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

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

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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-1β, IL-6, TNF-α) expression. HIV-infected (HIV+) animals treated daily with 15 mg/kg body weight. DHEA-S treatment reduced inflammatory gene transcripts (IL-1β, TNF-α, CD3ɛ, GFAP) in brain compared to vehicle-(β-cyclodextrin)-treated HIV+ animals similar to levels found in vehicle-treated HIV− animals. DHEA-S treatment also increased CD4+ T-cell levels and prevented neurobehavioral deficits and neuronal loss among HIV+ animals, compared to vehicle-treated HIV+ 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 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; hIFN, 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

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number 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 σ1 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 impairments remain 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) 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 coupled 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 deuteriated DHEA-S by liquid chromatography-mass spectrometry (LC-MS) using a Waters 2695 separations module connected to a Waters micromass ZQ. 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% CO₂ 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% CO₂, 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% CO₂. 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% CO₂. 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 FACSCanT (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 iCycler 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 [−32P]TPP 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.
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 TCID50/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.

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 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

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence, 5’–3’</th>
<th>(T_m) (°C)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH for</td>
<td>AGCCCTTCTGGAGTTGCTGGA</td>
<td>56–60</td>
<td>Human/feline</td>
</tr>
<tr>
<td>GAPDH rev</td>
<td>AGCAGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Human/feline</td>
</tr>
<tr>
<td>IL-1β for</td>
<td>CGAAGAGAGAGAGGGGACTTTGGG</td>
<td>56</td>
<td>Human/feline</td>
</tr>
<tr>
<td>IL-1β rev</td>
<td>GCAGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Human/feline</td>
</tr>
<tr>
<td>TNFα for</td>
<td>ACGGACGGATCCCTGTGTAATTCCG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>TNFα rev</td>
<td>GCAGAAGAGAGAGGGGACTTTGGG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>CD3e for</td>
<td>GATGCTACGGGCAGTATGGG</td>
<td>58</td>
<td>Human</td>
</tr>
<tr>
<td>CD3e rev</td>
<td>CATTAGACGGCTAGCCGCGCA</td>
<td>58</td>
<td>Human</td>
</tr>
<tr>
<td>HIVpol for</td>
<td>TTGAAGACAGCATGCAATGCGGACT</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>HIVpol rev</td>
<td>AGTCCTGGACGCCGATTCA</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-10 for</td>
<td>GCGGACGGATCCCTGTGTAATTCCG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-10 rev</td>
<td>GCAGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-6 for</td>
<td>AGTCCTGGACGCCGATTCA</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-6 rev</td>
<td>GCGGACGGATCCCTGTGTAATTCCG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-4 for</td>
<td>GGCGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>IL-4 rev</td>
<td>GCGGACGGATCCCTGTGTAATTCCG</td>
<td>56</td>
<td>Human</td>
</tr>
<tr>
<td>TNFα for</td>
<td>GCCAGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Feline</td>
</tr>
<tr>
<td>TNFα rev</td>
<td>CGAGGCTACGGGCAGTATGGG</td>
<td>56</td>
<td>Feline</td>
</tr>
<tr>
<td>CD3e for</td>
<td>AACAGAGAGAGAGGGGACTTTGGG</td>
<td>56</td>
<td>Feline</td>
</tr>
</tbody>
</table>

for, forward; rev, reverse.
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 HSD5B1 (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, Carpentry, 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 xylen, 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. 1A), 3β-HSD (Fig. 1C) and P450sc (Fig. 1E) in tissue sections from HIV− patients, while neurons in HIV+ sections exhibited less immunoreactivity for the same neurosteroid synthesis enzymes (Fig. 1B, 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. 1G). 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. 2A, C) and astrocytes (Fig. 2B, 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. 2A), but conversely, the same HIV+ supernatants did not affect 5α-reductase and 3α-HSD expression in astrocytes (Fig. 2B). 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. 2C). 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...
inhibited HIV pol transcripts with concurrent DHEA-S treatment (Fig. 2E), which was confirmed by a reduction in reverse transcriptase activity in culture supernatants (Fig. 2F) 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. 4A), 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. 4B). 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. 4C, 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. 5A). 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 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. Conversely, levels of CD8⁺ T cells were modestly elevated by FIV infection, but CD8⁺ T cells from untreated animals treated with DHEA-S displayed a nonsignificant increase in blood CD8⁺ T-cell levels (Fig. 5B). 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. 5C). 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.
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 CD3ε (Fig. 6A), F4/80 (a myeloid cell activation marker; Fig. 6B), GFAP (Fig. 6C), IL-10 (Fig. 6D), IL-1β (Fig. 6E), and TNF-α (Fig. 6F) 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. 7C), which was prevented with DHEA-S therapy (Fig. 7D). These observations were extended by quantifying the preservation of cortical neurons in DHEA-S-treated...
DHEA-S-treated FIV animals (Fig. 7M). GFAP immunoreactivity revealed activation of astrocytes in FIV infection (Fig. 7G), which was decreased in DHEA-S-treated FIV animals. FIV animals showed few Iba-1-immunopositive monocytic cells and rare activated cells (Fig. 7I). In contrast, the FIV animals’ brains showed more monocytoid (Iba-1-immunopositive) cells with hypertrophy (Fig. 7K), which was suppressed by DHEA-S treatment (Fig. 7L). 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. 8A). 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. 8B). 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 (37) 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. 8C). Overall, these neurobehavioral tests were suggestive of impairment of 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: γ = −3.34 + 43.1; correlation coefficient 0.999; PCR efficiency 99.2%. βCD-treated FIV animals, n = 12; DHEA-S treated FIV animals, n = 10; 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.
specific tasks in the FIV+/H11001 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. 8D).

**DISCUSSION**

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

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. DHEAS 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. 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. 

B. DHEAS-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 animal’s hind limbs tracked the trajectory and height of the cat’s foot steps. 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. 

C. 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 treated FIV− animals, n = 5–6; βCD-treated FIV+ animals, n = 10–14; DHEA-treated FIV+ animals, n = 6–10. Values are expressed as means ± se. *P < 0.05, **P < 0.01, ***P < 0.001.

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, DHEAS, 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 neuropsychiatric 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 allopregnanolone, 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 P450sc 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 DHEAS levels are reduced in HIV-infected persons (61, 64).

The present study focused on DHEAS, 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 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...
meable 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.

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