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S-EQUOL: A NEUROPROTECTIVE THERAPEUTIC FOR CHRONIC NEUROCOGNITIVE IMPAIRMENTS IN PEDIATRIC HIV

Kristen A. McLaurin, Hailong Li, Anna K. Cook, Rosemarie M. Booze, Charles F. Mactutus

Program in Behavioral Neuroscience, Department of Psychology, University of South Carolina, Columbia, SC 29208

Abstract

Chronic neurocognitive impairments, commonly associated with pediatric human immunodeficiency virus type 1 (PHIV), are a detrimental consequence of early exposure to HIV-1 viral proteins. Strong evidence supports S-Equol (SE) as an efficacious adjunctive neuroprotective and/or neurorestorative therapeutic for neurocognitive impairments in adult ovariectomized female HIV-1 transgenic (Tg) rats. There remains, however, a critical need to assess the therapeutic efficacy of SE when treatment occurs at an earlier age (i.e., resembling a therapeutic intervention for children with PHIV) and across the factor of biological sex. Utilization of a series of signal detection operant tasks revealed prominent, sex-dependent neurocognitive deficits in the HIV-1 Tg rat, characterized by alterations in stimulus-reinforcement learning, the response profile, and temporal processing. Early (i.e., Postnatal Day 28) initiation of SE treatment precluded the development of chronic neurocognitive impairments in all (i.e., 100%) HIV-1 Tg animals; albeit not for all neurocognitive domains. Most notably, the therapeutic effects of SE generalized across the factor of biological sex, despite the presence of endogenous hormones. Results support, therefore, the efficacy of SE as a neuroprotective therapeutic for chronic neurocognitive impairments in the post-cART era; an adjunctive therapeutic that demonstrates high efficacy in both males and females. Optimizing treatment conditions by evaluating multiple factors (i.e., age, neurocognitive domains, and biological sex) associated with chronic neurocognitive impairments and HAND affords a key opportunity to improve the therapeutic efficacy of SE.

Keywords

S-Equol; Biological Sex; Stimulus-Reinforcement Learning; Temporal Processing; Signal Detection

Address proofs and correspondence to: Charles F. Mactutus, Ph.D., Department of Psychology, 1512 Pendleton Street, University of South Carolina, Columbia, SC 29208, PH: +1 (803) 777-4137, FAX: +1 (803) 777-9558, mactutus@mailbox.sc.edu.

AUTHOR CONTRIBUTIONS

R.M.B. and C.F.M. designed the study. K.A.M., H.L., R.M.B., and C.F.M. planned the study. K.A.M., H.L., and A.K.C. collected the behavioral and neuroanatomical data. K.A.M., A.K.C., and C.F.M. analyzed the data. K.A.M., R.M.B., and C.F.M. wrote the early drafts of the manuscript and K.A.M. and C.F.M. updated and revised the final draft of the manuscript. All authors critically appraised and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INTRODUCTION

Pediatric human immunodeficiency virus type 1 (PHIV), an often neglected disease (Lallemant et al., 2011), afflicts approximately 1.7 million children (<15 years of age) worldwide (UNAIDS, 2019). Combination antiretroviral therapy (cART), the primary treatment regimen for HIV-1, suppresses viral replication in the periphery, but poorly penetrates the blood-brain barrier (Gimenez et al., 2004; Letendre et al., 2008). Due to the ineffective treatment of HIV-1 in the central nervous system (CNS), chronic neurocognitive impairments are a commonly reported consequence of PHIV (Franklin et al., 2005; Parameswaran et al., 2010). Although early initiation of cART has a beneficial effect on neurocognitive development (e.g., Laughton et al., 2012; Crowell et al., 2015), HIV-1 seropositive children continue to perform below their HIV-uninfected peers (Lowick et al., 2012; Puthanakit et al., 2013) and exhibit an altered developmental trajectory (Van den Hof et al., 2020). There remains, therefore, a critical need to develop adjunctive therapeutics that penetrate the CNS and preclude the development of chronic neurocognitive impairments associated with PHIV.

Since the advent of cART, there has been a quest to develop adjunctive therapeutics to mitigate neurocognitive impairments associated with HIV-1; the focus, however, has been in HIV-1 seropositive adults (for review, Bougea et al., 2019). Therapeutics evaluated in clinical trials have primarily targeted neural mechanisms implicated in HIV-1 associated neurocognitive disorders (HAND), including neurotransmitter system alterations (e.g., Schifitto et al., 2006; Schifitto et al., 2007; Simioni et al., 2013), neuroinflammation (e.g., Sacktor et al., 2011) and oxidative stress (e.g., Dana Consortium, 1997). HIV-1 seropositive adults, however, have exhibited little, if any, cognitive improvement following treatment. Therapeutics targeting synaptic dysfunction, which has also been implicated in the pathogenesis of HAND (e.g., Gelman et al., 2012; McLaurin et al., 2019a), have, at least in preclinical studies of adult rodents, shown greater promise (e.g., Raybuck et al., 2017; Moran et al., 2019; McLaurin et al., 2020).

Given their role in synaptic function (e.g., Hao et al., 2006, Khan et al., 2013, Wang et al., 2018), gonadal hormones, including the ovarian steroid hormone 17 β -estradiol, may offer an efficacious target for novel therapeutics for chronic neurocognitive impairments. Phytoestrogens, are plant-derived compounds possessing a phenolic ring and chemical structure resembling 17 β -estradiol; structural properties which enable them to bind to either estrogen receptor (ER) α or ER β (Glazier & Bowman, 2001). Isoflavones, including daidzein (DAI) and genistein, are a subclass of phytoestrogens that selectively bind to ER β (Casanova et al., 1999; Mersereau et al., 2008). Equol, which can exist in either the R- or S-conformation, is a metabolite produced by the gut microbiota following the ingestion of DAI (Setchell et al., 1984). Relative to its precursor (i.e., DAI), S-Equol (SE), the only enantiomer produced by humans (Setchell et al., 2005), has a slower clearance rate, higher bioavailability, and stronger affinity for ER β (Setchell et al., 2010). In adult ovariectomized female HIV-1 transgenic (Tg) rats, SE serves as an efficacious therapeutic for a subset of animals by enhancing neurocognitive function across multiple cognitive domains (e.g., preattentive processes, attention, executive function) commonly altered in HAND (Moran et al., 2019; McLaurin et al., 2020). To date, however, the therapeutic efficacy of SE when

treatment occurs at an earlier age (i.e., resembling a therapeutic intervention for children with PHIV) and across the factor of biological sex has not yet been evaluated.

Thus, the aims of the present study were twofold. First, to assess whether treatment with SE early (i.e., Postnatal Day (PD) 28) in the course of HIV-1 viral protein exposure precludes the development of chronic neurocognitive impairments. Second, to determine whether SE is an efficacious therapeutic for both male and female HIV-1 Tg animals; a critical consideration given that few studies are sufficiently powered to address sex differences in HIV (Rubin et al., 2019). The aims were addressed by conducting a series of operant signal detection tasks, tapping neurocognitive domains commonly altered in HIV-1 seropositive children with chronic neurocognitive impairments (e.g., Koekkoek et al., 2008; Ruel et al., 2012; Phillips et al., 2016; McLaurin et al., 2017a). Critically testing multiple factors (i.e., age (Moran et al., 2019; McLaurin et al., 2020), neurocognitive domains, biological sex) associated with chronic neurocognitive impairments in HIV-1 seropositive children affords a key opportunity to optimize treatment conditions and improve the therapeutic efficacy of SE.

METHODS

Experimental Design

A series of operant tasks, tapping multiple neurocognitive domains, were utilized to critically test the therapeutic efficacy of S-Equol (Figure 1). Three dependent variables were derived from each operant task to evaluate stimulus-reinforcement learning, the response profile (reflecting the key components of attention), and temporal processing

Animals

Between PD 7 and PD 9, Fischer (F344/N; Envigo, Inc., Indianapolis, IN) HIV-1 Tg ($N=24$ litters) and control ($N=19$ litters) animals, housed with their biological dam, were received at the animal vivarium. At approximately PD 24, animals were randomly sampled from each litter (HIV-1 Tg: male, $n=33$, female, $n=34$; Control: male $n=38$; female, $n=34$), weaned and pair- or group-housed with animals of the same sex for the duration of experimentation. To control for independence of observations, the goal was to select one rat for each treatment group from each litter (i.e., two male rats per litter and two female rats per litter). Rodent food (Pro-Lab Rat, Mouse, Hamster Chow #3000) and water were available *ad libitum* throughout the duration of the study.

The hemizygous HIV-1 Tg rat, originally developed by Reid et al. (2001), produces seven of the nine HIV-1 genes constitutively throughout development (Peng et al., 2010; Abbondanzo & Chang, 2014) under the control of the natural HIV-1 promoter. The current derivation of the HIV-1 Tg rat, on the F344/N background, exhibits growth rates similar to F344/N control animals (e.g., Peng et al., 2010; Moran et al., 2013), a lifespan of approximately 21 months (Peng et al., 2010) and intact sensory and motor system function (McLaurin et al., 2018). F344/N controls, rather than littermates, were used to assure the most developmentally appropriate and genetically stable baseline.

Recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health were used for the maintenance of HIV-1 Tg and control animals in

AAALAC-accredited facilities. The targeted environmental conditions for the animal vivarium were $21^{\circ} \pm 2^{\circ}\text{C}$, $50\% \pm 10\%$ relative humidity and a 12-h light:12-h dark cycle with lights on at 0700 h (EST). The Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina approved the project protocol (Federal Assurance # D16-00028).

S-Equol

Beginning at approximately PD 28, HIV-1 Tg and control animals began daily treatment with either SE or placebo (i.e., sucrose pellets (Bio-Serv, Inc., Flemington, NJ)). SE, obtained from Cayman Chemical Company (Ann Arbor, MI), was incorporated into sucrose pellets by Bio-Serv, with each pellet containing 0.2 mg SE.

Animals were randomly assigned to either the SE or placebo group (Control: SE, $n=35$ (male, $n=18$, female, $n=17$), Placebo, $n=37$ (male, $n=20$, female, $n=17$); HIV-1 Tg: SE, $n=35$ (male, $n=17$, female, $n=18$), Placebo, $n=32$ (male, $n=16$, female, $n=16$)). HIV-1 Tg and control animals treated with SE received a daily oral dose of 0.2 mg of SE; a dose which was previously established as the most effective dose using a dose-response experimental design (Moran et al., 2019). Furthermore, the dose selected yielded a daily amount of 0.25–1.0 mg/kg SE; an amount equivalent to a 2.5–10 mg dose in a 60 kg human (Cf., most elderly Japanese have a daily isoflavone intake of 30–50 mg, Akaza, 2012). The placebo group received a sucrose pellet. Treatment was administered daily, after the completion of neurocognitive testing. HIV-1 Tg and control animals were treated through PD 90.

Neurocognitive Assessments

Apparatus—Thirty-two operant chambers, located inside sound-attenuating chambers (Med Associates, Inc., Fairfax, VT) were used to assess animals in a series of operant tasks. The front wall of the operant chamber included a pellet dispenser (45 mg), located between two retractable levers, and three panel lights. The panel light (LED, 11 lux) located above the pellet dispenser was utilized for stimulus presentation. The top of the rear wall of the operant chamber included a house light (incandescent, 5.5 lux). Signal presentation, lever operation, reinforcement delivery, and data collection were controlled using PC and Med-PC for Windows software (V 4.1.3; Med Associates, Inc., Fairfax, VT).

Procedure

Shaping.: A standard shaping response protocol was used to train animals to lever-press at approximately two months of age, described in detail by McLaurin et al. (2017b) with minor modifications. Specifically, all animals were trained in the shaping response protocol for 45 days prior to beginning the signal detection operant task.

Sustained Attention: Signal Detection Operant Task (1000, 500, 100 msec).: All HIV-1 Tg and control animals were trained in a signal detection operant task, originally described by McGaughy & Sarter (1995), tapping sustained attention. Methodology utilized to train animals in the signal detection operant task is similar to our prior publication (McLaurin et al., 2019a).

In brief, the task consisted of a series of three vigilance programs that required animals to attend to a randomly presented stimulus (i.e., illumination of the central panel) across 160–162 testing trials; the presence or absence of which indicated which response to make (i.e., which lever to press) to receive a reinforcer (i.e., sucrose pellet). On each trial, an animal may emit one of four response choices, indicative of attention to the stimulus (hits, correct rejection), lapses of attention (misses) or failure of response inhibition (false alarms). The systematic manipulation of the stimulus duration (i.e., 1000, 500, 100 msec) in the third vigilance program, afforded an opportunity to assess the temporal components of sustained attention.

HIV-1 Tg and control animals were trained on each program until achieving at least 70% accuracy on five consecutive, or seven non-consecutive, test sessions. Accuracy was calculated using the equation reported in McLaurin et al. (2019a). Statistical analyses and figures represent the first five days in the task.

Increased Attentional Load: Signal Detection Operant Task (1000, 100, 10 msec):

HIV-1 Tg and control animals were subsequently assessed with shorter stimulus durations (i.e., 1000, 100, 10 msec) to increase attentional load. Animals were trained until achieving at least 70% accuracy on five consecutive, or seven non-consecutive, test sessions. Statistical analyses and figures represent the first five days in the task.

Selective Attention: Visual Distractor Task (1000, 100, 10 msec): Subsequently, HIV-1 Tg and control animals were challenged for five test sessions in a visual distractor task, tapping selective attention. The signal detection operant task with varying signal durations (1000, 100, 10 msec) was divided into three trial blocks, each containing 54 trials. A 1.5 sec visual distractor stimulus (1 sec prior to and 0.5 sec after the stimulus (center panel light) onset) was presented at the beginning of each trial during the second trial block. The house light was used as the visual distractor. Statistical analyses and figures represent all five days in the task.

Flexibility and Inhibition: Reversal Task (1000, 100, 10 msec): A subset of HIV-1 Tg (SE, $n=25$ (male, $n=11$, female, $n=14$), Placebo, $n=23$ (male, $n=12$, female, $n=11$) and control (SE, $n=24$ (male, $n=11$, female, $n=13$), Placebo, $n=24$ (male, $n=13$, female, $n=12$)) animals were subsequently challenged in a reversal task, tapping flexibility and inhibition. Response contingencies were reversed from those learned in the signal detection operant task. Animals completed the reversal task by either meeting criteria (i.e., 70% accuracy for five consecutive or seven non-consecutive test sessions) or completing at least 60 test sessions. Statistical analyses and figures represent either the days meeting criteria or, when an animal had completed at least 60 test sessions, the seven days with the highest percent accuracy.

Neuroanatomical Assessment

RNA in situ hybridization—A subset of HIV-1 Tg and control animals ($n=3$ per group) were sacrificed after the completion of the visual distractor task (Age, 47 ± 1.8 weeks (Mean $\pm$ SEM)) to evaluate dopamine (DA; *Drd1 α*) and norepinephrine (NE; *Adra2a*) receptor

expression in the medial prefrontal cortex (mPFC). The total number of days to criterion in the series of four vigilance programs was utilized to select a subset of animals with the goal of representing the spectrum of cognitive function observed in HIV-1 Tg and control animals.

Procedure.: A novel RNA *in situ* hybridization technique was used to evaluate DA and NE receptor expression in the mPFC. The D1-alpha subtype receptor probe (Rn-Drd1 α) and alpha-2 NE receptor probe (Rn-Adra2a) were used to evaluate DA and NE, respectively.

Methodology for RNA *in situ* hybridization was described in detail by Li et al. (2018), with minor modifications for the present study. Specifically, brain sections were cut from the mPFC (approximately 3.7 mm to 2.2 mm anterior to Bregma; Paxinos & Watson, 2014) and hybridized with the Rn-Drd1 α probe and Rn-Adra2a probe.

Analysis and Quantification.: A Nikon TE-2000E confocal microscope, utilizing Nikon's EZ-C1 software (version 3.81b), was used to acquire two Z-stack images (60x; n.a.=1.4) for each animal.

DA and NE receptor expression were characterized by a "discrete dots" staining pattern (Li et al., 2018). Given the observed staining pattern, a cell region was defined and fluorescent dots, reflecting a single copy of DA or NE mRNA expression, were quantified. Each cell was assigned a score ranging from 1 to 9 based on the number of fluorescent dots, whereby higher categories reflect a greater number of mRNA molecules within the cell.

Statistical Analysis

Analysis of variance (ANOVA) and regression techniques were used to statistically analyze all data (SAS/STAT Software 9.4, SAS Institute, Inc., Cary, NC; SPSS Statistics 26, IBM Corp., Somers, NY; GraphPad Software, Inc., La Jolla, CA). For variables that violate the assumption of compound symmetry, orthogonal decompositions or the Greenhouse-Geisser *df* correction factor (Greenhouse & Geisser, 1959) were implemented. Statistical significance was established at an alpha criterion of $p < 0.05$. *A priori* planned comparisons were conducted to establish the role of the HIV-1 transgene in neurocognitive function (i.e., Control Placebo vs. HIV-1 Tg Placebo) and the magnitude of the SE effect (i.e., HIV-1 Tg Placebo vs. HIV-1 Tg SE and/or Control Placebo vs. HIV-1 Tg SE). Specifically, planned comparisons were either conducted using simple effects, in the context of the overall analysis, or by conducting complementary analyses.

Body weight was analyzed using a mixed-factor ANOVA (SPSS Statistics 26, IBM Corp., Somers, NY) independently for each sex. Three dependent variables were derived from each operant task (i.e., Signal Detection Operant Task (1000, 500, 100 msec or 1000, 100, 10 msec), Visual Distractor Task, Reversal Task) to evaluate stimulus-reinforcement learning, the response profile and temporal processing.

Stimulus-reinforcement learning was derived by assessing the number of days to reach criterion (i.e., third vigilance program, fourth vigilance program, reversal task) or the number of days meeting criterion (i.e., visual distractor task) on each task. A curve-fitting

analysis, fit with a 95% confidence intervals (CI), was utilized to directly assess functional form (GraphPad Software, Inc., La Jolla, CA, USA).

The response profile illustrates the number of correct rejections, hits, misses and false alarms emitted by an animal during each task. A mixed-factor ANOVA (i.e., third vigilance program, fourth vigilance program, visual distractor task; SPSS Statistics 26, IBM Corp., Somers, NY) or generalized linear mixed model with a Poisson distribution and an unstructured covariance matrix (i.e., reversal task; PROC GLIMMIX, SAS/STAT Software 9.4, SAS Institute, Inc., Cary, NC) were utilized to analyze the response profile.

Temporal processing is a construct that was directly assessed by systematically manipulating the stimulus duration. More specifically, the number of hits and misses (i.e., response types occurring during signal trials) at each stimulus duration (e.g., 1000, 500, 100 msec) were evaluated. A prominent shift in the time at which the number of hits and misses intersect, reflecting the loss of stimulus detection, supports alterations in temporal processing. A mixed-factor ANOVA (SPSS Statistics 26, IBM Corp., Somers, NY; PROC MIXED; SAS/STAT Software 9.4, SAS Institute, Inc., Cary, NC) was utilized to statistically evaluate temporal processing.

For all statistical analyses, genotype (HIV-1 Tg vs. control), biological sex (male vs. female), and treatment (SE vs. placebo) served as between-subject's factors, as appropriate. Response type (correct rejections, hits, misses and false alarms), signal duration (e.g., 1000, 500, 100 msec), and age served as the within subject's factors, as appropriate.

Simple linear regression analyses were conducted to examine the relationship between the mean cell score, reflecting DA (Drd1 α) and NE (Adra2a) receptor expression in the mPFC, and the number of misses in the signal detection operant task tapping sustained attention (GraphPad Software, Inc., La Jolla, CA, USA). Two male HIV-1 Tg animals were removed from the linear regression, because they were statistical outliers with a total number of misses greater than three standard deviations away from the mean. To account for the nested experimental design (i.e., two images per animal), cell scores were averaged between the two images.

RESULTS

Somatic Growth: Body Weight

Body weight, an assessment of somatic growth, was measured weekly in HIV-1 Tg and control animals from 4 weeks of age through 40 weeks of age (Figure 2). HIV-1 Tg animals, independent of biological sex and/or treatment, weighed significantly less than control animals throughout the duration of experimentation. The growth trajectory for HIV-1 Tg and control animals, independent of biological sex and/or treatment, was well-described by a one-phase association (R^2 s 0.99).

Statistically significant differences were observed in the rate of somatic growth, evidenced by a genotype $\times$ age interaction (Male: [$F_{36, 2412}$]=47.0, p_{GG} 0.001, η_p^2 =0.412] with a prominent linear component [$F_{1, 67}$]=95.7, p 0.001, η_p^2 =0.588]; Female: [$F_{36,$

2232)=18.3, p_{GG} 0.001, $\eta_p^2=0.228$] with a prominent linear component [$F(1, 62)=52.4$, p 0.001, $\eta_p^2=0.458$]. Treatment with SE, however, did not alter the rate of somatic growth in either HIV-1 Tg or control animals evidenced by the failure to observe a statistically significant main effect of treatment ($p>0.05$) or any statistically significant higher-order interactions with treatment ($p>0.05$).

Neurocognitive Assessments

Presence of the HIV-1 Transgene

Stimulus-Reinforcement Learning: Days to Criterion.: Stimulus-reinforcement learning, a neurocognitive domain commonly altered in chronic neurocognitive impairments (e.g., Nichols et al., 2016), was evaluated across multiple operant tasks in the HIV-1 Tg rat. In all tasks, criterion was established at 70% accuracy for five consecutive or seven non-consecutive test sessions. All HIV-1 Tg and control animals, independent of biological sex, were able to successfully acquire the third vigilance program, tapping sustained attention (Figure 3A), and the fourth vigilance program, requiring increased attentional demands (Figure 3B). Meeting criteria in the signal detection operant task (i.e., all four vigilance programs) afforded an opportunity to conduct a visual distractor task, tapping selective attention, and a reversal task, tapping flexibility and inhibition. In the visual distractor task, approximately 16.2% of control animals and 9.4% of HIV-1 Tg animals met criteria during all five test sessions (Figure 3C). In the reversal task, approximately 64% and 47.8% of control and HIV-1 Tg animals, respectively, met criteria in the reversal task (Figure 3D).

Findings support prominent alterations in stimulus-reinforcement learning in the HIV-1 Tg rat, albeit with boundary conditions. Presence of the HIV-1 transgene most profoundly disrupted stimulus-reinforcement learning in tasks requiring higher cognitive demands, including an increased attentional load (i.e., fourth vigilance program) and flexibility and inhibition (i.e., reversal task). Specifically, under increased attentional demands, HIV-1 Tg animals acquired the fourth vigilance program at a significantly slower rate (i.e., Best-Fit: One-Phase Association, Rs^2 0.97; Genotype Differences in Rate Constant, K : [$F(1,38)=17.5$, p 0.001]) than control animals. Furthermore, statistically significant differences in the best-fit function (i.e., First-Order Polynomial, Rs^2 0.97) describing the cumulative proportion of HIV-1 Tg and control animals acquiring the reversal task were observed [$F(2,33)=6.7$, p 0.003]. No alterations in stimulus-reinforcement learning were observed in either sustained (i.e., third vigilance program) or selective (i.e., visual distractor task) attention. Thus, HIV-1 Tg animals displayed deficits in stimulus-reinforcement learning, albeit only under conditions requiring higher cognitive demands.

Response Profile: Number of Correct Rejections, Hits, Misses, and False Alarms.: On each trial, independent of task, an animal may emit one of four response choices, indicative of attention to the stimulus (i.e., hit or correct rejection), lapses of attention (i.e., miss) or failure of response inhibition (i.e., false alarm).

Findings support alterations in the response profile for the HIV-1 Tg rat, dependent upon biological sex, when challenged with learning a new task (i.e., reversing response contingencies) at a more advanced age (Figure 4D). Presence of the HIV-1 transgene

significantly altered the response profile for flexibility and inhibition in male, but not female, animals (Genotype $\times$ Sex $\times$ Response Type Interaction, [$F(3,129)=5.6$, p 0.001]). Specifically, male HIV-1 Tg animals exhibited an increased number of correct rejections [$t(129)=-2.4$, p 0.02], and misses [$t(129)=-2.6$, p 0.01], relative to male control animals. In sharp contrast, female HIV-1 Tg animals displayed no impairments in the response profile for flexibility and inhibition ($p>0.05$).

No genotypic alterations in the response profile were observed in sustained attention (i.e., third vigilance program, Figure 4A), under increased attentional load (i.e., fourth vigilance program, Figure 4B), or selective attention (i.e., visual distractor task, 4C). The absence of genotypic alterations in either the third or fourth vigilance may result from the rigorous training of HIV-1 Tg and control animals in the first two vigilance programs; training which utilized a stringent criterion (i.e., 70% accuracy for five consecutive or seven non-consecutive days) to ensure that animals have learned the response contingencies. Presence of a floor effect during the second trial block of the visual distractor task precluded the ability to evaluate the effect of the HIV-1 transgene. However, HIV-1 Tg animals, dependent upon biological sex, displayed alterations in the response profile under conditions not previously trained (i.e., reversing response contingencies) at an advanced age.

Temporal Processing: Systematic Manipulation of Stimulus Duration. The systematic manipulation of stimulus duration (e.g., 1000, 500, 100 msec) provided a critical opportunity to evaluate temporal processing (Figure 5), which has been implicated as an elemental dimension of HAND (e.g., Chao et al., 2004; Moran et al., 2013). The number of hits and misses (i.e., response types occurring during signal trials) were examined at each signal duration, whereby the time at which the number of hits and misses intersect reflects the loss of stimulus detection.

Prominent alterations in temporal processing were observed in the HIV-1 Tg rat, albeit with boundary conditions. Presence of the HIV-1 transgene most profoundly disrupted temporal processing under novel conditions, including the first systematic manipulation of stimulus duration (i.e., third vigilance program; Figure 5A) and reversed response contingencies (i.e., reversal task; Figure 5C). Specifically, in the third vigilance program, HIV-1 Tg animals displayed a pronounced rightward shift in the loss of stimulus detection (i.e., where the number of hits and misses intersect) relative to control animals treated with placebo (approximately 720 msec vs. 420 msec, respectively; Genotype $\times$ Response Type $\times$ Duration with a prominent linear-quadratic component, [$F(1,65)=5.3$, p 0.03, $\eta_p^2=0.075$]); results which support an alteration in the temporal components of sustained attention.

In the reversal task, a sex-dependent deficit in the temporal components of flexibility and inhibition was observed in HIV-1 Tg animals (Genotype $\times$ Sex $\times$ Response Type Interaction, [$F(1, 43)=15.5$, p 0.001]). Male [$t(43)=-2.1$, p 0.05], but not female ($p>0.05$), HIV-1 Tg animals displayed a deficit in the temporal components of flexibility and inhibition relative to their control counterparts. Specifically, male HIV-1 Tg animals exhibited a prominent rightward shift in the loss of stimulus detection relative to male control animals (i.e., failure to reliably detect the stimulus at any duration assessed vs. 520 msec, respectively); a rightward shift resulting from the dramatic increase in the number of misses [$t(43)=-3.3$,

$p < 0.002$]. Furthermore, no genotypic alterations in temporal processing were observed under increased attentional demands (i.e., fourth vigilance program; $p > 0.05$; Figure 5B). Thus, HIV-1 Tg animals displayed prominent deficits in the temporal components of sustained attention, as well as flexibility and inhibition; results which support that temporal processing is most profoundly disrupted in HIV-1 Tg animals under novel conditions.

Therapeutic Efficacy of S-Equol

Stimulus-Reinforcement Learning: Days to Criteria.: Treatment with SE failed to preclude deficits in stimulus-reinforcement learning in either the fourth vigilance program (Figure 6A, 6B) or the reversal task (Figure 6C, 6D); tasks which occurred beyond the cessation of SE.

Specifically, under increased attentional demands (i.e., fourth vigilance program), the rate (i.e., K) of task acquisition in HIV-1 Tg animals treated with SE was statistically indistinguishable from HIV-1 Tg animals treated with placebo ($p > 0.05$) and remained significantly slower than control animals treated with placebo (i.e., Genotype Differences in Rate Constant, K : [$F(1,40)=16.7$, $p < 0.001$]). Furthermore, only 40% of the HIV-1 Tg animals treated with SE successfully acquired the reversal task, well-described by a segmental linear regression ($R^2 = 0.98$; Figure 6C, 6D); a proportion of animals that was lower than either HIV-1 Tg (i.e., 47.8%) or control (i.e., 64%) animals treated with placebo.

Response Profile: Number of Correct Rejections, Hits, Misses, and False

Alarms.: Treatment with SE altered the response profile in flexibility and inhibition by increasing responding in male HIV-1 Tg animals (Figure 6E). Comparison of male HIV-1 Tg animals treated with SE and male HIV-1 Tg animals treated with placebo revealed a statistically significant treatment $\times$ response type interaction [$F(3,63)=3.3$, $p < 0.03$]. Specifically, HIV-1 Tg animals treated with SE exhibited a statistically significant increase in the number of hits [$t(63)=2.5$, $p < 0.02$] relative to HIV-1 Tg animals treated with placebo. Treatment with SE did not alter the number of correct rejections ($p > 0.05$), misses ($p > 0.05$) or false alarms ($p > 0.05$) in male HIV-1 Tg animals.

Furthermore, comparison of HIV-1 Tg animals treated with SE and control animals treated with placebo revealed a main effect of genotype [$F(1,22)=9.9$, $p < 0.005$] (Figure 6E.2), but no statistically significant interactions ($p > 0.05$). Independent of response type, HIV-1 Tg animals treated with SE exhibited a greater rate of responding. In male HIV-1 Tg animals, therefore, treatment with SE increases the response rate, with a specific effect on the number of hits, supporting enhanced attention to the stimulus.

Temporal Processing: Systematic Manipulation of Stimulus Duration.: Treatment with SE during the formative period enhanced the temporal components of sustained attention (Figure 6F; Genotype $\times$ Treatment $\times$ Response Type $\times$ Duration interaction with a prominent linear-quadratic component [$F(1,131)=5.7$, $p < 0.02$, $\eta_p^2=0.042$]) and flexibility and inhibition (Figure 6G; Genotype $\times$ Treatment $\times$ Response Type, [$F(1, 43)=5.9$, $p < 0.02$]).

Specifically, in the third vigilance program, tapping sustained attention, HIV-1 Tg animals treated with SE exhibited a loss of signal detection at approximately 570 msec relative to

420 msec and 720 msec in control and HIV-1 Tg animals treated with placebo, respectively. The temporal components of sustained attention in HIV-1 Tg animals treated with SE were statistically indistinguishable from either HIV-1 Tg animals treated with placebo (Treatment $\times$ Response Type $\times$ Duration, $p > 0.05$) or control animals treated with placebo (Genotype $\times$ Response Type $\times$ Duration, $p > 0.05$).

In the reversal task, tapping flexibility and inhibition, male HIV-1 Tg animals treated with SE displayed a robust leftward shift in the loss of signal detection (i.e., approximately 190 msec) relative to either male control (i.e., approximately 520 msec) or male HIV-1 Tg (i.e., failed to reliably detect the stimulus at any duration assessed) animals treated with placebo. Treatment with SE enhanced the temporal components of flexibility and inhibition in HIV-1 Tg animals by increasing the number of hits, independent of stimulus duration, relative to HIV-1 Tg animals treated with placebo [$t(43) = 2.6$, $p = 0.01$]. Relative to control animals treated with placebo, HIV-1 Tg animals treated with SE exhibited increased responding, independent of either response type or stimulus duration [$t(43) = -3.12$, $p = 0.003$]. Thus, SE enhances the temporal components of sustained attention and flexibility and inhibition, thereby precluding the development of temporal processing deficits.

Neuroanatomical Assessments

In situ hybridization: Dopamine and norepinephrine receptor expression—

Regression analyses support the sensitivity of sustained attention to both DA and NE receptor expression. For DA (Figure 7A, 7B, 7C), a linear regression provided a well-described fit for the relationship between the total number of misses in the third vigilance program, tapping sustained attention, and mean cell score, revealing a regression coefficient (r) of 0.495. The relationship between the total number of misses in the third vigilance program and mean cell score for NE (Figure 7D, 7E, 7F) was also described by a linear regression, revealing a regression coefficient (r) of 0.615. Therefore, 24.5% of the variance in the mean cell score for Drd1 α and 37.9% of the variance in the mean cell score for Adra2a was explained by total number of misses in the third vigilance program, reflecting lapses in attention. For both DA and NE, as mean cell score decreased, the total number of misses increased, supporting suboptimal neurotransmitter system function.

DISCUSSION

The HIV-1 Tg rat was utilized to critically test the therapeutic efficacy of SE for chronic neurocognitive impairments associated with PHIV. At the genotypic level, a series of signal detection operant tasks revealed prominent neurocognitive deficits in the HIV-1 Tg rat, characterized by alterations in stimulus-reinforcement learning, the response profile (reflecting the key components of attention), and temporal processing. With regards to the therapeutic efficacy of SE, early (i.e., PD 28) treatment initiation precluded the development of chronic neurocognitive impairments in all (i.e., 100%) HIV-1 Tg animals; albeit only in select neurocognitive domains. Furthermore, the therapeutic effects of SE generalize across the factor of biological sex, despite the presence of endogenous hormones. Results support, therefore, the efficacy of SE as a neuroprotective therapeutic for chronic neurocognitive impairments in the post-cART era; an adjunctive therapeutic that demonstrates high efficacy

in both males and females. Critically testing multiple factors (i.e., age (Moran et al., 2019; McLaurin et al., 2020), neurocognitive domains, and biological sex) associated with chronic neurocognitive impairments and HAND affords a key opportunity to optimize treatment conditions and improve the therapeutic efficacy of SE.

A series of signal detection operant tasks were employed to assess the effect of HIV-1 viral proteins, biological sex and/or SE treatment on two types of attention (i.e., sustained attention and selective attention), attentional load, and executive function (i.e., flexibility and inhibition). As such, the experimental design afforded an opportunity to derive three key dependent variables from each signal detection task. At the genotypic level, HIV-1 Tg animals developed prominent deficits, albeit with boundary conditions, in stimulus-reinforcement learning, the response profile, and temporal processing; results which extend those previously reported (e.g., Moran et al., 2014; McLaurin et al., 2019a). Most notably, treatment with SE altered the response profile in flexibility and inhibition by increasing responding, with a specific emphasis on increasing attention to the stimulus (i.e., hits), in male HIV-1 Tg animals. Furthermore, SE precluded the development of neurocognitive impairments in the temporal components of both sustained attention and executive function (i.e., flexibility and inhibition) in all (i.e., 100%) HIV-1 Tg animals; an effect which is noteworthy given that alterations in the perception of time are significantly altered in PHIV (e.g., Phillips et al., 2016; McLaurin et al., 2017a) and have been implicated as an elemental dimension of HAND (e.g., Chao et al., 2004; Matas et al., 2010; Moran et al., 2013; McLaurin et al., 2019b). Treatment with SE, however, was unable to preclude deficits in stimulus-response learning under increased attentional demands or flexibility and inhibition.

The construct of time, in the broadest sense, has been implicated as a critical factor in the development and treatment of chronic neurocognitive impairments associated with PHIV. First, strong evidence supports the importance of the time of transmission (i.e., during pregnancy, delivery, or breastfeeding) in the development of neurocognitive deficits (Fitting et al., 2008; McLaurin et al., 2017a; Fitting et al., 2018). Specifically, early viral protein exposure, mimicking HIV-1 infection in pregnancy (Fitting et al., 2008; McLaurin et al., 2017a) has more deleterious effects on neurocognitive development than late viral protein exposure, resembling HIV-1 infection at labor/delivery (Fitting et al., 2018). Second, earlier initiation of cART, effectively suppressing HIV-1 in the periphery, is associated with improved neurocognitive outcomes (Laughton et al., 2012; Crowell et al., 2015). Furthermore, initiating SE treatment earlier (i.e., PD 28 vs. 2 to 3 months of age (McLaurin et al., 2020) vs. 6 to 8 months of age (Moran et al., 2019)) in the course of HIV-1 viral protein exposure increased therapeutic efficacy. However, although SE is most efficacious when utilized as a neuroprotective therapeutic (i.e., PD 28), it may also serve as a neurorestorative therapeutic, albeit with decreased efficacy (i.e., 40%; Moran et al., 2019). Thus, even under conditions of more deleterious neurocognitive impairments (i.e., early viral protein exposure), SE selectively precluded the development of chronic neurocognitive impairments.

Inclusion of biological sex as an integral component of the experimental design merits further discussion. In HIV-1 seropositive adults, prominent sex differences in the development and pattern of neurocognitive impairments have been reported (for review,

Rubin et al., 2019). In PHIV, however, the effect of biological sex on chronic neurocognitive impairments remains relatively understudied with the exception of a few recent manuscripts (McLaurin et al., 2016; Bangirana et al., 2017). Given the presence of notable sex differences in neurocognitive impairments, an efficacious adjunctive therapeutic for both male and female HIV-1 seropositive individuals would be advantageous. As demonstrated in the present study, SE is a neuroprotective therapeutic for both male and female HIV-1 Tg animals; results which are unsurprising given the presence of ER β in the PFC of both male and female rats (Zhang et al., 2002). Furthermore, although the phytoestrogen SE exhibits strong affinity ER β (Setchell et al., 2010), Equol also binds 5 α -dihydrotestosterone (Lund et al., 2004; Lund et al., 2011). Notably, both 17 β -estradiol (e.g., Hao et al., 2006, Khan et al., 2013, Wang et al., 2018) and 5 α -dihydrotestosterone are involved in synaptic function (Hajszan et al., 2007; Soma et al., 2018) supporting a convergent mechanism, independent of biological sex, by which SE may exert its effects.

The prefrontal cortex (PFC), located at the anterior pole of the mammalian brain, is well-recognized for its roles in the temporal organization of behavior and executive functions, including attention, flexibility, and inhibition (Fuster, 2008). Anatomically, the PFC can be subdivided into three distinct regions, including the mPFC, orbital PFC (oPFC) and lateral PFC (IPFC); subdivisions which each display some degree of functional specialization (i.e., mPFC: sustained attention (Kim et al., 2016); oPFC: stimulus-reinforcement learning (Rolls, 2004; Tsuchida et al., 2010), flexibility and inhibition (McAlonan & Brown, 2003); IPFC: selective attention (Kam et al., 2018)). Furthermore, the operant signal detection task, tapping sustained attention, is sensitive to DA and NE receptor expression in the mPFC; an effect that should be further investigated with sufficient statistical power to examine genotype, sex and/or treatment effects. Specifically, as the mean cell score for either DA or NE receptor expression decreased, animals exhibited a greater number of lapses in attention, evidenced by an increased total number of misses. Given the observed neurocognitive impairments, the constitutive expression of HIV-1 viral proteins likely has the most adverse effects on the mPFC and oPFC, whereas the IPFC remains relatively spared.

Although the pathophysiology of neurocognitive impairments associated with HIV-1 is multidimensional, synaptic dysfunction has been implicated as a key underlying neural mechanism (e.g., Gelman et al., 2012; Desplats et al., 2013; Roscoe et al., 2014; McLaurin et al., 2019a). Specifically, in the mPFC, synaptic dysfunction, accounting for 64.6% of the genotypic variance, is characterized by prominent alterations in dendritic branching complexity, synaptic connectivity, and dendritic spine morphology (McLaurin et al., 2019a). Mechanistically, SE, which penetrates the CNS via the blood-brain barrier and distributes most significantly to the PFC (Lund et al., 2001), may protect against the adverse consequences resulting from HIV-1 viral protein exposure by targeting synaptic dysfunction (Bertrand et al., 2014; Bertrand et al., 2015). Strong *in vitro* evidence supports the utility of SE to prevent synapse loss (Bertrand et al., 2014) and restore synaptic connectivity (Bertrand et al., 2015) induced by the HIV-1 viral protein, Tat. Elucidating the precise neural mechanism underlying the neuroprotective and neurorestorative effects of SE *in vivo*, however, remains a critical knowledge gap.

The rigor and reproducibility crisis in the behavioral and biomedical sciences stems, at least in part, from concern regarding the failure to effectively translate preclinical studies into the clinic. A multitude of factors, including poor experimental design (Collins & Tabak, 2014), low statistical power (Button et al., 2013), and inappropriate statistical analyses (Collins & Tabak, 2014) contribute to the reproducibility challenges in preclinical research. In light of these concerns, the present study was designed to target key aspects of translational relevance. First, the biological system (i.e., HIV-1 Tg rat) utilized to model key aspects of chronic neurocognitive impairments in PHIV, which expresses HIV-1 viral proteins constitutively throughout development (Peng et al., 2010; Abbondanzo & Chang, 2014), the dose of SE, which yields a daily amount less than the daily isoflavone intake of most elderly Japanese individuals (i.e., 30–50 mg; Akaza, 2012), and the inclusion of biological sex, reflect the clinical population of interest. Second, animals were sampled with the goal of controlling for independence of observations; an assumption that underlies many common statistical techniques (e.g., *t*-tests, analysis of variance). Violating the independence of observation assumption results in spuriously significant effects, evidenced by inflated type I error rates (e.g., Haseman & Hogan, 1975; Holson & Pearce, 1992; Aarts et al., 2014). Finally, the present study replicates and extends a series of cross-sectional *in vivo* studies (Moran et al., 2019; McLaurin et al., 2020).

In conclusion, treatment with SE during the formative period (i.e., PD 28 to PD 90) selectively precluded the development of chronic neurocognitive impairments, a consequence of PHIV, independent of biological sex. A series of cross-sectional studies support SE as both a neuroprotective and/or neurorestorative therapeutic for neurocognitive impairments associated with HIV-1; a longitudinal experimental design, however, needs to be utilized to evaluate whether SE can modify disease progression. Most critically, optimizing treatment conditions by evaluating multiple factors (i.e., age, neurocognitive domains, and biological sex) associated with chronic neurocognitive impairments and HAND affords a key opportunity to improve the therapeutic efficacy of SE.

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FIGURE 1.
Schematic of the Experimental Design.

FIGURE 2.

Mean body weight ($\pm 95\%$ CI), a measure of somatic growth, is illustrated for males (**A**) and females (**B**) as a function of genotype (HIV-1 Tg vs. control), treatment (SE vs. placebo) and age. Independent of biological sex, HIV-1 Tg animals weighed significantly less and exhibited a slower rate of growth relative to control animals [Genotype $\times$ Age interaction, $p < 0.001$]. Treatment with SE did not alter the growth trajectory of either HIV-1 Tg or control animals.

FIGURE 3.

Stimulus-reinforcement learning, presented as a function of genotype (HIV-1 Tg vs. control; $\pm 95\%$ CI), was assessed for sustained attention (**A**), under increased attentional load (**B**), selective attention (**C**), and flexibility and inhibition (**D**). HIV-1 Tg rats, independent of biological sex, exhibited prominent deficits in stimulus-reinforcement learning, albeit only under conditions requiring higher cognitive demands (i.e., Increased Attentional Load and Flexibility and Inhibition). No significant alterations ($p > 0.05$) in stimulus-reinforcement learning were observed in operant tasks tapping sustained or selective attention.

FIGURE 4.

The number of correct rejections, hits, misses, and false alarms, reflecting the four response choices an animal may emit on each trial, are presented as a function of genotype (HIV-1 Tg vs. control; $\pm$ SEM). No statistically significant ($p > 0.05$) genotypic differences in the response profile were observed in operant tasks tapping sustained attention (**A**), the effects of increased attentional load (**B**) or selective attention (**C**). For selective attention, Block 2 reflects when the visual distractor was present. HIV-1 Tg animals, however, exhibited prominent alterations in the response profile, dependent upon biological sex, when challenged with learning a new task (i.e., reversing response contingencies) at a more advanced age [Genotype $\times$ Sex $\times$ Response Type interaction, $p = 0.001$] (**D**). Specifically, in the operant task tapping flexibility and inhibition, male, but not female, HIV-1 Tg animals exhibited an increased number of correct rejections and misses relative to male control animals. * $p < 0.05$ relative to respective Controls.

FIGURE 5.

Temporal processing was assessed by evaluating the number of hits and misses at each stimulus duration as a function of genotype (HIV-1 Tg vs. control; $\pm$ SEM) for signal detection operant tasks tapping sustained attention (**A**), the effect of increased attentional demands (**B**) and flexibility and inhibition (**C**). Presence of the HIV-1 transgene most profoundly disrupted temporal processing under novel conditions, including the first systematic manipulation of stimulus duration, tapping sustained attention [Genotype $\times$ Response Type $\times$ Duration interaction with a prominent linear-quadratic component, p 0.05] and reversed response contingencies, tapping flexibility and inhibition, albeit in a sex-dependent manner [Genotype $\times$ Sex $\times$ Response Type interaction, p 0.001]. Specifically, HIV-1 Tg animals exhibited a prominent rightward shift in the loss of signal detection (i.e.,

where the number of hits and misses intersect) relative to control animals. No statistically significant ($p>0.05$) genotypic alterations in temporal processing were observed under conditions requiring increased attentional demands.

FIGURE 6.

The utility of S-Equal (SE) as an innovative therapeutic to preclude the development of chronic neurocognitive impairments in the HIV-1 transgenic rat is presented for stimulus-reinforcement learning (A-D), the response profile (E) and temporal processing (F-G). Treatment with SE failed ($p > 0.05$) to preclude deficits in stimulus-reinforcement learning in operant tasks requiring either an increased attentional load (A-B) or tapping flexibility and inhibition (C-D); tasks which occurred beyond the cessation of SE. However, SE significantly altered the response profile in HIV-1 Tg male animals in the reversal task, tapping flexibility and inhibition, by increasing the response rate relative to either HIV-1 Tg animals treated with placebo [Treatment $\times$ Response Type interaction, $p = 0.05$] (E) or control animals treated with placebo [Main Effect: Genotype, $p = 0.01$] (E.2, inset). Furthermore,

treatment with SE enhanced the temporal components of sustained attention [Genotype $\times$ Treatment $\times$ Response Type $\times$ Duration interaction with a prominent linear-quadratic component, $p < 0.05$] (**F**) and flexibility and inhibition [Genotype $\times$ Treatment $\times$ Response Type interaction, $p < 0.05$] (**G**) evidenced by a prominent leftward shift in the loss of signal detection relative to HIV-1 Tg animals treated with placebo. Thus, treatment with SE selectively precluded the development of chronic neurocognitive impairments, independent of biological sex, in the HIV-1 Tg rat. Planned comparisons: HIV-1 Tg SE vs. HIV-1 Tg Placebo, * $p < 0.05$; HIV-1 Tg SE vs. Control Placebo & $p < 0.05$.

Dopamine (Drd1 α) Receptor Expression

Norepinephrine (Adra2a) Receptor Expression

FIGURE 7.

In situ hybridization was utilized to assess dopamine (Drd1 α , **A-C**) and norepinephrine (Adra2a, **D-F**) receptor expression in the medial prefrontal cortex. A subset of animals were selected for *in situ* hybridization based on the total number of days to criterion in the series of four vigilance programs. Representative images for Drd1 α are shown for animals with a lower (**A**) and higher (**B**) number of total misses; images reflecting the linear regression which provided a well-described fit for the relationship between the total number of misses in the third vigilance program and mean cell score for dopamine (**C**; correlation coefficient, r , 0.495). Representative images for Adra2a are also shown for animals with a lower (**D**) and higher (**E**) total number of misses; images reflecting the linear regression which provided a well-described fit for the relationship between the total number of misses in the third vigilance program and mean cell score for norepinephrine (**F**; r , 0.615). Results support, therefore, the sensitivity of the signal detection operant task, tapping sustained attention, to alterations in Drd1 α and Adra2a receptor expression.