopregnanolone (AlloP; i.e., 5 α -pregnan-3 α -ol-20-one), typically fluctuate to greater concentrations within females compared to males (Kancheva et al., 2007). Progesterone can attenuate microglial activation, microgliosis, astrogliosis, and can suppress cytokine release (Labombarda et al., 2011; Munroe, 1971; Robinson & Klein, 2012). Unlike progesterone, AlloP is a potent, positive allosteric modulator of GABA_A receptors (Majewska et al., 1986; Paul & Purdy, 1992), a potential negative allosteric modulator of NMDA receptors (Johansson & Le Grevès, 2005; Maurice et al., 2006), is produced in response to immune challenge (Billiards et al., 2002; Ghezzi et al., 2000), and can reduce excitotoxicity partly through rapid increases of tonic inhibition in models of CNS insult (Baulieu & Schumacher, 2000; Brunton et al., 2014; Mellon et al., 2008; Sayeed & Stein, 2009). Allopregnanolone is produced rapidly in response to stress challenges to restore sympathetic and parasympathetic tone (Barbaccia et al., 1996; Patchev et al., 1994, 1996), functions which are observed to be dysregulated among some HIV⁺ individuals (Chittiprol et al., 2007, 2008; Hurwitz et al., 2005). In ovariectomized female mice, supra-physiological progesterone attenuated the anxiety-like effects of HIV-1 Tat exposure (Paris et al., 2014a); however, the mechanisms underlying behavioral protection, the involvement of pregnane steroids on neuroinflammation, and the importance of neurosteroid formation were not assessed.

We hypothesized that HIV-1 Tat would increase both anxiety-like behavior of ovariectomized female mice and protein oxidation in brain, and progesterone administration would ameliorate these effects when unopposed by estrogens. Further, we expected that pharmacological blockade of progesterone metabolism to AlloP would attenuate behavioral protection against HIV-1 Tat *in vivo*. In concurrent experiments, we hypothesized that AlloP would ameliorate HIV-1 Tat-induced pathology *in vitro*, including effects on neurodegeneration, microgliosis, and intracellular calcium ([Ca²⁺]_i).

2. Materials and Methods

The use of mice in these studies was pre-approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and the experiments were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

2.1. Subjects and housing

Adult, male (n=16) and female (n = 77) mice expressing an HIV-1 *tat* transgene as previously described (Hauser et al. 2009; Bruce-Keller et al. 2008) were generated in the

vivarium at Virginia Commonwealth University (MCV campus). Briefly, HIV-1 Tat₁₋₈₆ is conditionally expressed in a CNS-targeted manner via a GFAP-driven Tet-on promoter (activated via consumption of chow containing doxycycline). Mice (approximately 70 days of age) were housed 4 – 5/cage and were maintained in a temperature- and humidity-controlled room on a 12:12 h light/dark cycle (lights off at 18:00 h) with *ad libitum* access to food and water. A subset of gonadally-intact female mice (n = 16) had their estrous cycles tracked as previously described (Paris et al., 2014a).

2.2. Surgical manipulation

Some female mice (n = 61) underwent bilateral ovariectomy (OVX) under isoflurane (4 %) anesthesia as previously reported (Paris et al., 2014a). Following surgery, mice were monitored to ensure weight gain, muscle tone, and proper neurological response and general health (Crawley and Paylor, 1997). One mouse failed to recover and was excluded from the study. Mice were allowed 14 days for surgical recovery and endogenous hormone washout prior to experimental hormone manipulation.

2.3. Chemicals

To induce HIV-1 Tat₁₋₈₆ expression, transgenic mice were placed on doxycycline chow (Dox Diet #2018; 6,000 mg/kg) obtained from Harlan Laboratories (Madison, WI). In order to assess the influence of steroid hormones on experimental endpoints *in vivo*, mice were administered s.c. vehicle (10 % EtOH in oil), progesterone (4 mg/kg in vehicle; Sigma-Aldrich Co., P0130; Paris et al., 2014a) or finasteride (50 mg/kg in vehicle; Sigma-Aldrich Co., F1293; Paris et al., 2011) per prior protocols.

To assess the interactions of AlloP and Tat *in vitro*, cells were treated with HIV-1 Tat₁₋₈₆ (50 nM in ddH₂O; ImmunoDx, 1002-2) and/or AlloP (100 nM in 1:10,000 DMSO diluted in media; Sigma-Aldrich Co., P8887). The chosen Tat concentration reflects one from a range that is observed to elicit functional deficits in glia and neurons similar to those occurring in HIV infection (Kruman et al., 1998; Nath et al., 1999; Singh et al., 2004; El-Hage et al., 2005, 2008; Perry et al., 2010). The chosen AlloP dosing reflects a physiological concentration that has previously been found to confer protection from several neurotoxic insults (Ardeshiri et al., 2006; Lockhart et al., 2002; Waters et al., 1997) and is preferred to lower concentrations given the high instability of the neurosteroid due to its rapid clearance (Carter et al., 1997; Phillipps, 1975). Higher concentrations may directly activate GABA_A receptors (Lambert et al., 1990) and were considered non-optimal. Moreover, others have utilized the present dosing regimen to determine potential efficacy for clinical trials aimed at assessing AlloP-protection over Alzheimer's related cognitive impairment (Irwin & Brinton, 2014; Irwin et al., 2015).

2.4. Doxycycline and steroid hormone replacement regimen

Immediately following surgery, mice were placed on Dox Diet for the duration of the experiment (30 days total). Fourteen days following OVX, mice began a progestogen hormone replacement therapy regimen. Once every 5 days for 15 days, some mice were pretreated with vehicle or finasteride (50 mg/kg, s.c.), a 5 α -reductase inhibitor that blocks progesterone's capacity to metabolize to AlloP. One and a half hours later, mice were

administered either vehicle or progesterone (4 mg/kg, s.c.). Given that mice were ovariectomized, finasteride effects were not independently assessed. On the day of testing, mice were assessed 4 h following the last injection of vehicle or progesterone. This hormone regimen replicates the circulatory levels observed on the day of proestrus (Walf et al., 2006) and allows examination of pregnane steroid effects in the absence of circulating estrogens (Paris et al., 2014a).

2.5. Behavioral assays

Testing was conducted in a behavioral battery of anxiety-like assays (open field, followed by elevated plus maze, followed by marble burying). All mice were tested in the same order of tasks, given that the use of multiple anxiety assays increases confidence of the behavioral constructs assessed and carry-over effects are observed to be minimal in similar testing batteries (Crawley and Paylor, 1997; Lad et al., 2010; McIlwain et al., 2001). A subset of animals was additionally assessed in a rotarod task to provide a motor measure that was not confounded by anxiety-like responding. Prior to all behavioral testing, mice were acclimated to the testing room for 1 h. All behaviors were recorded and digitally encoded by an ANY-maze behavioral tracking system (Stoelting Co., Wood Dale, IL).

2.5.1. Open field—The open field test assesses anxiety-like behavior and ataxia (Hall & Ballachey, 1932). Mice were placed in the lower left corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co., Wood Dale, IL) and allowed to explore for 5 min. A longer latency to enter the brightly-lit (inner 20 cm square) center of the field, less time spent in the center of the field, and greater time spent immobile, were considered indices of greater anxiety-like behavior. The total distance (in meters) traveled, as well as the time spent rearing, were utilized as indices of motor/exploratory behavior (Bailey & Crawley, 2009).

2.5.2. Elevated plus maze—The elevated plus maze assesses anxiety-like behavior (Lister, 1987). Mice were placed in the center of a maze (Columbus Instruments, Columbus, OH, USA) comprised of four arms (two open arms: 30 × 5 × 0 cm each; two closed arms: 30 × 5 × 15.25 cm each) elevated 40 cm off the ground and allowed to explore for 5 min. A longer latency to enter the open arms and a greater amount of time spent in the closed, vs. open, arms were used as indices of greater anxiety-like behavior (File et al., 2005).

2.5.3. Marble burying—The marble burying test utilizes spontaneous digging behavior, characteristic of rodents, to assess anxiety/compulsive-like behavior (Broekkamp et al. 1986; Paris et al., 2014b; Poling et al. 1981). Briefly, mice were individually placed in a standard mouse housing cage (28 × 16 × 13 cm) with 20 marbles (1.5 cm diameter; evenly spaced in 5 rows of 4) located on a 5 cm layer of woodchip bedding. After 30 min, the number of marbles that were completely buried was counted. A greater number of buried marbles was considered an index of greater anxiety/compulsive-like behavior.

2.5.4. Rotarod—Locomotor behavior was assessed on an accelerated rotarod as previously described (Paris et al., 2013). Briefly, mice were trained to balance on an immobile rotarod (3 cm in diameter and suspended 44.5 cm high; Columbus Instruments, Columbus, OH) for 30 s. Mice were then trained to navigate the task across two 30 s fixed speed trials (10 rpm)

and two 180 s fixed speed trials (10 rpm). Lastly, mice were tested on two accelerated speed trials (180 s max. latency at 0 – 20 rpm). The mean latency to fall from the rotarod, and the maximum RPM achieved, across the two accelerated trials were utilized as indices for locomotor performance. Decreased latencies to fall and lower maximal RPM on the accelerated test indicate an impaired motor phenotype.

2.6. Protein oxidation assessment via OxyBlot

Whole brains were collected from mice tested in behavioral assays following completion of the last task. Striata (caudate/putamen) were grossly dissected at the time of tissue collection, flash-frozen in liquid nitrogen, and stored at -80°C until assay (~ 3 mo). At the time of assay, tissues were homogenized in RIPA buffer with a protease/phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail, Pierce, Rockford, IL) using a Precellys 24 Homogenizer (MO BIO Laboratories, Inc., Carlsbad, CA; 3×10 seconds). Protein concentrations were determined via bicinchoninic acid (BCA) assay per kit manufacturer instructions (Pierce Biotechnology, Rockford, IL).

An OxyBlot detection kit (EMD Millipore Corp., S7150, Darmstadt, Germany) was used to assess protein oxidation in striata. Briefly, carbonyl groups in homogenized lysates (20 μg protein) were, or were not, derivatized to 2,4-dinitrophenylhydrazine (DNP-hydrazone) by addition of DNP-hydrazine or a control solution, respectively. Derivatized and non-derivatized lysates were loaded onto 4 – 20 % Tris-HCl, Criterion TGX Stain-Free Gels (Bio-Rad Laboratories, Hercules CA) and proteins were transferred to PVDF membranes. DNP proteins were detected using the provided DNP antibody (rabbit, 1:150) and GAPDH was detected as a loading control using an anti-GAPDH antibody (mouse, EMD Millipore Corp., MAB360; 1:1000). Primary antibodies were visualized via application of appropriate secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 594. Five bands weighted by provided protein standards (21.0 – 97.4 kDa) were detected and intensity was quantified using Image Lab software (Bio-Rad Laboratories, Hercules, CA).

2.7. Circulating progesterone assessment via ELISA

In addition to brain, trunk blood was collected from mice at the time of sacrifice. Circulating progesterone was assessed in serum via enzyme-linked immunosorbent assay (ELISA; Neogen Corp., 402310, Lexington, KY). Consistent with previous methods (Paris et al., 2011), blood was collected in a chilled 1.5 mL aliquot tube and centrifuged at $3,000 \times g$ at 4°C for 10 min prior to freezing for later assay (~ 3 mo). Serum (100 μL) was incubated with ice-cold ether in glass borosilicate culture tubes, snap frozen, and evaporated to dryness. Steroid was reconstituted to 500 μL with kit extraction buffer and assayed per manufacturer instructions. The antibody supplied exerts a reported 100% cross-reactivity with progesterone and negligible (0.2 – 2.5 %) cross-reactivity for additional steroids (17 α -hydroxyprogesterone, androstenedione, corticosterone, cortisol, cortisone, dehydroepiandrosterone, deoxycorticosterone, estradiol, estriol, estrone, pregnenolone, testosterone). Intra-assay variance was 5.1 %.

2.8. Primary neuron-glia co-cultures and BV-2 microglia cultures

Neuron-glia co-cultures were prepared as previously described (Zou et al., 2011). In brief, primary mixed-glia cultures were derived from the striatum of postnatal day 0–1 ICR (CD-1) outbred mice. Dissected striata were minced before being incubated (37°C, 5% CO₂) with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA) for 30 min. Tissues were triturated and sequentially filtered through 100-µm and 40-µm diameter pore cell strainers (Greiner Bio-One). Cells were plated at a density of 50,000 cells/well onto poly-L-lysine-coated (Sigma-Aldrich, St. Louis, MO) 12-well culture plates and maintained for 6 days in DMEM supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT).

Primary striatal neurons were cultured from embryonic day 15–16 mouse pups. Tissues were dissociated as described for primary glial cells, except for the pore size of the two cell strainers, which were both 70 µm in diameter. After dissociation and filtration, neurons were plated at a density of 25,000 cells/well onto previously established 6-day-old mixed-glia cultures. Neuron-glia co-cultures were maintained in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen, Carlsbad, CA), L-glutamine (0.5 mM; Invitrogen, Carlsbad, CA), glutamate (25 mM; Sigma-Aldrich, St. Louis, MO), and an antibiotic mixture (Invitrogen, Carlsbad, CA), and incubated in a humidified incubator (37°C, 5% CO₂) for 6 days prior to repeated-measures experiments. For [Ca²⁺]_i assessment, dissociated neurons were plated on poly-L-Lysine coated 35-mm glass-bottom dishes at a density of 15,000 cells/dish and cultured for 6 days before fura-2 AM was loaded.

BV-2-derived microglia were prepared as previously described (Hahn et al., 2010). Briefly, BV-2 cells were cultured in T-75 flasks in medium comprised of RPMI-1645 (Gibco, Grand Island, NY) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA), and antibiotics, and incubated in a humidified incubator (37°C, 5% CO₂) for 3 days prior to experiments. Cells were harvested at 90 % confluency with a non-enzymatic dissociation agent (Cellstripper, Mediatech Inc., Manassas, VA), washed and re-suspended in media. For [Ca²⁺]_i assessment, BV-2 cells were plated on poly-L-lysine coated 35-mm glass-bottom dishes with a density of 600 cells/dish one day prior to fura-2 AM loading.

2.8.1. Neuronal viability in neuron-glia co-cultures—Computer-assisted, time-lapse imaging was used to track individual neurons for 60 h following treatment with vehicle (ddH₂O applied at hour 0), AlloP (100 nM applied at hour 0 and hour 30), and/or HIV-1 Tat_{1–86} (50 nM applied at hour 0) as previously described (Zou et al., 2011). Briefly, 12-well plates were transferred to a heat insert MXX holder (PeCon Instruments, Houston, TX) and set on the scanning stage of a Zeiss Axio Observer Z.1 inverted microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY). For each well, 10 non-overlapping fields were randomly selected. A total of 32–43 (mean = 37 ± 1) individual medium spiny striatal neurons were identified in each field on the basis of their distinctive morphology in digital images. Time-lapse images of the same series of fields were recorded at 30 min intervals for 60 h following treatments, using an automated, computer-controlled stage encoder and Axiovision 4.8 software (Carl Zeiss Microscopy, LLC, Thornwood, NY). During the course

of the experiment, cells were maintained in an XL S1 environment incubator (PeCon) at 37°C in 5% CO₂/95% air at high humidity. At the end of each experiment, we assessed all preselected neurons for viability in digital images taken at each time-point during the entire treatment period. Neuronal death was assessed using rigorous morphological criteria, including the disintegration of neurites, the involution and/or the complete fragmentation of the cell body. Findings were recorded as the mean percentage of surviving neurons, relative to pretreatment numbers $\pm$ SEM from $n = 3$ independent experiments.

2.8.2. Intracellular calcium in neuron or microglia culture—Dissociated primary striatal neurons, or BV-2-derived microglia, were cultured on 35-mm glass-bottom dishes (MatTek, Ashland, MA) and were loaded with fura-2 AM (2.5 μ m, Invitrogen, Carlsbad, CA) for 20 min (37°C, 5% CO₂). Cells were then washed once and incubated in the medium for another 20 min to allow the de-esterification of the acetoxymethyl ester (AM) group. After that, the culturing dish was transferred to an automated, computer-controlled stage embedded on the Zeiss Axio Observer Z1 microscope and imaged using the physiology module of the Zeiss Zen software (Carl Zeiss Microscopy, LLC, Thornwood, NY). A series of fluorescent images (excitation at 340 and 380 nm, emission at 510 nm) were taken using an AxioCam MRm digital camera (Carl Zeiss Microscopy, LLC, Thornwood, NY) at a frame rate of 1 Hz during the first 90 s, 0.2 Hz during the next 60 s, 0.033 Hz from 2.5 min to 10 min, and 0.0166 Hz from 10 to 20 min. For each cell, 3 regions of interest (ROIs) were randomly selected in the cytoplasm. [Ca²⁺]_i of each cell was calculated from the average F340/F380 ratio (Grynkiewicz et al, 1985) of 3 ROIs, using a standard curve generated by a calcium calibration buffer kit (Life Technologies, Carlsbad, CA).

2.8.3. Immunocytochemistry—Striatal neuron-glia co-cultures were fixed in 4% paraformaldehyde for 20 min, and then permeabilized with Triton X-100 for 20 min. Co-cultures were double-stained for Iba-1 (rabbit, Wako Pure Chemical Industries, 019-19741; 1:100) and GFAP (mouse, EMD Millipore Corp., MAB360; 1:200). Primary antibodies were detected using appropriate secondary antibodies conjugated to either AlexaFluor 488 or AlexaFluor 594, respectively. Cell nuclei were visualized with Hoechst 33342.

2.9. Statistical analyses

Calibration of behavioral tasks using gonadally intact mice was assessed via one-tailed Student's *t*-tests. Dependent measures for ovariectomized mice on behavioral tasks, OxyBlots, progesterone ELISA, and immunocytochemistry were assessed via separate two-way analyses of variance (ANOVA) with steroid treatment and Tat-tg genotype as factors. Simple, linear regressions were performed to assess the amount of variance that oxidatively modified striatal proteins accounted for on behavioral outcome measures. Dependent measures in the time-lapse experiments *in vitro* were assessed via repeated measures ANOVAs with treatment and time as factors. Fisher's Protected Least Significant Difference *post-hoc* tests determined group differences following main effects. Interactions were delineated via simple main effects and main effect contrasts with alpha controlled for multiple comparisons. Analyses were considered significant when $p < 0.05$.

3. Results

3.1. Anxiety-like behavior is exacerbated by central HIV-1 Tat and attenuated, in a 5 α -reductase-dependent manner, by progesterone

The influence of central HIV-1 Tat on anxiety-like behavior was assessed using mice that were transgenic for Tat₁₋₈₆ and the rtTA transcription factor necessary to express Tat (Tat⁺) or their control littermates that lacked the *tat* transgene (Tat⁻). Prior investigation of pregnane steroid influence (Paris et al., 2014a) was conducted in a similar, but distinct Tat transgenic murine model that is a high-expressing line of HIV-1 Tat (Kim et al., 2003). To assess a commensurate behavioral profile, a subset of gonadally intact male and female mice were assessed for anxiety-like behavior following a 30 d Dox-induction of the transgene. Consistent with prior results (Hahn et al., 2015; Paris et al., 2014ab, 2015), Tat⁺ mice demonstrated significantly greater anxiety-like behavior than their Tat⁻ counterparts (Table 1), thus validating the use of the present model for ovariectomy and assessment of hormone replacement manipulations. As such, female Tat⁺ and Tat⁻ mice were ovariectomized (day 1) and placed on Dox Diet for 2 weeks (a timeframe appropriate for endogenous hormone washout and the duration of Tat induction, before which, we have not observed anxiety-like effects) as depicted (Fig. 1A). On day 15, mice began hormone treatments (vehicle or finasteride pretreatment 1.5 h prior to vehicle or progesterone treatment) once every 5 days (Fig. 1A). On day 30, mice were tested 4 h after the last hormone treatment (Fig. 1A).

Inducing HIV-1 Tat significantly influenced anxiety-like responding among ovariectomized mice. Compared to Tat⁻ controls, Tat⁺ mice (i) had a longer latency to enter [$F(1,54) = 4.84$, $p < 0.05$] (Table 2), and (ii) spent less time in [$F(1,54) = 4.57$, $p < 0.05$] (Fig. 1B), the brightly-lit center of an open field, (iii) spent more time immobile [$F(1,54) = 4.74$, $p < 0.05$] (Table 2), (iv) less time on the open arms of an elevated plus maze [$F(1,54) = 4.03$, $p < 0.05$] (Fig. 1C), and (v) buried more marbles in an anxiogenic marble-burying task [$F(1,54) = 8.04$, $p < 0.05$] (Fig. 1D). Progesterone attenuated anxiety-like behavior in the open field and elevated plus maze (but not marble burying), significantly increasing the amount of time spent in the center of an open field [$F(2,54) = 4.73$, $p < 0.05$] (Fig. 1B) and the time spent on the open arms of an elevated plus maze [$F(2,54) = 5.35$, $p < 0.05$] (Fig. 1C), but not when preceded by finasteride. Notably, pretreatment with finasteride prior to progesterone, significantly increased the latency for mice to enter the open arms of the elevated plus maze [$F(2,54) = 5.80$, $p < 0.05$] (Table 2), independent of Tat-genotype.

Significant effects on locomotor behavior were not observed in the total distance traveled in an open field (Table 2), time spent rearing in the open field (Table 2), the latency to fall from (Fig. 1E), or the maximum RPM achieved on (Table 2), an accelerated rotarod. There was a notable, but non-significant, reduction in the open field distance traveled among Tat⁺ mice that were pretreated with finasteride (Table 2). As expected, circulating progesterone content was significantly [$F(2,34) = 9.38$, $p < 0.05$] greater among progesterone administered mice pretreated with vehicle ($p = 0.001$) or finasteride ($p = 0.0003$) compared to those treated only with two applications of vehicle (Table 3). Tat-genotype did not significantly influence circulating progesterone concentrations (Table 3).

3.2. Tat promotes, and progesterone mitigates, protein oxidation in striatum concurrent with anxiety-like behavior

Striatal protein oxidation was assessed in brains harvested from behaviorally-tested mice via OxyBlot assays (Fig. 2A). Hormone treatment and Tat genotype significantly interacted [$F(2,38) = 3.67, p < 0.05$] such that vehicle-treated Tat⁺ mice demonstrated greater striatal protein oxidation than did vehicle-treated Tat⁻ mice ($p = 0.002$; Fig. 2B). Tat-promoted oxidation was significantly attenuated by administration of progesterone, as observed in all progesterone-administered groups ($p = 0.007 - 0.01$); however, this effect was not influenced by pretreatment with finasteride (Fig. 2B).

Notably, oxidatively modified protein content significantly correlated with several measures of anxiety-like performance as assessed via linear regressions. Striatal protein oxidation significantly predicted the latency to enter an open arm on the elevated plus maze [$\beta = 45.13, t(42) = 2.09, p < 0.05$] (Fig. 2C), the amount of time spent on the open arms [$\beta = 3.89, t(42) = -2.08, p < 0.05$] (Fig. 2D), and the number of whole marbles buried in the marble burying task [$\beta = 4.12, t(42) = 2.02, p < 0.05$] (Fig. 2E). Protein oxidation accounted for a significant proportion of variance in these measures [open arm latency: $R^2 = 0.10, F(1,42) = 4.38, p < 0.05$; open arm time: $R^2 = 0.10, F(1,42) = 4.33, p < 0.05$, marbles buried: $R^2 = 0.09, F(1,42) = 4.01, p < 0.05$]. The four highest DNP-expressers were not from the same group (2× Tat⁺ mice administered vehicle/vehicle, 1× Tat⁻ mouse administered vehicle/progesterone, and 1× Tat⁻ mouse administered finasteride/progesterone). No additional correlations were revealed between protein oxidation and remaining behavioral measures.

3.3. Tat promotes neurotoxicity in primary neuron-glia co-cultures; effects are protected by allopregnanolone

To directly assess the neuroprotective potential of AlloP on HIV-1 Tat-mediated degeneration, mixed neuron/glia co-cultures were treated with vehicle, AlloP (100 nM), and/or Tat₁₋₈₆ (50 nM). Time-lapse imaging was used to track the fate of neurons for 60 h (Fig. 3A). All treatments were added at the start of the experiment (see Fig. 3B; left arrow at 0 h). Given the elimination phase half-life of AlloP *in vivo* (~4.5 h; Timby et al., 2006), the protective potential was further assessed via addition of the neurosteroid a second time, 30 h after treatment began (see Fig. 3B; right arrow at 30 h).

The percentage of neuronal survival was influenced by treatments over the time of observation [$F(45,120) = 16.23, p < 0.05$] (Fig. 3B). In wells treated with HIV-1 Tat₁₋₈₆, the proportion of surviving neurons was significantly reduced compared to all other treatments by 8 h ($p < 0.0001$ compared to any other group; Fig. 3B). Tat-treated cells demonstrated significantly less survival compared to control wells at every time-point examined thereafter ($p < 0.0001 - 0.002$; Fig. 3B). However, co-treatment with AlloP rescued Tat-induced neuronal cell death. Compared to wells treated with Tat alone, in the presence of AlloP, neurons in the Tat-treated wells demonstrated significantly greater viability from 8 h – 28 h ($p < 0.0001 - 0.008$) following the first AlloP application, and again from 40 h – 52 h ($p = 0.01 - 0.03$) following the second AlloP application (Fig. 3B). Notably, wells treated with AlloP alone demonstrated significantly greater viability than did control wells from 36 h –

48 h ($p = 0.02 - 0.04$; Fig. 3B) supporting the notion that AlloP is neuroprotective even in the absence of a direct insult.

3.4. HIV-1 Tat increases, and allopregnanolone attenuates, microglia numbers *in vitro*

In order to assess the influence that Tat and/or AlloP may exert on microgliosis and astrogliosis, neuron-glia co-cultures were labeled for Iba-1 and GFAP following the 60 h time-lapse treatment with vehicle, AlloP (100 nM), and/or Tat₁₋₈₆ (50 nM). The number of Hoechst 33342⁺ cells that were Iba-1⁺ or GFAP⁺ were counted and analyzed.

Treatment significantly influenced microglia numbers at the end of 60 h [$F(3,8) = 5.60$, $p < 0.05$]. Exposure to Tat significantly increased the proportion of Iba-1⁺ cells compared to vehicle-exposure ($p = 0.004$; Fig. 3C). Co-administration of AlloP significantly attenuated this effect ($p = 0.01$; Fig. 3C). Neither AlloP-treatment group (alone or in conjunction with Tat) significantly differed from vehicle-exposed controls (Fig. 3C). No significant differences were observed in the proportion of cells that were GFAP⁺ (Control: 65 ± 2 , AlloP: 66 ± 5 , Tat: 62 ± 3 , AlloP+Tat: 69 ± 3).

3.5. Allopregnanolone partially attenuates Tat-induced increases in neuronal [Ca²⁺]_i

Given that co-treatment with AlloP reduced Tat-induced neurodegeneration *in vitro*, we aimed to assess whether AlloP could attenuate the effects of HIV-1 Tat to dysregulate [Ca²⁺]_i, which we and others have observed to be an initial step in Tat-induced toxicity (El-Hage et al., 2005, 2008; Hu, 2016; Krogh et al., 2014, 2015; Fitting et al., 2014). Neuronal cultures were incubated with fura-2 AM and assessed via ratiometric imaging (Fig. 4A).

Intracellular calcium was significantly influenced by the treatments applied in a time-dependent manner [$F(438,7938) = 1.37$, $p < 0.05$] (Fig. 4B). HIV-1 Tat₁₋₈₆ significantly increased [Ca²⁺]_i compared to treatment with control vehicle or AlloP alone by 6 min following application, and this effect was maintained throughout the duration of the experiment ($p < 0.0001 - 0.05$; Fig. 4B). However, co-treatment with AlloP partially rescued Tat-induced [Ca²⁺]_i elevation. Neurons treated with AlloP and Tat together did not demonstrate significantly increased [Ca²⁺]_i compared to control neurons or neurons treated with AlloP at any time-point, and had significantly lower [Ca²⁺]_i than those treated with Tat alone from 13.5 min after application throughout the duration of the experiment ($p < 0.01 - 0.05$; Fig. 4B). Neurons treated with AlloP alone did not significantly differ from control neurons at any time-point.

3.6. Allopregnanolone partially attenuates Tat-induced increases in microglial [Ca²⁺]_i

The extent to which AlloP's neuroprotective effects could be due, in part, to direct actions on microglia were not known. To begin to assess this, microglia derived from a BV-2 cell line were loaded with fura-2 AM, exposed to vehicle, Tat₁₋₈₆ (50 nM) and/or AlloP (100 nM), and assessed via ratiometric imaging as described above.

Similar to effects in primary striatal neurons, HIV-1 Tat exacerbated, and AlloP partially prevented, elevations in microglial [Ca²⁺]_i, however, these factors did not interact. Rather, there was a main effect [$F(3,12791) = 8.29$, $p < 0.05$] for Tat to significantly increase [Ca²⁺]_i

compared to vehicle or AlloP-treatment alone ($p < 0.0001$) and for AlloP in conjunction with Tat to significantly differ from all other groups ($p < 0.0001$; Fig. 5). A main effect of time was also observed [$F(146,12791) = 9.22$, $p < 0.05$] with all time-points after 3 min significantly differing from initial observation (Fig. 5).

4. Discussion

The present findings upheld the hypothesis that HIV-1 Tat would increase, and exogenous progesterone administration would attenuate, anxiety-like behavior in ovariectomized female mice. These data are consistent with prior reports demonstrating Tat-induced anxiety-like behavior in mice (Hahn et al., 2015; Paris et al., 2014ab) and extend prior findings to reveal that blocking 5α -reduction attenuates progesterone's anxiolytic effects in the open field and elevated plus maze (but not marble burying). Notably, we have also observed endogenous hormone fluctuations to modestly influence Tat-induced-anxiety-like behavior in the open field and elevated plus maze, but not marble burying (the latter of which required greater progesterone replacement to reach amelioration; Paris et al., 2014a). In the present work, Tat induction significantly increased, and progesterone ameliorated, protein oxidation in striatum; a region highly implicated for pathology in the clinical HIV/AIDS population (Nath, 2015). However, these effects were independent of 5α -reductase blockade suggesting that protection involved actions of the pro-hormone, progesterone, over its metabolites. The mechanisms that may underlie these effects are not known, but parallel experiments *in vitro* upheld the hypothesis that the progesterone metabolite, AlloP, would attenuate Tat effects to promote neurotoxicity. This protection may have been conferred, in part, via AlloP's capacity to mitigate Tat-driven $[Ca^{2+}]_i$ in primary neurons or BV-2-derived microglia, as well as effects to attenuate Tat-mediated increases of microglia in neuroglia co-cultures. Together, these data support the notion that actions of 5α -reduced pregnane steroids may confer protection against HIV-1 Tat-mediated pathology.

Perturbations of immune cells within the CNS of HIV-infected individuals are associated with greater behavioral pathology. Macrophage recruitment and microglial activation were observed to co-occur with HIV encephalitis and HIV-associated dementia during the pre-HAART era (Glass et al., 1995; Tyor et al., 1995) and remain distinguishing features of neuroAIDS today, despite HAART (Burdo et al., 2013; Spudich & González -Scarano, 2012). Macrophage recruitment and microglial activation are associated with the production of cytokines, chemokines, proteases, reactive oxygen and nitrosative species, in addition to several excitotoxins (Colton & Gilbert, 1987; Kaul et al., 2001; Kraft-Terry et al., 2011; Meléndez et al., 2011). In pre-HAART observations of disease progression, microglial nodules, astrogliosis, and neurodegeneration characterized the clinical histopathology accompanying HIV-associated dementia (Everall et al., 1993; Kure et al., 1991; Navia et al., 1986). Recently, in a cohort of Hispanic women, cathepsins and cystatins, lysosomal proteins secreted from macrophages and microglia, were found to be elevated in the plasma of HIV seropositive individuals, compared to uninfected women (Cantres-Rosario et al., 2013). These lysosomal biomarkers were increased to a greater degree in monocytes and CSF among women with HIV-associated dementia (Cantres-Rosario et al., 2013). Therapeutics with the safety and bioavailability of steroid hormones, that can ameliorate the damaging effects of microglial reactivation, would be a useful adjunctive therapy.

Allopregnanolone is perhaps the most well characterized neurosteroid in respect to its known pharmacodynamic targets and behavioral effects. It is only recently that the potential influence of neurosteroids on HIV-pathology has been systematically considered (Perumal & Dhanasekaran, 2012). The expression of neurosteroid-synthesizing enzymes is reduced in HIV⁺ brains compared to uninfected brains (Maingat et al., 2013). This includes reductions in p450_{scc}, which catalyzes formation of pregnenolone (a progesterone precursor), and decreases in 5 α -reductase, the rate-limiting step in converting progesterone to AlloP (Maingat et al., 2013). Moreover, supernatant from HIV⁺ macrophages reduces expression of 5 α -reductase and 3 α -hydroxysteroid dehydrogenase (the enzymes required to convert progesterone to DHP, and DHP to AlloP, respectively) in human fetal neurons (but not astrocytes; Maingat et al., 2013). Another pregnenolone-derived neurosteroid, dehydroepiandrosterone (DHEA), has been assessed for its effects on HIV-toxicity. Sulfated DHEA levels are lower among individuals infected with clade-C HIV-1 (Chittiprol et al., 2009). As well, FIV⁺ animals demonstrate lower cortical DHEA and pregnenolone compared to FIV⁻ or mock-infected FIV⁻ animals (Maingat et al., 2013). These neurosteroids may exert important anti-inflammatory and antiviral effects. In support, application of sulfated DHEA to HIV⁺ human macrophages partially attenuated cytokine (TNF- α , IL-1 β , and IL-6) gene expression concurrent with down-regulated HIV replication (Maingat et al., 2013). Moreover, daily administration of this steroid ameliorated weight loss among FIV⁺ animals and improved CD4⁺ (but not CD8⁺) T-cell counts (Maingat et al., 2013). Notably, it did not influence viremia *in vivo* at the dosing regimen utilized but it did attenuate FIV-related increases in transcripts for GFAP, a marker of myeloid cell activation (F4/80), elevations in cytokine profile (including IL-1 β and TNF- α), and Iba-1⁺ cell counts as revealed by IHC (Maingat et al., 2013). These data add to what is known about steroid interactions in immunodeficiency viral infections and underscore their potential value as novel neuroimmune modulators.

The mechanisms by which AlloP may attenuate Tat's neurotoxic effects are not yet known; however, several overlapping avenues are likely involved. AlloP may exert anti-inflammatory effects that are independent of progesterone. In support, high concentrations of AlloP alone (10 μ M) attenuate LPS-induced nitric oxide release in microglial BV-2 cells by ~40%, efficacy commensurate to that of progesterone itself (Müller & Kerschbaum, 2006). Moreover, this protection was reduced by about half when dihydroprogesterone was used (a progestin receptor-preferring steroid with virtually no affinity for AlloP-neurotransmitter targets; Müller & Kerschbaum, 2006). Physiological concentrations of AlloP are also observed to inhibit LPS-promoted TNF production in mouse peritoneal macrophages to the same extent as physiological concentrations of progesterone (Ghezzi et al., 2000). Even low physiological concentrations of AlloP may protect blood-brain barrier integrity in response to an insult, such as a pathophysiological concentration of ammonia (Jayakumar et al., 2013). Others have utilized a mouse model of Niemann-Pick C, a disorder characterized by dysregulated cholesterol trafficking, to reveal a role for AlloP to inhibit microglial activation *in vivo* (Ahmad et al., 2005). As such, AlloP-mediated quiescence of central immune regulators, including microglia, may contribute to its neuroprotective efficacy.

To begin to elucidate the mechanisms of AlloP-neuroprotection in response to HIV-1 Tat, we first examined its influence on neuronal and microglial $[Ca^{2+}]_i$ regulation. We, and others, have observed Tat to drive Ca^{2+} influx in several CNS-resident cell types (El-Hage et al., 2005; Fitting et al., 2014; Haughey et al., 2001; Hu, 2016; Hui et al., 2014; Sorrell & Hauser, 2014; Zou et al., 2015). These effects have been observed in human microglia wherein $[Ca^{2+}]_i$ increases were rapid (as was also observed herein using BV-2 cells) and chemokine-dependent (Hegg et al., 2000). Notably, effects in human microglia could be blocked via L-type Ca^{2+} channel inhibition (Hegg et al., 2000). AlloP may exert similar effects to regulate $[Ca^{2+}]_i$ and has been shown to dampen glutamatergic activity in mPFC via inhibition of L-type Ca^{2+} channels (Hu et al., 2007). In particular, $Ca_v1.2$ and $Ca_v1.3$ L-type channels are identified as targets of AlloP inhibition (Earl & Tietz, 2011). Moreover, 5α -reduced steroid metabolites, including AlloP, are demonstrated to block T-type Ca^{2+} currents in sensory neurons (Todorovic & Lingle, 1998; Todorovic et al., 1998; Pathirathna et al., 2005; Nakashima et al., 1998). These effects have been observed with little influence over voltage-gated Na^+ or K^+ currents (Pathirathna et al., 2005). Given recent demonstrations that HIV-1 Tat can upregulate L-type Ca^{2+} channel expression in mPFC pyramidal cells (Wayman et al., 2012), and Tat actions at these targets promote neuronal excitability (Napier et al., 2014), neurosteroid therapeutics that ameliorate these actions may be highly effective. In support, AlloP in combination with diazepam confers protection from lethal seizure activity in mice, reducing microgliosis in hippocampus and cortex, and normalizing Ca^{2+} in cultured hippocampal neurons (Cao et al., 2012; Bruun et al., 2015). Future investigations should assess AlloP efficacy over Tat-mediated pathology in additional brain regions associated with anxiety, including the hippocampus.

Clinically, it is important to identify the naturally metabolizing steroid hormones that may underlie protection from HIV-pathology that is observed among women over men in some populations. While an influence of gender on HIV-viremia was not observed in a small sample of antiretroviral-naïve patients ($n = 40$; Bush et al., 1996), a recent sample of over 1,500 HIV⁺, treatment-naïve patients from 4 continents, found women to have higher CD4⁺ T-cell counts and lower viral loads, compared to men (Grinsztejn et al., 2011). Notably, these sex differences were independent of the country of origin. Moreover, the safety of some antiretroviral regimens may favor women over men (Campbell et al., 2012), although the extent to which the hormonal milieu accounts for variance in therapeutic response is not known. However, synthetic steroids that do not metabolize to neuroprotective pregnane compounds may be detrimental to HIV progression. In a cross-section of Kenyan and South African women, injectable synthetic progestins (depot medroxyprogesterone acetate or norethisterone oenanthate) were associated with a greater proportion of vaginal pro-inflammatory cytokines (Deese et al., 2015). Similar findings are observed in U.S. women on non-metabolizing, synthetic progestin therapies (Ghanem et al., 2005) and several investigations find HIV progression to be exacerbated by such therapies (Baeten et al., 2005; Stringer et al., 2007, 2009), whereas natural progesterone may be beneficial (Vassiliadou et al., 1999).

Although the current studies focused on identifying a pregnane steroid metabolite that may confer protection to the effects of HIV-1 Tat, several lines of research also support a role for estrogens. For example, estrogen may attenuate neurotoxicity in response to the HIV-1

proteins, Tat or gp120 (Howard et al., 2001; Wilson et al., 2006; Zemlyak et al., 2002), particularly when toxicity is catalyzed by psychostimulant exposure (Kendall et al., 2005; Turchan et al., 2001; Wallace et al., 2006). In these studies, the pro-hormone, progesterone, was found to be minimally protective (Kendall et al., 2005; Wallace et al., 2006); whereas, estrogen's anti-oxidant properties were suggested to attenuate microglial activation in response to Tat or gp120 (Bruce-Keller et al., 2001; Corasaniti et al., 2005; Zemlyak et al., 2005). As such, the interactions between estrogens and 5 α -reduced pregnane steroids, particularly AlloP, will be important considerations for future work.

While novel, the present data must be considered with some caveats. Anxiety-like behavior is increased when peripheral steroid glands are surgically removed (i.e., gonads and/or adrenals) such that gonadectomy/adrenalectomy produces more anxiogenesis than gonadectomy or adrenalectomy alone, and gonadally-intact proestrous rodents demonstrate less anxiogenic behavior than any aforementioned model (Frye & Paris, 2011). As such, ceiling effects can be observed when assessing additional anxiogenic manipulations (such as HIV-1 Tat expression) and have been reported previously (Paris et al., 2014a). To this end, future experiments may assess the effects of finasteride in gonadally-intact Tat-tg mice which demonstrate less anxiety-like behavior than OVX mice (Table 1). As well, the use of linear regression across behavioral and biochemical measures can be influenced by several high responders. While the present regressions demonstrated significant correlation between protein oxidation in the striatum and some anxiety measures, the amount of variance explained (~10 %) must be taken into consideration. Lastly, the progesterone-dosing regimen utilized herein reinstates circulating physiological progesterone at the time of progesterone administration and demonstrates that some anti-anxiety-like effects are 5 α -reductase-dependent. However, reversal of Tat-mediated affective disorders (extending beyond anxiety) will likely require greater progestogen dosing to be clinically efficacious, particularly considering the AlloP concentrations that have been used in preclinical studies aimed at treating dementia (Irwin & Brinton, 2014; Irwin et al., 2015). In support, Tat-induced aberrations in marble burying behavior, which models aspects of anxiety and compulsive affective disorders, can be modulated by multiple neurotransmitter systems (Albelda & Joel, 2012), and requires greater progesterone exposure to be ameliorated than that used herein (Paris et al., 2014a).

These data are the first to demonstrate a protective role for the progesterone metabolite, AlloP, on HIV-1-mediated toxicity. We find that HIV-1 Tat expression promotes anxiety-like behavior of mice *in vivo* and progesterone's anxiolytic effects are 5 α -reductase-dependent. The 5 α -reduced progestogen, AlloP, ameliorated Tat-induced neuronal death, restored calcium ion homeostasis, and quiesced microglial reactivity in culture. The therapeutic advantages to utilizing neurosteroids clinically for neuroinflammatory disease states should be further investigated.

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Highlights

- HIV-1 Tat exacerbates, and progesterone attenuates, anxiety-like behaviors in mice
- Inhibiting 5 α -reductase blocks progesterone-anxiolysis, not Tat-protein oxidation
- The 5 α -reduced progestogen, allopregnanolone, attenuates Tat-neurotoxicity *in vitro*
- Tat increases, and allopregnanolone attenuates, microglial number *in vitro*
- Allopregnanolone attenuates Tat-driven intracellular Ca²⁺ in neurons and microglia

Figure 1.

(A) Female mice ($n = 10/\text{group}$) were ovariectomized (day 0) and placed on doxycycline chow which conditionally-induces HIV-1 Tat₁₋₈₆ transgene expression in the CNS of those that are Tat⁺ (but not control Tat⁻ counterparts). After two weeks of recovery, mice received pretreatment with oil vehicle (10 % EtOH, s.c.) or finasteride (50 mg/kg, s.c.), followed 1.5 h later by vehicle or progesterone (4 mg/kg, s.c.) once every 5 days for 15 days. On day 15, 4 h after the last injection, mice were assessed for anxiety-like behavior via the (B) time spent in the brightly lit center of an open field, (C) time spent on the open arms of an elevated plus maze, and (D) number of marbles buried in an anxiogenic marble burying task. (E) Motor behavior was assessed in a subset of mice ($n = 7 - 8/\text{group}$) via the latency to fall from an accelerated rotarod. * indicates significant difference between Tat⁻ and Tat⁺ mice. † indicates significant difference between progesterone treatment vs. all other treatments, $p < 0.05$.

Figure 2.

(A) Representative OxyBlot on striatal tissues from behaviorally tested Tat-transgenic mice. Carbonyl groups in tissues were either derivatized to 2,4-dinitrophenylhydrazine (DNP; left lanes) via DNP-hydrazine, or not, using a control solution (right lanes). (B) DNP/GAPDH content in striatum of behaviorally-tested Tat⁺ or Tat⁻ ovariectomized mice, treated with oil vehicle (10 % EtOH, s.c.), finasteride (50 mg/kg, s.c.) and/or progesterone (4 mg/kg, s.c.) once every 5 days for 15 days. Striatal DNP content significantly correlated with the (C) latency to enter an open arm on the elevated plus maze, (D) the time spent in the open arms, and (E) the number of marbles buried in an anxiogenic marble burying task, $p < 0.05$.

Figure 3.

(A) Images of neurons plated in a neuron-glia co-culture following treatment with vehicle control (ddH₂O), allopregnanolone (AlloP; 100 nM), and/or HIV-1 Tat₁₋₈₆ (50 nM). Neuronal fate was followed (see white arrows) over a 60 h time-course. (B) Time lapse, repeated measures analyses were taken after all treatments were applied at hour 0 (left black arrow). AlloP was applied again at hour 30 to re-assess potential protective effects over Tat-neurotoxicity (second black arrow). (C) Percentage of cells, fixed at hour 60, that were Hoechst/Iba-1⁺. * indicates significant difference between control vs. Tat-treated neurons. † indicates significant difference between co-treated AlloP+Tat neurons vs. Tat-treated neurons. ‡ indicates significant difference between control vs. AlloP-treated neurons, $p < 0.05$. Scale bar = 30 μ m.

Figure 4.

(A) Pseudocolor images of Tat-increased $[Ca^{2+}]_i$ as assessed by ratiometric imaging of fura-2, AM following application of vehicle control (ddH₂O), allopregnanolone (AlloP; 100 nM), and/or HIV-1 Tat₁₋₈₆ (50 nM). (B) Pretreatment with AlloP protected neurons from Tat-induced increases in $[Ca^{2+}]_i$ in a time-dependent manner. Dotted line indicates application of Tat at 30 s. * indicates significant difference between control vs. Tat-treated neurons. † indicates significant difference between neurons co-treated with AlloP+Tat vs. Tat-treated neurons, $p < 0.05$. Scale bar = 10 μ m.

Figure 5.

Allopregnanolone (100 nM) pretreatment protected BV-2-derived microglia from HIV-1 Tat (50 nM)-increased $[Ca^{2+}]_i$, assessed via ratiometric imaging of fura-2 AM. Dotted line indicates application of Tat at 30 s. * indicates significant difference between control vs. Tat-treated microglia. † indicates significant difference between co-treated AlloP+Tat microglia vs. Tat-treated microglia. § indicates timepoints differ from 0 min, $p < 0.05$.

Open field, elevated plus maze, and anxiogenic marble burying performance of gonadally-intact male and female mice (n = 8/grp) that conditionally-expressed the HIV-1 Tat₁₋₈₆ transgene in the CNS (Tat⁺) or lacked the transcription factor necessary for Tat expression (Tat⁻).

Table 1

	Male		Proestrous Female		Diestrous Female	
	Tat ⁻	Tat ⁺	Tat ⁻	Tat ⁺	Tat ⁻	Tat ⁺
<i>Open Field</i>						
Total Distance (m)	9 ± 1	8 ± 1	9 ± 1	9 ± 2	7 ± 2	8 ± 1
Time Rearing (s)	15 ± 5	14 ± 4	16 ± 5	15 ± 5	13 ± 4	14 ± 3
Time in Center (s)	9 ± 2	4 ± 2 [*]	8 ± 2	3 ± 1 [*]	3 ± 1	2 ± 1
Latency to Center (s)	57 ± 24	134 ± 53	70 ± 32	150 ± 48	101 ± 46	167 ± 59
Time Immobile (s)	134 ± 17	189 ± 21 [*]	127 ± 20	200 ± 18 [*]	210 ± 22	208 ± 18
<i>Elevated Plus Maze</i>						
Latency to Open Arm (s)	105 ± 23	113 ± 34	155 ± 55	270 ± 10	158 ± 52	183 ± 48
Open Arm Time (s)	22 ± 4	5 ± 1 [*]	29 ± 9	9 ± 2 [*]	15 ± 4	6 ± 2 [*]
Closed Arm Time (s)	235 ± 27	243 ± 21	262 ± 9	279 ± 4	281 ± 3	284 ± 4
<i>Marble Burying</i>						
Whole Marbles Buried	3 ± 1	9 ± 1 [*]	5 ± 2	12 ± 2 [*]	7 ± 2	14 ± 2 [*]

* indicates significant difference between Tat⁻ and Tat⁺ mice, $p < 0.05$.

Open field, elevated plus maze, and rotarod performance of ovariectomized (OVX) mice that conditionally-expressed the HIV-1 Tat₁₋₈₆ transgene in the CNS (Tat⁺) or lacked the transcription factor necessary for Tat expression (Tat⁻). Mice were treated with oil vehicle (10 % EtOH, s.c.) or finasteride (50 mg/kg, s.c.), followed 1.5 h later by vehicle or progesterone (4 mg/kg, s.c.) once every 5 days for 15 days.

Table 2

	Vehicle/Vehicle		Vehicle/Progesterone		Finasteride/Progesterone	
	OVX Tat ⁻	OVX Tat ⁺	OVX Tat ⁻	OVX Tat ⁺	OVX Tat ⁻	OVX Tat ⁺
<i>Open Field</i>						
Total Distance (m)	5.9 ± 1.6	5.2 ± 0.4	7.0 ± 1.1	4.8 ± 1.1	4.7 ± 1.0	2.4 ± 1.0
Time Rearing (s)	7 ± 3	3 ± 1	9 ± 2	6 ± 2	3 ± 1	4 ± 2
Latency to Center (s)	179 ± 42	229 ± 24 [*]	160 ± 42	198 ± 34 [*]	149 ± 46	259 ± 28 [*]
Time Immobile (s)	181 ± 28	187 ± 9 [*]	155 ± 18	198 ± 20 [*]	183 ± 24	244 ± 18 [*]
<i>Elevated Plus Maze</i>						
Latency to Open Arm (s)	103 ± 40	80 ± 33	61 ± 35	101 ± 45	165 ± 46 [‡]	243 ± 38 [‡]
Closed Arm Time (s)	284 ± 3	277 ± 5	281 ± 3	267 ± 20	289 ± 3	288 ± 4
<i>Rotarod</i>						
Max. Velocity (RPM)	12 ± 2	11 ± 1	13 ± 1	12 ± 1	13 ± 2	9 ± 2

^{*} indicates significant main effect of genotype (Tat⁻ vs. Tat⁺).

[‡] indicates significant main effect of hormone treatment (finasteride/progesterone-treated mice vs. all other treatments), $p < 0.05$.

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Table 3

Circulating progesterone content among ovariectomized (OVX) mice (n = 6–7/grp) that conditionally-expressed the HIV-1 Tat_{1–86} transgene in the CNS (Tat⁺) or lacked the transcription factor necessary for Tat expression (Tat⁻) and were treated with oil vehicle (10 % EtOH, s.c.) or finasteride (50 mg/kg, s.c.), followed 1.5 h later by vehicle or progesterone (4 mg/kg, s.c.) on the day of behavioral testing.

	Vehicle/Vehicle		Vehicle/Progesterone		Finasteride/Progesterone	
	OVX Tat ⁻	OVX Tat ⁺	OVX Tat ⁻	OVX Tat ⁺	OVX Tat ⁻	OVX Tat ⁺
Progesterone (ng/mL)	1.0 ± 0.4	1.9 ± 0.6	17.2 ± 4.2 [‡]	23.9 ± 6.5 [‡]	23.0 ± 8.7 [‡]	22.4 ± 3.7 [‡]

[‡] indicates significant main effect of hormone treatment (significant difference from vehicle/vehicle-treated mice), *p* < 0.05.