10-1-2012

Neurosteroid-Mediated Regulation of Brain Innate Immunity in HIV/Aids: DHEA-S Suppresses Neurovirulence

Amber Paul
University of Alberta, paula6@erau.edu

Ferdinand G. Maingat
University of Alberta

Maria J. Polyak
University of Alberta

Pornpun Vivithanaporn
Mahidol University, University of Alberta

Farshid Noorbakhsh
University of Alberta

See next page for additional authors

Scholarly Commons Citation

Paul, A., Maingat, F. G., Polyak, M. J., Vivithanaporn, P., Noorbakhsh, F., Ahboucha, S., Baker, G. B., Pearson, K., & Power, C. (2012). Neurosteroid-Mediated Regulation of Brain Innate Immunity in HIV/Aids: DHEA-S Suppresses Neurovirulence. *The FASEB Journal, 27*(2). https://doi.org/10.1096/fj.12-215079

Neurosteroid-mediated regulation of brain innate immunity in HIV/AIDS: DHEA-S suppresses neurovirulence

Ferdinand G. Maingat,* Maria J. Polyak,* Amber M. Paul,* Pornpun Vivithanaporn,*,§ Farshid Noorbakhsh,* Samir Ahboucha,‖ Glen B. Baker,† Keir Pearson,‡ and Christopher Power*,†,1

*Department of Medicine, †Department of Psychiatry, and ‡Department of Physiology; University of Alberta, Edmonton, Alberta Canada; §Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok, Thailand; and ‖Department of Pharmacology and Environment, Faculty of Sciences, Université Cadi Ayyad, Marrakech, Morocco

ABSTRACT **Neurosteroids are cholesterol-derived molecules synthesized within the brain, which exert trophic and protective actions. Infection by human and feline immunodeficiency viruses (HIV and FIV, respectively) causes neuroinflammation and neurodegeneration, leading to neurological deficits. Secretion of neuroinflammatory host and viral factors by glia and infiltrating leukocytes mediates the principal neuropathogenic mechanisms during lentivirus infections, although the effect of neurosteroids on these processes is unknown. We investigated the interactions between neurosteroid-mediated effects and lentivirus infection outcomes. Analyses of HIV-infected (HIV^+) and uninfected human brains disclosed a reduction in neurosteroid synthesis enzyme expression. Human neurons exposed to supernatants from HIV^+ macrophages exhibited suppressed enzyme expression without reduced cellular viability. HIV^+ human macrophages treated with sulfated dehydroepiandrosterone (DHEA-S) showed suppression of inflammatory gene (*IL-1*β, *IL-6*, *TNF*-α) expression. FIV-infected (FIV^+) animals treated daily with 15 mg/kg body weight. DHEA-S treatment reduced inflammatory gene transcripts (*IL-1*β, *TNF*-α, *CD3*ε, *GFAP*) in brain compared to vehicle-(β-cyclodextrin)-treated FIV^+ animals similar to levels found in vehicle-treated FIV^- animals. DHEA-S treatment also increased $CD4^+$ T-cell levels and prevented neurobehavioral deficits and neuronal loss among FIV^+ animals, compared to vehicle-treated FIV^+ animals. Reduced neuronal neurosteroid synthesis was evident in lentivirus infections, but treatment with DHEA-S limited neuroinflammation and prevented neurobehavioral deficits. Neurosteroid-derived therapies could be effective in the treatment of virus- or inflammation-mediated neurodegeneration.—Maingat, F. G., Polyak, M. J., Paul, A. M., Vivithanaporn, P., Noorbakhsh, F., Ahboucha S., Baker, G. B., Pearson, K., Power, C. Neurosteroid-mediated regulation of brain innate immunity in HIV/AIDS: DHEA-S suppresses neurovirulence. *FASEB J.* 27, 725–737 (2013). www.fasebj.org**

Key Words: FIV · inflammation · glia · neuron

Steroid hormones are widely acknowledged to exert their effects by binding to intracellular receptors that act as transcriptional regulators of gene expression (1, 2). Investigations over the past 2 to 3 decades have demonstrated that some steroids also bind to specific neurotransmitter receptors and alter neuronal excitability, producing nongenomic effects (3–5). Steroids with these properties have been termed "neuroactive steroids" (NASs; ref. 3) and include pregnenolone and pregnenolone sulfate (6), dehydroepiandrosterone (DHEA), and DHEA sulfate (DHEA-S), and progesterone, as well as some 3α-reduced neurosteroids, including allopregnenolone and tetrahydrodeoxycorticosterone (THDOC). Neuroactive steroids can either originate as circulating steroid hormones or be produced locally within the brain from cholesterol and in the latter case are termed neurosteroids (7). Conversion of cholesterol to pregnenolone is catalyzed by the enzyme P450 side-chain cleavage (P450scc; ref. 8), and through the actions of several enzymes, pregnenolone is converted to a num-

Abbreviations: βCD, β-cyclodextrin; AIDS, acquired immunodeficiency syndrome; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; FIV, feline immunodeficiency virus; GABA, γ-aminobutyric acid; GC-MS, gas chromatography-mass spectrometry; GFAP, glial fibrillary acidic protein; hFA, human fetal astrocyte; hFN, human fetal neuron; HIV, human immunodeficiency virus; hMDM, human monocyte-derived macrophage; LC-MS, liquid chromatography-mass spectrometry; MEM, minimum essential medium; NAS, neuroactive steroid; OMT, object memory test; P450scc, P450 side-chain cleavage; PBMC, peripheral blood mononuclear cell; SCC, side-chain cleavage; SIV, simian immunodeficiency virus

[1] Correspondence: Department of Medicine, 6-11 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2, Canada. E-mail: chris.power@ualberta.ca

doi: 10.1096/fj.12-215079

ber of other neurosteroids, including those mentioned above.

Neuroactive steroids can act as allosteric modulators at several neurotransmitter receptors, including γ-aminobutyric acid (GABA) (9, 10), NMDA glutamate (11–14), 5-HT3 (15, 16), and σ_1 receptors (17), with the GABA-A receptor and the NMDA receptor receiving the most attention in this regard. A large body of evidence now implicates NASs in the pathophysiology and/or pharmacotherapy of a number of neuropsychiatric conditions (18–27).

The immunosuppressive lentiviruses, including human, simian, and feline immunodeficiency viruses (HIV, SIV, and FIV, respectively) are defined by systemic immune disruption followed by immunosuppression leading to the acquired immunodeficiency syndrome (AIDS) with the concurrent development of neurological disorders, termed neurovirulence (28). Despite the increased accessibility to antiretroviral therapies, neurocognitive impairment remains an ongoing problem among HIV-infected persons receiving requisite antiretroviral treatments for systemic immunosuppression (29, 30). This situation might be caused by insufficient brain penetration of the current antiretroviral drugs, the development of drug-resistant viruses within the brain, or a cascade of innate immune activation, initiated (but not promulgated) by virus replication. As a consequence, the virus exerts its pathogenic effects, including induction of aberrant neuroinflammatory responses, as well as the expression and release of cytotoxic viral proteins. The collective actions of these pathogenic host and viral processes result in neuronal injury; for example, synaptodendritic retraction followed by neuronal injury and eventual death typify the disease process in HIV-associated neurocognitive disorders. These circumstances call for examining new disease pathways, which could be targeted with rational therapies and might also enhance the effects of existing antiretroviral therapies.

FIV is structurally similar to HIV and causes immunosuppression and neurological disorders in domestic cats. Like HIV, FIV infects T cells, causing CD4 T-cell suppression in blood together with high virus levels in blood. Moreover, FIV infects the brain, causing memory and learning impairments, gait abnormalities, seizures, and agitation, which resemble many aspects of HIV-associated neurocognitive disorders (31–33). Like HIV and SIV, FIV infects microglial cells, resulting in the induction of neuroinflammatory processes, including production and secretion of free radicals, cytokines, and chemokines, which promote the entry of activated lymphocytes into the brain (34–36). These events likely contribute to neurodegeneration, defined by neuronal injury and loss in FIV (and HIV) infection.

Given that neurosteroids influence neurocognitive performance (37), regulate innate immune activation (38), and modulate neuronal growth and survival (39, 40), we hypothesized that neurosteroid synthesis was disrupted during HIV infection, contributing to HIV-related neurodegeneration. Moreover, neurosteroid treatment might be beneficial in lentivirus-related neurological diseases. The present studies indicated that enzymes responsible for neurosteroid synthesis in the brain were suppressed in HIV infection, particularly in neurons and that treatment with the long-acting neuroactive steroid DHEA-S improved neurological performance and reduced *in vivo* neuroinflammation and neurodegeneration.

MATERIALS AND METHODS

Ethics statement

The use of autopsied brain tissues and blood were approved under the protocol number 2291 by the University of Alberta Human Research Ethics Board (Biomedical), and written informed consent documents were signed before or at the collection time. Human fetal tissues were obtained from 15- to 19-wk aborted fetuses with written consent approved under the protocol 1420 by the University of Alberta Human Research Ethics Board (Biomedical). All animals were housed and monitored on a regular schedule, and the behavioral tests performed adhered to the Center for Animal Care and Control Guidelines. All surgery was performed after pentobarbital sodium administration, and all efforts were made to minimize suffering. This study was approved under the protocol number 449 by the University of Alberta Animal Care and Use Committee for Health Sciences.

Viruses

Culture supernatants from feline peripheral blood mononuclear cells (PBMCs) infected with an infectious molecular clone (FIV-Ch) served as sources of infectious FIV (41) for the *in vivo* experiments. Supernatants from human PBMCs infected with an HIV-1 strain (HIV-1 SF162) were prepared similarly and used for *ex vivo* infection experiments. Viruses were titered by limiting dilution, as previously reported (42–44).

Human brain samples

Human CNS tissue (frontal lobe) was collected at autopsy with consent and stored at −80°C from HIV-infected (HIV encephalitis, $n=2$; toxoplasmic encephalitis, remote from lesion, $n=3$; normal brains, $n=2$) and noninfected (stroke, $n=2$; sepsis, $n=3$; leukemia, $n=2$) control patients with consent. All HIV-infected individuals were AIDS defined and had died of AIDS-related causes (43, 44).

Analysis of neurosteroids

Gas chromatography-mass spectrometry (GC-MS) analysis was used to measure levels of DHEA and pregnenolone in CNS tissues, using a modification of a procedure we described previously (45). Protein was precipitated by the addition of methanol followed by centrifugation. The supernatant was retained, and the steroids were isolated using solid-phase extraction with Oasis 30-mg HLB plates (Waters, Mississauga, ON, Canada). The samples were then eluted with dichloromethane:methanol (90:10 v/v), taken to dryness, and derivatized with heptafluorobutyrylimidazole (Fisher, Mississauga, ON, Canada) and the resultant derivatives were analyzed by GC combined with negative ion chemical ionization MS (an Agilent 6890 GC coupled to a 5973N mass selective detector;

Agilent Technologies, Santa Clara, CA, USA). For analysis of DHEA-S, the extraction procedure was carried out using an HLB microElution plate followed by elution with acetonitrile: methanol (60:40) and direct measurement of levels of underivatized DHEA-S by liquid chromatography-mass spectrometry (LC-MS) using a Waters 2695 separations module connected to a Waters micromass Z_Q. Standard curves were run in parallel with each assay.

Cell culture

Primary human fetal neurons (hFNs) were obtained in accordance with the University of Alberta Ethics Committee, prepared as previously reported (46) and cultured in polyornithine (Sigma-Aldrich, St. Louis, MO, USA)-coated plates with supplemented minimum essential medium (MEM) with 10% fetal bovine serum (FBS; Life Technologies, Burlington, ON, Canada) (47). Primary human fetal astrocytes (hFAs) were prepared using the same method but were grown in the absence of cytosine arabinoside (Sigma-Aldrich). Human monocyte-derived macrophages (hMDMs) were prepared from healthy HIV-seronegative controls by initially isolating PBMCs on a Histopaque gradient and plastic adherence and thereafter maintained in RPMI (Life Technologies) supplemented with 10% L929 medium and 20% FBS (47). MDMs were cultured at 37°C, 10% CO_2, for 1 wk before use.

HIV infection and DHEA-S treatment of macrophages

hMDMs were pretreated with DHEA-S (100 nM; Steraloids, Newport, RI, USA) or DMSO in growth medium 2 h prior to infection. hMDMs were subsequently infected with HIV-SF162 or mock infected for 6 h, after which the medium was replaced with fresh growth medium, as described previously (48). The cultures were maintained for 72 h at 37°C, 5% CO_2, after which a reverse transcriptase assay was performed on the harvested supernatants to monitor levels of viral release, as previously reported (47). The supernatants harvested at d 3, 6, and 9 were applied to hFNs and hFAs for 48 h at 37°C, 5% CO_2. Cultures were then fixed and processed for in-cell Western blot analysis of β-tubulin immunoreactivity and DAPI staining.

In-cell Western blot analysis

hFNs and hFAs were cultured for 24 h with supernatants from HIV-mock or HIV-infected MDM at 37°C, 10% CO_2. An in-cell Western ELISA (LI-COR Biosciences, Lincoln, NE, USA) using antibodies against β-tubulin was used to assess neuronal viability. After treating hFNs and hFAs with MDM-derived supernatants, cells were fixed in 2% formalin, washed in PBS, permeabilized with 0.5% Triton X-100 in PBS, and blocked in LI-COR blocking buffer. Fixed cells were incubated overnight with anti-β-tubulin (1:800; Sigma-Aldrich), human P450scc [1:100; a generous gift from Dr. Walter Miller, University of California, San Francisco (UCSF), San Francisco, CA, USA; ref. 49], 5α-reductase (1:100; Dr. Synthia Mellon, UCSF), or HSD3B1 (3βHSD, 1:100; Abcam, Cambridge, MA, USA) antibodies. Then, the cells were washed and incubated with secondary Alexa Fluor 680 (Invitrogen, Burlington, ON, Canada)-conjugated or IRDye 800 (Rockland Immunochemicals, Gilbertsville PA, USA)-conjugated antibody. After the final washing, neuronal viability or staining was quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences).

DAPI staining

Fixed cells were exposed to 0.01 mg/ml DAPI (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature, followed by 3 washes in PBS. After drying, DAPI fluorescence (excitation at 360 nm) was quantified using Gen5 software on a Synergy HT Microplate Reader (Biotek, Winooski, VT, USA).

Western blot analysis

Cortical tissue from HIV^+ and HIV^- patients was prepared for Western blotting by homogenizing in Lysing Matrix D tubes using the Fast-Prep-24 apparatus (MP Biomedicals, Eschwege, Germany) and boiling in Laemmli sample buffer, followed by separation by 12% SDS-polyacrylamide at 120 V for 2 h. Proteins were transferred overnight at 4°C onto nitrocellulose membranes, followed by blocking with 5% skimmed milk to prevent nonspecific binding. Western blot analysis was performed using a rabbit polyclonal antibody to 5α-reductase (1:100; Dr. Synthia Mellon, UCSF) followed by peroxidase conjugated secondary antibody and processing with Pierce ECL Western blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

Flow cytometry analysis

PBMCs were isolated from blood of FIV^- and FIV^+ animals at wk 8 and 12, as previously reported (50). PBMCs were labeled with anti-feline CD4 or CD8 monoclonal antibodies, and FITC-conjugated goat anti-mouse IgG1 antibody was used as a secondary antibody. Omitting the primary antibody served as a control. FACS analysis was performed using the FACSCanto (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer.

Host gene analyses

First-strand cDNA was synthesized by using aliquots of 1 μg of total RNA prepared from brain and cultured hMDMs together with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random primers (51). Specific genes were quantified by real-time RT-PCR using an i-Cycler IQ system (Bio-Rad, Mississauga, ON, Canada). cDNA prepared from total RNA derived from plasma, brain, and cell cultures was diluted 1:2 with sterile water and 5 μl were used as a template for a PCR reaction. The specific primers used in the real-time PCR are summarized in **Table 1**. Semiquantitative analysis was performed by monitoring, in real time, the increase of fluorescence of the SYBR Green dye on the Bio-Rad detection system, as previously reported (52), and was expressed as relative fold change (RFC) compared to mock-infected samples.

Reverse transcriptase assay

Reverse transcriptase activity in culture supernatants was measured using a protocol described previously (53). Briefly, 10 μl of culture supernatant was cleared of cellular debris by high-speed centrifugation and incubated with 40 μl of reaction cocktail containing [$-^{32}$P]TTP for 2 h at 37°C. Samples were blotted on DE81 Ion Exchange Chromatography Paper (Whatman International, Clifton, NJ, USA) and washed 3 times for 5 min in 2× SSC and twice for 5 min in 95% ethanol (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Reverse transcriptase levels were measured by liquid scintillation counting. All assays were performed in duplicate and repeated ≥2 times.

TABLE 1. *Oligonucleotide primers used in real-time RT-PCR analyses*

Primer	Sequence, 5′–3′	T_m (°C)	Species
GAPDH for	AGCCTTCTCCATGGTGGTGAA	56–60	Human/feline
GAPDH rev	CGGAGTCAACGGATTTGGTCG	56–60	Human/feline
IL-1β for	CCAAAGAAGAAGATGGAAAAGCG	56	Human/feline
IL-1β rev	GGTGCTGATGTACCAGTTGGG	56	Human/feline
TNFα for	CCCAGGGACCTCTCTCTAATCA	56	Human
TNFα rev	GCTACAGGCTTGTCACTCGG	56	Human
CD3ε for	GATGCAGTCGGGCACTCACT	58	Human
CD3ε rev	CATTACCATCTTGCCCCCAA	58	Human
HIVpol for	TTAAGACAGCAGTACAAATGGCAGT	56	Human
HIVpol rev	ACTGCCCCTTCACCTTTCCA	56	Human
IL-10 for	CCTCTCACCGTCTTGCTTTC	56	Human
IL-10 rev	GCAGAGGTTGCTTGTTCTCC	56	Human
IL-6 for	AGCTGCGCAGAATGAGATGAGTT	56	Human
IL-6 rev	ACCCCTGACCCAACCACAAAT	56	Human
IL-4 for	GGCTGACTTAGGAGCTGGTG	56	Human
IL-4 rev	GTGTTCCCTGCCATACTCGT	56	Human
TNFα for	CCCCAGGGCTCCAGAAGG	56	Feline
TNFα rev	TGGGGCAGAGGGTTGATTAGTTG	56	Feline
CD3ε for	AAGCAAGAGTGTGTCAGAACT	56	Feline

for, forward; rev, reverse.

Animals and virus infection

Adult specific pathogen-free pregnant animals (queens) were housed according to University of Alberta and University of Calgary animal care facilities' guidelines in agreement with Canadian Council on Animal Care (CCAC) guidelines. All queens were negative for feline retroviruses [FIV and feline leukemia virus (FeLV)] by PCR analysis and serological testing. At postnatal d 1, animals were infected with 200 μl of FIV-Ch29 at 10^4 $TCID_{50}$/ml in accordance with CCAC guidelines, as described previously (42, 54). Control animals (FIV^-) received heat-inactivated virus. Animals were weaned at 6 wk, at which point treatment began with daily subcutaneous injections of DHEA-S (15 mg/kg body wt; Steraloids) or vehicle β-cyclodextrin (βCD; Sigma-Aldrich). Dosage was determined by taking into account past *in vitro* and *in vivo* trials in studies previously reviewed (55). Animals were monitored for 12 wk postinfection, during which time changes in body weight were recorded, and samples were taken for analyses of $CD4^+$ and $CD8^+$ T-cell populations. Neurobehavioral tests were conducted at 12 wk prior to the animals being euthanized by pentobarbital overdose; brain tissue and plasma were harvested at this time. Samples were frozen immediately at −80°C for subsequent protein or total RNA extractions. Brain tissue was fixed in 4% buffered (pH 7.4) paraformaldehyde for immunocytochemical analysis.

Behavioral tests

Gait analysis

Gait was assessed by inking the rear footpads and luring the animals across a suspended 6-inch-wide by 8-foot-long plank covered with a sheet of paper, as described previously (32). Briefly, a straight line bisecting the left and right footprints was drawn down the length of the paper. The distance between two lines (running parallel to the bisecting line) originating from the middle of each footprint (right and left) was measured as the gait width. Only trials with a continuous forward walking motion across the plank were scored, but repeated in triplicate. The gait width of an animal consisted of an average of a minimum of 10 of these measurements. The measurement of gait was assessed as the SD or variance in gait width.

Maze test

A modified T maze (56) was used to test spatial memory, cognitive learning capacity, and performance speed, as described previously (32). For the first 3 sessions, animals were adapted to the maze with all of the doors open to permit exploration and facilitate habituation. For 3 training sessions, 10 consecutive trials were conducted for each side. Animals were unfed overnight before training days and were trained to walk through the maze to obtain food. On testing days, ≥8 trials were collected for each animal, during which the total time to enter and exit the maze and errors in navigation were determined. A timer was started once the animal started forward movement after being placed in the maze opening and was stopped immediately on the animal exiting. During testing, all doors in the maze were open, and errors in navigation were scored as instances where the animal had turned into a dead end, if the animal reversed motion (when not required), and if the animal continued past the exit and turned back into the maze. After each successful trial, animals were fed.

Object memory test (OMT)

Animals were tested for their ability to remember the position of a moveable barrier over a series of trials, as described previously (32, 57). Briefly, animals were fitted with reflective markers on their hind paws and trained to walk down a narrow alley toward a 6-cm-high removable barrier to obtain food. The food was positioned such that the animal would have to step over the barrier with only its forelimbs to obtain the food. While feeding, the barrier was lowered, and after 10 s, the food was moved forward, luring the animal forward. Trials were recorded on video and analyzed with Peak Motus software (Peak Performance Technologies, Centennial, CO, USA) using the reflective markers on the hind paws and the top of the barrier as reference points. Maximum step height and trajectory were recorded for a series of 10 trials in which

the barrier was dropped down and 4 control trials in which the barrier was unmoved and the animal was allowed to step over the barrier continuously without stopping for food. All data were analyzed by an examiner unaware of the individual animal's identity.

Plasma and neural tissue viral load

Using a quantitative real-time RT-PCR protocol in which the oligonucleotide primers were derived from the FIV pol gene, the number of copies of viral RNA in plasma, cortex, and basal ganglia (per microgram RNA) was determined (58).

Immunodetection in tissue sections

Immunohistochemical and immunofluorescent labeling was performed using 6-μm paraffin-embedded serial feline and human brain sections prepared as described previously (59). Briefly, brain sections were deparaffinized and hydrated using decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the slides in 0.01 M trisodium citrate buffer (pH 6.0) for 10 min. Sections were blocked in PBS containing 10% normal goat serum (48), 2% BSA, and 0.1% Triton X-100 overnight at 4°C. Human brain sections were incubated overnight at 4°C with antibodies against human side-chain cleavage (SCC) enzyme (1:100; a generous gift from Dr. Walter Miller, UCSF; ref. 49), 5α-reductase (1:100; a generous gift from Dr. Synthia Mellon, USCF), and HSD3B1 (3βHSD, 1:100; Abcam) followed by washing in PBS. Feline tissue sections were incubated overnight at 4°C with antibodies against feline glial fibrillary acidic protein (GFAP; 1:200; Dako, Carpentaria, CA, USA) and ionized calcium binding adaptor molecule (Iba-1; 1:200; Wako, Tokyo, Japan) followed by washing in PBS. Sections were then incubated with biotin-conjugated goat antibodies followed by avidin-biotin-peroxidase amplification (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature followed by repeated washing in PBS. Subsequent immunoreactivity was detected by 3,3′-diaminobenzidine tetrachloride staining (Vector Laboratories). The specificity of staining was confirmed by omitting the primary antibody. Slides were dehydrated in increasing amounts of alcohol and mounted. All sections were examined with a Zeiss Axioskop 2 upright microscope (Carl Zeiss, Oberkochen, Germany).

Nissl staining and counting of neurons

To visualize neurons for quantitation, 6-μm paraffin-embedded serial feline brain sections were deparaffinized and hydrated using decreasing concentrations of ethanol. Sections were stained in warm 0.1% cresyl violet solution (Sigma-Aldrich) for 10 min, followed by differentiation in 95% ethanol and subsequent dehydration in 100% ethanol and xylene, and then mounted. All sections were examined with a Zeiss Axioskop 2 upright microscope. To assess neuronal abundance in cortex of FIV- and mock-infected animals, Nissl-stained neurons in the left parietal cortex were counted at +10 mm from bregma. Immunopositive cells were counted at ×400 view in all layers of the cortex in 5 separate nonoverlapping fields for each animal. The total number of cells was summed for each animal and averaged across groups.

Statistical analysis

Statistical analyses were performed by the Student's *t* test when comparing 2 different groups or by ANOVA with Tukey-Kramer or Bonferroni as *post hoc* tests, using GraphPad Instat 3.0 (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

RESULTS

Neurosteroid enzyme expression in human brain

Neuronal injury and loss are common features of HIV infection, often occurring later on in the disease course (6). To investigate the levels of neurosteroid synthesis enzymes in the brain, cerebral tissue sections from uninfected (HIV^-) and HIV-infected (HIV^+) persons were immunostained for several enzymes involved in neurosteroid synthesis (**Fig. 1**). Numerous cells resembling neurons in multiple fields were immunopositive for 5α-reductase (Fig. 1*A*), 3β-HSD (Fig. 1*C*) and P450scc (Fig. 1*E*) in tissue sections from HIV^- patients, while neurons in HIV^+ sections exhibited less immunoreactivity for the same neurosteroid synthesis enzymes (Fig. 1*B*, *D*, *F*), suggesting an overall down-regulation of the synthesis pathway. Immunoblotting of cerebral cortical specimens from HIV^- ($n=5$) and HIV^+ ($n=5$) patients disclosed significantly lower 5α-reductase/β-actin immunoreactivity ratios in HIV^+ brains compared to HIV^- brains (Fig. 1*G*). These observations highlighted the differences in neurosteroid synthesis capacity in brain between patients with and without HIV infection.

Neurosteroid synthesis in primary human neural cells

The cellular sources of neurosteroids are assumed to be largely neurons and astrocytes (60). To define the effects of HIV infection on neural cell expression of neurosteroid synthesis enzymes, we applied supernatants from HIV^+ or HIV^- macrophages to human neurons (**Fig. 2*A*, *C***) and astrocytes (Fig. 2*B*, *D*). Supernatants from HIV^+ macrophages applied to neurons caused a reduction in 5α-reductase and 3α-HSD protein expression in neurons compared with HIV^- supernatants (Fig. 2*A*), but conversely, the same HIV^+ supernatants did not affect 5α-reductase and 3α-HSD expression in astrocytes (Fig. 2*B*). In both astrocytes and neurons, β-tubulin immunoreactivity was not affected. Treatment of neurons or astrocytes with DHEA-S prior to the application of HIV^+ supernatants did not influence neuronal or astrocyte β-tubulin immunoreactivity (Fig. 2*C*). These findings indicated that neurosteroid synthesis was reduced in neurons with exposure to HIV infection, which was not accompanied by reduced cellular viability.

Neurosteroid regulation of inflammation and viral replication

Neurosteroids are increasingly recognized to influence multiple brain functions and processes, including innate immune activation (21, 61). Cellular responses were assessed in hMDMs infected with HIV-1 SF162 and

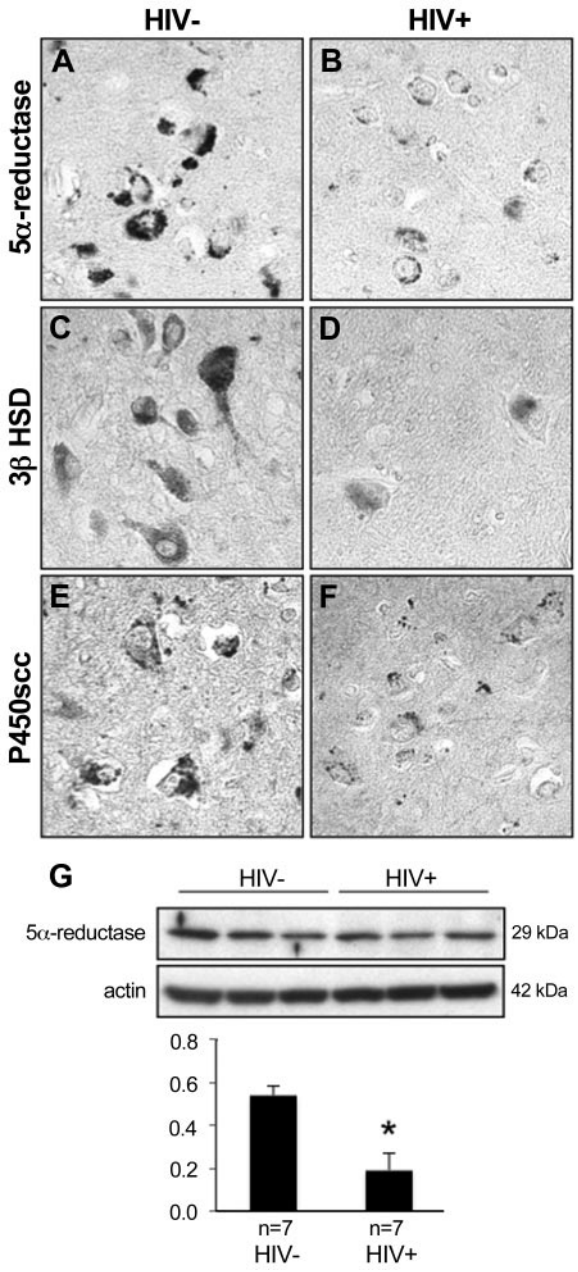

Figure 1. Neurosteroid synthesis enzyme immunoreactivity is down-regulated in HIV$^+$ brains. *A–F*) HIV$^+$ brain (n=7) showed reduced immunolabeling of the neurosteroid synthesis enzymes, 5α-reductase (*B*), 3βHSD (*D*), and scc (*F*) when compared to HIV$^-$ brain sections (n=7; *A*, *C*, and *E*, respectively). *G*) Representative Western blot analysis of cerebral cortex specimens showed decreased levels of 5α-reductase in HIV$^+$ (n=7) specimens when compared with HIV$^-$ cortex (n=7). Values are expressed as mean ± SE. *$P < 0.05$.

treated with DHEA-S or DMSO (as a vehicle). Infected cells showed an induction of innate immune responses, as evidenced by significantly increased levels of *IL-1*β (**Fig. 3*A***), *TNF*-α (Fig. 3*B*) and *IL-6* (Fig. 3*D*), which were significantly suppressed when the cells were pretreated with DHEA-S. Transcript levels encoding *IL-10*, a largely anti-inflammatory cytokine, did not change significantly with HIV infection or DHEA-S treatment (Fig. 3*C*). Infected hMDMs exhibited significantly reduced HIV *pol* transcripts with concurrent DHEA-S treatment (Fig. 2*E*), which was confirmed by a reduction in reverse transcriptase activity in culture supernatants (Fig. 2*F*) at d 4 postinfection. These observations suggested that DHEA-S suppressed neuroinflammation and viral production in HIV-infected MDMs.

In vivo detection of DHEA-S

Following confirmation of significantly reduced DHEA and pregnenolone levels in cortical samples from FIV-infected (FIV$^+$) animals compared to mock-infected (FIV$^-$) animals (**Fig. 4*A***), we extended our studies of the effect of DHEA-S on neuroinflammation by utilizing our established feline model of HIV/AIDS (32, 34, 42). We based the DHEA-S dosage and dosing frequency on previous animal studies (62, 63), in which a range of beneficial dosages was established depending on the behavioral or cognitive task assessed. The uptake and circulation of DHEA-S were analyzed in FIV$^+$-animal plasma levels, showing a significant elevation in treated animals (Fig. 4*B*). To verify the ability of DHEA-S to cross the blood-brain barrier, we measured DHEA and DHEA-S in the cortex and basal ganglia of animals by GC-MS and LC-MS, respectively. Both DHEA and its sulfated form were significantly elevated in each of the sampled sites, but only in those animals that were FIV$^+$ and receiving DHEA-S therapy (Fig. 4*C*, *D*, respectively). However, these *in vivo* DHEA-S concentrations were below those used for the *ex vivo* studies described in Fig. 3, but these findings confirmed that DHEA-S levels were increased in both brain and blood with treatment in FIV$^+$ animals.

In vivo actions of DHEA-S

In the present *in vivo* model, weight gain was significantly lower in FIV$^+$ than in FIV$^-$ animals, which became more apparent during the course of the studies (data not shown and ref. 48). This deficit in weight gain among FIV$^+$ animals was reversed with daily subcutaneous DHEA-S therapy (**Fig. 5*A***). CD4$^+$ and CD8$^+$ T-cell levels in blood were measured by FACS analysis of PBMCs. As in HIV and SIV infections, CD4$^+$ T-cell levels were significantly depressed in FIV$^+$ animals compared to FIV$^-$ animals at 12 wk postinfection. However, among FIV$^+$ animals treated with DHEA-S, elevated blood CD4$^+$ T-cell levels were evident (Fig. 5*B*). Conversely, levels of CD8$^+$ T cells were modestly elevated by FIV infection, but FIV$^+$ animals treated with DHEA-S displayed a nonsignificant increase in blood CD8$^+$ T-cell levels (Fig. 5*B*). Analyses of viral RNA levels disclosed that FIV *pol* sequences were detected in equal levels in cortex, basal ganglia, and plasma of FIV$^+$ and DHEA-S-treated FIV$^+$ animals (Fig. 5*C*). These studies indicated that systemic and immune abnormalities present in the FIV$^+$ animals, namely weight gain and CD4$^+$ T-cell levels, were restored with DHEA-S therapy, but viral burden remained unaffected.

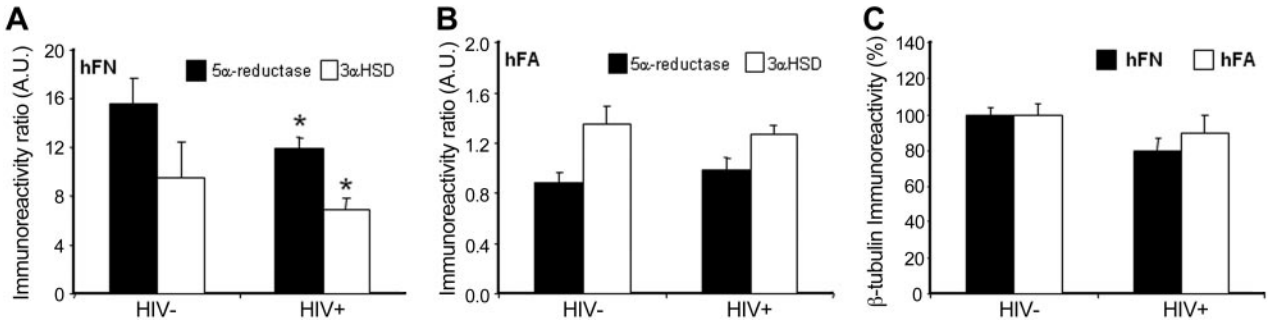

Figure 2. DHEA-S treatment of HIV$^+$ cultured cells *ex vivo* down-regulates neurosteroid enzyme synthesis pathway enzymes in neurons. *A*) The neurosteroid synthesis enzymes 5α-reductase and 3αHSD in human fetal neurons displayed reduced immunoreactivity after exposure to supernatants from HIV-infected hMDMs. *B*) Human fetal astrocytes revealed no change in 5α-reductase and 3αHSD enzyme expression following exposure to supernatants from HIV-infected hMDMs. *C*) Supernatants from HIV-infected hMDMs were not cytotoxic to neurons or astrocytes. Each enzyme was averaged over 5 wells and repeated in triplicate using supernatants from 3 separate HIV-infected hMDM cultures. Values are expressed as means ± SE. $^*P < 0.05$.

In vivo host immune brain responses

Increased innate immune responses are key aspects of HIV-1 infection and AIDS-related inflammation, particularly in the brain. Host immune transcript levels were analyzed in the cerebral cortex and basal ganglia, which are centers for memory and learning and motor control. Real-time RT-PCR was performed using cDNA synthesized from mRNA isolated from cortex and basal ganglia as a template in the present *in vivo* model. These studies revealed that *CD3*ε (**Fig. 6*A***), *F4/80* (a myeloid cell activation marker; Fig. 6*B*), *GFAP* (Fig. 6*C*), *IL-10* (Fig. 6*D*), *IL-1*β (Fig. 6*E*), and *TNF*-α (Fig. 6*F*) transcript levels were significantly increased in the cortex and basal ganglia of FIV$^+$ animals, compared to FIV$^-$ animals. Significant suppression of all of these transcripts was observed in FIV$^+$ animals treated with DHEA-S. Conversely, transcript levels of 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGCR*), the rate-controlling enzyme in the pathway producing cholesterol, were unchanged during FIV infection or DHEA-S treatment, suggesting that total brain cholesterol synthesis is not affected by FIV (data not shown). These observations highlighted the regulatory effects of DHEA-S treatment on inflammation in an *in vivo* model of AIDS.

In vivo neuropathological changes

Since pathophysiological and molecular changes were evident in the present AIDS model, the neuropathological correlates of these perturbations were investigated. Nissl staining of brain sections revealed fewer neurons in parietal cortical tissue from FIV$^+$ animals (**Fig. 7*C***), which was prevented with DHEA-S therapy (Fig. 7*D*). These observations were extended by quantifying the preservation of cortical neurons in DHEA-S-treated

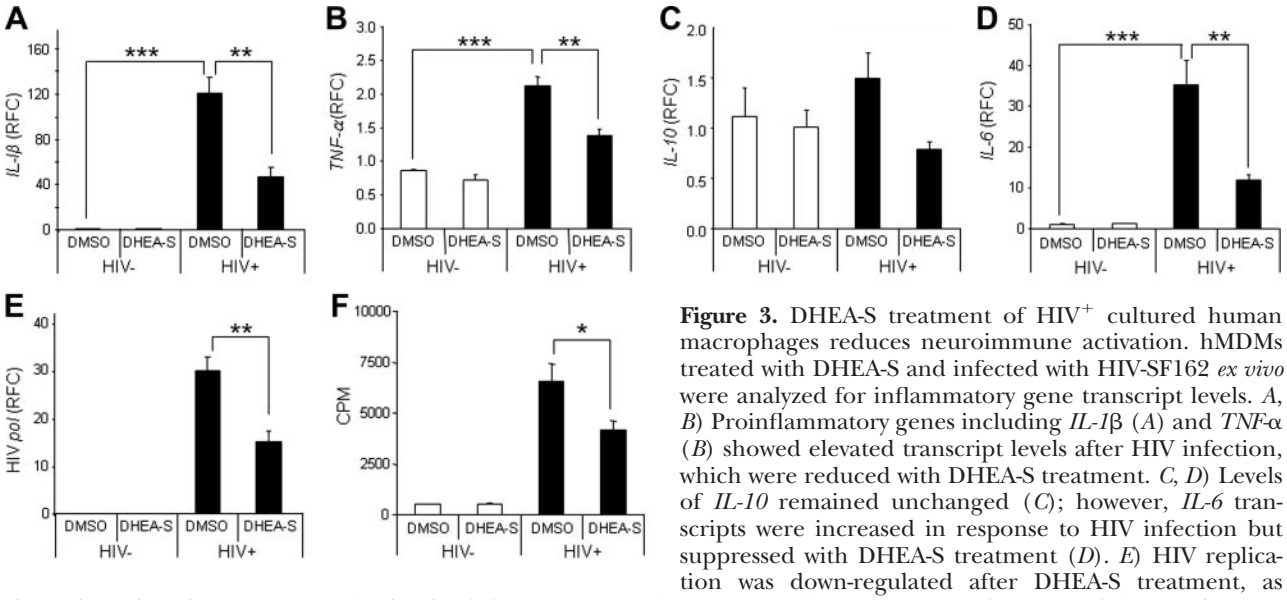

Figure 3. DHEA-S treatment of HIV$^+$ cultured human macrophages reduces neuroimmune activation. hMDMs treated with DHEA-S and infected with HIV-SF162 *ex vivo* were analyzed for inflammatory gene transcript levels. *A*, *B*) Proinflammatory genes including *IL-1*β (*A*) and *TNF*-α (*B*) showed elevated transcript levels after HIV infection, which were reduced with DHEA-S treatment. *C*, *D*) Levels of *IL-10* remained unchanged (*C*); however, *IL-6* transcripts were increased in response to HIV infection but suppressed with DHEA-S treatment (*D*). *E*) HIV replication was down-regulated after DHEA-S treatment, as shown by reduced HIV *pol* transcript levels. *F*) A reverse transcriptase assay using supernatants from DHEA-S-treated MDMs infected with HIV-SF162 *ex vivo* revealed down-regulation of HIV replication. Each treatment was averaged over 5 wells and repeated in triplicate using supernatants from 3 separate HIV-infected/mock PBL supernatants and 3 separate primary MDM cultures. Values are expressed as means ± SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

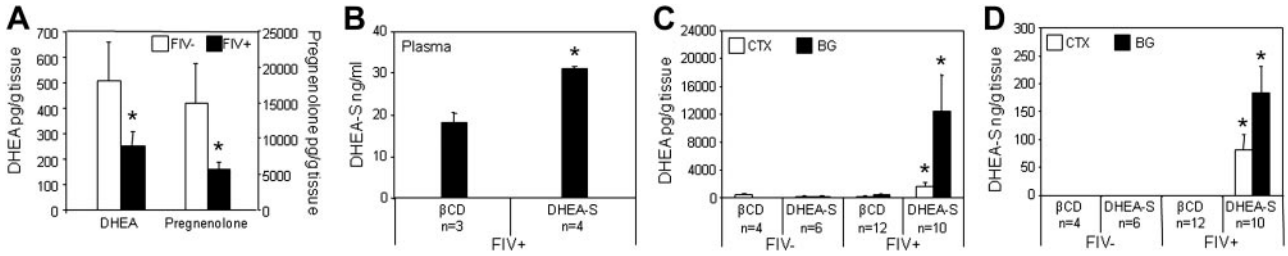

Figure 4. DHEA-S treatment of FIV$^+$ animals results in elevation of DHEA and DHEA-S levels in brain and blood. *A*) Feline cortical samples were analyzed by GC-MS to measure DHEA and pregnenolone levels and revealed significantly reduced levels in FIV$^+$ animals (n=11) compared to FIV$^-$ animals (n=4). *B*) Plasma was isolated from feline blood samples and analyzed by LC-MS to measure levels of DHEA-S. FIV$^+$ animals treated daily with DHEA-S (n=4) exhibited elevated levels of DHEA-S compared to βCD (vehicle)-treated FIV$^+$ animals (n=3). *C, D*). Cerebral cortical and basal ganglia samples were processed and analyzed by GC-MS to detect levels of DHEA (*C*) and DHEA-S (*D*). Elevated levels of DHEA and DHEA-S were detected in cortex and basal ganglia, but only in DHEA-S-treated FIV$^+$ animals. βCD-treated FIV$^-$ animals, n = 4; DHEA-S treated FIV$^-$ animals, n = 6; βCD-treated FIV$^+$ animals, n = 12; DHEA-S treated FIV$^+$ animals, n = 10. Values are expressed as means ± SE. *$P < 0.05$.

FIV$^+$ animals (Fig. 7*M*). GFAP immunoreactivity revealed activation of astrocytes in FIV infection (Fig. 7*G*), which was decreased in DHEA-S-treated FIV$^+$ animals. FIV$^-$ animals showed few Iba-1-immunopositive monocytic cells and rare activated cells (Fig. 7*I*). In contrast, the FIV$^+$ animals' brains showed more monocytoid (Iba-I-immunopositive) cells with hypertrophy (Fig. 7*K*), which was suppressed by DHEA-S treatment (Fig. 7*L*). These neuropathological studies further defined the neuroprotective effects of DHEA-S treatment and the dampening of host neuroimmune activation within the brain.

In vivo neurobehavioral studies

HIV infection affects psychomotor and neurocognitive processing (64). We used a maze task to evaluate both speed and memory in the present animals (32). The mean time taken to complete the maze was greater in the FIV$^+$ group compared to FIV$^-$ at 12 wk postinfection, which was significantly restored to levels similar to FIV$^-$ animals with DHEA-S treatment of FIV$^+$ animals (**Fig. 8*A***). Similarly, the mean number of errors was significantly less in the FIV$^-$ group and the DHEA-S treated FIV$^+$ groups relative to the FIV$^+$ animals (Fig. 8*B*). Thus, DHEA-S therapy reduced the adverse effects of FIV infection on psychomotor and cognitive processing.

Memory deficits are among the greatest impediments to patients with HIV infection (64). We used an object memory task (57) to evaluate this memory deficit in our *in vivo* model. FIV$^-$ animals revealed an increased mean number of successful trials compared to the FIV$^+$ groups, which, when treated with DHEA-S, showed an improvement in this measure (Fig. 8*C*). Overall, these neurobehavioral tests were suggestive of impairment of

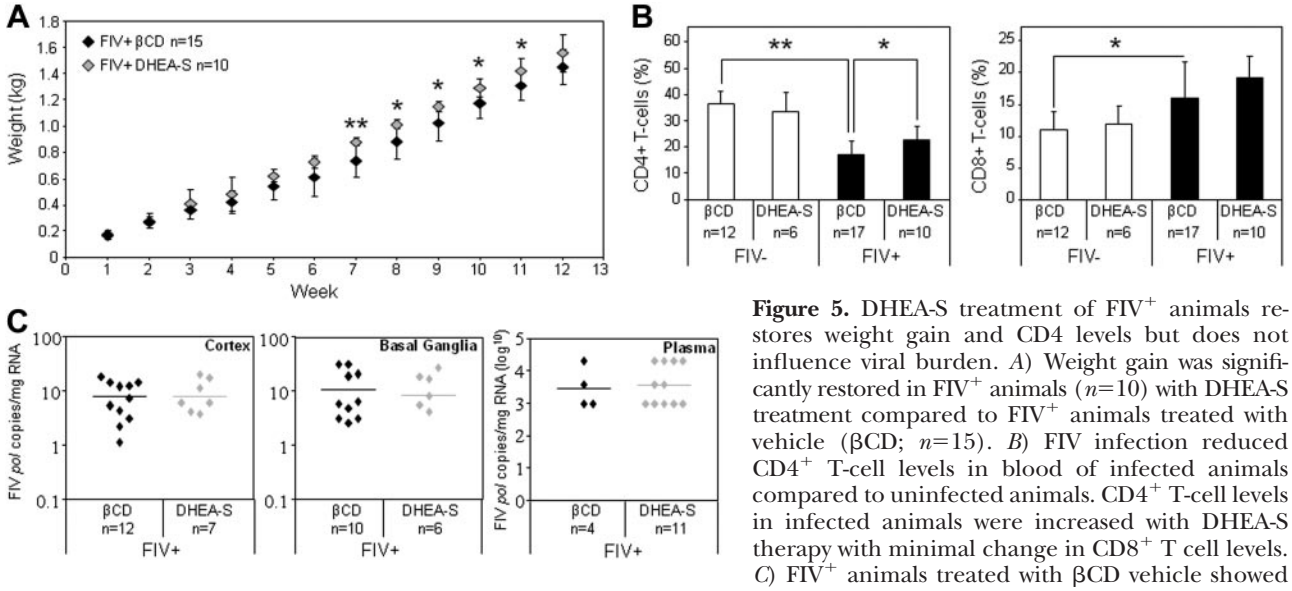

Figure 5. DHEA-S treatment of FIV$^+$ animals restores weight gain and CD4 levels but does not influence viral burden. *A*) Weight gain was significantly restored in FIV$^+$ animals (n=10) with DHEA-S treatment compared to FIV$^+$ animals treated with vehicle (βCD; n=15). *B*) FIV infection reduced CD4$^+$ T-cell levels in blood of infected animals compared to uninfected animals. CD4$^+$ T-cell levels in infected animals were increased with DHEA-S therapy with minimal change in CD8$^+$ T cell levels. *C*) FIV$^+$ animals treated with βCD vehicle showed detectable levels of virus in cortex, basal ganglia, and plasma, which remained unchanged with DHEA-S treatment. Copy numbers in blood and brain were derived from a standard curve: $y = -3.34 + 43.1$; correlation coefficient 0.999; PCR efficiency 99.2%. βCD-treated FIV$^-$ animals, n = 12; DHEA-S treated FIV$^-$ animals, (n=6); βCD-treated FIV$^+$ animals, n = 17; DHEA-S treated FIV$^+$ animals, n = 10. Values are expressed as means ± SE. *$P < 0.05$, **$P < 0.01$.

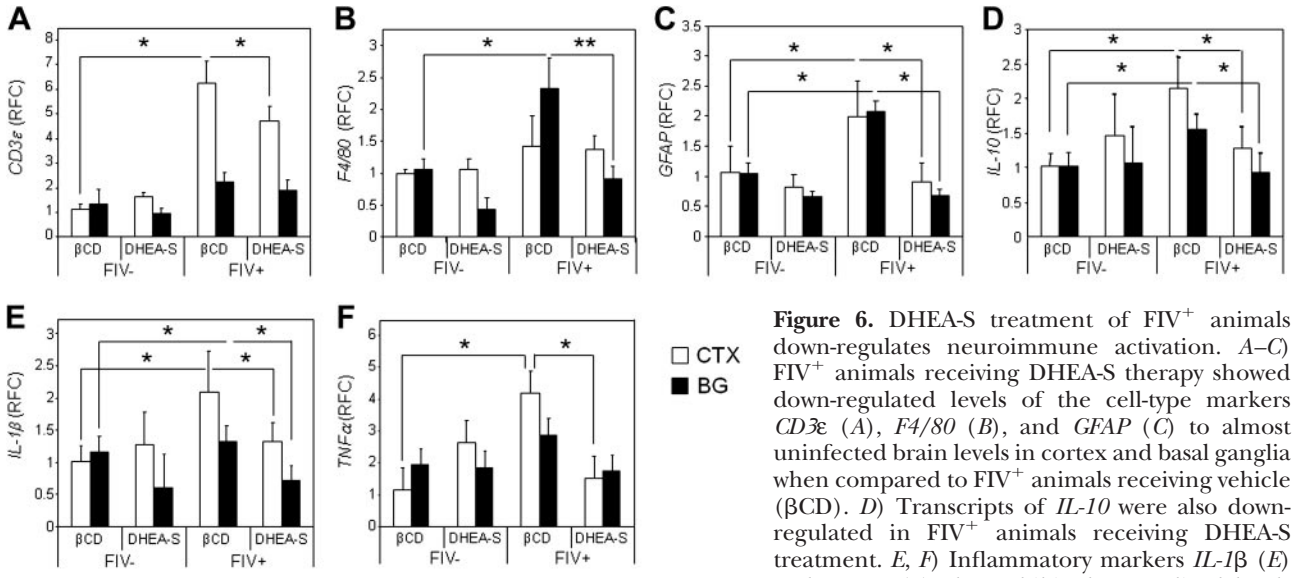

Figure 6. DHEA-S treatment of FIV$^+$ animals down-regulates neuroimmune activation. *A–C*) FIV$^+$ animals receiving DHEA-S therapy showed down-regulated levels of the cell-type markers *CD3*ε (*A*), *F4/80* (*B*), and *GFAP* (*C*) to almost uninfected brain levels in cortex and basal ganglia when compared to FIV$^+$ animals receiving vehicle (βCD). *D*) Transcripts of *IL-10* were also down-regulated in FIV$^+$ animals receiving DHEA-S treatment. *E, F*) Inflammatory markers *IL-1*β (*E*) and *TNF*-α (*F*) also exhibited normalized levels with DHEA-S treatment. βCD-treated FIV$^-$ animals, $n = 8$; DHEA-S treated FIV$^-$ animals, $n = 6$; βCD-treated FIV$^+$ animals, $n = 8$; DHEA-S-treated FIV$^+$ animals, $n = 10$. Values are expressed as mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$.

specific tasks in the FIV$^+$ groups, which were improved with DHEA-S therapy.

Patients with HIV infection often present with impaired gait, leading to falls and reduced ambulation (65). We investigated the variation of gait width, while the animals were walking across a single plank of constant width (32). These studies showed that the mean diversity in gait width, analyzed as variance in multiple trials within individual animals, was greater in the FIV$^+$ than in FIV$^-$ groups and that DHEA-S therapy significantly decreased this deficit in FIV$^-$-infected animals (Fig. 8*D*).

DISCUSSION

The present study is the first report of DHEA-S-mediated benefits in virus-induced neuropathogenesis, in-

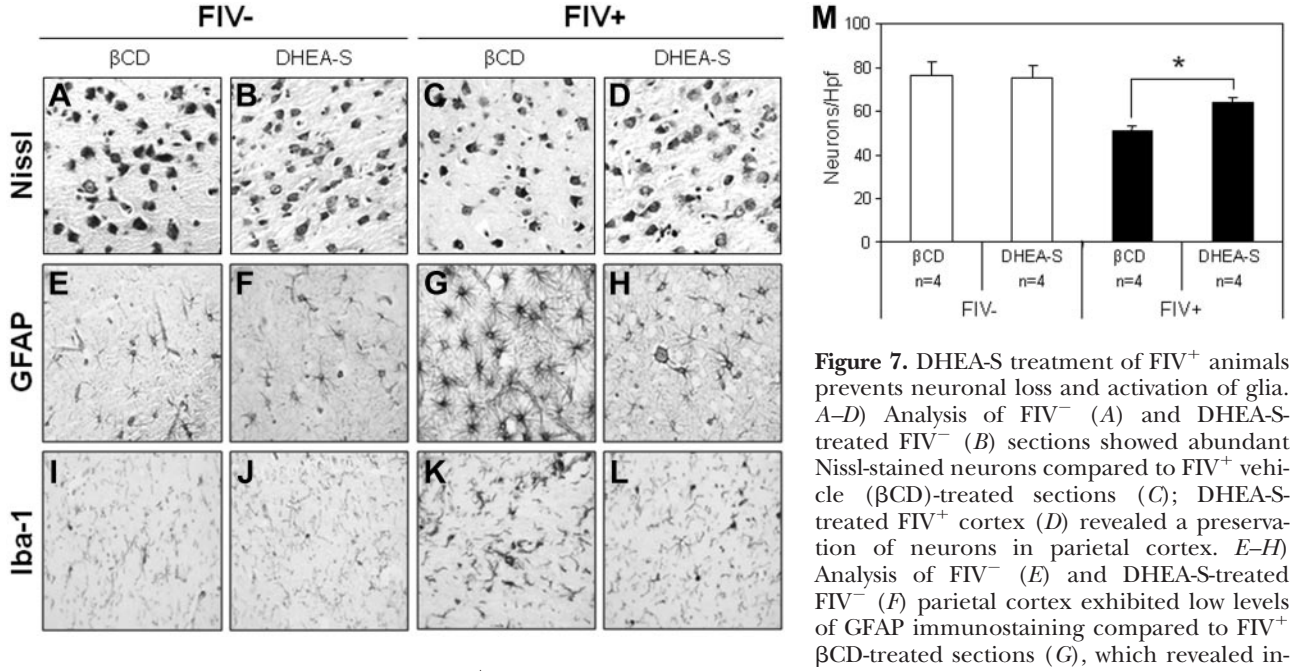

Figure 7. DHEA-S treatment of FIV$^+$ animals prevents neuronal loss and activation of glia. *A–D*) Analysis of FIV$^-$ (*A*) and DHEA-S-treated FIV$^-$ (*B*) sections showed abundant Nissl-stained neurons compared to FIV$^+$ vehicle (βCD)-treated sections (*C*); DHEA-S-treated FIV$^+$ cortex (*D*) revealed a preservation of neurons in parietal cortex. *E–H*) Analysis of FIV$^-$ (*E*) and DHEA-S-treated FIV$^-$ (*F*) parietal cortex exhibited low levels of GFAP immunostaining compared to FIV$^+$ βCD-treated sections (*G*), which revealed increased GFAP staining; DHEA-S-treated FIV$^+$ cortex (*H*) showed decreased levels of GFAP staining comparable to FIV$^-$ sections. *I–L*) FIV$^-$ (*I*) and DHEA-S-treated FIV$^-$ (*J*) sections revealed low levels of ionized calcium-binding adaptor molecule 1 (Iba-1) immunoreactivity compared to FIV$^+$ βCD-treated sections (*K*); DHEA-S-treated FIV$^+$ cortex (*L*) showed decreased levels of Iba-1 immunostaining comparable to FIV$^-$ sections. *M*) Neuronal loss was confirmed by quantifying Nissl-stained neurons in 5–8 randomly selected fields averaged over 4 animals/group. Values are expressed as means ± SE. $^*P < 0.05$.

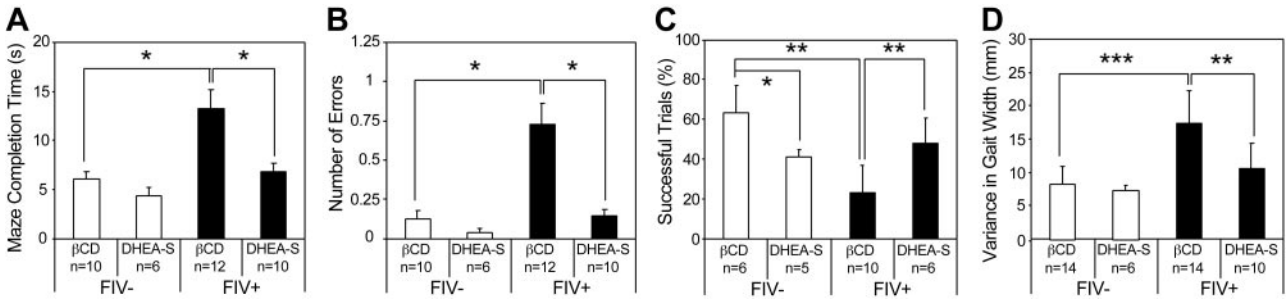

Figure 8. DHEA-S treatment of FIV$^+$ animals improves neurobehavioral performance. Maze testing demonstrated deficiencies in spatial memory and cognitive abilities. Animals were trained to complete a modified T maze and were tested for maze completion times and navigational errors. *A, B*) Maze completion times (*A*) and navigational errors (*B*) during trials were increased in FIV$^+$ vehicle (βCD)-treated animals but were restored to control levels with DHEA-S treatment. *C*) DHEA-S-treated FIV$^+$ animals exhibited an improved spatial memory in the OMT. Animals were lured by food to approach a 6-cm-high barrier and step over it with their fore limbs. The gate was lowered, and the animal was allowed to feed for 10 s, after which the food was moved forward to lure the animal to step forward. Reflective dots placed on the outside of the animals' hind limbs tracked the trajectory and height of the cat's footsteps. FIV$^+$ βCD-treated animals showed a deficit in recalling the height and position of the barrier, as demonstrated by the lower percentage of successful trials. This deficit in spatial memory was partially restored with DHEA-S treatment. *D*) FIV$^+$ animals exhibited impairment in locomotor tasks. Analysis of ink footprints made by the animals as they walked across a suspended plank revealed an increase in the variance of gait width of FIV$^+$ animals, which was decreased to control levels in DHEA-S-treated FIV$^+$ animals. βCD-treated FIV$^-$ animals, n = 6–14; DHEA-S treated FIV$^-$ animals, n = 5–6; βCD-treated FIV$^+$ animals, n = 10–14; DHEA-S-treated FIV$^+$ animals, n=6–10. Values are expressed as means ± SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cluding models of the closely related lentiviruses, HIV, and FIV. A recent study outlined the various benefits of DHEA and DHEA-S in human health, including reducing inflammation, providing neuroprotection, and improving cellular immunity, cognitive function and memory, while highlighting that the clinical efficacy of DHEA and DHEA-S supplementation in relieving pathophysiological circumstances needs further study (66). We observed that neurosteroid synthesis enzyme expression was reduced, specifically in neurons, during HIV infection and also in HIV-exposed neurons. Notably, the neurosteroid, DHEA-S, suppressed the *ex vivo* and *in vivo* induction of proinflammatory genes, such as IL-1β, as well as TNF-α, both assumed to contribute directly to neuronal injury and death. The latter results were supported by *in vivo* corollary observations of reduced neuropathology and neurobehavioral deficits with DHEA-S treatment in the FIV model. Collectively, the findings point to neurosteroids as potentially valuable components of therapeutic regimens for patients with HIV-related neurological disorders and perhaps other neuroinflammatory diseases.

While steroids are generally regarded as products of prototypic steroidogenic organs (gonads or adrenals), it is apparent that the brain possesses a full complement of enzymatic capacity machinery for synthesizing steroids (3, 19, 40, 44, 50). Brain-derived steroids contribute to neural cell growth and development (23, 67, 68) and can alter neurotransmitter-regulated ion channels by very rapid and specific modulatory mechanisms depending on the individual steroid; neurosteroids producing these effects are termed neuroactive steroids. Steroidogenic pathways in the brain are diverse in that neurosteroids use different receptors depending on the individual steroid molecule. For example, we have recently shown that allopregnenolone, which acts as a positive allosteric modulator of the GABA-A receptor, regulates neuroinflammation and prevents demyelination and axonal loss in models of multiple sclerosis (69). Other neurosteroids exert their effects by modulating glutamate or sigma receptors, as well as by acting on nuclear steroid receptors (70).

DHEA is a 19-carbon steroid and is the most abundant circulating steroid in humans, often acting as a cortisol antagonist in humans (71). Like other neurosteroids, DHEA is derived from cholesterol through conversion of cholesterol to pregnenolone by P450scc enzyme, followed by metabolism by another enzyme, CYP17A1, to 17α-hydroxypregnenolone and then to DHEA. DHEA can be sulfated by sulfotransferase (SULT2A1) to form DHEA-S, which is present at levels >100-fold higher than DHEA in plasma of humans and has a longer half-life. DHEA has been reported to influence microglial activation and ensuing release of free radicals (72) and perhaps to reduce neuronal death and increase glial differentiation *in vitro* (73). Consistent with our study, DHEA treatment is also known to improve quality of life among individuals with HIV/AIDS who are aviremic (74) and might also enhance mood in the same population of patients but does not seem to affect *in vivo* blood CD4 T-cell or viral load levels (75). Moreover, blood DHEA-S levels are reduced in HIV-infected persons (61, 64).

The present study focused on DHEA-S, not only because it has a longer half-life than DHEA, but also because of its success in other animal models in improving behavioral and cognitive performance. DHEA-S treatment has been to shown to improve recovery of function after traumatic brain injury in rats (62), and its repeated treatment of mice with mild traumatic brain injury improves cognitive and behavioral performance

(63). Indeed, the features of mild traumatic brain injury also include neurodegeneration and inflammation (76), which ultimately lead to deficits in behavior and cognitive performance.

From the present studies, it is clear that treatment with neurosteroids does not increase immunodeficiency, unlike glucocorticoids, and nor do neurosteroids adversely affect renal metabolism or body mass. However, our studies suggest that neurosteroids are effective modulators of innate immunity, as evidenced by reduced cytokine transcript expression. These effects could be mediated by two processes, namely, acting as negative modulators of gene transcription through nuclear receptors or by enhancing GABA-A receptor-mediated actions. Recently, both macrophages and lymphocytes have been shown to express the GABA-A receptor (77, 78). The precise mechanism by which DHEA-S acts on microglia and macrophages remains uncertain but GABA-A receptor modulation is theoretically possible. Recent data show an up-regulation of GABA-A receptor activity in patients with HIV-1-associated neurocognitive disorders treated with antiretroviral therapy *vs.* untreated patients (79). It will be important to understand the effect of neurosteroids on different aspects of innate immunity, such as Toll- and NOD-like receptors, which regulate cytokine production. However, other GABA-A receptor-targeting neuroactive steroids, such as the synthetic molecule, ganaxolone, might also have therapeutic benefits in lentivirus infections and other inflammatory neurological diseases. Indeed, 16α-bromo-epiandrosterone, a synthetic DHEA analog, improved systemic immunity and reduced IL-12 p40 expression in FIV^+ animals (80).

The effects of neurosteroids on viral infection and replication remain largely uncharacterized, although small clinical studies indicate that DHEA-S and allopregnenolone did not affect blood viral levels in HIV infection (74, 81). An experimental study of rabies virus infection showed that DHEA treatment worsened outcomes largely because viral clearance was diminished, likely because of reduced inflammation (82). This latter study underscores the importance of the specificity and timing of neurosteroids as therapeutic agents. However, the present studies showed that DHEA-S reduced virus expression and release *ex vivo* in HIV-infected macrophages but did not affect *in vivo* blood or brain FIV RNA levels. Several explanations might underlie these results: *ex vivo* viral replication might be more susceptible to DHEA-S because of better bioavailability and/or greater dependence on concurrent proinflammatory molecule expression, which is also controlled by DHEA-S. In addition, tissue penetration by DHEA-S *in vivo* might be limited despite detectable levels by LC-MS and apparent suppression of inflammatory gene expression in the brain of DHEA-S-treated animals. As with conventional antiretroviral drugs, tissue penetration is a critical determinant of drug efficacy in the brain and further analyses will be required to fully understand these effects. Nonetheless, a noninflamed blood-brain barrier, which is relatively impermeable to a sulfated neurosteroid, together with the hydrophilic nature of DHEA-S might account for the absence of DHEA-S in mock-infected (FIV^-) animals.

Future studies will need to investigate the effects of different neurosteroids on specific innate immune processes, including the induction of the inflammasome, endoplasmic reticulum stress, and individual danger or stranger signals recognizing molecular complexes, as well as relevant neurotransmitter receptors. Given that there are no accepted or validated treatments for HIV-associated neurocognitive disorders except highly active antiretroviral therapy (HAART), these observations raise the exciting possibility of using a range of endogenous brain-derived small molecules and/or analogues as future treatments added to existing HAART regimens. FJ

C.P. holds a Canada Research Chair (CRC; tier 1) in Neurological Infection and Immunity and is an Alberta Innovates–Health Solutions Senior Scholar. P.V. holds a fellowship from Alberta Innovates–Health Solutions. These studies were supported by the Canadian Institutes of Health Research (C.P., G.B.B.). The authors thank Dr. Synthia Mellon (University of California, San Francisco, CA, USA) for generously providing antibodies for these studies and helpful discussions. The authors are grateful to Ms. Gail Rauw for her expert technical assistance in assaying levels of the neurosteroids. The authors declare no conflicts of interest.

REFERENCES

1. Truss, M., and Beato, M. (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr. Rev.* **14,** 459–479
2. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889–895
3. Paul, S. M., and Purdy, R. H. (1992) Neuroactive steroids. *FASEB J.* **6,** 2311–2322
4. Rupprecht, R. (2003) Neuroactive steroids: mechanisms of action and neuropsychopharmacological properties. *Psychoneuroendocrinology* **28,** 139–168
5. Rupprecht, R., and Holsboer, F. (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci.* **22,** 410–416
6. Epstein, L. G., and Gelbard, H. A. (1999) HIV-1-induced neuronal injury in the developing brain. *J. Leukoc. Biol.* **65,** 453–457
7. Baulieu, E. E., Robel, P., and Schumacher, M. (2001) Neurosteroids: beginning of the story. *Int. Rev. Neurobiol.* **46,** 1–32
8. Warner, M., and Gustafsson, J. A. (1995) Cytochrome P450 in the brain: neuroendocrine functions. *Front. Neuroendocrinol.* **16,** 224–236
9. Lambert, J. J., Belelli, D., Hill-Venning, C., and Peters, J. A. (1995) Neurosteroids and GABAA receptor function. *Trends Pharmacol. Sci.* **16,** 295–303
10. Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., and Paul, S. M. (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* **232,** 1004–1007
11. Park-Chung, M., Wu, F. S., and Farb, D. H. (1994) 3 alpha-hydroxy-5 beta-pregnan-20-one sulfate: a negative modulator of the NMDA-induced current in cultured neurons. *Mol. Pharmacol.* **46,** 146–150
12. Weaver, C. E., Jr., Marek, P., Park-Chung, M., Tam, S. W., and Farb, D. H. (1997) Neuroprotective activity of a new class of steroidal inhibitors of the *N*-methyl-D-aspartate receptor. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10450–10454
13. Weaver, C. E., Jr., Park-Chung, M., Gibbs, T. T., and Farb, D. H. (1997) 17beta-Estradiol protects against NMDA-induced excito-

toxicity by direct inhibition of NMDA receptors. *Brain Res.* **761,** 338–341
14. Wu, F. S., Gibbs, T. T., and Farb, D. H. (1991) Pregnenolone sulfate: a positive allosteric modulator at the *N*-methyl-D-aspartate receptor. *Mol. Pharmacol.* **40,** 333–336
15. Dong, L., Zhu, Y., Dong, Y., Yang, J., Zhao, Y., Qi, Y., Wu, P., and Zheng, P. (2009) Neuroactive steroid dehydroepiandrosterone sulfate inhibits 5-hydroxytryptamine (5-HT)-evoked glutamate release via activation of sigma-1 receptors and then inhibition of 5-HT3 receptors in rat prelimbic cortex. *J. Pharmacol. Exp. Ther.* **330,** 494–501
16. Wetzel, C. H., Hermann, B., Behl, C., Pestel, E., Rammes, G., Zieglgansberger, W., Holsboer, F., and Rupprecht, R. (1998) Functional antagonism of gonadal steroids at the 5-hydroxytryptamine type 3 receptor. *Mol. Endocrinol.* **12,** 1441–1451
17. Maurice, T., Urani, A., Phan, V. L., and Romieu, P. (2001) The interaction between neuroactive steroids and the sigma1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res. Brain Res. Rev.* **37,** 116–132
18. Dubrovsky, B. (2006) Neurosteroids, neuroactive steroids, and symptoms of affective disorders. *Pharmacol. Biochem. Behav.* **84,** 644–655
19. Dubrovsky, B. O. (2005) Steroids, neuroactive steroids and neurosteroids in psychopathology. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **29,** 169–192
20. Eser, D., Schule, C., Baghai, T. C., Romeo, E., Uzunov, D. P., and Rupprecht, R. (2006) Neuroactive steroids and affective disorders. *Pharmacol. Biochem. Behav.* **84,** 656–666
21. MacKenzie, E. M., Odontiadis, J., Le Melledo, J. M., Prior, T. I., and Baker, G. B. (2007) The relevance of neuroactive steroids in schizophrenia, depression, and anxiety disorders. *Cell. Mol. Neurobiol.* **27,** 541–574
22. Strous, R. D., Maayan, R., and Weizman, A. (2006) The relevance of neurosteroids to clinical psychiatry: from the laboratory to the bedside. *Eur. Neuropsychopharmacol.* **16,** 155–169
23. Le Melledo, J. M., and Baker, G. B. (2002) Neuroactive steroids and anxiety disorders. *J. Psychiatry Neurosci.* **27,** 161–165
24. Ahboucha, S., and Butterworth, R. F. (2007) The neurosteroid system: an emerging therapeutic target for hepatic encephalopathy. *Metab. Brain Dis.* **22,** 291–308
25. Marx, C. E., Bradford, D. W., Hamer, R. M., Naylor, J. C., Allen, T. B., Lieberman, J. A., Strauss, J. L., and Kilts, J. D. (2011) Pregnenolone as a novel therapeutic candidate in schizophrenia: emerging preclinical and clinical evidence. *Neuroscience* **191,** 78–90
26. Luchetti, S., Huitinga, I., and Swaab, D. F. (2011) Neurosteroid and GABA-A receptor alterations in Alzheimer's disease, Parkinson's disease and multiple sclerosis. *Neuroscience* **191,** 6–21
27. Schule, C., Eser, D., Baghai, T. C., Nothdurfter, C., Kessler, J. S., and Rupprecht, R. (2011) Neuroactive steroids in affective disorders: target for novel antidepressant or anxiolytic drugs? *Neuroscience* **191,** 55–77
28. Van Marle, G., and Power, C. (2005) Human immunodeficiency virus type 1 genetic diversity in the nervous system: evolutionary epiphenomenon or disease determinant? *J. Neurovirol.* **11,** 107–128
29. Rackstraw, S. (2011) HIV-related neurocognitive impairment. A review. *Psychol. Health Med.* **16,** 548–563
30. Wright, E. (2011) Neurocognitive impairment and neuroCART. *Curr. Opin. HIV AIDS* **6,** 303–308
31. Bendinelli, M., Pistello, M., Lombardi, S., Poli, A., Garzelli, C., Matteucci, D., Ceccherini-Nelli, L., Malvaldi, G., and Tozzini, F. (1995) Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin. Microbiol. Rev.* **8,** 87–112
32. Maingat, F., Vivithanaporn, P., Zhu, Y., Taylor, A., Baker, G., Pearson, K., and Power, C. (2009) Neurobehavioral performance in feline immunodeficiency virus infection: integrated analysis of viral burden, neuroinflammation, and neuronal injury in cortex. *J. Neurosci.* **29,** 8429–8437
33. Zink, M. C., Laast, V. A., Helke, K. L., Brice, A. K., Barber, S. A., Clements, J. E., and Mankowski, J. L. (2006) From mice to macaques–animal models of HIV nervous system disease. *Curr. HIV Res.* **4,** 293–305
34. Power, C., Moench, T., Peeling, J., Kong, P. A., and Langelier, T. (1997) Feline immunodeficiency virus causes increased glutamate levels and neuronal loss in brain. *Neuroscience* **77,** 1175–1185
35. Poli, A., Pistello, M., Carli, M. A., Abramo, F., Mancuso, G., Nicoletti, E., and Bendinelli, M. (1999) Tumor necrosis factor-alpha and virus expression in the central nervous system of cats infected with feline immunodeficiency virus. *J. Neurovirol.* **5,** 465–473
36. Noorbakhsh, F., Tang, Q., Liu, S., Silva, C., van Marle, G., and Power, C. (2006) Lentivirus envelope protein exerts differential neuropathogenic effects depending on the site of expression and target cell. *Virology* **348,** 260–276
37. Ritsner, M. S., and Strous, R. D. (2010) Neurocognitive deficits in schizophrenia are associated with alterations in blood levels of neurosteroids: a multiple regression analysis of findings from a double-blind, randomized, placebo-controlled, crossover trial with DHEA. *J. Psychiatr. Res.* **44,** 75–80
38. Ritsner, M. S. (2011) The clinical and therapeutic potentials of dehydroepiandrosterone and pregnenolone in schizophrenia. *Neuroscience* **191,** 91–100
39. Charalampopoulos, I., Remboutsika, E., Margioris, A. N., and Gravanis, A. (2008) Neurosteroids as modulators of neurogenesis and neuronal survival. *Trends Endocrinol. Metab.* **19,** 300–307
40. Schumacher, M., Robel, P., and Baulieu, E. E. (1996) Development and regeneration of the nervous system: a role for neurosteroids. *Dev. Neurosci.* **18,** 6–21
41. Zhu, Y., Vergote, D., Pardo, C., Noorbakhsh, F., McArthur, J. C., Hollenberg, M. D., Overall, C. M., and Power, C. (2009) CXCR3 activation by lentivirus infection suppresses neuronal autophagy: neuroprotective effects of antiretroviral therapy. *FASEB J.* **23,** 2928–2941
42. Power, C., Buist, R., Johnston, J. B., Del Bigio, M. R., Ni, W., Dawood, M. R., and Peeling, J. (1998) Neurovirulence in feline immunodeficiency virus-infected neonatal cats is viral strain specific and dependent on systemic immune suppression. *J. Virol.* **72,** 9109–9115
43. Bhat, R. K., Ellestad, K. K., Wheatley, B. M., Warren, R., Holt, R. A., and Power, C. (2011) Age- and disease-dependent HERV-W envelope allelic variation in brain: association with neuroimmune gene expression. *PLoS One* **6,** e19176
44. Noorbakhsh, F., Vergnolle, N., McArthur, J. C., Silva, C., Vodjgani, M., Andrade-Gordon, P., Hollenberg, M. D., and Power, C. (2005) Proteinase-activated receptor-2 indiction by neuroinflammation prevents neuronal death during HIV infection. *J. Immunol.* **174,** 7320–7329
45. Ahboucha, S., Butterworth, R. F., Pomier-Layrargues, G., Vincent, C., Hassoun, Z., and Baker, G. B. (2008) Neuroactive steroids and fatigue severity in patients with primary biliary cirrhosis and hepatitis C. *Neurogastroenterol. Motil.* **20,** 671–679
46. Afkhami-Goli, A., Noorbakhsh, F., Keller, A. J., Vergnolle, N., Westaway, D., Jhamandas, J. H., Andrade-Gordon, P., Hollenberg, M. D., Arab, H., Dyck, R. H., and Power, C. (2007) Proteinase-activated receptor-2 exerts protective and pathogenic cell type-specific effects in Alzheimer's disease. *J. Immunol.* **179,** 5493–5503
47. Power, C., McArthur, J. C., Nath, A., Wehrly, K., Mayne, M., Nishio, J., Langelier, T., Johnson, R. T., and Chesebro, B. (1998) Neuronal death induced by brain-derived human immunodeficiency virus type 1 envelope genes differs between demented and nondemented AIDS patients. *J. Virol.* **72,** 9045–9053
48. Maingat, F., Halloran, B., Acharjee, S., van Marle, G., Church, D., Gill, M. J., Uwiera, R. R., Cohen, E. A., Meddings, J., Madsen, K., and Power, C. (2011) Inflammation and epithelial cell injury in AIDS enteropathy: involvement of endoplasmic reticulum stress. *FASEB J.* **25,** 2211–2220
49. Black, S. M., Szklarz, G. D., Harikrishna, J. A., Lin, D., Wolf, C. R., and Miller, W. L. (1993) Regulation of proteins in the cholesterol side-chain cleavage system in JEG-3 and Y-1 cells. *Endocrinology* **132,** 539–545
50. Zhu, Y., Jones, G., Tsutsui, S., Opii, W., Liu, S., Silva, C., Butterfield, D. A., and Power, C. (2005) Lentivirus infection causes neuroinflammation and neuronal injury in dorsal root ganglia: pathogenic effects of STAT-1 and inducible nitric oxide synthase. *J. Immunol.* **175,** 1118–1126
51. Boven, L. A., Noorbakhsh, F., Bouma, G., van der Zee, R., Vargas, D. L., Pardo, C., McArthur, J. C., Nottet, H. S., and Power, C. (2007) Brain-derived human immunodeficiency vi-

rus-1 Tat exerts differential effects on LTR transactivation and neuroimmune activation. *J. Neurovirol.* **13,** 173–184
52. Power, C., Henry, S., Del Bigio, M. R., Larsen, P. H., Corbett, D., Imai, Y., Yong, V. W., and Peeling, J. (2003) Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. *Ann. Neurol.* **53,** 731–742
53. Johnston, J., and Power, C. (1999) Productive infection of human peripheral blood mononuclear cells by feline immunodeficiency virus: implications for vector development. *J. Virol.* **73,** 2491–2498
54. Johnston, J. B., Silva, C., Hiebert, T., Buist, R., Dawood, M. R., Peeling, J., and Power, C. (2002) Neurovirulence depends on virus input titer in brain in feline immunodeficiency virus infection: evidence for activation of innate immunity and neuronal injury. *J. Neurovirol.* **8,** 420–431
55. Maninger, N., Wolkowitz, O. M., Reus, V. I., Epel, E. S., and Mellon, S. H. (2009) Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). *Front. Neuroendocrinol.* **30,** 65–91
56. Levine, M. S., Lloyd, R. L., Fisher, R. S., Hull, C. D., and Buchwald, N. A. (1987) Sensory, motor and cognitive alterations in aged cats. *Neurobiol. Aging* **8,** 253–263
57. McVea, D. A., and Pearson, K. G. (2006) Long-lasting memories of obstacles guide leg movements in the walking cat. *J. Neurosci.* **26,** 1175–1178
58. Kennedy, J. M., Hoke, A., Zhu, Y., Johnston, J. B., van Marle, G., Silva, C., Zochodne, D. W., and Power, C. (2004) Peripheral neuropathy in lentivirus infection: evidence of inflammation and axonal injury. *AIDS* **18,** 1241–1250
59. Tsutsui, S., Vergote, D., Shariat, N., Warren, K., Ferguson, S. S., and Power, C. (2008) Glucocorticoids regulate innate immunity in a model of multiple sclerosis: reciprocal interactions between the A1 adenosine receptor and beta-arrestin-1 in monocytoid cells. *FASEB J.* **22,** 786–796
60. Mellon, S. H. (2007) Neurosteroid regulation of central nervous system development. *Pharmacol. Ther.* **116,** 107–124
61. Griffin, L. D., Gong, W., Verot, L., and Mellon, S. H. (2004) Niemann-Pick type C disease involves disrupted neurosteroidogenesis and responds to allopregnanolone. *Nat. Med.* **10,** 704–711
62. Hoffman, S. W., Virmani, S., Simkins, R. M., and Stein, D. G. (2003) The delayed administration of dehydroepiandrosterone sulfate improves recovery of function after traumatic brain injury in rats. *J. Neurotrauma* **20,** 859–870
63. Milman, A., Zohar, O., Maayan, R., Weizman, R., and Pick, C. G. (2008) DHEAS repeated treatment improves cognitive and behavioral deficits after mild traumatic brain injury. *Eur. Neuropsychopharmacol.* **18,** 181–187
64. McArthur, J. C., Brew, B. J., and Nath, A. (2005) Neurological complications of HIV infection. *Lancet Neurol.* **4,** 543–555
65. Power, C., Boisse, L., Rourke, S., and Gill, M. J. (2009) NeuroAIDS: an evolving epidemic. *Can. J. Neurol. Sci.* **36,** 285–295
66. Traish, A. M., Kang, H. P., Saad, F., and Guay, A. T. (2011) Dehydroepiandrosterone (DHEA)—a precursor steroid or an active hormone in human physiology (CME). *J. Sex Med.* **8,** 2960–2982
67. Mellon, S. H., and Griffin, L. D. (2002) Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol. Metab.* **13,** 35–43
68. Mellon, S. H., and Griffin, L. D. (2002) Synthesis, regulation, and function of neurosteroids. *Endocr. Res.* **28,** 463
69. Noorbakhsh, F., Ellestad, K. K., Maingat, F., Warren, K. G., Han, M. H., Steinman, L., Baker, G. B., and Power, C. (2011) Impaired neurosteroid synthesis in multiple sclerosis. *Brain* **134,** 2703–2721
70. Losel, R. M., Falkenstein, E., Feuring, M., Schultz, A., Tillmann, H. C., Rossol-Haseroth, K., and Wehling, M. (2003) Nongenomic steroid action: controversies, questions, and answers. *Physiol. Rev.* **83,** 965–1016
71. Kalimi, M., Shafagoj, Y., Loria, R., Padgett, D., and Regelson, W. (1994) Anti-glucocorticoid effects of dehydroepiandrosterone (DHEA). *Mol. Cell. Biochem.* **131,** 99–104
72. Barger, S. W., Chavis, J. A., and Drew, P. D. (2000) Dehydroepiandrosterone inhibits microglial nitric oxide production in a stimulus-specific manner. *J. Neurosci. Res.* **62,** 503–509
73. Bologa, L., Sharma, J., and Roberts, E. (1987) Dehydroepiandrosterone and its sulfated derivative reduce neuronal death and enhance astrocytic differentiation in brain cell cultures. *J. Neurosci. Res.* **17,** 225–234
74. Abrams, D. I., Shade, S. B., Couey, P., McCune, J. M., Lo, J., Bacchetti, P., Chang, B., Epling, L., Liegler, T., and Grant, R. M. (2007) Dehydroepiandrosterone (DHEA) effects on HIV replication and host immunity: a randomized placebo-controlled study. *AIDS Res. Hum. Retroviruses* **23,** 77–85
75. Piketty, C., Jayle, D., Leplege, A., Castiel, P., Ecosse, E., Gonzalez-Canali, G., Sabatier, B., Boulle, N., Debuire, B., Le Bouc, Y., Baulieu, E. E., and Kazatchkine, M. D. (2001) Double-blind placebo-controlled trial of oral dehydroepiandrosterone in patients with advanced HIV disease. *Clin. Endocrinol. (Oxf.)* **55,** 325–330
76. Gao, X., and Chen, J. (2011) Mild traumatic brain injury results in extensive neuronal degeneration in the cerebral cortex. *J. Neuropathol. Exp. Neurol.* **70,** 183–191
77. Nigam, R., El-Nour, H., Amatya, B., and Nordlind, K. (2010) GABA and GABA(A) receptor expression on immune cells in psoriasis: a pathophysiological role. *Arch. Dermatol. Res.* **302,** 507–515
78. Lombardi, G., Dianzani, C., Miglio, G., Canonico, P. L., and Fantozzi, R. (2001) Characterization of ionotropic glutamate receptors in human lymphocytes. *Br. J. Pharmacol.* **133,** 936–944
79. Borjabad, A., Morgello, S., Chao, W., Kim, S. Y., Brooks, A. I., Murray, J., Potash, M. J., and Volsky, D. J. (2011) Significant effects of antiretroviral therapy on global gene expression in brain tissues of patients with HIV-1-associated neurocognitive disorders. *PLoS Pathog.* **7,** e1002213
80. Pedersen, N. C., North, T. W., Rigg, R., Reading, C., Higgins, J., Leutenegger, C., and Henderson, G. L. (2003) 16alpha-bromoepiandrosterone therapy modulates experimental feline immunodeficiency virus viremia: initial enhancement leading to long-term suppression. *Vet. Immunol. Immunopathol.* **94,** 133–148
81. Scott-Sheldon, L. A., Kalichman, S. C., Carey, M. P., and Fielder, R. L. (2008) Stress management interventions for HIV+ adults: a meta-analysis of randomized controlled trials, 1989 to 2006. *Health Psychol.* **27,** 129–139
82. Roy, A., and Hooper, D. C. (2007) Lethal silver-haired bat rabies virus infection can be prevented by opening the blood-brain barrier. *J. Virol.* **81,** 7993–7998

Received for publication July 10, 2012.
Accepted for publication October 1, 2012.