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ORIGINAL ARTICLE

Effects of Morphine on Gp120-induced Neuroinflammation Under Immunocompetent Vs. Immunodeficient Conditions

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Abstract

HIV-associated neurocognitive disorder (HAND) is a common complication of HIV infection, whose development is known to be facilitated by inflammation and exacerbated by morphine. Previously, using the gp120 transgenic (tg) mouse model in combination with LP-BM5 (a murine retrovirus that can cause systemic immunodeficiency in susceptible mouse strains) we demonstrated differential gp120-associated central nervous system (CNS) neuroinflammatory responses under immunocompetent (-LP-BM5) vs. immunocompromised (+LP-BM5) conditions. Here, we further investigated the effects of morphine on gp120-associated neuroinflammatory response within the hippocampus under differential immune status. First, we confirmed that morphine treatment (2×25 mg pellets) did not significantly affect the development of immunodeficiency induced by LP-BM5 and all brain regions examined (hippocampus, striatum, and frontal lobe) had detectable LP-BM5 viral gag genes. Morphine notably reduced the performance of gp120tg+ mice in the alteration T-maze assay when 2-minute retention was used, regardless of LP-BM5 treatment. Morphine further enhanced GFAP expression in gp120tg+ mice regardless of host immune status, while promoted CD11b expression only in immunocompetent mice, regardless of gp120tg expression. In immunocompetent gp120tg+ mice, morphine increased the RNA expression of CCL2, CCL5, CXCL10, IL-12p40, and IFNβ; while under the immunodeficient condition, morphine downregulated the expression of CCL2, CCL5, CXCL10, IL-12p40, and IL-1β. Further, expression of TNFα and IFNγ were enhanced by morphine regardless of host immune status. Altogether, our results suggest that the effects of morphine are complex and dependent on the immune status of the host, and host immune status-specific, targeted anti-neuroinflammatory strategies are required for effective treatment of HAND.

Keywords HIV-associated neurocognitive disorder (HAND) · gp120 transgenic mice · Opioid · LP-BM5 · Immune status · Cytokine

Introduction

Approximately 37.9 million people around the world are living with Human Immunodeficiency Virus (HIV); which has claimed more than 33 million lives since the beginning of the epidemic (World Health Organization Myanmar 2019). Currently there is no cure and the only known treatment is antiretroviral therapy (World Health Organization Myanmar 2019). Combined antiretroviral therapy (cART) can decrease the concentration of plasma viral loads to undetectable and has been found to suppress HIV infection in the periphery, while HIV-1 viral proteins in the central nervous system (CNS) could still thrive and contribute to destruction of neuronal processes (Heaton et al. 2010; Kelly et al. 2014; Maartens et al. 2014). Over 30% of HIV cases can be attributed to intravenous (IV) drug use (Mimiaga et al. 2013) and addition of prescription opioids could lead to risky behaviors that would increase HIV transmission (Mateu-Gelabert et al. 2015; Peters et al. 2016; Young and Havens 2012). Thus the current opioid epidemic could further facilitate the HIV epidemic (Reviewed in detail in (Murphy et al. 2019)). Yet, opioids are often prescribed as treatment for persons suffering from HIV, and long-term opioid use has been shown to amplify the risk of death (Weisberg et al. 2015). So, it is critical to understand the association

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between opioids, such as morphine, and HIV infection, and their roles in contributing to HIV-associated pathologies.

It is estimated that approximately one third to one half of the currently infected population will develop HIV-1 associated neurocognitive disorders (HAND), which is found to be more prevalent in older individuals (McArthur 2004; Watkins and Treisman 2015). Persons suffering from HAND present with decreased cognitive function, disrupted memory and learning and reduced quality of life (Letendre et al. 2009). The severity of HAND symptoms varies depending on variables such as progression of infection, treatment, age and use of opioid drugs (Anthony et al. 2008; Hauser and Knapp 2014; Letendre et al. 2009; Weisberg et al. 2015). Importantly, morphine is capable of accelerating HIV infection into acquired immunodeficient syndrome (AIDS), which inflates the risk and severity of HAND (Bell et al. 1998; McArthur 2004; Nath et al. 2002; Robertson et al. 2007). Chronic morphine usage was seen to activate immune cells and increase viral loads in the hippocampus of patients infected with HIV (Bell et al. 1998; Reddy et al. 2012), potentially through its promotion of synaptodendritic damage and modulation of neuroinflammation (Murphy et al. 2019; Sil et al. 2021).

Previously, using a murine retrovirus (LP-BM5) to inflict a state of immunodeficiency in gp120 transgenic (gp120tg) mice, we demonstrated that differential neuroinflammatory micro-environments under immunocompetent vs. immunodeficient conditions could affect how gp120 acts in various brain regions particularly, in the hippocampus, and the consequent neuroinflammatory responses (Arabatzis et al. 2020). Opioids have been shown to potentiate gp120-induced cytokine release from astrocytes and exacerbate gp120 toxicity in neurons (Mahajan et al. 2005). Opioid withdrawal can disrupt homeostatic synaptic repair in gp120tg mice (Bandaru et al. 2011). Further, gp120 and morphine can modulate the expression of each other's receptors, opioid receptors and HIV-1 co-receptors respectively, indicating a complex synergism between opioid drugs and gp120 (Podhaizer et al. 2012; Turchan-Cholewo et al. 2008). Therefore, in this current study, we further examined morphine's effects on gp120-induced neuroinflammation within hippocampus, under differential immune status using the established model system. Spontaneous alteration T-maze test was used to determine animals learning and memory functions. Expression of selected cytokines/chemokines and synaptophysin were examined.

Materials and Methods

Animals and Sample Collection

Gp120tg mice were originally obtained from Dr. Marcus Kaul (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA) and bred at UNE's animal facility. All mice were housed at the University of New England (UNE) animal facility with 12:12-hr light:dark cycle and were provided food and water *ad libitum*. Prior studies have demonstrated that HAND-like pathology significantly increases with age in gp120tg+ mice (Arabatzis et al. 2020; D'Hooge et al. 1999; Maung et al. 2014; Toggas et al. 1994). Thus, in this current study, mice at 5-6 months of age at day 0 (LP-BM5 treatment, see below) were used. Gp120tg- littermate mice were used as controls. Both male and female mice were included in each group (males:females = 50:50). All mice were sacrificed for tissue and blood collection 8 weeks post-LP-BM5 injection following the spontaneous alternation T-maze test (during the 8th week). Upon sacrifice, mice are about 8-months old. Eight weeks post infection was chosen based on our previous study with C57BL/6 mice treated with LP-BM5 and morphine (Cao et al. 2012; McLane et al. 2014). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the UNE and followed the Animal Welfare Act and NIH Guide for the Care and Use of Laboratory Animals.

LP-BM5 Viral Infection and Pellet Implantation

LP-BM5 viral stock was originally obtained from Dr. William Green (Dartmouth College Geisel School of Medicine, Hanover, NH). The LP-BM5 stock was maintained in our laboratory and the viral load was titered by plaque assay as described previously (Cao et al. 2012). On day 0, gp120tg+ and gp120tg- littermates were intraperitoneally (i.p) injected with 5×10^4 plaque-forming unit (p.f.u.) of LP-BM5. This viral dosage can induce immunodeficiency and CNS infection in gp120tg mice (Arabatzis et al. 2020). The non-infected mice were not injected with the LP-BM5 virus. HIV-1 is known to be immunosuppressive and causes immunodeficiency in humans. Murine retrovirus, LP-BM5, is a murine model of acquired immunodeficiency (Li and Green 2006), which was used in this study to inflict a state of immunodeficiency in gp120tg mice. Similar to HIV-1, LP-BM5 retroviral infection induces severe immunodeficiency and is capable of infiltrating the CNS rapidly within 6-8 weeks of infection, inflicting a state of immunodeficiency in selected mice (Cao et al. 2012; Kustova et al. 1999; McLane et al. 2014; Morse et al. 1992; Sei et al. 1998).

Morphine treatment was performed as previously described. Briefly, on day 49 post-LP-BM5, a placebo or morphine (25 mg) pellet (NIDA, Bethesda, MD, USA) was subcutaneously implanted into all mice. Three days later, without removing the first pellet, a second pellet of the same type was implanted in all mice (McLane et al. 2014, 2018). This morphine pellet treatment regimen has been used in our previous published studies (McLane et al. 2014, 2018). We have previously conducted a pharmacokinetics study in

C57BL/6 mice comparing the effects of morphine delivered via slow release pellets, osmotic pump, and subcutaneous injections on morphine plasma concentrations, analgesic effects, and naloxone-precipitated withdrawal behaviors (McLane et al. 2017). Following a single 25 mg pellet, morphine's plasma concentration peaked at 24 h post implantation followed by a rapid decline, however maintained at a significant level for 3 days before returning to baseline on day 4 (McLane et al. 2017). With the addition of the second pellet on day 3, after another peak at day 4 (similar level as on day 1), morphine plasma concentration maintained at the similar level as that on day 3 until day 10 (unpublished data).

Development of Systemic Immunodeficiency

Indicators of LP-BM5 induced systemic immunodeficiency are splenomegaly and hyperimmunoglobulinemia (Li and Green 2006). At 8 weeks post-LP-BM5 injection, mice were sacrificed and blood and spleens were collected. Spleen to body weight ratios were calculated to measure the development of splenomegaly. Serum was collected from blood after clotting, and was stored at -20 °C until used for enzyme-linked immunosorbent assays (ELISAs) (Arabatzis et al. 2020; Li and Green 2006; McLane et al. 2014).

RNA Isolation and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from the hippocampus, striatum, and frontal lobe tissues (left side) and spleen using the RNeasy Lipid Tissue Kit (Qiagen, Germantown, MD, USA) and cDNA was synthesized using qScript cDNA supermix (Quanta, Gaithersburg, MD USA). qRT-PCR was performed as described previously (Arabatzis et al. 2020). Briefly, viral RNA (BM5Def and BM5Eco) were measured in spleens and various brain tissues and β-actin was used as the control (Cook et al. 2003). Since the results for BM5Def and BM5Eco expression were similar, only the expression of BM5Def, the pathological viral gag RNA, are presented here. Hippocampal cytokine/chemokine, CD11b (used as a marker for microglia), Glia fibrillary acidic protein (GFAP, used as a marker for astrocytes), gp120 RNA, and OPRM1 (opioid receptor Mu 1) expressions were measured using GAPDH as control. All primers (synthesized by Integrated DNA Technologies, Inc, IDT, Coralville, IA) and qRT-PCR cycles used are summarized in Table 1. Relative expressions for all markers were calculated using the ΔΔCt method.

Synaptophysin ELISA

Hippocampal samples from the right side of the brain of each mouse were collected and homogenized to determine levels of synaptophysin via an ELISA kit (AVIVA systems biology, San Diego, CA; catalog number OKCD02915) as previously described following the manufacturer's instructions (Arabatzis et al. 2020). Levels of synaptophysin were normalized based on the total protein concentration of each sample. A Bicinchoninic Acid assay (Pierce™ BCA assay; Thermo Fisher Scientific, Waltham, MA) was used to determine the total protein concentration. Data are presented as nanogram (ng) of synaptophysin per milligram (mg) of protein.

Spontaneous Alternation T-maze Assay

In order to evaluate animals' learning/memory functions, the spontaneous alternation T-maze behavioral test was used as adapted by Deacon and Rawlins (Deacon and Rawlins 2006). The T-maze assay is a behavioral test that takes advantage of the fact that mice enjoy exploring new environments and is used to assess primarily hippocampal learning and memory function (Deacon and Rawlins 2006). This behavioral assay was conducted during the 8th week post-LP-BM5 injection (days 53-55). T-maze apparatus and the testing details were described previously by our group (Arabatzis et al. 2020; McLane et al. 2018). Data presented include (1) the percentage of correct responses (in total of 9 trials) to represent each mouse's working memory and (2) the time-to-choice which indicated whether there were motivational or motor function complications for the animals.

Statistical Analysis

All graphs were generated using SigmaPlot 10.0 (Systat Software Inc. San Jose, CA). After completing initial analysis, no sex differences were detected, so the data from males and females were combined for analysis. Log-transformations were used for some data sets in order to obtain normal distribution before completing statistical analysis. Analysis of outliers was completed using the Grubbs Test through the GraphPad online resources, QuickCalcs (GraphPad Software Inc., La Jolla, CA). All data (except for viral RNA) were analyzed using Two-Way Analysis of Variance (ANOVA) with group (4 levels; classified based on animals' genotype and LP-BM5 treatment) and treatment (placebo or morphine) as between subject factors followed by the Tukey *post-hoc* test. Due to non-infected groups present with no detectable level of LP-BM5 viral RNA, data sets for viral loads were analyzed using two-way ANOVA with genotype (2 levels) and treatment (morphine or placebo) as between subject factors. As previously described, if a main effect was detected from at least one factor but no interaction was identified, when necessary analysis of one factor was conducted separately for each level of the other factor in order to analyze individual treatment group differences (Wei et al. 2012). As in our

Table 1 Primers and Run methods for PCR

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Run Method
BM5eco *gag*[a]	CCAATGTGTCCATGTCATTT	CTTTCTCTCTCTGCTCATCGC	• 95 °C for 8 min • 80 cycles of o 94 °C for 15 s o 63 °C for 45 s o 72 °C for 15 s
BM5def[b]	CCTTTTCCTTTATCGACACT	ACCAGGGGGGGAATACCTCG	
β-actin[a]	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	
IFN-α[c]	ATGGCTAGGCTCTGTGCTTTTCCTC	AGGGCTCTCCAGACTTCTGCTCTG	• 95 °C for 3 min • 40 cycles of o 95 °C for 30 s o 65 °C for 30 s o 72 °C for 30 s
IFN-β[c]	TTGCCATCCAAGAGATGCTCCAGA	TCAGAAACACTGTCTGCTGGTGGA	
gp120[f]	TGAGCCAATTCCCATACATTATTG	CCTGTTCCATTGAAC GTCTTATTATTAC	• 95 °C for 10 min • 40 cycles of o 95 °C for 30 s o 59 °C for 1 min o 72 °C for 1 min • Denaturation step: o 95 °C for 1 min o 59 °C for 30 s o 95 °C for 30 s
LCN2[f]	CCAGTTCGCCATGGTATTTT	GGTGGGGACAGAGAAGATGA	
IL-1β[g]	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA	• 94 °C for 30 s • 30 cycles of o 55 °C for 30 s o 72 °C for 1 min
iNos[d]	TGGTGGTGACAAGCACATTTG	CATTGGAAGTGAAGCGTTTCG	• 95 °C for 15 s • 40 cycles of o 95 °C for 15 s o 60 °C for 1 min
CCL2/ MCP-1[d]	GTATGTCTGGACCCATTCCTTC	GCTGTAGTTTTTGTCACCAAGC	
CCL3/ MIP-1α[d]	CAGCCAGGTGTCATTTTCCT	AGGCATTCAGTTCCAGGTCA	
CCL5/ RANTES[d]	AGCTGCCCTCACCATCATC	CTCTGGGTTGGCACACACTT	
IL-6[d]	CAGAGGATACCACTACCAACAG	TCTCATTTCCACCACGATTTCCC	
Il-12 p40[d]	CCATTGAACTGGCGTTGGAAG	CGGGTCTGGTTTGATGATGTCC	
TNF- α[d]	TGAACTTCGGGGTGATCGGTC	AGCCTTGTCCCTTGAAGAGAAC	
IFN-ϒ[d]	GCCATCAGCAACAACATAAGC	CAGCAGCGACTCCTTTTCC	
CXCL10[d]	TTTCTGCCTCATCCTGCTG	CTCATCATTCTTTTTCATCGTG	
GAPDH[d]	ACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	
Arg-1[e]	TTTCTCAAAAGGACAGCCTC	GTGAGCATCCACCCAAATG	• 95 °C for 30 s • 40 cycles of o 95 °C for 3 s o 60 °C for 30 s with fast ramp speed
CCL4[e]	GTCTCATAGTAATCCATCACAAAGC	CTCTCTCTCCTCTTGCTCGT	
CD11b[e]	TGTCCAGATTGAAGCCATGA	CCACAGTTCACACTTCTTTCAG	
GFAP[e]	AACCGCATCACCATTCCTG	GCATCTCCACAGTCTTTACCA	
OPRM1[e]	CAACATGAGTCGGAGAAGGAT	CGGCTAATACAGTGGATCGAA	
GAPDH[e]	GTGGAGTCATACTGGAACATGTAG	AATGGTGAAGGTCGCTGTG	

[Citation: a=(Cook et al. 2003), b=(Giese et al. 1994), c=(Kundu et al. 2013), d=(Christophi et al. 2009), e=PrimeTime® Primers from IDT (Coralville, IA, CA), f=(Kaul lab, personal communication), g=(Zhao et al. 2009)]

previous study with gp120tg mice and LP-BM5 treatment (Arabatzis et al. 2020), all data are presented as interaction graphs for easy viewing of any interactions between factors (as interaction graphs are the most appropriate for visualizing interactions between main factors, i.e. the less parallel the lines are, the more likely there is to be a significant interaction; while bar graphs are not efficient for this purpose). Main effects from ANOVA analysis are marked in each graph, while results of *post-hoc* analyses are reported in the text to reduce visual confusion (except for Fig. 10 OPRM1 in which individual group differences are noted). All data are presented as mean ± SEM, with $p \leq 0.05$ considered to be statistically significant.

Results

Development of Immunodeficiency

Previously, we have demonstrated that both gp120tg mice and morphine-treated C57BL/6 wild type mice (LP-BM5-susceptable strain) developed LP-BM5-induced

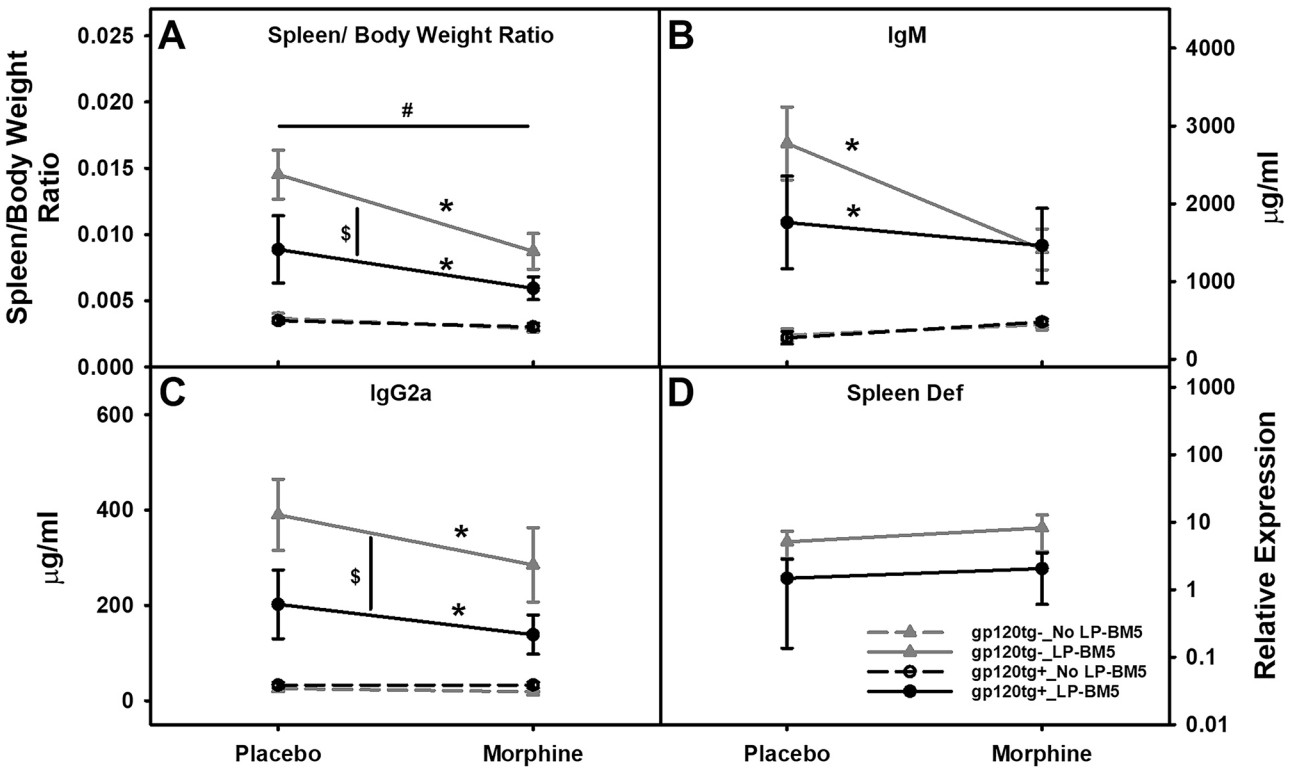

Fig. 1 Development of Immunodeficiency. Spleen/Body weight ratio (**A**), serum IgM (**B**), serum IgG2a (**C**), and spleen BM5Def expression (**D**) are illustrated in interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc test were performed. # indicates main effects of morphine ($p<0.05$), * indicates main group effects: $p<0.05$ between the indicated group (gp120tg-_LP-BM5 or gp120+_LP-BM5) and both "no LP-BM5" groups (gp120tg-_no LP-BM5 and gp120+_no LP-BM5), and $ indicates main group effects: $p<0.05$ between the indicated groups

immunodeficiency (Arabatzis et al. 2020; McLane et al. 2014). Here we first tested whether gp120tg mice treated with morphine and LP-BM5 would also develop immunodeficiency. Evidence of LP-BM5-induced systemic immunodeficiency include splenomegaly and hyperimmunoglobulinemia, along with the detection of viral gag RNAs in the spleen (Li and Green 2006). First, main group (based on gp120tg expression and LP-BM5 injection status) effects were seen for spleen/body weight ratio, and serum levels of IgM and IgG2a (Two-way ANOVA (log-transformed data): Fig. 1A, spleen/body weight ratio, $p_{group}<0.001$, $p_{mor}=0.008$, $p_{groupxmor}=0.453$; Fig. 1B, serum IgM, $p_{group}<0.001$, $p_{mor}=0.445$, $p_{groupxmor}=0.157$; Fig. 1C, serum IgG2a, $p_{group}<0.001$, $p_{mor}=0.194$, $p_{groupxmor}=0.857$). Specifically, regardless of morphine treatment, LP-BM5 induced significant increase in spleen/body weight ratio, and serum levels of IgM and IgG2a in both gp120tg+ and gp120tg- mice ($p<0.05$ for all with Tukey *post-hoc* test). Interestingly

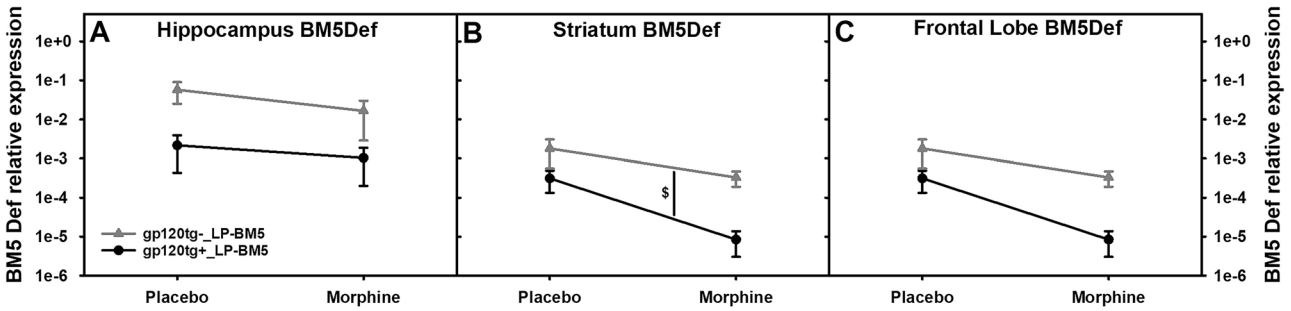

Fig. 2 Brain viral load in the CNS of gp120tg mice. Relative expressions of BM5Def viral loads in the hippocampus (**A**), striatum (**B**), and frontal lobe (**C**) are presented as interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. $ indicates main group effects: $p<0.05$ between the indicated groups

with this cohort of study, presence of gp120tg (gp120tg+ vs. gp120tg-) also significantly reduced respective measures for spleen/body weight ratio (Fig. 1A) and serum IgG2a (Fig. 1C) ($p<0.05$ between LP-BM5-treated gp120tg+ vs. gp120tg-groups with Tukey *post-hoc* test). Although a statistically significant main effect of morphine was only detected with spleen/body weight ratio, morphine treatment, overall, appeared to reduce LP-BM5-induced increases in all of the above measures. Consistent with the development of the immunodeficiency, all groups regardless of gp120tg expression or morphine treatment, expressed comparable levels of BM5Def viral RNA in the spleen (Two-way ANOVA: Fig. 1D, spleen BM5Def, p_{group}=0.646, p_{mor}=0.679, $p_{groupxmor}$=0.843). Altogether, these results support that immunodeficiency can be established by LP-BM5 and maintained in both gp120tg+ and gp120tg- mice following morphine treatment.

Brain Viral Load in the CNS of gp120tg Mice

We have shown that LP-BM5 can be detected in various brain regions of gp120tg mice (Arabatzis et al. 2020). We further examined the effects of morphine on LP-BM5 levels within the brain regions that are known to be affected by HAND: hippocampus, frontal lobe and striatum. Overall, consistent with peripheral responses, morphine-treated groups showed lower levels of LP-BM5 BM5Def RNA, but only in the striatum morphine statistically significantly reduced BM5Def in gp120tg+ mice (Two-way ANOVA (log-transformed data): Fig. 2A, hippocampus BM5Def, p_{group} =0.394 p_{mor}=0.252, $p_{groupxmor}$=0.494; Fig. 2B, striatum, BM5Def, p_{group}=0.004 p_{mor}=0.626, $p_{groupxmor}$=0.513, p<0.05 between gp120tg+_with LP-BM5 vs. gp120tg-_with LP-BM5 groups with Tukey *post-hoc* test; Fig. 2C, front lobe, BM5Def, p_{group} =0.140 p_{mor}=0.979, $p_{groupxmor}$=0.953). These results confirmed the existence of LP-BM5 virus within the CNS under morphine treatment. As our previous study indicated more substantial changes in gp120-related neuroinflammatory responses were observed in the hippocampus when comparing immunocompetent vs. immunodeficient status, our subsequent investigation in this study focused primarily on hippocampus.

Gp120 expression in the hippocampus of gp120tg mice

We also verified the expression of gp120 in the hippocampus of gp120tg mice via qRT-PCR as we have shown previously (Arabatzis et al. 2020). As expected, regardless of LP-BM5 or morphine treatment, gp120tg+ mice expressed significantly higher levels of gp120 than gp120tg- mice (Two-way ANOVA (log-transformed data): Fig. 3, hippocampus gp120 RNA, p_{group} <0.001, p_{mor}=0.205, $p_{groupxmor}$=0.127; $p<0.05$ between either one of the gp120tg+ groups and both gp120tg- groups with Tukey *post-hoc* test), and neither LP-BM5 nor morphine significantly affected hippocampal gp120 expression.

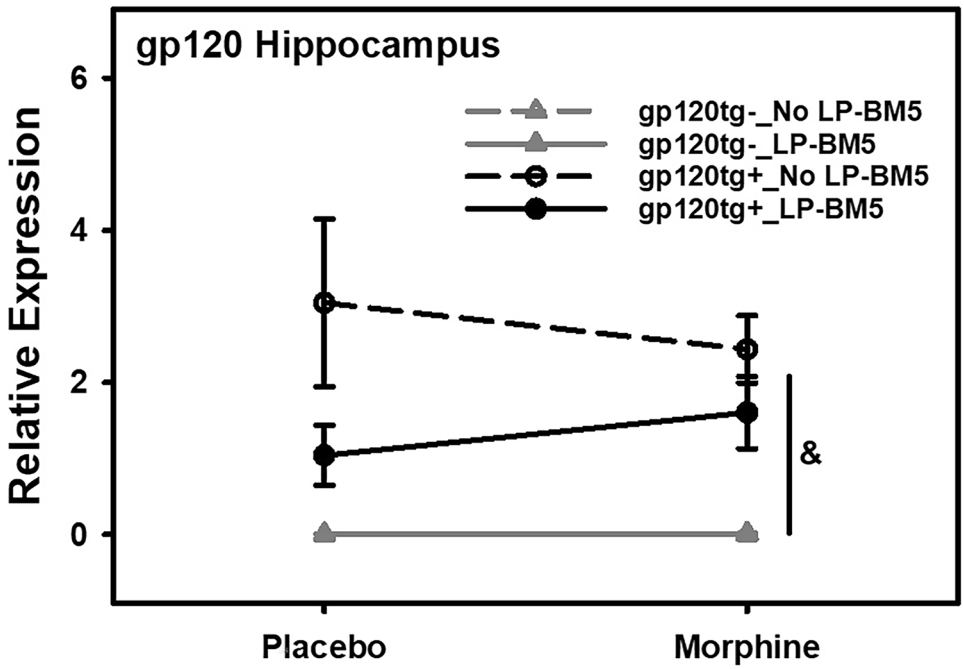

Fig. 3 Gp120 expression in the hippocampus of gp120tg mice. RNA expression of gp120 in the hippocampus is shown as an interaction plot. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. & represents a p<0.05 between either one of the gp120tg+ groups (gp120+_no LP-BM5 or gp120+_LP-BM5) and both gp120tg- groups (gp120-_no LP-BM5 and gp120-_LP-BM5)

Evaluation of Cognitive Functioning Through Alternating T-maze Behavioral Assay

Next, we evaluated hippocampal related cognitive function using the alternation T-maze behavioral assay, which we have used in the past when studying the effects of LP-BM5, gp120 and morphine (Arabatzis et al. 2020; McLane et al. 2018) and is known to be primarily determined by the hippocampal learning functions (Deacon and Rawlins 2006; Lalonde 2002). When a 0-min interval was used, there were no group differences in either percent-correct or time-to-choice (Two-way ANOVA: Fig. 4A, 0 min percent-correct, p_{group}=0.515 p_{mor}=0.207, $p_{groupxmor}$=0.636; Fig. 4C, 0 min time-to-choice, log-transformed data, p_{group}=0.541 p_{mor}=0.426, $p_{groupxmor}$=0.961). When 2-min interval was used to increase the burden on hippocampus (Deacon and Rawlins 2006; Lee and Kesner 2003), although there was a main effect of morphine on time-to-choice, further Tukey *post-hoc* test did not reveal any group differences (Two-way ANOVA: Fig. 4D 2 min time-to-choice, p_{group}=0.105, p_{mor}=0.033, $p_{groupxmor}$=0.775), suggesting that morphine did not significantly modify animals motivation and/or mobility to make a choice. A main effect of morphine on 2-min percent-correct was also detected and *post-hoc* analysis identified the significant differences between morphine- vs. placebo- treated gp120tg+_LP-BM5 groups despite that both gp120tg-_LP-BM5 and gp120tg+_no LP-BM5 groups appeared to be affected by morphine in the similar way (Two-way ANOVA: Fig. 4B 2 min percent-correct, p_{group}=0.128, p_{mor}=0.010, $p_{groupxmor}$=0.652, and Tukey test: p<0.05 between morphine vs. placebo treated gp120tg+_LP-BM5 groups). Therefore, presence of gp120tg and being under immunocompromised condition rendered mice to be more susceptible to morphine's deteriorating effects on hippocampal learning and memory.

Expression of Synaptophysin (SYP) in the Hippocampus of gp120tg Mice

Abnormal synaptic connectivity in the brain is one of the hallmarks of HAND (McArthur 2004; McArthur et al. 2010; Sil et al. 2021). The hippocampus of gp120tg mice has been shown to express reduced levels of presynaptic terminal protein, SYP (Arabatzis et al. 2020; Maung et al.

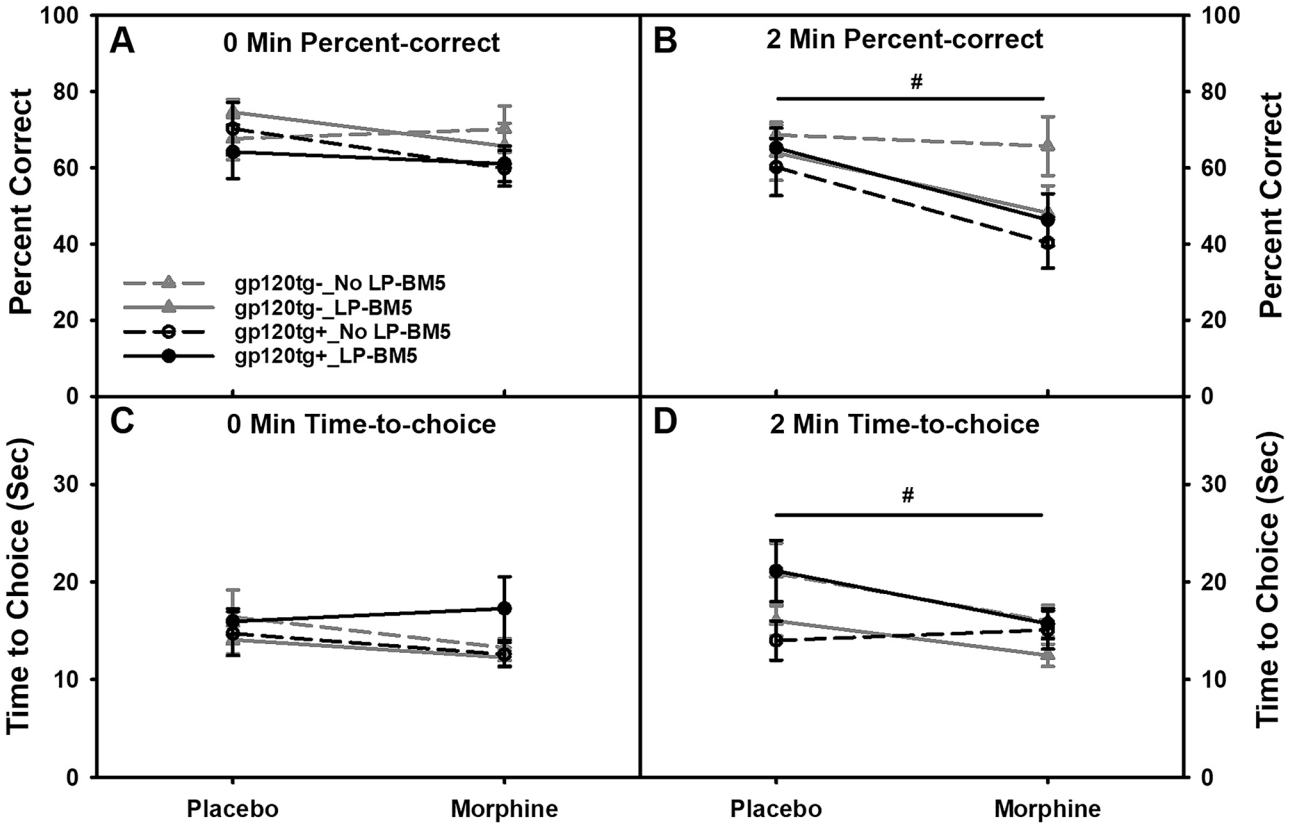

Fig. 4 Evaluation of cognitive functioning through alternation T-maze behavioral assay T maze assay measures: 0-minute average percent-correct (**A**) and 2-minute retention average percent-correct (**B**) along with the corresponding time-to-choice for both 0-minute (**C**) and 2-minute (**D**) intervals are shown in interaction plots. Two-way ANOVA followed by Tukey post-hoc analysis was performed. Data are presented as mean ± SEM; n=6-17 per group. # indicates main morphine effects (p<0.05)

2014). To assess whether the observed reduced hippocampal learning/memory function is associated with a reduction of SYP expression, we measured the levels of SYP via ELISA. Morphine main effects on SYP expression were detected. However, not positively correlating with the morphine's effects on 2 min percent-correct in Fig. 4B, morphine slightly increased SYP expression at the protein level with the statistical significance observed only in the gp120tg+_no LP-BM5 group (Two-way ANOVA: Fig. 5, hippocampus SYP (log-transformed data), p_{group}=0.411, p_{mor}=0.007, $p_{groupxmor}$=0.706, and Tukey test: p<0.05 between morphine and placebo treatments for gp120tg+_no LP-BM5 mice).

RNA Expression of CD11b and GFAP in the CNS of gp120tg Mice

Glial contribution to HAND pathophysiology has been well established (Sil et al. 2021). To investigate the involvement of neuroinflammatory responses in observed hippocampal learning/memory function deficit, we first examined the responses of microglia/infiltrating macrophage population and astrocytes within the hippocampus by examining RNA expression of CD11b (microglia/infiltrating macrophage) and GFAP (astrocytes). As expected from our previous data (Arabatzis et al. 2020), the level of GFAP expression but not CD11b expression is significantly associated with higher gp120tg expression (Two-way ANOVA (log-transformed data): Fig. 6A, hippocampus CD11b, p_{group}=0.051, p_{mor}=0.008, $p_{groupxmor}$=0.139; Fig. 6B, hippocampus GFAP, p_{group}<0.001, p_{mor}=0.004, $p_{groupxmor}$=0.366, and p<0.05 between either one of the gp120tg+ vs. both gp120tg- groups with Tukey *post-hoc* test). For both CD11b and GFAP expression, main effects of morphine were detected. *Post-hoc* analysis indicated further induction of GFAP expression with morphine treatment in gp120tg+ groups regardless of LP-BM5 treatment, yet small morphine-induced increases in CD11b expression in non-LP-BM5 treated groups regardless of gp120tg status (Tukey test: Fig. 6A, CD11b, p<0.05 between morphine and placebo treated non-LP-BM5 groups, regardless of gp120tg expression; Fig. 6B, GFAP, p<0.05 between morphine and placebo treatments for gp120+ groups, regardless of LP-BM5 treatment). Thus, together, both gp120 expression and morphine treatment can significantly enhance astrocytic responses.

RNA Expression of Pro-inflammatory Mediators in the Hippocampus of gp120tg Mice

In our previous study examining the gp120-induced inflammatory responses under immunocompetent vs. immunodeficient conditions, we observed various expression patterns of different cytokines/chemokines in the hippocampus (Arabatzis et al. 2020). Here, we further examined effects of morphine on various inflammatory mediators. As expected, LP-BM5 alone induced CCL5 in both gp120tg+ and gp120tg- mice. Similar to our previous study with B6 mice, the expression levels of CCL5 in both gp120tg+_LP-BM5 and gp120tg-_LP-BM5 mice trended decreasing upon morphine treatment while gp120tg+_no LP-BM5 group showed slight but significant increase in CCL5 expression with morphine treatment (Two-way ANOVA (log-transformed data): Fig. 7A, CCL5, p_{group}<0.001, p_{mor}=0.430, $p_{groupxmor}$=0.004;

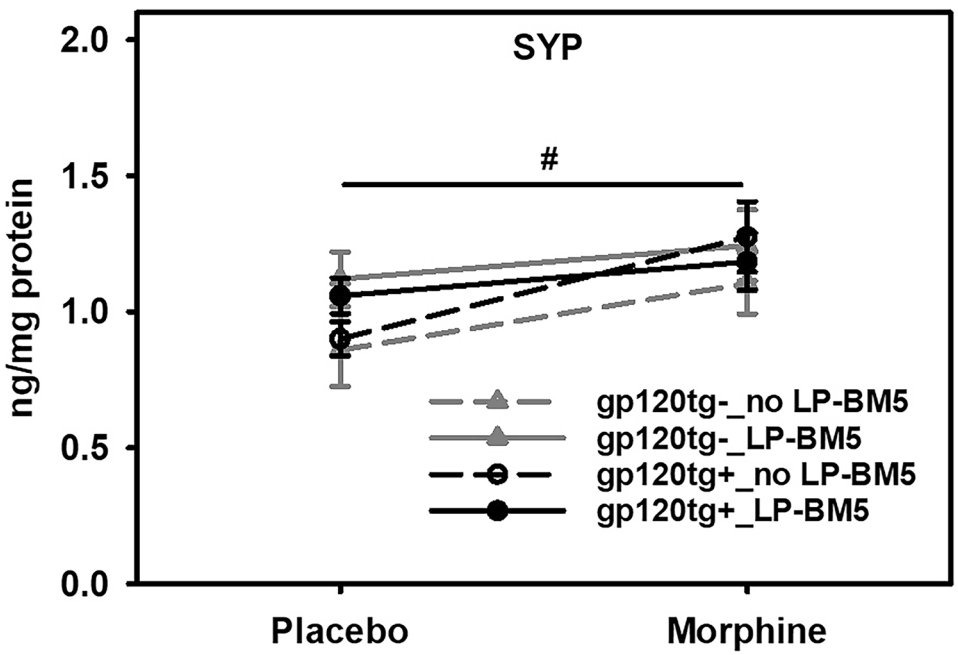

Fig. 5 Expression of Synaptophysin in the hippocampus of gp120tg mice. Expression of hippocampal synaptophysin at protein level is shown in an interaction plot. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. # indicates main morphine effects (p<0.05)

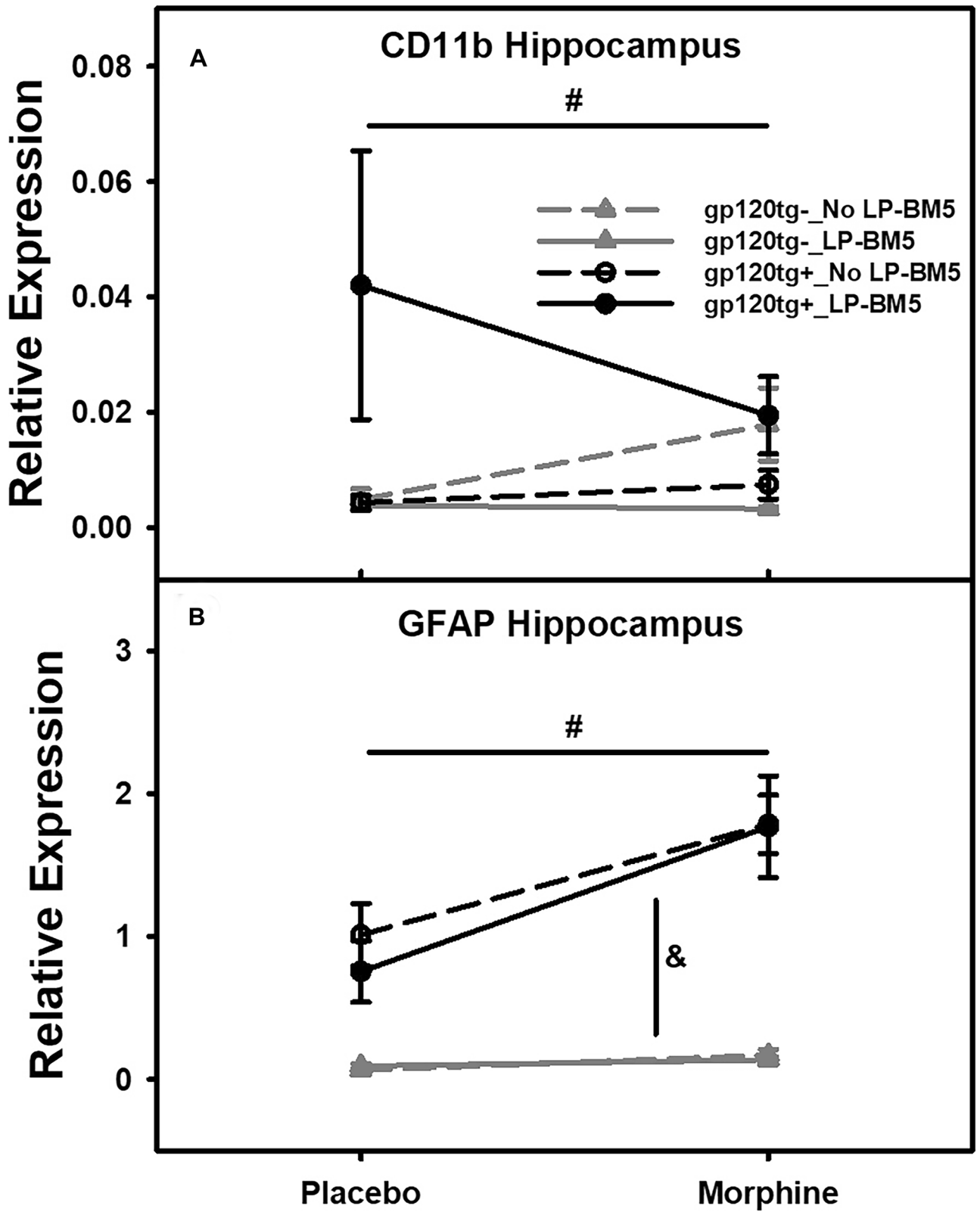

Fig. 6 RNA expression of CD11b and GFAP in the CNS of gp120tg mice. RNA expression of CD11b (**A**) and GFAP (**B**) in the hippocampus are shown in interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. # indicates morphine main effects ($p<0.05$) and & indicates $p<0.05$ between either one of the gp120tg+ groups (gp120+_no LPBM5 or gp120+_LP-BM5) and both gp120tg- groups (gp120-_no LP-BM5 and gp120-_LP-BM5)

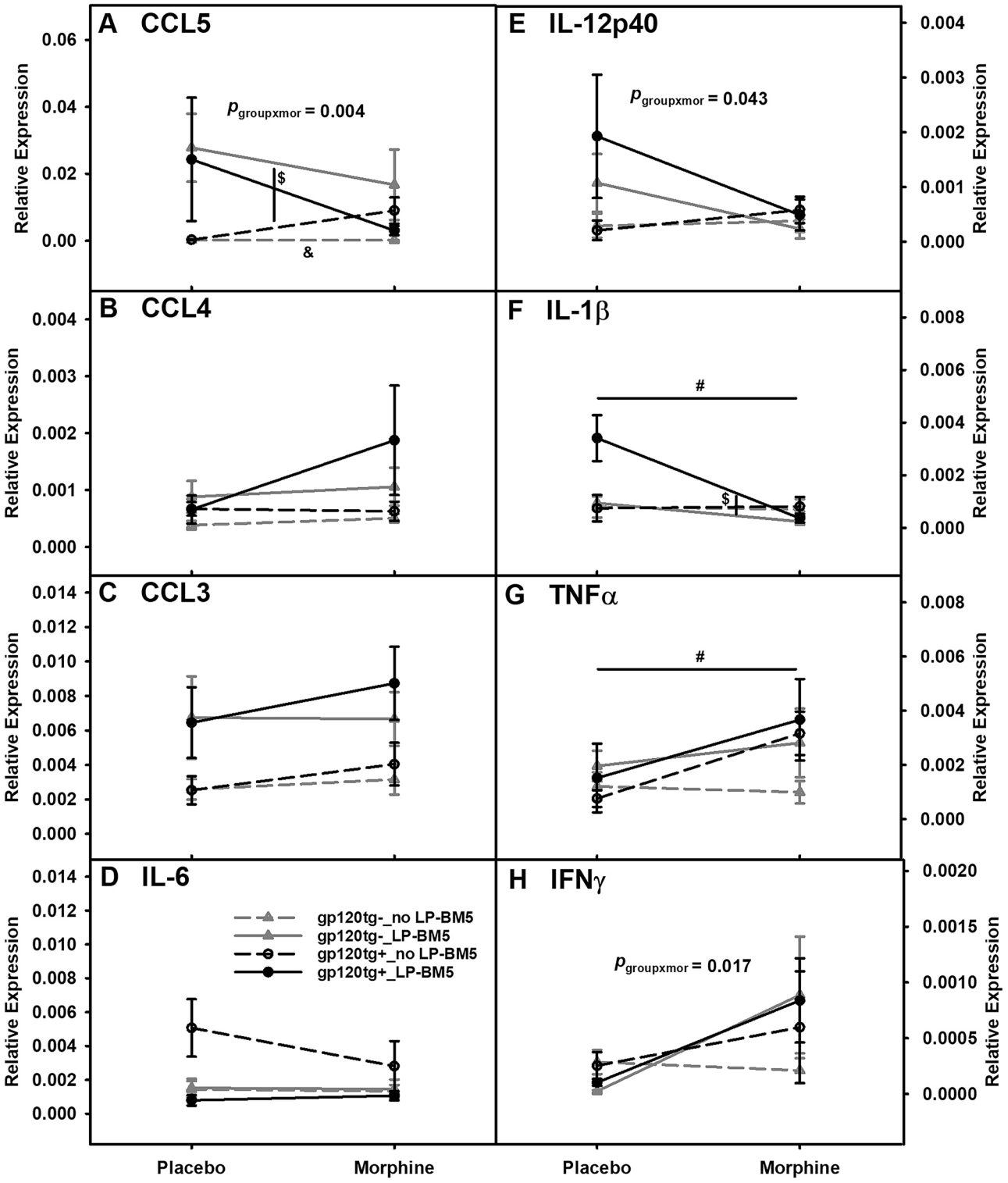

Fig. 7 RNA expression of pro-inflammatory mediators in the hippocampus of gp120tg mice. RNA expressions of CCL5 (**A**), CCL4 (**B**), CCL3 (**C**), IL-6 (**D**), IL12-p40 (**E**), IL-1β (**F**), TNFα (**G**), and IFNγ (**H**) are presented as interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. $ indicates main group effects: $p<0.05$ between the indicated groups, & indicates main group effects: $p<0.05$ between "gp120tg-_no LP-BM5" group vs. all other groups, and # indicates morphine main effects ($p<0.05$). p value for significant interactions between morphine and group are marked within respective graphs

Tukey test: within placebo groups, $p<0.05$ between all LP-BM5 treated groups vs. LP-BM5 non-treated groups, and $p<0.05$ between morphine and placebo treatments for gp120tg+_no LP-BM5 group). Similar trends in morphine effects were observed in IL-12p40 expression (also did not reach statistical significance for all comparisons) (Two-way ANOVA (log-transformed data): Fig. 7E, IL-12p40, $p_{group}=0.622$, $p_{mor}=0.946$, $p_{groupxmor}=0.043$, and Tukey test: $p<0.05$ between morphine and placebo treatments for gp120tg-_LP-BM5 group). Morphine also significantly reduced IL-1β expression in gp120tg+_LP-BM5 group (Two-way ANOVA (log-transformed data): Fig. 7F, IL-1β, $p_{group}=0.011$, $p_{mor}=0.038$, $p_{groupxmor}=0.108$, and Tukey test: $p<0.05$ between morphine and placebo treatments for gp120tg+_LP-BM5 group). For some other cytokines/chemokines we examined, although not all reached statistical significance, morphine appears to increase their expression, particularly for gp120tg+ and/or LP-BM5 treated groups (Two-way ANOVA (log-transformed data): Fig. 7G, TNFα, $p_{group}=0.600$, $p_{mor}=0.023$, $p_{groupxmor}=0.491$ with Tukey test showing $p<0.05$ between morphine and placebo treatments for gp120tg+_no LP-BM5 group, and Fig. 7H, IFNγ, $p_{group}=0.214$, $p_{mor}=0.324$, $p_{groupxmor}=0.017$ with Tukey test showing $p<0.05$ between morphine and placebo treatments for gp120tg-_LP-BM5 group). No significant changes were observed for CCL4, CCL3 or IL-6 regardless of treatment (Two-way ANOVA (log-transformed data): Fig. 7B, CCL4, $p_{group}=0.508$, $p_{mor}=0.787$, $p_{groupxmor}=0.500$, Fig. 7C, CCL3, $p_{group}=0.092$, $p_{mor}=0.143$, $p_{groupxmor}=0.786$, Fig. 7D (log-transformed data), IL-6, $p_{group}=0.666$, $p_{mor}=0.210$,

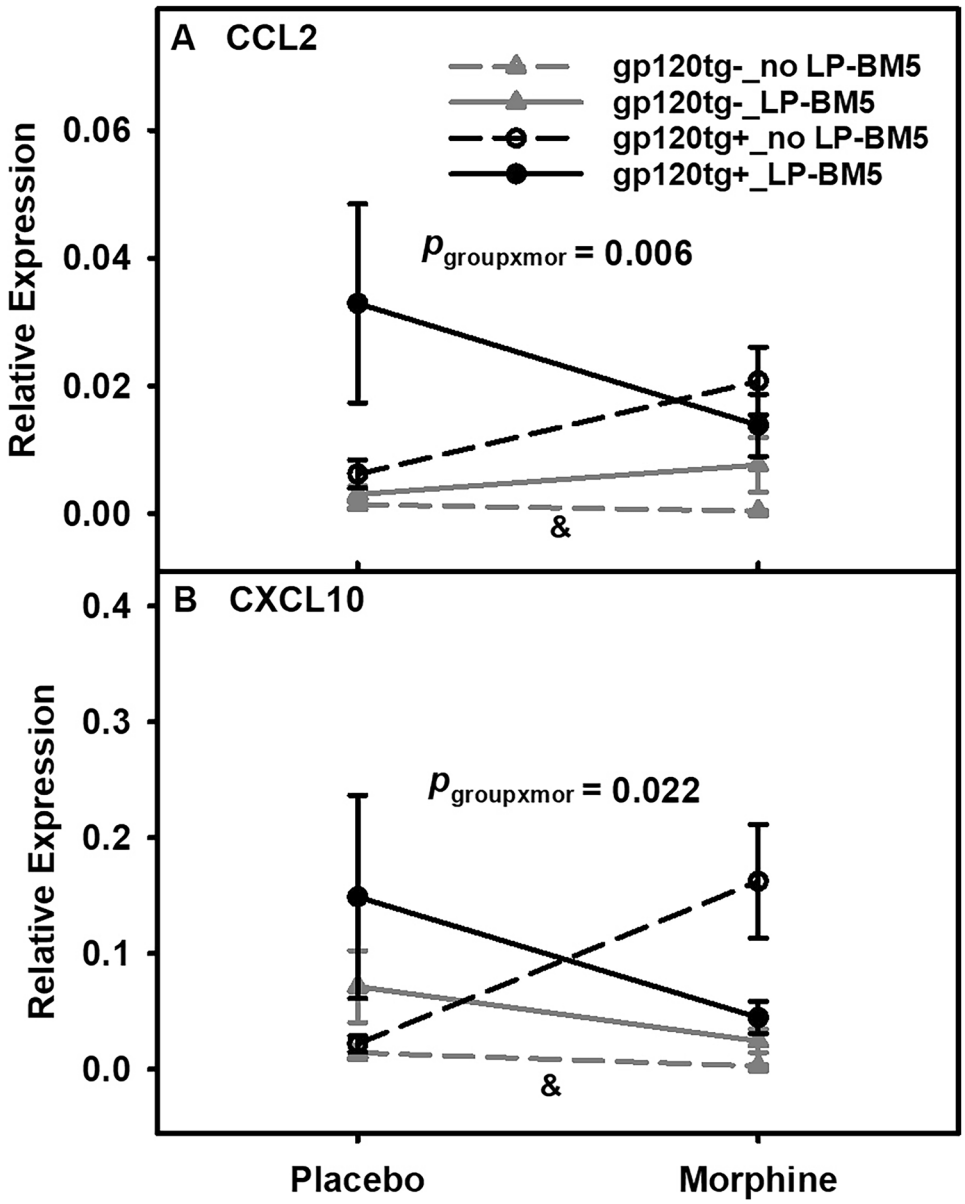

Fig. 8 RNA expression of CCL2 and CXCL10 in the CNS of gp120tg mice. RNA expression of CCL2 (**A**) and CXCL10 (**B**) in the hippocampus that are presented as interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. & indicates main group effects: $p<0.05$ between "gp120tg-_no LP-BM5" group vs. all other groups. p value for significant interactions between morphine and group are marked within respective graphs

$p_{groupxmor}$=0.165). In addition, no remarkable changes in hippocampal iNOS and Arg-1 expression were observed (data not shown).

As CCL2 and CXCL10 have been suggested to be biomarkers for the development of HAND (Arabatzis et al. 2020; Burlacu et al. 2019; Lee et al. 2015; Ploquin et al. 2016), we report these two chemokines together. CCL2 and CXCL10 showed similar pattern of changes in that (1) gp120tg-_no LP-BM5 group are significantly different from the other 3 groups and (2) for gp120tg+ mice, without LP-BM5 treatment, morphine increased the expression of CCL2 and CXCL10, while with LP-BM5 treatment, levels of CCL2 and CXCL10 were not significantly modified by morphine (Two-way ANOVA (log-transformed data): Fig. 8A, CCL2, p_{group}<0.001, p_{mor}=0.976, $p_{groupxmor}$=0.006, Tukey *post-hoc* test p<0.05 between morphine and placebo groups for both gp120tg-_no LP-BM5 and gp120tg+_no LP-BM5 groups. Figure 8B, CXCL10, p_{group}<0.001, p_{mor}=0.63, and $p_{groupxmor}$=0.022. Tukey *post-hoc* test p<0.05 between morphine and placebo groups for gp120tg+_no LP-BM5 group). Overall, the trends of changes in CXCL10 among all groups were similar to that of CCL5 and IL-12p40 although however with various degrees.

It is known that type I interferon response, a critical antiviral response, can be impaired by both HIV infection and morphine treatment (Acchioni et al. 2015; McLane et al. 2018; Wang et al. 2012), and IFNβ supplementation was beneficial in reducing gp120tg-induced cognitive functional deficits (Thaney et al. 2017). When type I IFNs were measured, although neither LP-BM5 nor morphine

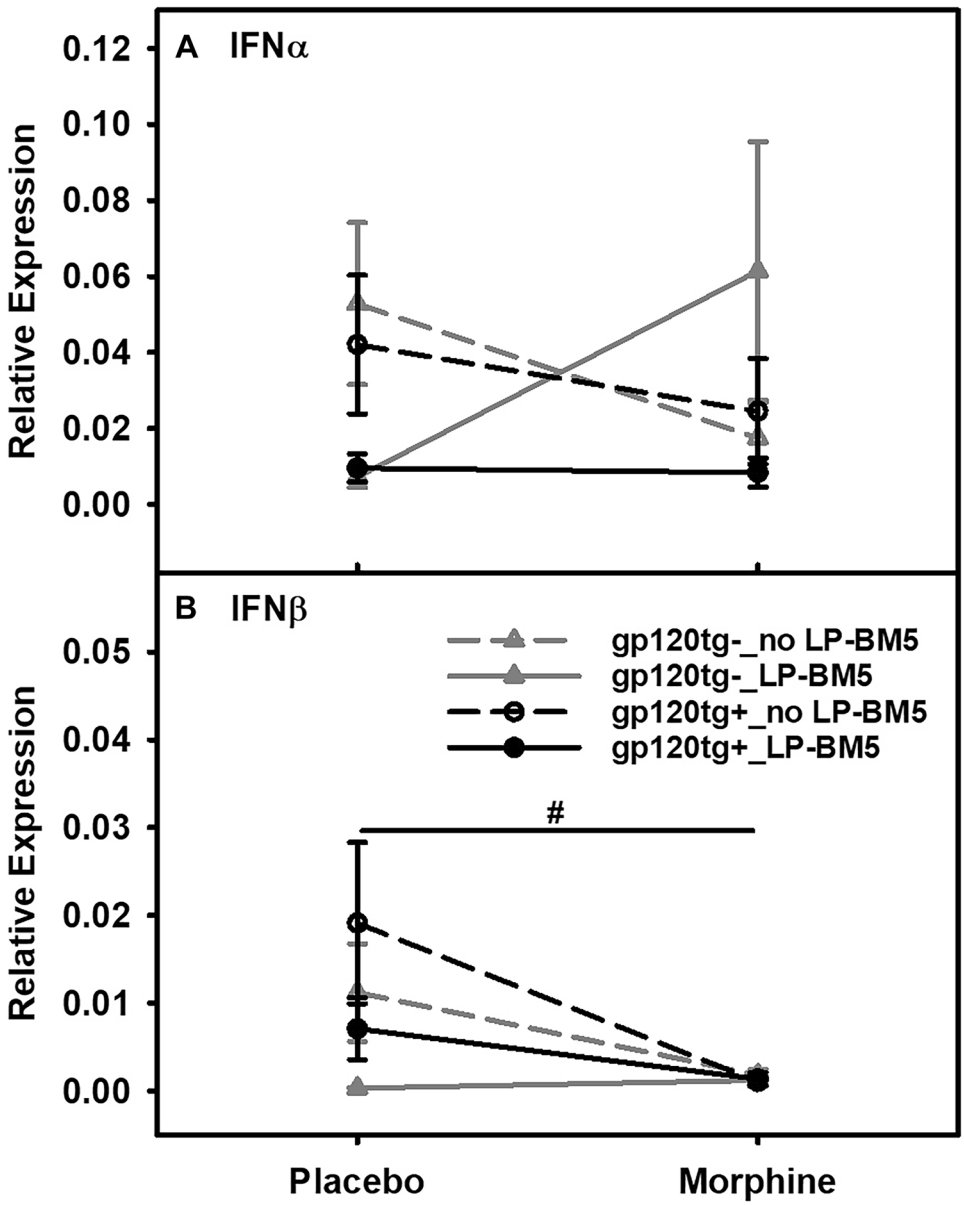

Fig. 9 RNA expression of type I interferons in the CNS of gp120tg mice. RNA expression of IFNα (**A**) and IFNβ (**B**) in the hippocampus are shown in interaction plots. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey *post-hoc* tests were performed. # indicates morphine main effects (p<0.05)

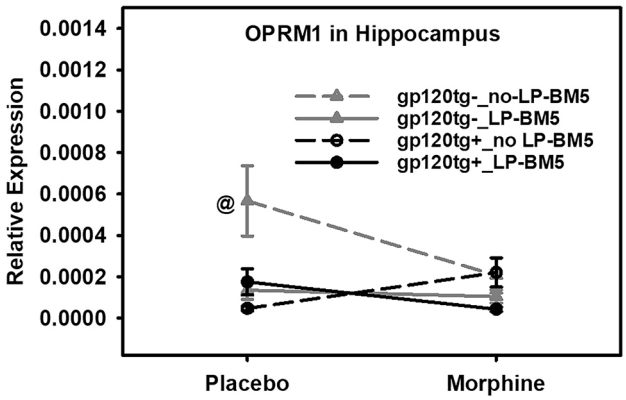

Fig. 10 RNA expression of the Mu opioid receptor in the hippocampus of gp120tg mice. RNA expression of OPRM1 in the hippocampus is displayed as an interaction plot. Data are presented as mean ± SEM; n=6-17 per group. Two-way ANOVA followed by Tukey post-hoc tests were performed. @ indicates $p<0.05$ at placebo level between gp120tg-_no LP-BM5 vs. all other groups and $p<0.05$ between morphine and placebo treatments for the gp120tg-_no LP-BM5 group

affected IFNα expression significantly, a significant morphine main effect was detected with IFNβ expression. Morphine significantly reduced the levels of IFNβ in both non-LP-BM5 treated groups regardless of gp120 expression (Two-way ANOVA (log-transformed data): Fig. 9A, IFNα, p_{group}=0.355, p_{mor}=0.177, $p_{groupxmor}$=0.553. Figure 9B, IFNβ, p_{group}=0.093, p_{mor}=0.014, and $p_{groupxmor}$=0.070, and Tukey *post-hoc* test $p<0.05$ between morphine and placebo groups for both gp120tg-_no LP-BM5 and gp120tg+_no LP-BM5 group).

RNA Expression of the Mu Opioid Receptor in the Hippocampus of gp120tg Mice

Morphine acts through its receptor, mainly the Mu opioid receptor. Therefore, we also examined the expression of OPRM1 in hippocampus. Within placebo groups, gp120tg-_no LP-BM5 group had the highest levels of OPRM1 expression, which was significantly reduced by morphine (Two-way ANOVA: Fig. 10, hippocampus MOR, p_{group}=0.049, p_{mor}=0.148, $p_{groupxmor}$=0.130, and *post-hoc* Tukey test $p<0.05$ at placebo level between gp120tg-_no LP-BM5 vs. all other groups, and $p<0.05$ between morphine and placebo treatments for gp120tg-_no LP-BM5 group). That is, all factors used in our experiments, gp120tg, LP-BM5, and morphine significantly reduced the RNA expression of OPRM1.

Discussion

In 2020, HIV-1 remains to be a public health crisis as there are over 37 million individuals infected and nearly 40,000 new cases each year in the United States (Murphy et al. 2019). Since combination antiretroviral therapy (CART) became available in 1996, many HIV+ patients have achieved peripheral viral suppression and exhibited improved immune functions, leading to improvements in morbidity and life expectancy as well as less impairment due to HIV infection (Heaton et al. 2010). Other studies have found that the prevalence of HIV patients with HAND that progressed into AIDS has also decreased from approximately 16% to under 5% since the initiation of CART (Heaton et al. 2010; McArthur et al. 2010). Prior to antiretroviral therapy, factors including low CD4+ T-cell counts, HIV AIDS-related symptoms (such as anemia, low body weight, and fatigue), co-existence of neuropsychological disorders, and high HIV RNA levels within plasma and CSF are known to be associated with the development and severity of HAND (Sacktor 2018). In the CART era, with the peripheral viral suppression, systemic disorders including hypertension, hypercholesterolemia, and diabetes (often related to systemic chronic inflammation) have been recognized to be risk factors of HAND (Sacktor 2018). In addition, CNS viral load continues to be a risk factor for HAND. Positive CNS viral detection is common among individuals who had well controlled HIV replication in the periphery (Spudich et al. 2019). HIV infection often become latent in the CNS and contributes to destruction of neuronal processes (Anthony et al. 2008; Bradley et al. 2014; Heaton et al. 2010; Kelly et al. 2014; Maartens et al. 2014). Furthermore, prior studies have found that CART effectively decreased the plasma viral loads to undetectable and suppressed HIV infection in the periphery only for approximately 30% of individuals infected with HIV-1 in the US (Bradley et al. 2014; Maartens et al. 2014); therefore, even with CART, individuals' immune status could vary significantly, which may differentially affect the development and pathogenesis of HAND. All of these highlight the needs of assessing HIV-CNS pathophysiology under differential host immune statuses and degrees of viral replication in the CNS, which become more critical when evaluating morphine's effects as morphine is a known regulator of immune responses (Chen et al. 2019; Eisenstein 2019). For example, it has been reported that morphine's antinociceptive effects were significantly enhanced when inflammation was enhanced (Perrot et al. 2001). However, in the CART era, studies regarding HAND have largely ignored the host immune status due to the assumption that peripheral viremia is well-suppressed by CART. Previously, we have shown that gp120-induced neuroinflammatory responses were brain region and immune status specific (Arabatzis et al. 2020). Therefore, in this current study we further evaluated morphine's effects on gp120-induced neuroinflammation within the hippocampus under immunocompetent vs. immunodeficient conditions. Gp120tg mice were used to determine effects of HIV gp120 and the systemic immunodeficient condition was achieved through active

infection of LP-BM5, a murine retrovirus. LP-BM5 infected mice are known to develop immunodeficiency and express virus in the CNS (McLane et al. 2014). We first established that gp120tg mice (gp120tg+ or gp120tg-) could become immunodeficient after LP-BM5±morphine treatment and viral RNA could be detected in all experimental conditions (Figs. 1 and 2). Morphine notably reduced learning/memory functions measured via alternation T-maze assay, when higher hippocampal burden was used, in gp120tg+ alone, LP-BM5 alone, and gp120tg+_LP-BM5 groups, while statistical significance was only detected with gp120tg+_LP-BM5 group (Fig. 4). Gp120 is a viral envelope glycoprotein known to contribute to damage, particularly synaptodendritic injury, in the hippocampus; a region of the brain that regulates executive function, emotion, learning, memory, and motivation (Ellis et al. 2007). Hippocampal synaptophysin at protein levels were found to be slightly but significantly increased by morphine (Fig. 5). Similar morphine-induced effects have been reported by others (Iqbal O'Meara et al. 2020). Thus, measuring synaptic functions (rather than expression levels of selected synaptic proteins) would be critical in order to delineate morphine's damaging effects on cognitive functions.

Patients suffering from HAND display severe inflammation in the CNS (McArthur et al. 2010; Nasi et al. 2017; Saylor et al. 2016). In order to better understand the involvement of neuroinflammatory responses in hippocampal function deficits, we first measured the RNA expression levels of CD11b and GFAP attempting to examine the responses of both microglia/infiltrating macrophages and astrocytes within the hippocampus, respectively. It is known that gp120tg mice express increased numbers of microglia and display profound astrocytosis (Thaney et al. 2018). Our previous study demonstrated that the RNA expression of GFAP was significantly higher in gp120tg+ mice in the hippocampus (but not in the striatum or frontal lobe) (Arabatzis et al. 2020). Here we showed that morphine could further enhance GFAP expression in gp120tg+ mice regardless of the immune status induced by LP-BM5 (Fig. 6B), while morphine's promoting effects on CD11b was only observed in immunocompetent mice regardless of gp120tg expression (Fig. 6A). These results demonstrated that morphine could potentiate pro-inflammatory effects, through different cellular contributors depending on the immune status of the host (potentially also the status of CNS viral replication). Differential contributions from microglia/macrophages vs. astrocytes activations to neuroinflammation may lead to differential CNS pathology (Lu et al. 2014; Serrano-Pozo et al. 2013), which may require differential treatment strategies.

Gp120 can mediate inflammatory responses by up-regulating or down-regulating levels of inflammatory cytokines (Asensio et al. 2001; Ronaldson and Bendayan 2006; Yeung et al. 1995). Cytokines that are found to be altered in the CNS of infected patients include but not limited to: monocyte chemoattractant protein-1 (MCP-1 or CCL2), regulated upon activation normal T-cell expressed and secreted (RANTES or CCL5), interleukin-1 beta (IL-1β), as well as antiviral interferons such as interferon alpha (IFNα), interferon beta (IFNβ) and interferon gamma (IFNγ) (Brabers and Nottet 2006; Deshmane et al. 2009; Gonzalez et al. 2002; Kelder et al. 1998; Sivro et al. 2014; Watanabe et al. 2010). In our study, when various inflammation-related cytokines/chemokines were examined, immune status-dependent differential morphine effects were also observed. In immunocompetent (non-LP-BM5 treated) gp120tg+ mice, morphine increased the RNA expression of CCL2, CCL5, CXCL10, IL-12p40, and IFNβ; while under the immunodeficient condition (LP-BM5 treated, along with CNS viral replication), morphine downregulated the expression of CCL2, CCL5, CXCL10, IL-12p40, and IL-1β. Further, expression of TNFα and IFNγ were enhanced by morphine regardless of host immune status (Figs. 7, 8 and 9), suggesting consistent contribution of TNFα and IFNγ in morphine-induced CNS inflammation. Together, under immunocompetent conditions, at least related to gp120-associated neuroinflammation, one could propose that both microglia/macrophage and astrocyte activation and their activation-mediated multiple cytokine responses are contributing to morphine-potentiated CNS inflammation; while under immunodeficient condition, morphine-potentiated inflammation could be attributed mainly to astrocytic responses. This hypothesis requires further testing in the future studies.

In addition, RNA expression of mu opioid receptor was down-regulated upon morphine treatment regardless of gp120 expression or LP-BM5 status. In fact, gp120tg presence and LP-BM5 treatment significantly reduced its expression at RNA level as well (Fig. 10), which may indicate a potential downstream reduction of receptor synthesis after stimulations by these factors. This is consistent with the previous observation that reported a 50% reduction in mu-opioid receptor protein quantity measured by Western blot following chronic morphine treatment in mice (Bernstein and Welch 1998). However, regulation of opioid receptor expression can occur at both transcriptional and translational levels, and can vary depending on cell type, sub-cellular compartment (cell surface vs. cytoplasma), cell activation status, presence of HIV proteins, and microenvironment (Beltran et al. 2006; Cadet et al. 2001; Raehal and Bohn 2005; Turchan-Cholewo et al. 2008), and may not be related to the levels of morphine (as we have observed here). Therefore, the observed down-regulation of OPRM1 in hippocampus is a collective phenomenon and requires further investigation in order to be fully understood. Although it is not the goal for the current study, morphine levels within specific brain tissues could be examined via mass spectrometry in the future.

We realized that one of the limitations of this study is that many analytes were measured at the RNA levels. However, due to the availability of samples and the numbers of analytes we wished to measure, qRT-PCR was the most efficient methods at the time. Future studies could evaluate the expression of selected analytes at protein levels. Additionally, in our current study, we examined the effects of morphine on gp120-induced neuroinflammatory responses under immunocompetent vs. immunodeficient conditions. Many other HIV proteins may also behave differently at different immune status. Other HIV HAND models (such as HIV-transgenic rats, HIV Tat-transgenic mice) could be used to analyze other HIV components besides gp120 (Sil et al. 2021).

In summary, this study was the first step in examining morphine's differential effects under immunocompetent vs. immunodeficient conditions. We found that morphine-induced neuroinflammatory responses are likely to be mediated by different cells and cytokines/chemokines depending on the host's immune status (potentially also viral replication status within the CNS), which will be further delineated in future studies. As the result, the immune status of individual hosts needs to be in consideration when designing the strategies for managing and treating HAND in opioid users.

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Author Contributions Ling Cao contributed to the study conception and design, supervised the conduction of the study, and participated in data analysis and manuscript preparation. Dalton Canonico participated in data collection, statistical analysis and manuscript preparation. Sadie Casale and Tristan Look participated in data collection, data input, and reviewed the manuscript.

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Declarations

Conflict of Interest All authors declare no conflict of interest.

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