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Glutamine antagonist JHU083 improves psychosocial behavior and sleep deficits in EcoHIV-infected mice

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ABSTRACT

Combined antiretroviral therapy ushered an era of survivable HIV infection in which people living with HIV (PLH) conduct normal life activities and enjoy measurably extended lifespans. However, despite viral control, PLH often experience a variety of cognitive, emotional, and physical phenotypes that diminish their quality of life, including cognitive impairment, depression, and sleep disruption. Recently, accumulating evidence has linked persistent CNS immune activation to the overproduction of glutamate and upregulation of glutaminase (GLS) activity, particularly in microglial cells, driving glutamatergic imbalance with neurological consequences. Our lab has developed a brain-penetrant prodrug of the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON), JHU083, that potently inhibits brain GLS activity in mice following oral administration. To assess the therapeutic potential of JHU083, we infected mice with EcoHIV and characterized their neurobehavioral phenotypes. EcoHIV-infected mice exhibited decreased social interaction, suppressed sucrose preference, disrupted sleep during the early rest period, and increased sleep fragmentation, similar to what has been reported in PLH but not yet observed in murine models. At doses shown to inhibit microglial GLS, JHU083 treatment ameliorated all of the abnormal neurobehavioral phenotypes. To explore potential mechanisms underlying this effect, hippocampal microglia were isolated for RNA sequencing. The dysregulated genes and pathways in EcoHIV-infected hippocampal microglia pointed to disruptions in immune functions of these cells, which were partially restored by JHU083 treatment. These findings suggest that upregulation of microglial GLS may affect immune functions of these cells. Thus, brain-penetrable GLS inhibitors like JHU083 could act as a potential therapeutic modality for both glutamate excitotoxicity and aberrant immune activation in microglia in chronic HIV infection.

1. Introduction

Combined antiretroviral therapies (cART) have drastically reduced the mortality associated with human immunodeficiency virus (HIV)-infection (Matinella et al., 2015; Nedelcovych et al., 2017a). However, for people living with HIV (PLH), extended lifespans often include comorbidities that lower quality of life. The incidence rate of depression is doubled in long-term PLH compared to an uninfected population (Ciesla and Roberts, 2001), and which is associated with worse clinical outcomes and increased mortality (Anagnostopoulos et al., 2015). PLH disproportionally exhibit cognitive impairment, which contributes to declining quality of life along with poor disease prognosis (Doyle et al., 2012; Thaler et al., 2015; Tozzi et al., 2003). Sleep disturbances are also overrepresented among PLH (Faraut et al., 2018; Wu et al., 2015a). These sleep disturbances manifest as both reduced sleep abundance over each 24-h period and disrupted sleep architecture that includes increased fragmentation and nighttime restlessness. These abnormalities result in low-quality, less restorative sleep (Faraut et al., 2018; Wibbeler et al., 2012). There is a high concordance between these comorbid phenotypes in PLH, which broadly interact to impact mental,

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emotional, and physiological health (Tozzi et al., 2003; Davis, 2004; Shimizu et al., 2011; Gutierrez et al., 2019; Rogers et al., 2020).

Previous investigations have highlighted the role of dysfunctional glutamatergic metabolism in the neurological pathology common with HIV infection. PLH exhibit elevated cerebrospinal fluid glutamate levels as well as dysregulation of genes involved in glutamate production, metabolism, and glutamatergic synaptic transmission (Zhao et al., 2012; Gelman et al., 2012a, 2012b). Mechanistically, this phenomenon may in part arise from the persistent activation of resident microglia and invading macrophages throughout the period of HIV-infection in the central nervous system (CNS). Activated immune cells in the brain, specifically microglia, upregulate the activity of glutaminase (GLS), the enzyme responsible for glutamate biosynthesis. This perturbation results in the production of excess extracellular glutamate (Wu et al., 2015b; Potter et al., 2013; Erdmann et al., 2006; Huang et al., 2011).

Our lab has discovered JHU083 (ethyl (R)-2-((R)-2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoate), a lipophilic prodrug of the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON), which inhibits brain GLS activity in mice following oral administration (Rais et al., 2016; Zhu et al., 2019). GLS inhibition by JHU083 has been shown to restore normal CSF glutamate levels and improve measures of hippocampal-memory function in the EcoHIV murine model of HIV-associated cognitive impairment (Nedelcovych et al., 2019). In this model, mice are infected with the chimeric virus EcoHIV, in which the HIV envelope gene fragment expressing human-specific HIV gp120 protein has been replaced with the ecotropic murine virus gene expressing rodent-specific gp80 envelope protein, enabling it to infect mouse $CD4^+$ T cells, macrophages, and microglia (Gu et al., 2018; He et al., 2014). EcoHIV-infected mice replicate many of the hallmarks of a human HIV infection controlled with cART, including blood-brain barrier disruption (Berger and Avison, 2004; Jones et al., 2016), neuroinflammation (Jones et al., 2016; Kelschenbach et al., 2019; Tavazzi et al., 2014), elevated glutamate levels (Nedelcovych et al., 2019; Ferrarese et al., 2001), dopaminergic neuron injury (Nickoloff-Bybel et al., 2020; Olson et al., 2018), synaptodendritic injury (Kelschenbach et al., 2019; Masliah et al., 1997), elevated risk of ischemic stroke (Benjamin and Khoo, 2018; Bertrand et al., 2019), and altered lipid metabolism (Zhu, 2019). The neurocognitive phenotypes these mice exhibit are restored by GLS inhibition via JHU083, which speaks to the importance of glutamate metabolism in cognitive function and highlights the pathway as a potential therapeutic target.

In this study, we found that the EcoHIV-infected mouse model of cART-controlled chronic HIV exhibited several psychosocial behavior deficits and sleep disturbances, and that these abnormal neurobehavioral phenotypes were improved with JHU083 treatment. Transcriptome analyses of isolated $CD11b^+/CD45^{Low}$ microglia identified multiple immune-related genes and pathways disrupted by EcoHIV infection, as well as the degree to which these were restored following JHU083 therapy.

2. Materials and methods

2.1. Mice

Male 8-12-week-old C57BL/6J mice were purchased from Jackson Laboratories and acclimated to the Johns Hopkins animal facility for 1–2 weeks before experimental use. Only males were used to avoid behavioral and sleep confounds present at different stages of the mouse oestrus cycle (Chari et al., 2020; Koehl et al., 2003). Unless explicitly noted otherwise, animals were maintained at 21 °C with 40–60% humidity under a 12-h light-dark (LD) cycle with water and chow access *ad libitum*. Mouse body weight was monitored weekly throughout the experiment. All animals were group-housed, split by EcoHIV-infection status, except for single housing during sleep-recording periods.

2.2. EcoHIV preparation and inoculation of mice

Mice were infected with EcoHIV as previously described (Nedelcovych et al., 2019; Gu et al., 2018; Kim et al., 2019; Potash et al., 2005). Briefly, mice received an intraperitoneal (i.p.) injection of 4×10^6 pg viral particles as measured by p24 ELISA (Advanced Biosciences Laboratory, Rockville, MD) or an equivalent volume of control saline solution. Infection of each cohort was confirmed by evaluating the presence of high levels of EcoHIV in protein in the spleen via p24 ELISA at 2 days post-inoculation.

2.3. Drug treatment

Dosing was initiated at 3 weeks post-inoculation, the time when EcoHIV infection becomes chronic (Gu et al., 2018), and continued throughout the course of all experiments until sacrifice. Mice were dosed i.p. with 1.83 mg/kg of JHU083 dissolved freshly in 50 mM HEPES buffered saline (Research Products International, Mt. Prospect, Illinois) on alternating days, as previously described (Rais et al., 2016), a dosing schedule that has been shown to be capable of inhibiting microglial glutaminase (Zhu et al., 2019; Nedelcovych et al., 2019). Injections were delivered at the same time of the day, specifically at 60 min before lights-on time in a dim red-lit room for the sleep behavior recordings.

2.4. Behavioral studies

Behavior tests for locomotion, social process, consummatory anhedonia, and sleep phenotypes were conducted at 4.5 weeks post-inoculation, after 10–12 days of drug administration (Fig. 1A, F).

2.4.1. Social interaction test (SIT)

SIT was performed to evaluate social avoidance, which belongs to social processes, a domain relevant to the psychopathology of depression (Carcone and Ruocco, 2017). As we previously described (Zhu et al., 2019; Sakamoto et al., 2021), mice were placed in a three-chamber apparatus (40 cm width × 20 cm height × 26 cm depth) containing a small, porous cage in the corner of each side chamber. Each trial consisted of 3 sessions: 1) The mouse was placed in the center chamber to habituate for 10 min, 2) the mouse was allowed to freely explore the empty side-chambers for an additional 10 min, and 3) a stranger mouse (stranger) of the same strain was placed in the plastic cage in one chamber, while a novel inanimate object (object) was placed in the opposing chamber's cage, and the test mouse was again allowed to explore all chambers freely for 10 min. The activity was recorded from a top-down video camera, and behaviors such as sniffing and interaction with the stranger/object, as well as residence time in each chamber were quantified via TopScan 3.0 (CleverSys, Reston, Virginia).

2.4.2. Sucrose preference test (SPT)

SPT was performed to examine consummatory anhedonia, which is in the positive valence system domain and observed in patients with depression. As described previously (Zhu et al., 2019), the mice were singly caged before the test. On day 1, their regular water bottles were replaced with two 50 ml tubes (bottle 'A' and bottle 'B') fitted with bottle stoppers containing two-balled sipper tubes. The positions of bottles A and B were switched daily to avoid a side bias, and the fluid consumed from each bottle was measured daily. During days 1 and 2, bottles A and B were filled with normal drinking water (W/W). During days 3 and 4, both bottles were filled with a solution of 1.5% sucrose dissolved in drinking water (S/S). On days 5–8, bottle A contained 1.5% sucrose, and bottle B contained drinking water (S/W). Sucrose preference on each day for each mouse was calculated as 100% x (Vol A/(Vol A + Vol B)) and averaged across the days for a given condition (W/W, S/S, or S/W).

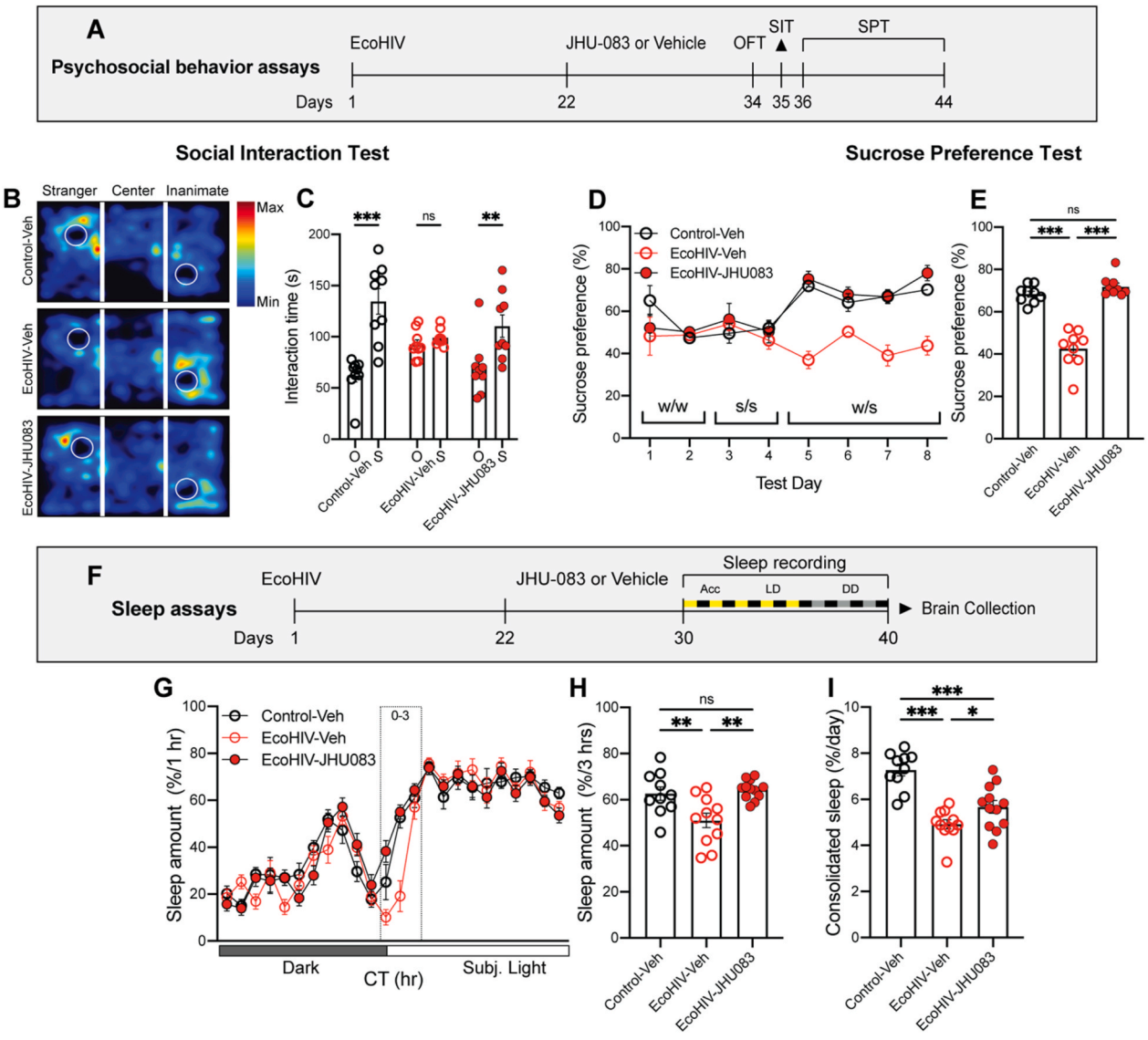

Fig. 1. JHU083 rescues depressive and sleep phenotypes in EcoHIV-infected mice. A. Experimental timeline for the psychosocial behavior assays. B. SIT representative heat maps of mouse location within each of the 3 chambers during 10 min of the assay. White circle depicts the location of the cage holding the stranger mouse or inanimate object, color scale represents time at each position. C. Quantification of cumulative time/mouse spent interacting with the stranger or object. D. Sucrose water consumed as a percentage of total water consumed on each SPT day. w/w indicates both bottles contain water (2 days), s/s indicates both bottles contain sucrose (2 days), w/s indicates one bottle sucrose, one bottle water, swapped cage sides each day (4 days). E. Intra-animal mean of sucrose preference across all w/s days. F. Experimental timeline for the sleep assays. G. Trace of sleep amount over 24 h in DD in 1 h bins. H. Sleep amount (%) in the first 3 h of the mouse sleep period (CT 0–3). I. Consolidated sleep (bouts >15 min) amount as % of total sleep in one 24-h day. For B-E, Control-Veh, black open circles, n = 9; EcoHIV-Veh, red open circles, n = 9; EcoHIV-JHU083, red filled circles, n = 9. For G-I, Control-Veh, black open circles, n = 10; EcoHIV-Veh, red open circles, n = 11; EcoHIV-JHU083, red filled circles, n = 12. All data represent mean ± SEM; 2-way ANOVA with multiple comparisons (Holm-Sidak) for C,D,G; 1-way ANOVA with multiple comparisons (Holm-Sidak) for E,H,I; $p > 0.05$,ns; $p < 0.05$,*; $p < 0.01$,**; $p < 0.005$,***. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.4.3. Open field test (OFT)

OFT was performed to examine overall activity levels and mouse health. Mice were introduced to a novel arena (40 × 40 cm), and allowed to freely explore for 30 min. A grid of 16 x 16 infrared lasers and detectors measured the locomotor activity of the mouse in 2 dimensions, and the broken beams, activity, locomotion, and distance were quantified via the Photobeam Activity System software for the PAS-Open Field (San Diego Instruments, San Diego, California).

2.4.4. Sleep recording

The PiezoSleep system (Signal Solutions, Lexington, KY) was used to record mouse sleep activity over 24 h and across several days. In brief, a piezoelectric pad was placed underneath a singly-housed mouse in a custom cage with a thin plastic bottom. As the mouse moved around the cage or slept, locomotor and breathing activity was translated into a singular waveform by the piezoelectric sensor and identified as an epoch of wakefulness or sleep within each 2 s sliding window by the closed-loop software, resulting in an automated sleep-wake score that has been previously shown to highly correlate to electroencephalography (EEG) measures (Mang et al., 2014). Each mouse was acclimated to the LD cycle in the recording facility for 14–20 days, and to each recording cage for 5 days. To prevent behavioral masking of light, sleep/wake was recorded for 3 days in LD, and 3 days in all dark (DD) conditions, with JHU083 dosing at ZT/CT 23 (1 h before lights on/putative lights on). All sleep/wake values were averaged across intra-animal days by time, and compared between groups for the analyses. Sleep amount was quantified

as the percentage of total time scored as sleep over the total time per bin, and consolidated sleep was scored by bouts of contiguous sleep lasting longer than 15 min before disruption by a bout of wakefulness longer than 10 s.

2.5. Microglia isolation and fluorescence-activated cell sorting (FACS)

The bilateral mouse hippocampus was removed using our established method (Zhu et al., 2019). Microglia ($CD45^{low}$ $CD11b^{+}$) were stained from dissociated brain tissue by a FACS Aria Flow Cytometer using our published method with minor modifications (Nedelcovych et al., 2019). Briefly, dissociated cells were resuspended in FACS staining buffer (1x PBS with 0.5% BSA) and incubated for 30 min on ice with the following antibodies: BV421 conjugated Rat anti-CD45 (1:100, Cat. No. 103133, BioLegend), APC conjugated Rat anti-CD11b (1:100, Cat. No. 101212, BioLegend). Cells were then washed once, resuspended in 300 μL FACS staining buffer, and filtered. The population of cells containing microglia could be readily identified based on the forward scattering (FSC) and side scattering (SSC) properties. Primary gating was performed on FSC/SSC to exclude debris, followed by FSC-A/FSC-H plot to gate singlets, and live cells were gated using 7-AAD. $CD45^{low}CD11b^{+}$ cells within this population were then gated and sorted. Cell suspensions from wild-type brain tissue were used as a negative control for establishing CD45 and CD11b negative gates using fluorescence minus one staining.

2.6. RNA sequencing

For RNA sequencing experiments, total RNA was isolated from cells using the Qiagen Allprep DNA/RNA kit (Cat. No./ID: 80284) according to the manufacturer's specifications. RNA samples were converted to double-stranded cDNA using the Ovation RNA-Seq System v2.0 kit (Tecan Cat. No./ID: 7102–32), which utilizes a proprietary strand displacement technology for linear amplification of mRNA without rRNA/tRNA depletion. Quality and quantity of the resulting cDNA were monitored using the Bioanalyzer High Sensitivity kit (Agilent Cat. No./ID: 5067–4626), which yielded a characteristic smear of cDNA molecules ranging in size from 500 to 2000 nucleotides in length. After shearing 500 ng of cDNA to an average size of 250 nucleotides with the Covaris S4 (Covaris Inc. Woburn, MA), library construction was completed with the Illumina Truseq Nano kit (Cat. No./ID: 20015964) according to the manufacturer's instructions. mRNA libraries were sequenced on an Illumina Novaseq 6000 instrument using 50 bp paired-end dual indexed reads and 1% of PhiX control. Reads were aligned to GRCh38 using rsem version 1.3.0 with the following options: star-calc-ci-star-output-genome-bam-forward-prob 0.5. Differential expression analysis and statistical testing was performed using DESeq2 software.

2.7. Expression and pathways analysis

Data cleaning on raw RNAseq expression values was performed for all genes across all samples using interquartile range to remove outliers and the baseline noise, and then the data were log10 transformed to increase the skewness. Greater than 50% missing values (gene expression abundance) of a gene in each sample category were removed, and missing values below 50% in each sample group were imputed by 1/5th of the minimum positive value within the group using the MetaboAnalyst web-based comprehensive data analysis tool (Pang et al., 2021). A total of 2463 genes were identified across the experimental groups, and statistically significantly differentially expressed genes were identified by applying either a p-value < 0.05 (unpaired parametric *t*-test) cut-off or a combination of p-value < 0.05 and fold change (FC) >2 or < 2 cut-offs between EcoHIV-infected and uninfected controls. Associated pathways for EcoHIV dysregulated genes (combination of p < 0.05 and FC > 2 or <2 cut-offs) were identified via Reactome Pathway Database (reactome.org) (Griss et al., 2020), with the contributing genes reported as a percentage of associated factors over total dysregulated genes. Restored pathways were identified using the same approach, where genes directionally restored towards uninfected control were collected from the comparison of EcoHIV-Veh and EcoHIV-JHU083 groups.

2.8. Statistical methods

Multiple group comparisons between uninfected Control-Veh, EcoHIV-infected-Veh, and EcoHIV-JHU083 mice were conducted via 1-way or 2-way ANOVA, as appropriate, with all multiple comparisons corrected via Holm-Sidak test. Student's t-tests were applied to determine significantly dysregulated genes in pairwise comparisons (Control-Veh vs. EcoHIV-Veh, and EcoHIV-Veh vs. EcoHIV-JHU083). All data are presented as mean +standard error of the mean (SEM), and all p value notations follow the same rubric: $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.005$, ***. Statistical analyses and figure production were performed using Microsoft Excel and GraphPad Prism 9.

3. Results

To investigate the role of EcoHIV infection on psychosocial behavior phenotypes and the therapeutic potential of GLS inhibition via JHU083, we conducted two commonly used rodent behavioral assays for evaluating social avoidance and consummatory anhedonia: the SIT and the SPT (Kaidanovich-Beilin et al., 2011; Liu et al., 2018). In the SIT, uninfected mice spent more than twice as long interacting with the 'stranger' mouse than with the inanimate object (stranger, 134.6 s; object, 62.3 s; $p < 0.001$), while EcoHIV-infected mice showed no preference (stranger, 92.6 s; object, 99.0 s) (Fig. 1B and C). In contrast, treatment of infected mice with JHU083 restored preference for social interactions (stranger, 110.5 s; object, 69.8 s; $p = 0.008$). To control for potential confounds stemming from infection- or treatment-related differences in locomotion, we performed a standard OFT with equivalent cohorts and observed no group differences in total distance traveled during testing (**Supp. Fig. 1A).**

For an orthogonal behavior measure of consummatory anhedonia, we performed the SPT to characterize the loss of consummatory pleasure, a component of anhedonia, by measuring taste reward (Scheggi et al., 2018) with an additional EcoHIV-infected cohort ± JHU083. Uninfected mice preferred the sucrose solution for (68.3 ± 9.4%) of their total liquid consumption on average across all testing days, while the EcoHIV-infected mice consumed only (42.5 ± 12.6%) from the sucrose bottle during an equivalent period. However, EcoHIV-infected mice treated with JHU083 exhibited restored sucrose preference, resulting in (72.0 ± 11.1%) consumption (Fig. 1D and E). For each behavioral assay, we included a group of uninfected control mice that had been identically treated with JHU083 to control for the effects of the drug itself, and observed no meaningful effects of treatment in the SPT or the SIT (Supp. Fig. 1B).

While sleep disturbances are a well-established comorbidity for PLH (Faraut et al., 2018; Wu et al., 2015a; Wibbeler et al., 2012), sleep-related phenotypes have not been characterized in any mouse models of HIV infection. Using a non-surgical piezoelectric sleep-sensing system, daily sleep/wake amount and architecture were quantified in EcoHIV infected mice and uninfected controls ± JHU083 following the same infection, dosing, and behavioral regimen as detailed above (Fig. 1F). Although each group maintained circadian rhythmicity and slept equivalent amounts when averaged across the entire day or night, a significant reduction in sleep amount was observed in EcoHIV-infected mice (51.0 ± 10.4%) during the first 3 h of the subjective day (the sleep onset period for nocturnal mice) compared to uninfected controls (62.6 ± 9.3%; $p = 0.006$) in both all-dark (DD) and 12 h light:12 h dark (LD) conditions. JHU083 treatment of EcoHIV-infected mice normalized sleep amount during this period (64.4 ± 4.1%) (Fig. 1G and H; Supp. Fig. 1C). To explore disruptions in sleep architecture, we evaluated the

daily fragmentation of the sleep cycle for each mouse. Over the course of 3 recording days, EcoHIV-infected mice spent less time in consolidated sleep bouts (>15 min) than did uninfected mice ($p < 0.001$). This loss of sleep consolidation in EcoHIV-infected mice was partially reversed by treatment with JHU083 (Fig. 1I). As with the depressive phenotypes, no significant effects of JHU083 dosing on sleep were observed in uninfected mice (Supp. Fig. 1B).

To begin to explore the molecular basis of the observed behavioral changes and how JHU083 ameliorates them in EcoHIV-infected animals, $CD45^{low}/CD11b^{+}$ microglia were isolated from murine hippocampi using FACS and subjected to gene transcription analysis by bulk RNA-sequencing (Fig. 2A). Interestingly, more viable microglia were recovered from hippocampi of EcoHIV-infected mice compared with controls, and this effect was normalized in infected mice treated with JHU083 (Fig. 2B). We found 83 genes that were significantly up- or down-regulated in the microglia of mice infected with EcoHIV (41 upregulated, 42 downregulated, $p < 0.05$) (Supp. Fig 2A and B). Of these, 63 exhibited greater than 2-fold change between infected mice and uninfected controls, with approximately 65% of dysregulated genes completely or partially normalized in the EcoHIV-infected mice treated with JHU083 (Fig. 2C, Supp. Fig. 2C). To better understand the role of these affected genes in EcoHIV infection pathophysiology, we conducted biological pathway analysis of dysregulated microglial genes using gene enrichment in the Reactome Pathway Database (Jassal et al., 2020). The pathways with the highest proportion of dysregulated genes included the immune system (both adaptive and innate), signal transduction, cytokine signaling, and MHC antigen presentation (Fig. 2D). In the same analysis, JHU083 treatment of EcoHIV infected mice normalized expression of >50% of dysregulated genes in 89% of pathways (Fig. 2E). These results link gene dysregulation in microglia associated with EcoHIV infection to the normalization of expression via GLS inhibition, and by extension, to the reversal of behavioral abnormalities observed in infected mice. The drug-associated changes in gene expression were largely related to lipid metabolism and vesicle-mediated transport, suggesting that GLS may mediate some of the immune responses in microglia through mechanisms that involve vesicle transport. Indeed, GLS has been shown to be a positive regulator of extracellular biogenesis (Wu et al., 2018; Gao et al., 2020; Wang et al., 2017).

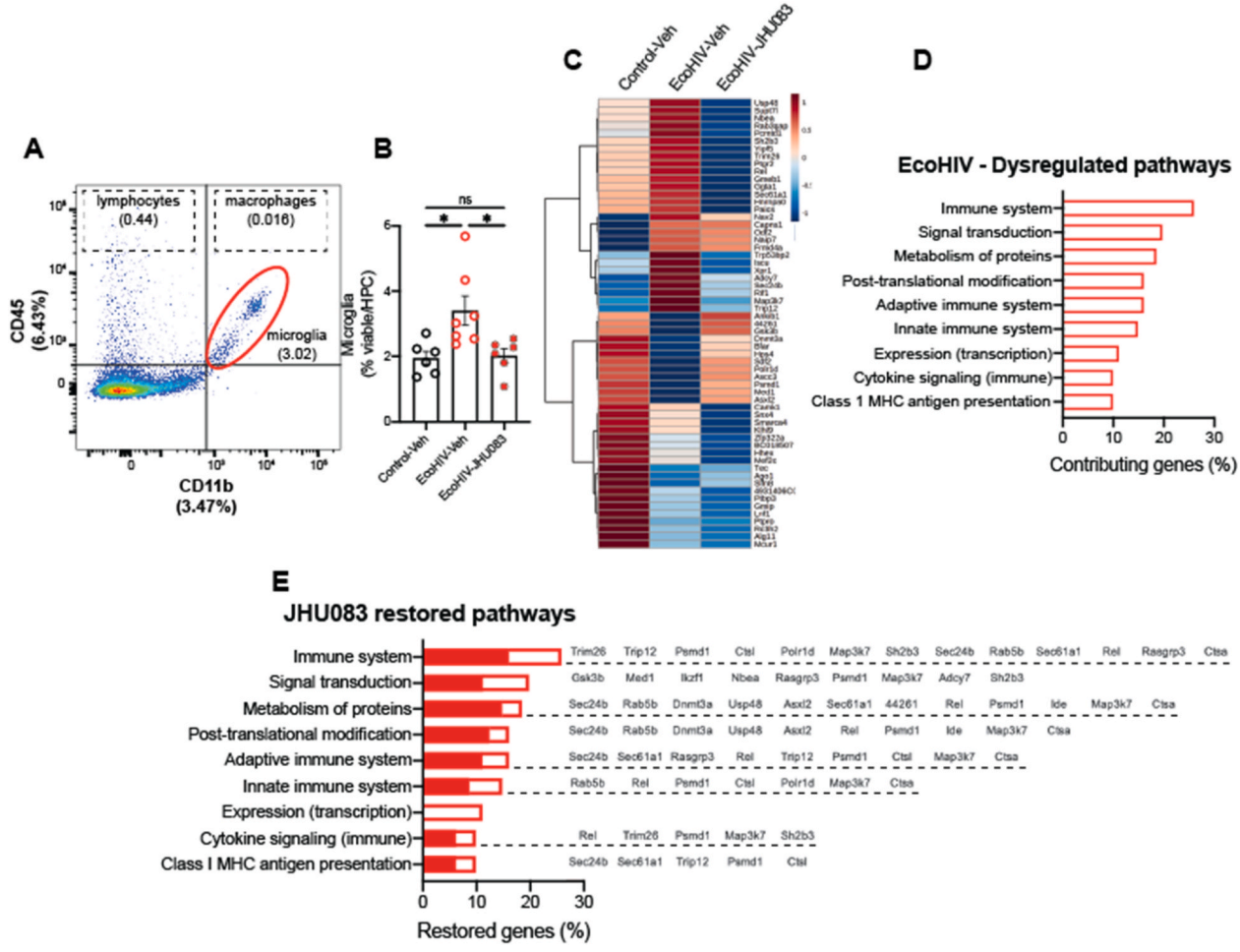

Fig. 2. EcoHIV-infection induces transcriptional dysregulation in isolated microglia. A. Representative FACS gating strategy for microglia isolation from mouse hippocampus. CD11b and CD45 are the microglial markers used for gating. The red oval depicts the live microglia. B. Viable microglia recovered from each mouse hippocampus following FACS, as a % of all viable recovered cells. C. Heatmap representing gene expression difference between Control-Veh and EcoHIV-Veh in 63 significantly altered genes with expression level fold-change >2, with additional column showing expression levels of the same genes in EcoHIV-JHU083. Color represents degree of up-regulation (warmer) and down-regulation (cooler) of each. D. Pathways enriched in dysregulated genes identified from Reactome Pathway Analysis Database. Bars reflect the number of dysregulated genes matched to each pathway as a percentage of the total dysregulated genes. E. Pathways restored by EcoHIV-JHU083 depicts the same dysregulated pathways as (D), with bars depicting the number of restored genes matched to each pathway as a percentage of the total restored genes. For B, Control-Veh, black open circles, n = 5; EcoHIV-Veh, red open circles, n = 7; EcoHIV-JHU083, red filled circles, n = 6. For C,D,E, sequencing performed on microglia isolated from n = 3/group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The results from these studies suggest a role for dysregulated glutamate metabolism in HIV-associated depression and sleep disturbances. Using the EcoHIV-infection model that simulates chronic HIV infection suppressed by ART, we identified behavioral manifestations of social avoidance and consummatory anhedonia, as well as disrupted and fragmented sleep patterns. To date neither of these phenotypes have been described in EcoHIV-infected mice. We then demonstrated that oral administration of the GLS inhibitor JHU083 effectively reduced these abnormal neurobehavioral phenotypes, suggesting that GLS inhibitors could be a useful adjuvant therapy for PLH with comorbid psychiatric conditions, and sleep disorders. These data support previous investigations which have highlighted the importance of upregulated GLS activity and overproduction of glutamate in driving brain immune dysfunction during HIV-infection (Zhao et al., 2012; Wu et al., 2015b; Huang et al., 2011).

The etiology of HIV-associated psychiatric comorbidities and impairments in sleep are active areas of research, as investigators attempt to disentangle the mechanisms of neurobehavioral impairments in treatment-suppressed PLH from behavioral and lifestyle confounds of this patient population (Galvan et al., 2002; Garin et al., 2017). Our findings support the idea that chronic HIV infection, even when effectively managed by cART, contributes to CNS dysfunction by promoting a persistent activation of microglia through mechanisms involving GLS and glutamate biosynthesis (Vazquez-Santiago et al., 2014; Serafini, 2015). This mechanistic understanding is integral to furthering development of effective therapeutics for the treatment of HIV-associated psychiatric conditions and sleep impairments that are overly represented in PLH (Faraut et al., 2018; Wu et al., 2015a; Wibbeler et al., 2012). Persistent microglial activation is not unique to HIV, and occurs in a broad range of CNS infectious diseases, psychiatric diseases, neurodegenerative diseases, and traumatic brain injuries (Kelschenbach et al., 2019; Lee et al., 2012; Schimmel et al., 2017). Perhaps JHU083, or a similar glutamate-modulating compound, may offer improvements for patients suffering from a wide variety of CNS perturbations which induce chronic dysregulation.

Animal models for emotion and mood disorders are inherently reductionist and interpretation to the analogous human conditions is limited. To mitigate this concern, we chose our behavioral measurements to explore both sociability and sucrose preference to encompass both the social processes and positive valence system domains as outlined in the National Institute of Mental Health Research Domain Criteria (RDoC) that are negatively impacted in PLH (Carcone and Ruocco, 2017; Nanni et al., 2015). In addition, we conducted a multi-day sleep and circadian analysis so as to include the arousal/regulatory system as an additional domain of investigation that is commonly impaired in PLH (Faraut et al., 2018; Wu et al., 2015a; Lee et al., 2012). To our knowledge, this study is the first to report sleep/-wake abnormalities in a mouse model of HIV-infection. Importantly, in all assessed domains of the present study, the infection-induced murine phenotypes we observed reflect phenotypes common in PLH (Faraut et al., 2018; Wu et al., 2015a; Lee et al., 2012). These results highlight the multi-factorial therapeutic potential for GLS inhibition in an established patient population with high unmet clinical need.

In addition to establishing the EcoHIV-infected mouse as a model for neurobehavioral dysregulations common in PLH, our investigation identified GLS, glutamate production, and immune activation as possible mechanisms contributing to psychiatric and sleep impairments in PLH. Despite only low levels of circulating virus after 3 weeks post-inoculation (Kelschenbach et al., 2019), flow cytometry and RNA sequencing revealed, respectively, an elevated number of microglia in the hippocampus and gene dysregulation in pathways involved in elements of immune response. Higher numbers of microglia in the hippocampi of infected mice compared to controls may reflect migration of these cells from other brain regions in response to the synaptodendritic injury in the hippocampus underlying memory impairment caused by EcoHIV in mice (Kelschenbach et al., 2019; Kim et al., 2019). At the same time, our transcriptional analysis of these cells indicates that at least some hippocampal microglia in infected mice could be deleterious rather than supportive to neurons. The observed normalization of the hippocampal microglia content with JHU083 treatment may suggest, in turn, normalization of the physiological balance between microglia and neurons necessary for normal brain function. Among the immune-related genes identified in our analyses to be significantly upregulated in EcoHIV-infected mice and either fully or partially normalized by JHU083 treatment are Trim26 and Trip12. Trim26 is a negative regulator of the Type 1 interferon pathway, activation of which induces antiviral effects at the transcription, translation and protein signaling levels (Gorska and Eugenin, 2020a; Ding et al., 2021a). It is therefore unsurprising that we observed upregulated Trim26 in EcoHIV-infected mice. However, previous studies have shown that treatment of EcoHIV-infected mice with the JHU083 parent drug DON does not decrease viral load (Nedelcovych et al., 2017b), so it is likely that any direct antiviral effects of JHU083 are muted by the fact that the drug also suppresses T cell activity (Hollinger et al., 2019; Baxter et al., 2017) and that neurobehavioral changes are through a secondary mechanism. Trip12 is a ubiquitin ligase, and its overexpression has been found in cancer and bacterial infections (Brunet et al., 2020). A target of Trip12, ADP ribosylation factors (ARF) are better characterized in cancer, but they have also been shown to regulate neuronal survival in the hippocampus (Kim et al., 2020), thereby suggesting a potential pro-cognitive pathway. Although studies linking Trip12 haploinsufficiency to intellectual disability (Brunet et al., 2020) are inverse to our findings, it is possible that any expression outside of a normal range could be pathogenic. Beyond the immune pathways, Dnmt3a, a DNA methyltransferase, was significantly reduced in EcoHIV-infected mice and normalized with JHU083 treatment. Multiple studies have reported associations between Dnmt3a and cognitive function in rodents and humans (Chouliaras et al., 2011, 2015; Wei et al., 2021). While the exact mechanism remains unknown, overall our results reflect the idea that even during cART-controlled infection, PLH still experience a persistent, low-level activation of the CNS immune system, and this continual activity contributes to behavioral dysregulation. As has been shown previously (Nedelcovych et al., 2019; Vazquez-Santiago et al., 2014; Ding et al., 2021b; Gorska and Eugenin, 2020b), elevated microglial GLS activity and glutamate overproduction drive neuronal excitotoxicity, likely through a broad swath of dysfunctional signaling. Previous experimentation has established that JHU083 attenuates these phenotypes by inhibiting GLS to normalize glutamatergic balance in the brain (Nedelcovych et al., 2019). Here, we show that JHU083 treatment restores the activity of several molecular pathways involved in immune response in microglia, suggesting their potential importance in the pathophysiology of disease. These findings highlight the potential for developing GLS inhibitors as therapeutics for HIV infection and other exogenous or endogenous etiologies which result in persistent activation of microglia and elevated GLS levels in the brain. In addition, microglia molecular signatures identified in this study may allow us to stratify HIV-associated depressive patients based on biotypes, contributing to the development of novel mechanism-based interventions that target specific patient populations.

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Declaration of competing interest

Authors B.S.S. and D.V. are inventors on patent applications filed by

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2022.100478.

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