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Activation of GPR55 induces neuroprotection of hippocampal neurogenesis and immune responses of neural stem cells following chronic, systemic inflammation

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Abstract

New neurons are continuously produced by neural stem cells (NSCs) within the adult hippocampus. Numerous diseases, including major depressive disorder and HIV-1 associated neurocognitive disorder, are associated with decreased rates of adult neurogenesis. A hallmark of these conditions is a chronic release of neuroinflammatory mediators by activated resident glia. Recent studies have shown a neuroprotective role on NSCs of cannabinoid receptor activation. Yet, little is known about the effects of GPR55, a candidate cannabinoid receptor, activation on reductions of neurogenesis in response to inflammatory insult. In the present study, we examined NSCs exposed to IL-1β *in vitro* to assess inflammation-caused effects on NSC differentiation and the ability of GPR55 agonists to attenuate NSC injury. NSC differentiation and neurogenesis was determined via immunofluorescence and flow cytometric analysis of NSC markers (Nestin, Sox2, DCX, S100β, βIII Tubulin, GFAP). GPR55 agonist treatment protected against IL-1β induced reductions in neurogenesis rates. Moreover, inflammatory cytokine receptor mRNA expression was down regulated by GPR55 activation in a neuroprotective manner. To determine inflammatory responses *in vivo,* we treated C57BL/6 and GPR55$^{-/-}$ mice with LPS (0.2 mg/kg/day) continuously for 14 days via osmotic mini-pump. Reductions in NSC survival (as determined by BrdU incorporation), immature neurons, and neuroblast formation due to LPS were attenuated by concurrent direct intrahippocampal administration of the GPR55 agonist, O-1602 (4μg/kg/day). Molecular analysis of the hippocampal region showed a suppressed ability to regulate immune responses by GPR55$^{-/-}$ animals manifesting in a prolonged inflammatory response (IL-1β, IL-6, TNFα) after chronic, systemic inflammation as compared to C57BL/6 animals. Taken together, these results suggest a neuroprotective role of GPR55 activation on NSCs *in vitro* and *in vivo* and

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Author Contributions J.D.H. designed and performed experiments, wrote the manuscript, and supervised the project. V.Z.R. designed animal experiments. S.G. and M.W. performed *in vitro* experiments and histology. U.S aided in transcriptome analysis. Y.P. supervised the project and refined the manuscript.

Competing Interests

None

that GPR55 provides a novel therapeutic target against negative regulation of hippocampal neurogenesis by inflammatory insult.

Keywords

GPR55; neurogenesis; inflammation; neuroprotection; cannabinoid; hippocampus; neural stem cell

1. Introduction

Within the adult brain, new neurons are generated from neural stem cells in two major sub-regions: the subventricular zone of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus(Zhao, Deng, and Gage 2008; van Praag et al. 2002; Spalding et al. 2013). New neurons formed within the hippocampus, a process known as hippocampal neurogenesis, integrate newly differentiating neural stem/progenitor cells into existing neural networks of the hippocampus and are integral for processes such as learning and memory(Deng, Aimone, and Gage 2010; Sahay and Hen 2007). Decrease of hippocampal neurogenesis can result in detrimental outcomes including cognitive impairment, stress-induced behavior changes, and depression(Coras et al. 2010; Lagace et al. 2010; Sahay and Hen 2007; Snyder et al. 2011). Thus, there is great interest in understanding of biological processes which alter hippocampal neurogenesis and therapeutic approaches to alleviate such dysregulation.

Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and Type 1 Interferons (interferon α and β), are essential soluble mediators of the innate immune response. Systemic inflammation that can be modeled by lipopolysaccharide (LPS) administration initiates molecular signaling and cellular processes leading to pathological neuroinflammation(Valero et al. 2014; Qin et al. 2007; Godbout et al. 2005). Neuroinflammation, cranial irradiation, and increased expression of pro-inflammatory cytokines within the hippocampal micro-environment suppress neurogenesis and can induce long lasting behavioral alterations including reduced memory formation and depression as well as alterations in the functional integration of adult born neurons(Ekdahl et al. 2003; Valero et al. 2014; Zonis et al. 2015; Monje et al. 2002; Iosif et al. 2006; Jakubs et al. 2008; Wood et al. 2011). In support of this, it has been found that blockade of inflammatory mediators upregulated by either systemic administration of LPS or cranial irradiation rescued disrupted neurogenesis rates within the hippocampus(Monje, Toda, and Palmer 2003). Importantly, selective activation of PPARγ was highly effective at protecting both hippocampal neurogenesis and memory formation during LPS-induced transient illness(Ormerod et al. 2013).

Recently, the cannabinoid system has been proposed to regulate the neural stem cell (NSC) niche within the hippocampus both under homeostatic and pathologic conditions. During homeostatic conditions, the cannabinoid 1 (CB_1) receptor has been implicated in neuronal differentiation of NSCs while activation of the cannabinoid 2 (CB_2) receptor increases NSC proliferation(Xapelli et al. 2013; Molina-Holgado et al. 2007; Palazuelos et al. 2012; Rodrigues et al. 2017). Importantly, evidence suggests that NSCs have bi-directional cross-

talk between the cannabinoid system (CB_1 and CB_2) and inflammatory cytokines (IL-1β, TNFα) that is necessary for NSC proliferation(Rubio-Araiz et al. 2008; Garcia-Ovejero et al. 2013). Activation of CB_2 receptors rescued impaired hippocampal neurogenesis caused by chronic insult by HIV-1 neurotoxic protein gp120 further suggesting a neuroprotective role of cannabinoid-like ligands and cannabinoid receptor activation in the NSC niche(Avraham et al. 2014).

The recently de-orphaned G-protein coupled receptor (GPCR), GPR55, is activated endogenously by L-α-lysophosphatidylinositol (LPI), a lipid signaling molecule, as well as N-arachidonoyl glycine (NAGly) and numerous endo, phyto, and synthetic cannabinoids(Oka et al. 2007; Henstridge et al. 2010; Heynen-Genel et al. 2010; Fakhouri et al. 2017; Ryberg et al. 2007; Console-Bram et al. 2017). Due to its activation by a number of cannabinoid compounds, GPR55 is considered a candidate cannabinoid receptor. Signaling through GPR55 in peripheral tissues has possessed pro- and anti-inflammatory effects depending on cell type and cause of inflammation. Activation of GPR55 was found to increase neutrophil chemotaxis and recruitment, increase pro-inflammatory cytokine release from monocytes and natural killer (NK) cells, and is involved in intestinal inflammatory responses(Balenga et al. 2011; Chiurchiu et al. 2015; Lin et al. 2011; Staton et al. 2008). In contrast to these pro-inflammatory data, it was also found that GPR55 reduced the release of nerve growth factor on inflammation-activated mast cells and attenuated neuroinflammation and chronic pain from colitis induced by intracolonic administration of dinitrobenzenesulfonic acid(Cantarella et al. 2011; Borrelli et al. 2015). In the central nervous system (CNS), the endogenous agonist for GPR55 (LPI) showed neuroprotective effects after excitotoxic lesion in a microglial and GPR55-dependent manner(Kallendrusch et al. 2013). Furthermore, GPR55 mRNA expression in microglia is down-regulated after treatment with LPS or IFNγ, suggesting that GPR55 is involved in neuroinflammation(Pietr et al. 2009).

Recent evidence has also suggested a significant role for GPR55 in the hippocampus in that GPR55 activation boosts neurotransmitter release and modulates synaptic plasticity in the hippocampal CA1 region(Hurst et al. 2017; Sylantyev et al. 2013). Importantly, expression of GPR55 has been found on both human and murine NSCs and administration of GPR55 agonists increased both NSC proliferation and differentiation along a neuronal lineage *in vitro* and *in vivo*(Hill et al. 2018). Of note, $GPR55^{-/-}$ animals displayed reduced rates of hippocampal NSC proliferation, survival, and neuronal differentiation, suggesting that GPR55 is a necessary facet of NSC physiology(Hill et al. 2018). However, the role GPR55 plays in immune responses within NSC niches has not been properly explored.

In this work, we sought to determine the effects of GPR55 activation on human and murine hippocampal NSCs under inflammatory conditions. We show here that treatment with GPR55 agonist protects against reduced neuron formation caused by IL-1β *in vitro* and chronic, low-level systemic inflammation induced by LPS administration *in vivo*. Activation of GPR55 demonstrated neuroprotective regulation of inflammatory cytokine receptor mRNA expression in both human and murine NSC cultures after IL-1β exposure. On the contrary, $GPR55^{-/-}$ mice displayed increased expression of inflammatory cytokine mRNA in the hippocampus after 14 days of continuous LPS treatment as compared to C57BL/6

animals, suggesting an immune-regulatory role for GPR55. Thus, GPR55 may present a yet unrecognized therapeutic target for alleviation of negative consequences of chronic peripheral inflammation on hippocampal neurogenesis and associated cognitive and behavioral alterations.

2. Methods

2.1 Animal studies

2.1.1 Animals—GPR55$^{-/-}$ mice were obtained from the Texas A&M Institute for Genomic Medicine (Dr. Andrei Golovko). Generation of these animals is described in detail by Wu et al.(Wu et al. 2010) and maintained as previously described at Temple University(Hill et al. 2018). C57BL/6 mice (wild type) used for this study were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Male and female mice aged 12–15 weeks of age at the beginning of the study were single-housed on a 12:12 light/dark cycle (7:00am – 7:00pm) at 22.1 ± 1°C with *ad libitum* access to food and water. Animals were randomly assigned to treatment group with equal numbers of male and female animals per group. Based on power analyses from previous experiments conducted by the laboratory, we determined that a minimum of 6 animals per group would provide sufficient power to detect a significant difference with 95% confidence.

2.1.2 Intrahippocampal infusion of O-1602—Surgery for implantation of cannulae and osmotic pumps for O-1602 delivery was performed as previously described(Hill et al. 2018). In short, osmotic mini-pumps (Alzet Durect, Cupertino, CA, USA; model 1002) were filled with either vehicle (artificial cerebral spinal fluid (ACSF, Tocris Bioscience, Bristol, UK), 0.05% EtOH) or O-1602 (O-1602 diluted in 100% EtOH; agonist was further diluted in ACSF with a final concentration of 0.05% EtOH). Pumps were connected to a stainless-steel cannula (Alzet Durect, brain infusion kit 3) connected through polyvinyl tubing. Infusion doses were set to 4 μg/kg/day continuously for 14 days.

2.1.3 Systemic LPS administration—LPS (O111:B4; Sigma Aldrich, St. Louis, MO, USA) was administered systemically via osmotic mini pump (Alzet Durect, model 1002) at a dose of 0.2 mg/kg/day for a total of 14 continuous days. Animals in the no insult group were implanted with osmotic mini pumps filled with sterile saline. Pumps with LPS or saline were implanted at the same time as pumps connected to cannulae and were set for subdermal administration. Low-dose continuous dispersion was chosen to better mimic a chronic, low-level systemic infectious state rather than an acute, high-level infection.

2.1.4 BrdU—Animals were injected with 100mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) i.p. twice per day for days 1 and 2 of the experimental schedule and once per day for days 3 and 4 for a total of six injections.

2.2 Pharmacological agents

5-Methyl-4-[(1*R*,6*R*)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol (O-1602, analog of cannabidiol and potent GPR55 agonist; Tocris Bioscience), 3-[[4-(2,3-dimethylphenyl)-1-piperazinyl]carbonyl]-N,N-dimethyl-4-(1-pyrrolidinyl)-benzenesulfonamide (ML184

[CID-2440433], synthetic GPR55 agonist; Cayman Chemical, Ann Arbor, MI, USA), N-[4-[[(3,4-dimethyl-5-isoxazolyl)amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide (ML193 [CID-1261822], GPR55 antagonist; Cayman Chemical), 4-{4,6-dihydro-4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxopyrrolo[3,4-*c*]pyrazol-5(1*H*)-yl]benzoic acid (CID-16020046, GPR55 antagonist; Tocris Bioscience). Master stock solutions of O-1602, CID-16020046, ML184, and ML193 were prepared in 100% dimethylsulfoxide (DMSO) according to their solubility and manufacturer's instructions. Working concentrations were generated in culture media and further diluted to final concentrations on the day of use. For *in vivo* experiments, master stock solutions of O-1602 were prepared in 100% EtOH. All master stock solutions were aliquoted and stored at −20°C. Recombinant human and murine IL-1β (PeproTech, Rocky Hill, NJ, USA) was reconstituted in 0.1% BSA in sterile saline. Cytokines were then aliquoted and stored at −20°C.

2.3 Cell culture

2.3.1 human NSCs—*In vitro* experiments utilized primary human NSCs (ReNcell VM) commercially obtained from Millipore Inc. (Billerica, MA, USA; Cat# SCC008, RRID:CVCL_E921). The ReNcell VM cell line is an immortalized human NSC line derived from the ventral mesencephalon region of human fetal brain. hNSC cultures were regularly characterized by flow cytometry for NSC markers (nestin, Sox2). Undifferentiated cells were maintained and cultured in ReNcell NSC maintenance medium (Millipore) supplemented with epidermal growth factor (EGF; 20 ng/ml; Millipore) and basic fibroblast growth factor (bFGF; 20 ng/ml; Millipore). The cells were grown as an adherent monolayer on laminin-coated (mouse laminin, Millipore, 10μg/ml in DMEM/F12 w/o HEPES, w/ L-glutamine) 75 cm^2 cell culture flasks, 6-well plates, or 8-well chamber slides at 37° C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged when flasks were ~80–90% confluent. Media was changed every 2–3 days. Differentiation of hNSCs was induced by removal of growth factors (bFGF, EGF) from the culture medium. Cultures were allowed to differentiate for 10 days.

2.3.2 murine NSCs—Primary hippocampal NSCs were obtained from C57BL/6 mice at 4–6 weeks of age as described previously by our lab which is based on a protocol kindly provided by Dr. Wenhui Hu at Temple University{Hill, 2018 #221;Zhang, 2012 #435;Xiao, 2018 #451}. Dissociated cells were cultured at a density of 2×10^5 cells/mL in proliferation medium (DMEM/F12 (1:1) supplemented with 0.2% heparin, 1xB27 supplement, 20ng/mL mouse epidermal growth factor (EGF), 10ng/mL mouse fibroblast growth factor basic (bFGF), penicillin/streptomycin, and L-glutamine). Neurospheres were allowed to grow for seven days with half medium changed every other day. For expansion as an adherent monolayer, neurospheres were dissociated with Accutase and plated on Matrigel (Corning, Corning, NY, USA) coated 6-well plates. Proliferation medium was changed every other day. To differentiate murine NSCs, cells were passaged and plated on Matrigel coated 8-well chamber slides (Millipore). Differentiation media consisted of DMEM/F12 (1:1) supplemented with 1xB27 supplement, 1xN2 supplement, penicillin/streptomycin, and L-glutamine. Media was changed every other day. For differentiation studies, mNSC samples were in differentiating conditions for 5 days.

2.3.3 In vitro inflammatory treatments—Recombinant human or murine IL-1β was added to cultures at a dose of either 10 ng/mL (for chronic experiments) or 100 ng/mL (for acute experiments). Doses were chosen based on previous literature showing deficits in neurogenesis *in vitro* as well as acute responses by NSCs(Zonis et al. 2015; Borsini et al. 2017; Green et al. 2012).

2.4 Histology

Animal tissue was harvested 14 days after pump/cannula implantation. In short, animals were anaesthetized with isofluorine and sacrificed by transcardial perfusion with 10 ml PBS. Brains were removed and fixed in 4% formaldehyde at 4°C for 24 hours. Brain samples were then transferred to a 30% sucrose solution and stored at 4°C until samples were saturated. Cannula placement was confirmed visually by gross anatomical disruption. Administration of compound and placement was originally determined in pilot studies using Evans Blue (0.05 mg/ml; Sigma Aldrich) for 14 days. Samples were then sectioned coronally on a Leica CM1860 cryostat (Leica Biosystems, Wetzlar, Germany) into 30μm sections. Samples were serially sectioned into six wells and stored in cryoprotectant at −20°C until use for immunofluorescence staining.

2.5 Immunofluorescence staining

Hippocampal samples were sectioned coronally on a Leica CM1860 cryostat (Leica Biosystems, Wetzlar, Germany) into 30 μM sections. Samples were serially sectioned into 6 wells. All sections were washed 3x with PBS to remove residual cryoprotectant. *In vitro* hNSCs and mNSCs attached to laminin and Matrigel, respectively, in 8-well chamber slides were fixed with 4% paraformaldehyde solution for 15 minutes and washed 3 times with PBS to remove residual paraformaldehyde. All samples were stained as previously described by our laboratory(Hill et al. 2018). For BrdU staining, after removal of cryoprotectant, sections were treated in 1 N HCl at 4°C for 10 min, 2 N HCl at 37°C for 30 min then washed twice in 0.1 M borate buffer (pH 8.5) for 5 min each. All samples were permeabilized with 0.1% Triton X-100 in PBS and then blocked for 2 hours in 1% BSA, 5% normal donkey serum in PBS/0.1% Triton X-100. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Samples were then washed four times in PBS/0.1% Triton X-100 and incubated is secondary antibody diluted in blocking solution for 2h at room temperature. Antibodies used were anti-Ki67 (1:500, Abcam, Cambridge, UK; Cat# ab15580 Lot# GR264777 RRID:AB_443209), anti-DCX (1:1000, Millipore Cat# AB2253 Lot# 2828588 RRID:AB_1586992), anti-BrdU (1:200, Abcam Cat# ab6326 Lot# GR267766–1 RRID:AB_305426), anti-βIII-tubulin (1:1000, Sigma Aldrich Cat# T2200 Lot#028M4759V RRID:AB_262133), anti-GFAP (1:500, Aves Labs, Tigard, OR, USA; Cat# GFAP Lot#87867983 RRID:AB_2313547), anti-IBA-1 (1:500, Wako Chemicals, Richmond, VA, USA; Cat# 019–19741 Lot#WDK2121 RRID:AB_839504), and anti-CD68 (1:1000, Bio-Rad Laboratories, Hercules, CA, USA; Cat# MLA1957 Lot#1708 RRID:AB_322219). Secondary antibodies used were Alexa-488® goat anti-guinea pig (Thermo Fisher Scientific, Waltham, MA, USA Cat# A-11073 Lot#1841755 RRID:AB_2534117), Alexa-594® donkey anti-rat(Thermo Fisher Scientific Cat# A-21209 Lot#45081A RRID:AB_2535795), Alexa-488® donkey anti-rabbit (Thermo Fisher Scientific Cat# A-21206 Lot#1608521 RRID:AB_2535792), Alexa-488® goat anti-chicken (Thermo Fisher Scientific Cat#

A-11039 Lot #1937504 RRID:AB_142924), Alexa-594® goat anti-chicken (Thermo Fisher Scientific Cat# A-11042 Lot#1899511 RRID:AB_142803). All secondary antibodies were used at a dilution of 1:400. Imaging for hNSCs was performed using a CoolSNAP EZ CCD camera (Photometrics, Tucson, AZ, USA) coupled to a Nikon i80 Eclipse (Nikon Instruments Inc., Melville, NY, USA). Confocal images were taken on a Nikon A1R confocal microscope (Nikon). Filters and laser lines used were as follows: for 488 we used 525/50; for 594 we used 632/60; for DAPI we used 450/50.

2.6 NSC quantification and cell count analysis

2.6.1 In vivo quantification—Quantification of positive immunofluorescent cells in the SGZ was performed as previously described(Hill et al. 2018). In short, one out of every six adjacent sections were chosen and processed for immunofluorescence staining. The number of Ki67+, BrdU+, or DCX+ cells was then counted under a fluorescent microscope (with CoolSNAP EZ CCD camera (Photometrics) coupled to a Nikon i80 Eclipse microscope in the area of the SGZ (defined as a two-cell layer in the borders of the granular cell layer, omitting those in the outermost focal planes). All counts were performed blinded to treatment group or genotype.

2.6.2 Cell quantification of in vitro staining—Determination of neuronal and glial differentiated cells (βIII-tubulin, GFAP) was assessed by confocal microscopy. Z-stacks of cultured cells were taken on a Nikon A1R confocal microscope under 40x magnification. For quantification analysis, at least 5 images were collected per treatment condition per experiment. Percentage of βIII-tubulin positive or GFAP positive cells was determined by the number of positive immunostained cells divided by the total number of DAPI positive nuclei in the image.

2.7 Microglial activation analysis

Hippocampal sections were immunostained for IBA-1 and CD68 as described above. Three sections per animal were chosen for analysis. Anatomical location was controlled for by including one section from three ranges according to Bregma; −1.22 to −2.30 mm representing the dorsal hippocampus, −2.30 to −2.70 mm representing the junction between dorsal and ventral hippocampus, and −2.70 to −3.64 mm representing the ventral hippocampus. These methods and coordinates, with slight modification by our lab, are based on previously published studies(Hoeijmakers et al. 2017; Hoeijmakers et al. 2018). Confocal 3D z-stack images were taken on a Nikon A1R confocal microscope at 0.5μM steps over a range of 24–26 μM per section using a 20x objective. Using NIS-Elements software (Nikon, Version 5.02.00 RRID:SCR_014329), images were processed and a fixed threshold was set to include all positive immunofluorescent signal in the region of interest (ROI). ROIs were set to a 600 μm x 600 μm area which included the dentate gyrus, hilus, and molecular layer. Next, we rendered a comprehensive 3D representation within the NIS-Elements software to determine the total number of IBA-1+ cells within each ROI as well as the percentage of volume within the ROI that was either IBA-1+, CD68+, or both IBA-1+/CD68+. To determine changes in microglial (IBA-1+) expression of CD68, we divided the total volume of IBA-1+/CD68+ by the total IBA-1+ volume giving us changes in the percent of IBA-1+ signal colocalized with CD68. We then averaged data from each image per animal together

to obtain a single biological replicate for analysis. Total animals analyzed per group are as follows; C57BL/6: vehicle/saline n=5, O-1602/saline n=6, vehicle/LPS n=5, O-1602/LPS n=6; GPR55$^{-/-}$: vehicle/saline n=6, O-1602/saline n=6, vehicle/LPS n=6, O-1602/LPS n=6.

2.8 Flow cytometry

Flow cytometric analysis was performed as previously described(Hill et al. 2018). Antibodies used were against human Sox-2 (BD Biosciences, San Jose, CA, USA, Cat# 561610 Lot# RRID:AB_10712763), nestin (BD Biosciences, Cat# 560393 Lot#3305985 RRID:AB_1645170), S100β (Abcam, Cat#ab196442 Lot#GR206303–4 RRID:AB_2722596), βIII-Tubulin (BD Biosciences, Cat# 560394 Lot# 7132512 RRID:AB_1645400), DCX (BD Biosciences, Cat# 561505 Lot# 7269884 RRID:AB_10643766), and GFAP (Biolegend, San Diego, CA, USA; Cat# 644710 Lot# B249852 RRID:AB_2566685). Cytometric acquisition was performed using a BD FACS Canto II flow cytometer and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA; RRID:SCR_008520).

2.9 qPCR

Total RNA from human NSCs was extracted using *RNeasy*® Plus Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Total RNA (1 mg) was converted to cDNA using high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Specific primers and probes (TaqMan) for human and mouse immune receptors were purchased from Life Technologies (Thermo Fisher Scientific) and analyses were performed using the StepOnePlus real-time PCR system (Thermo Fisher Scientific). Primer and probe sets for immune receptors are listed in Table 1 (human) and Table 2 (mouse). Data for human samples were normalized to GAPDH while data for mouse samples were normalized to Rn18s. qPCR analysis for *in vitro* immune receptor studies is represented as fold change using the $2^{-\Delta\Delta Ct}$ method. Statistics for these studies were performed on ΔC_T values.

2.10 RNAseq

C57BL/6 or GPR55$^{-/-}$ mice were chronically treated with a low dose LPS (0.2 mg/kg/day) or saline via osmotic mini-pump for 14 days as outlined previously in methods. These animals were not implanted with cannula to determine immune responses within the hippocampus without any disruption of the CNS. Animal numbers were as follows: C57BL/6 + saline n=5, C57BL/6 + LPS n=4, GPR55$^{-/-}$ + saline n=5, GPR55$^{-/-}$ + LPS n=4. Only male animals were used for this experiment. At the end of the treatment schedule, animals were perfused with saline and brains were harvested. RNA was prepared using the tissue from the hippocampal region and gene expression was analyzed by RNAseq using the mouse inflammation and immunity transcriptome array (RMM005Z) from Qiagen. Data from primary analysis from Qiagen was uploaded onto GeneGlobe Data Analysis Center (Qiagen). Data was normalized for Total Unique Molecular Index Count in each sample. This method was used to normalize the Unique Molecular Index count of every gene by the Total Unique Molecular Index count in each sample. Heatmaps were generated using this data to visualize changes in the proinflammatory profile. Specific genes within the LPS proinflammatory pathway were selected and bar graphs were plotted using the normalized Total Unique Molecular Index Count for each gene.

2.11 Statistics

For all analyses, data was processed randomly and quantification was performed by experimenters who were blinded to experimental groups. All values are expressed as mean ± SEM. Analyses were performed using GraphPad Prism 7.0 software (San Diego, CA, USA; RRID:SCR_002798). The significance of the differences between groups was evaluated by one-way analysis of variance (ANOVA; followed by Tukey's post-hoc test) or two-way ANOVA (followed by Tukey's post-hoc test). Post hoc tests were only performed when F achieved $p<0.05$. $p<0.05$ values were determined to be statistically significant. Statistics for qPCR data were performed on ΔC_T values.

3. Results

3.1 Activation of GPR55 on human neural stem cells is protective against reduced neuron formation due to IL-1β *in vitro*.

To determine the protective effects of GPR55 activation during neuroinflammation, we treated hNSCs for 10 days with 10 ng/mL recombinant IL-1β under differentiation conditions with or without ML184 (1 μM), a selective agonist for GPR55. To ensure that effects seen with ML184 were through activation of GPR55, we also treated cells with the GPR55 antagonist ML193 (5 μM). We determined the number of cells positive for the neuronal marker βIII-tubulin or glial marker GFAP using immunofluorescent staining and calculated the percentage of positive cells as the number of positively marked cells divided by the total number of DAPI nuclei in each image. Representative images are presented in Figure 1A. Treatment with IL-1β significantly reduced the number of cells positive for βIII-tubulin (0.994% ± 0.15) as compared to vehicle controls (3.918% ± 0.41; $F(4,20)=14.85$, $p<0.0001$, $n=5$). Pre-treatment with ML184 significantly protected against reduction in βIII-tubulin positive cells (2.709% ± 0.36), while treatment with IL-1β + ML193 (1.267% ± 0.24) or the combination IL-1β + ML184 + ML193 (1.218% ± 0.183) had no effect as compared to IL-1β treatment alone (Figure 1B). Unexpectedly, treatment with IL-1β reduced the number of GFAP positive cells (5.834% ± 0.44) as compared to vehicle treated controls (7.387% ± 0.51; $F(4,20)=19.61$, $p<0.0001$, $n=5$) yet post hoc analysis between vehicle and IL-1β treatments did not reach statistical significance (Figure 1C). ML184 treatment with IL-1β did significantly diminished the number of GFAP positive cells (4.525% ± 0.31) as compared to vehicle. IL-1β + ML193 (6.342% ± 0.46) and IL-1β + ML184 + ML193 (7.23% ± 0.22) did not differ from vehicle controls. Using flow cytometry, we obtained similar results as in the immunofluorescence studies and we normalized separate experiments as percent of vehicle control to account for inter-experiment variability. For βIII-tubulin (Figure 1D), IL-1β significantly reduced the percent positive cells (26.63% ± 3.37; $F(4,25) = 54.65$, $p<0.0001$, $n=6$) as compared to vehicle controls, while treatment with ML184 significantly protected against this reduction (49.97% ± 6.64). Treatment of IL-1β + ML193 (20.77% ± 2.26) or the combination IL-1β + ML184 + ML193 (22.21% ± 3.49) had no effect as compared to IL-1β treatment alone. GFAP positive cells trended in a similar manner to the IFA studies, yet results were not significantly different between groups (Figure 1E). Results for DCX (Figure 1F) showed similar trends as βIII-tubulin which is not surprising as DCX is a marker for immature, rather than mature, neurons. DCX positive cells were significantly reduced with IL-1β treatment (33.28% ± 1.49; $F(4,25) = 41.07$, $p<0.0001$,

n=6) when compared to vehicle control. ML184 application protected against this reduction (60.04% ± 4.70) while treatment with IL-1β + ML193 (42.74% ± 3.93) or the combination IL-1β + ML184 + ML193 (42.92% ± 6.02) had no effect as compared to IL-1β treatment alone. As with GFAP, staining for the mature astrocyte marker S100β showed reduced numbers of positive cells for all treatments as compared to vehicle-treated controls. These results were found to be statistically significant ($F(4,25) = 20.25$, $p<0.0001$, $n=6$) as represented in Figure 1G. Treatment with ML184 (57.51% ± 2.49) also reduced the number of S100β positive cells significantly as compared to IL-1β treatment alone (74.63% ± 4.63).

3.2 hNSC cytokine receptor expression is altered by GPR55 activation after inflammatory insult with IL-1β.

We subsequently investigated what, if any, effects activation of GPR55 had on pro- or anti-inflammatory cytokine receptor mRNA expression in hNSC. We chose to target receptors known to be expressed by NSCs within the hippocampus: IL-1R1, IL-1R2, TNFR1, TNFR2, IL-6st (gp130), and IL-10Rα. hNSC cultures were treated with the GPR55 agonist ML184 (1 μM) or antagonist ML193 (5 μM) 30 minutes prior to insult with IL-1β (100 ng/mL). For samples treated with the combination ML184 + ML193, antagonist was added to culture medium 30 minutes prior to agonist. Cells were collected 4 hours or 24 hours after addition of IL-β to the media. Neither treatment with ML184, ML193, nor ML184 + ML193 had any significant effects on cytokine receptor mRNA levels at 4 hours or 24 hours, suggesting that activation of GPR55 does not have any effect on immune responses of hNSCs in the absence of inflammatory mediators. Statistics for hNSC inflammatory response experiments are listed in Table 3, $n = 3$ independent experiments. After treatment with IL-1β for 4 hours, no treatment combination showed a significant change in comparison to vehicle-treated samples for any of the cytokine receptors selected, with the exception of TNFR1 after treatment with IL-1β + ML184. Results from 24 hour samples showed significant changes in receptor expression. After 24 hours of IL-1β, pre-treatment with ML184 significantly attenuated increased expression of mRNA for IL-1R1 and IL-6st as compared to IL-1β-treated samples. At the same time, pre-treatment with ML184 during insult with IL-1β significantly increased expression of TNFR2 and IL-10Rα. These results were mediated through GPR55 as concurrent treatment with ML193 attenuated all effects seen with administration of ML184, thus suggesting that activation of GPR55 during inflammatory insult with IL-1β *in vitro* is neuroprotective through immune mechanisms not directly related to neuronal differentiation.

3.3 Activation of GPR55 on murine neural stem cells increases neurogenesis and is protective against reduced neuron formation due to IL-1β *in vitro*.

Because activation of GPR55 increases hNSC neuronal differentiation under homeostatic conditions (Hill et al. 2018) and GPR55 activation is protective against IL-1β-induced reduction in neurogenesis, we next sought to determine if these effects were also observed in mouse primary hippocampal NSC cultures. Hippocampal NSCs were dissected out of 4–6 week-old C57BL/6 mice and cultured as an adherent monolayer. To determine if activation of GPR55 on mNSCs had effects similar to that seen with hNSCs, murine hippocampal cultures under differentiating conditions were treated with O-1602 (2 μM) for 5 days. Different pharmacological agents were utilized in murine experiments because ML184 has a piperazine structure which precludes this compound from activating the rodent receptor

(Brown et al. 2011; Lingerfelt et al. 2017). Samples were immunostained for βIII-tubulin and GFAP (Figure 3A) and assessed for total percentage of cells positively stained for either marker similar to studies performed with hNSC samples. O-1602 treatment significantly increased the percentage of βIII-tubulin positive cells (20.46% ± 0.92) as compared to vehicle treated samples (12.83% ±1.09; $F(3,12) = 24.91$, $p<0.0001$, $n=4$) as seen in Figure 3B. Consistent with results with hNSCs, blocking activation by the GPR55 antagonist CID-16020046 (10μM) significantly reduced βIII-tubulin positive cells (7.69% ± 0.76) as compared to vehicle. Combination treatment of O-1602 + CID-16020046 also resulted in reduced βIII-tubulin positive cells as compared to vehicle yet these results did not reach statistical significance. GFAP positive cells were reduced with O-1602 treatment (26.81% ± 1.15) in comparison to vehicle (32.94% ± 2.25; $F(3,12) = 5.139$, $p=0.0163$, $n=4$) yet post hoc analysis did not show significant differences between these two groups (Figure 3C). Cells treated with O-1602 did demonstrate significant differences in GFAP positive cells when compared to both CID-16020046 treatment alone (37.39% ± 3.24) or combination treatment of O-1602 + CID-16020046 (40.16% ± 2.22), yet these conditions did not differ significantly from vehicle-treated samples. Inflammatory insult (10 ng/mL of IL-1β) decreased βIII-tubulin expression (4.93% ± 0.64; $F(4,15) = 60.84$, $p<0.0001$, $n=4$) while pre-treatment with O-1602 attenuated this reduction (10.84% ± 0.66)(Figure 3D). Pharmacological blockade of GPR55 during insult displayed similar results to IL-1β treatment alone (4.52% ± 0.39) or treatment with IL-1β plus agonist (4.31% ± 0.41), suggesting that the neuroprotective effects of O-1602 are GPR55- dependent. As expected, treatment with IL-1β significantly increased the number of GFAP positive cells (50.59% ± 1.75; $F(4,15) = 20.56$, $p<0.0001$, $n=4$) in comparison to vehicle, while GPR55 activation attenuated these effects (27.86% ± 1.76) as seen in Figure 3E. Administration of CID-16020046 alone (47.84% ± 2.79) or in combination with O-1602 (43.84% ± 1.69) did not alter GFAP positive cell numbers in comparison to IL-1β, yet was significant in comparison to IL-1β + O-1602 treated samples. In summary, we demonstrate for the first time that GPR55 activation in cultured murine hippocampal NSCs increases neuronal differentiation under homeostatic conditions and is protective against reduced neurogenesis caused by insult with IL-1β.

3.4 mNSC cytokine receptor expression is altered by GPR55 activation after inflammatory insult with IL-1β.

We next determined whether murine NSC cytokine receptor mRNA expression was altered by GPR55 activation as seen in the hNSC experiments. Again, we treated mNSC cultures with O-1602 (2 μM) or CID-16020046 (10 μM) 30 minutes prior to inflammatory insult with IL-1β (100 ng/mL). In the case of treatment with both O-1602 and CID-16020046, CID-16020046 treatment was applied to cells 30 minutes prior to O-1602. Cells were collected at 4 or 24 hours post IL-1β treatment. In mNSC samples, we found a more rapid response in inflammatory cytokine mRNA upregulation due to IL-1β treatment as we did in the hNSC experiments. At the 4-hour time point, there was significant upregulation of all inflammatory cytokine receptor mRNA tested (IL-1R1, IL-1R2, TNFR1, TNFR2, IL-6st) (Figure 4 A-E) with IL-1β insult as compared to non-insulted vehicle control samples. Without inflammatory insult, neither O-1602 nor CID-16020046 administration had any significant effect on inflammatory receptor mRNA expression suggesting, like with human

cells, activation of GPR55 during non-inflammatory conditions either at 4- or 24-hour time points does not affect NSC immune responses. Interestingly, activation of GPR55 with O-1602 significantly attenuated the increase in IL-1R1 at 4 hours and 24 hours as compared to IL-1β treatment with or without antagonist. O-1602 treatment also attenuated IL-1β-induced increases in mRNA for IL-6st at 4 hours, but did not have significant effects at 24 hours even though there was a trend toward attenuated responses. Moreover, O-1602 significantly increased TNFR2 mRNA as compared to IL-1β treated samples. CID-16020046 abrogated the effects seen with O-1602, again suggesting that these responses are due to GPR55 activation. All statistics for mNSC mRNA studies can be found in Table 4. Together, these data suggest that during insult with IL-1β, activation of GPR55 suppresses pro-inflammatory responses within NSCs.

3.5 Direct intrahippocampal infusion of GPR55 agonist O-1602 is neuroprotective during chronic inflammation *in vivo*.

Previous work from our laboratory has reported that direct administration of O-1602 into the hippocampus of C57BL/6 mice increases hippocampal NSC proliferation and differentiation along a neuronal lineage (Hill et al. 2018). We wanted to test not only if administration of O-1602 would be neuroprotective during a state of inflammation, but also if a chronic low dose of LPS administered systemically would be sufficient to alter hippocampal neurogenesis. We treated male and female 12–15 week old C57BL/6 and GPR55$^{-/-}$ mice with LPS via osmotic mini-pump at a dose of 0.2 mg/kg/day for 14 days. Animals were also implanted with a second mini-pump connected to a stainless-steel cannula which administered O-1602 directly into the left hippocampus at a dose of 4 μg/kg/day. BrdU was administered twice per day on treatment days 1 and 2 and once per day on days 3 and 4. Animals were harvested after 14 days of continuous treatment and the hippocampus was sectioned for immunohistological analysis. Hippocampal NSC proliferation was assessed by positive staining for Ki67, a nuclear antigen expressed during all stages of the cell cycle except G_0(Fisher et al. 2002). Survival of NSCs was assessed by positive staining for BrdU and neuroblast/immature neuron formation was determined by staining for doublecortin (DCX) within the SGZ of the dentate gyrus within the hippocampus. Representative images can be seen in Figure 5A. Our results replicated data from our previous report concerning administration of O-1602 into the hippocampus, i.e., O-1602 increased NSC proliferation (Figure 5B; Interaction $F(1,28) = 0.028$, $p=0.869$; Inflammatory treatment $F(1,28) = 4.506$, $p=0.043$; Agonist treatment $F(1,28) = 21.9$, $p<0.0001$), survival (Figure 5C Interaction $F(1,28) = 1.518$, $p=0.228$; Inflammatory treatment $F(1,28) = 7.341$, $p=0.011$; Agonist treatment $F(1,28) = 30.57$, $p<0.0001$), and immature neurons (Figure 5D Interaction $F(1,28) = 0.1577$, $p=0.694$; Inflammatory treatment $F(1,28) = 19.93$, $p=0.0001$; Agonist treatment $F(1,28) = 28.06$, $p<0.0001$). There were also significant increases in the number of immature neurons formed(Interaction $F(1,28) = 1.023$, $p=0.321$; Inflammatory treatment $F(1,28) = 12.02$, $p=0.0017$; Agonist treatment $F(1,28) = 28.91$, $p<0.0001$). Interestingly, a low dose of chronic, systemic LPS negatively regulated neuronal differentiation and survival of NSCs. Animals treated with LPS had significantly reduced cell numbers that were DCX+ (7218 ± 399.1), BrdU+ (1243 ± 87.79), and DCX+/BrdU+ (643.5 ± 57.76)($n=8$) as compared to saline treated animals (DCX+ (9133 ± 445.5), BrdU+ (1680 ± 89.56), and DCX+/BrdU + (1176 ± 90.84) ($n=8$). To our surprise, the number of Ki67+ cells within the SGZ were not

significantly reduced due to LPS treatment (Saline 1734 ± 138.4; LPS 1410 ± 96.26). This suggests that animals treated with LPS become tolerant to the dose of LPS; data presented later corroborates this idea. Administration of O-1602 had significant neuroprotective effects when given to animals also receiving LPS. There were significant increases in the number of DCX+ (9525 ± 292.3), BrdU+ (2032 ± 182.1), and DCX+/BrdU+ (1403 ± 167) (n=8) cells as compared to animals given LPS with vehicle administration into the hippocampus. Similar to our previous report (Hill et al. 2018), GPR55$^{-/-}$ animals displayed significantly reduced rates of NSC proliferation (Ki67+, 890 ± 110.5), BrdU incorporation (BrdU+, 390.8 ± 65.5), immature neurons (DCX+, 1859 ± 220.7), and immature neuron formation (DCX+/BrdU+, 209.6 ± 35.9)(n=6) as compared to C57BL/6 animals. Our current study also shows that O-1602 treatment did not alter neurogenesis markers as compared to saline-treated GPR55$^{-/-}$ animals (Ki67+, 837 ± 55.1; BrdU+, 417.1 ± 60.2; DCX+, 1758 ± 94.7; DCX+/BrdU+, 231.1 ± 28.7)(n=7). LPS treatment of GPR55$^{-/-}$ mice showed significant reductions in immature neurons (DCX+, 1282 ± 52.3; Interaction $F(1,22) = 0.0012$, p=0.972; Inflammatory treatment $F(1,22) = 19.03$, p=0.0002; Agonist treatment $F(1,22) = 0.631$, p=0.435) and neuroblast formation (DCX+/BrdU+, 81.2 ± 9.1; Interaction $F(1,22) = 0.242$, p=0.627; Inflammatory treatment $F(1,22) = 42.02$, p<0.0001; Agonist treatment $F(1,22) = 0.156$, p=0.6968), yet results for proliferation (Ki67, 560.3 ± 102; Interaction $F(1,22) = 0.104$, p=0.749; Inflammatory treatment $F(1,22) = 11.66$, p=0.0025; Agonist treatment $F(1,22) = 0.1197$, p=0.733) and survival (BrdU+, 285.6 ± 40.3; Interaction $F(1,22) = 0.030$, p=0.864; Inflammatory treatment $F(1,22) = 5.837$, p=0.0244; Agonist treatment $F(1,22) = 0.157$, p=0.696)(n=6), although reduced, were not significant as compared to saline-treated GPR55$^{-/-}$ animals. O-1602 also did not have any effect in any conditions during LPS treatment (Ki67+, 558.3 ± 78.9; BrdU+, 296.7 ± 29.8; DCX+, 1182 ± 85.2; DCX+/BrdU+, 80.6 ± 9.6)(n=7). Thus, administration of the GPR55 agonist O-1602 displayed significant neuroprotective effects on hippocampal NSC survival and neuroblast formation. This effect was GPR55-dependent due to lack of effect seen in GPR55$^{-/-}$ animals.

3.6 Microglial activation is not attenuated by intrahippocampal administration of GPR55 agonist O-1602 during chronic inflammation *in vivo.*

Previous reports indicated that administration of LPI to hippocampal slice cultures from rats protected against neuronal damage due to excitotoxic lesion in a microglial-dependent manner (Kallendrusch et al. 2013). Based on this report, as well as data showing GPR55 expression on microglia, we sought to determine if treatment with O-1602 *in vivo* had any effect on microglial activation. In order to visualize activation changes of resident microglia, hippocampal sections were stained for IBA-1 and CD68, which is upregulated during inflammatory insult, and subsequently visualized by confocal microscopy (Ramprasad et al. 1996; Neumann, Kotter, and Franklin 2009). Z-stack images were acquired (600 μm x 600 μm field) which encompassed part of the dentate gyrus, hilus, and molecular layer. A fixed threshold was then used to include all positive fluorescent signal and 3D surfaces were generated using NIS-Elements software. Representative images are shown in Figure 6A. Evidence suggest that microglia increase in number in response to inflammatory conditions, so we investigated if there were any changes in microglia number or phagocytic activity between Saline- treated C57BL/6 and GPR55$^{-/-}$ animals (Nimmerjahn, Kirchhoff, and Helmchen 2005). Interestingly, there were no significant differences in the number of

IBA-1+ microglia (Figure 6B), percent of volume that was IBA-1+ (Figure 6C), percent of volume that was CD68+ (Figure 6D), percent of volume of colocalized IBA-1+/CD68+ (Figure 6E), or the percent of IBA-1+ microglia colocalized with CD68 (Figure 6F) between C57BL/6 and GPR55$^{-/-}$ animals. LPS-treated animals showed significant increases in all analyses as compared to saline-treated animals; importantly, these effects were not altered by administration of O-1602. Results showed significant increases in the number of IBA-1+ microglia within each ROI (C57BL/6 Interaction F(1,18) = 0.02381, p=0.8791, Inflammatory treatment F(1,18) = 63.84, p<0.0001, Agonist treatment F(1,18) = 0.2979, p=0.1015, GPR55$^{-/-}$ Interaction F(1,20) = 3.54e-004, p=0.9852, Inflammatory treatment F(1,20) = 24.55, p<0.0001, Agonist treatment F(1,20) = 0.6146, p=0.4422). We also saw significant increases in the percent of volume that was IBA-1+ within each ROI of LPS treated animals (C57BL/6 Interaction F(1,18) = 0.2547, p=0.619, Inflammatory treatment F(1,18) = 84.89, p<0.0001, Agonist treatment F(1,18) = 0.101, p=0.7542, GPR55$^{-/-}$ Interaction F(1,20) = 0.0356, p=0.8522, Inflammatory treatment F(1,20) = 44.43, p<0.0001, Agonist treatment F(1,20) = 1,145, p=0.2974). We then determined that CD68+ volume was increased significantly as well further suggesting an increase in inflammatory state within the brain of LPS-treated animals (C57BL/6 Interaction F(1,18) = 0.06321, p=0.8043, Inflammatory treatment F(1,18) = 22.74, p=0.0002, Agonist treatment F(1,18) = 1.07, p=0.3147, GPR55$^{-/-}$ Interaction F(1,20) = 6.157e-003, p=0.9382, Inflammatory treatment F(1,20) = 78.75, p<0.0001, Agonist treatment F(1,20) = 0.7335, p=0.4019). Next, we sought to determine the percentage of volume that was both IBA-1+ and CD68+ within each ROI. Results showed significant increases in the percent volume of IBA-1+ signal colocalized with CD68+ due to LPS treatment (C57BL/6 Interaction F(1,18) = 0.128, p=0.7247, Inflammatory treatment F(1,18) = 21.22, p=0.0002, Agonist treatment F(1,18) = 2.771e-005, p=0.9959, GPR55$^{-/-}$ Interaction F(1,20) = 3.955e-004, p=0.9843, Inflammatory treatment F(1,20) = 63.39, p<0.0001, Agonist treatment F(1,20) = 0.6291, p=0.4370). Lastly, we determined the percentage of IBA-1+ signal that was colocalized with CD68. Our results indicate that there was a significant increase in the percentage of IBA-1+ signal that was colocalized with CD68 due to inflammatory insult suggesting significant upregulation of CD68 by microglia (C57BL/6 Interaction F(1,18) = 0.3479, p=0.5626, Inflammatory treatment F(1,18) = 20.64, p=0.0003, Agonist treatment F(1,18) = 7.572e-003, p=0.9783, GPR55$^{-/-}$ Interaction F(1,20) = 1.812, p=0.1933, Inflammatory treatment F(1,20) = 42.07, p<0.0001, Agonist treatment F(1,20) = 0.1166, p=0.7364). Together, these results suggest that the neuroprotective effects of O-1602 treatment on NSCs were not due to reduced microglial activity within the hippocampus because O-1602 treatment did not alter increases in microglial number or expression of microglial activation markers after 14 days of low-level, systemic inflammation.

3.7 Lack of GPR55 induces altered inflammatory responses after 14 days of chronic, low-level systemic inflammation *in vivo*.

We next wanted to determine if lack of GPR55 has an effect on the immune response to chronic, low-level LPS administration. For this experiment, osmotic mini-pumps containing either LPS (0.2 mg/kg/day) or saline were implanted sub-dermally in C57BL/6 and GPR55$^{-/-}$ mice. These animals did not receive a second pump connected to cannula so as to not have any breach or trauma to the CNS. Brains were harvested after 14 days of treatment

and the hippocampal region was dissected out for RNA isolation. Samples were then analyzed for gene expression via the QIAseq mouse inflammation and immunity transcriptome array (RMM005Z, Qiagen). Heatmaps were generated (Figure 7A) from the resulting analysis and graphical analysis of molecular targets were grouped based on type of target; cytokines (Figure 7B), co-stimulators (Figure 7C), chemokines (Figure 7D), and LPS-related signaling (Figure 7E). C57BL/6 and GPR55$^{-/-}$ animals treated with saline showed no differences in any of the targets analyzed, except for significant reductions in GPR55$^{-/-}$ samples of the chemokine CCL3 and co-stimulator Bst2 and significant upregulation of CCL25. TLR4 in GPR55$^{-/-}$ animals was also upregulated, but results were not significant. LPS treatment in C57BL/6 mice showed a significant increase in CD86 while also displaying a trend in reduction of inflammatory cytokines (IL-1β, TNFα, IL-6, IL-12A, IL-12B), Myd88, and TRAF6. GPR55$^{-/-}$ animals treated with LPS exhibited significantly higher levels of inflammatory cytokines and the co-stimulators ICAM-1 and TICAM2 with a trend in upregulation of TRAF6. These animals also displayed significant reduction of co-stimulators Bst2 and H2-Dma. Statistics for hippocampal RNA expression data are listed in Table 5. Taken together, these data suggest that C57BL/6 mice were becoming tolerant to the low-level inflammation being administered, while GPR55$^{-/-}$ animals had an altered immune response resulting in higher levels of inflammatory mediators within the hippocampus.

4. Discussion

Dysregulation of adult hippocampal neurogenesis is implicated in numerous pathological conditions including Alzheimer's disease, HIV-1 associated neurocognitive disorder, virus-induced memory dysfunction, and depression (Mu and Gage 2011; Okamoto et al. 2007; Garber et al. 2018; Mahar et al. 2014). A common trait of each of these conditions is a chronic upregulation of inflammatory mediators leading to a neuroinflammatory state. Recently, the cannabinoid system has been implicated in NSC physiology and protection during pathophysiological conditions (Rodrigues et al. 2017; Palazuelos et al. 2006; Avraham et al. 2014). A potential member of the cannabinoid family, GPR55, has also been shown to regulate NSC proliferation and neuronal differentiation (Hill et al. 2018), yet comprehensive studies regarding GPR55 activation on NSCs during insult with inflammatory mediators are lacking.

In the present study, we showed that reduced neuron formation *in vitro* induced by IL-1β was ameliorated by activation of GPR55 in both human and mouse cultures of NSCs. Pre-treatment with GPR55 agonists also blocked upregulation of inflammatory cytokine receptor mRNA (IL-1R1, IL-6st) while increasing mRNA for anti-inflammatory or neuroprotective cytokine receptors (IL-10Rα, TNFR2) within NSCs. This was apparent in both human and mouse cells. We also show for the first time that GPR55 activation induces neuronal differentiation of murine primary hippocampal NSCs similar to results seen with human NSCs as reported previously (Hill et al. 2018). *In vivo*, we sought to determine if a chronic, low-level inflammation (systemic administration of LPS) would elicit negative effects on hippocampal neurogenesis similar to those seen after high level, acute infection. Interestingly, a dose of 0.2 mg/kg/day of LPS was sufficient to significantly reduce NSC survival (as measured by BrdU) and neuroblast formation within the SGZ of the

hippocampus. Direct intrahippocampal administration of O-1602, a potent GPR55 agonist, protected against reduced NSC survival and neuroblast formation during insult with chronic, low-level infusion of LPS. We determined that neuroprotective effects were elicited by direct involvement on NSCs as microglial activation (detected by comprehensive image analysis) did not differ between LPS animals that received vehicle as compared to O-1602 treatment. To further assess GPR55 function during the immune response, we treated animals with a low-level, chronic LPS paradigm and assessed inflammatory mRNA levels within the hippocampus showing an altered immune response in $GPR55^{-/-}$ animals. The presented data suggest that GPR55 can protect NSCs against inflammatory insult and poses a potent target with therapeutic potential.

Inflammatory insult with IL-1β on NSCs has been extensively studied showing reduced proliferation and neuronal differentiation rates both *in vitro* and *in vivo* (Wu et al. 2013; Zhang et al. 2013; Crampton et al. 2012). We chose to focus on IL-1β for *in vitro* studies since insult with IL-1β has also been studied in the context of cannabinoid signaling and neuroprotection (Garcia-Ovejero et al. 2013). Our results indicate that pre-treatment with the GPR55 agonists ML184 or O-1602 on human or mouse NSCs, respectively, is neuroprotective against reduced neuronal formation caused by insult with IL-1β. This effect was attenuated by concurrent treatment with GPR55 antagonist, suggesting that these effects are GPR55-dependent. These results are consistent with other studies of the effects of CB_1 and CB_2 receptors on protection against insult by IL-1β implicating GPR55 as another target for therapeutic intervention against NSC injury. O-1602 has also been implicated in protection of neuronal cell viability, yet the exact receptor necessary for this effect was not elucidated (Janefjord et al. 2014).

The number of GFAP positive cells was also decreased in samples treated with GPR55 agonist in combination with IL-1β indicating that this was not simply a blunting of gliogenesis, but rather a protective effect of GPR55 activation. Interestingly, we found that treatment of human NSCs with IL-1β reduced the number of GFAP positive cells in comparison to vehicle- treated samples. This observation goes against the notion that gliogenesis is increased during insult with inflammatory mediators. We speculate that this may be due to slowing of proliferation rates, thus resulting in fewer positive cells. Our studies in primary murine NSCs did show increases in GFAP-positive cells with IL-1β treatment similar to findings from other studies suggesting to us that our methods for quantification of neurogenesis were not faulty (Green and Nolan 2012; Crampton et al. 2012). Numerous mechanisms have also been linked to IL-1β-induced reductions in neurogenesis directly within NSCs, including regulation of the transcription factor TLX and p53-dependent apoptosis (Ryan et al. 2013; Guadagno et al. 2015). We did not examine directly intracellular mechanisms in which GPR55 activation protected against reduced neurogenesis facilitating a need for more in-depth study of the exact mechanisms necessary for the neuroprotective effects of GPR55.

In order for cytokines to exert any effect on the NSC population, they must first bind to specific receptors on the cell surface. Numerous pro- and anti-inflammatory cytokines are able to exert effects on the NSC population, including IL-1β through IL-1R1 and IL-1R2, TNFα through TNFR1 and TNFR2, IL-6 through the surface co-receptor IL-6st (gp130),

and the anti-inflammatory cytokine IL-10 through IL-10Rα (Green and Nolan 2012; Ben-Hur et al. 2003; Chen and Palmer 2013; Kotasova, Prochazkova, and Pachernik 2014; Perez-Asensio et al. 2013). To investigate if activation of GPR55 had any effect on the acute immune response by NSCs to IL-1β, we measured mRNA expression of known cytokine receptors expressed by NSCs (IL-1R1, IL-1R2, TNFR1, TNFR2, IL-6st, IL-10Rα) at 4 and 24 hours post insult. We found that activation of GPR55 reduced increases in pro-inflammatory receptor mRNA (IL-1R1, IL-6st) while further promoting anti-inflammatory receptor mRNA (IL-10Rα, TNFR2) during insult with IL-1β. It is interesting that activation of GPR55 induced effects on some specific cytokine receptors while not affecting others. IL-1R1 is the primary receptor through which IL-1β signals and GPR55 significantly attenuated increases due to IL-1β treatment while having no effect on IL-1R2, a decoy receptor with no signaling capacity (Wang et al. 2007; Koo and Duman 2008; Colotta et al. 1993). Activation of TNFR1 is known to induce detrimental effects on neurogenesis. GPR55 agonist treatment of NSCs during insult did not alter TNFR1 mRNA levels; however, TNFR2, which has been shown to be neuroprotective, was significantly upregulated as compared to IL-1β treatment alone (Chen and Palmer 2013). IL-6st increases were also attenuated by GPR55 activation yet this signal transducer functions not only for IL-6 mediated signaling, but also for IL-11, leukemia inhibitory factor (LIF), and oncostatin M (OSM), which are all part of the IL-6 family of cytokines (Kwak et al. 2010). Insult with IL-1β upregulated IL-10Rα mRNA in human samples and pre-treatment with ML184 significantly increased this upregulation. It should be noted that we could not determine expression of IL-10Rα on murine hippocampal NSC samples so analysis of effects of GPR55 activation could not be performed. Results from inflammatory response experiments are indicative of an anti-inflammatory, or neuroprotective, mechanism through which GPR55 is actively attenuating the ability of pro-inflammatory cytokines (IL-1β, IL-6) to signal while increasing the effect of anti-inflammatory and neuroprotective signals (TNFα via TNFR2, IL-10). It is unclear how activation of GPR55 affected changes to mRNA levels. The genes for these receptors, either human or mouse, are on different chromosomes (except IL-1R1 and IL-1R2; human-chromosome 2, mouse-chromosome 1) so it is unlikely that there is only one specific loci or promoter where these effects are taking place, yet treatment with GPR55 may be inducing upregulation of transcription factors necessary for cytokine receptor transcription. Another possibility is that GPR55 signaling increases post-transcriptional degradation of IL-1R1 and IL-6st mRNA through a yet unknown mechanism. IL-1R1 mRNA degradation has been shown during inflammatory states by microRNAs through targeting of the 3’UTR suggesting that the process by which GPR55 activation regulates inflammatory cytokine receptor mRNA expression could be microRNA-dependent (Skinner et al. 2017; Halappanavar et al. 2013). Regulation of microRNA by the cannabinoid system, specifically the CB_2 receptor, results in protective effects against acute liver failure caused by d-galactosamine/LPS, further supporting the possibility that GPR55 may act in a similar fashion(Tomar et al. 2015). Further study is needed to fully elucidate these mechanisms.

A major facet of neurodegenerative disorders and viral infection is a chronic upregulation of neuroinflammatory mediators and subsequent reductions in hippocampal neurogenesis leading to cognitive impairment (Ferguson et al. 2016; Zonis et al. 2015; Ferrell and Giunta

2014; Belarbi et al. 2012). We wanted to understand if low levels of chronic, systemic inflammation could exert negative effects on neurogenesis similar to studies utilizing acute, high dose, or direct administration of inflammatory mediators to the CNS. We therefore utilized osmotic mini-pumps to deliver LPS (0.2 mg/kg/day) sub-dermally for 14 days and found significant reductions in NSC survival and immature neuron formation within the hippocampus. These results indicate that even very low levels of systemic inflammation can, over time, induce negative regulation of hippocampal neurogenesis. Interestingly, NSC proliferation within the SGZ (Ki67+) was not significantly reduced 14 days after pump implantation. Taken together with data showing significant reduction of BrdU+ cells (BrdU was administered on days 1–4 of LPS treatment), these data suggest that the animals may be acclimating to the levels of LPS being administered and attenuating their immune responses. Tolerance of LPS-induced signals impairs the production of pro-inflammatory cytokines without inhibiting the expression of anti-inflammatory mediators and occurs through numerous mechanisms including reduction in TLR4, MyD88, IRAK-1, and TRAF6 (Nomura et al. 2000; Oak et al. 2006; Li, Wang, and Redmond 2006; Xiong et al. 2011). Indeed, we did see trends in downregulation of mRNA for MyD88 and TRAF6 in the hippocampus of C57BL/6 animals chronically treated with LPS suggesting an increase in tolerance. We did not see changes in mRNA expression of TLR4 or IRAK-1 in C57BL/6 animals, but that does not rule out changes in protein levels because LPS tolerance has been shown to decrease protein levels while maintaining mRNA expression (Li, Wang, and Redmond 2006). Moreover, we observed a trend in reduction of mRNA for major cytokines including IL1β, TNFα, IL6, IL12A, and IL12B, further suggesting LPS tolerance in C57BL/6 mice. GPR55$^{-/-}$ mice showed some reduction of TLR4 and MyD88, yet these animals also displayed higher levels of TLR4 in the hippocampus compared to C57BL/6 animals. All other tolerance-like effects due to LPS treatment in C57BL/6 mice were altered in GPR55$^{-/-}$ animals. GPR55$^{-/-}$ mice also had increased mRNA expression of pro-inflammatory cytokines (IL1β, TNFα, IL12A) and increased IRAK-1 and TRAF6, suggesting that lack of GPR55 in these animals is altering the chronic immune response due to LPS administration. It is important to note that the animals tested for hippocampal mRNA expression did not receive cannula implantation nor any agonist treatment. The exact contribution GPR55 has on this immune response is still not fully understood and requires further study.

To combat the detrimental effects of neuropathological conditions, additional therapeutic interventions that either dampen chronic neuroinflammation or attenuate reductions in hippocampal neurogenesis are critically needed. The cannabinoid system has already been shown to rescue impaired neurogenesis and reduce inflammation due to the HIV-1 protein Gp120 through activation of CB_2 receptors (Avraham et al. 2014). Using our chronic, systemic inflammatory paradigm, we investigated if treatment with the GPR55 agonist O-1602 could protect against LPS-induced dysregulation of hippocampal neurogenesis. O-1602 significantly protected against reduction in hippocampal NSC survival (BrdU+), the number of immature neurons (DCX+), and proliferating NSC that ultimately became neuroblasts (DCX+/BrdU+) within the SGZ of the hippocampus. GPR55$^{-/-}$ animals showed reduced rates of NSC proliferation, survival, and neuroblast formation under non-inflammatory conditions similar to our previous studies(Hill et al. 2018). LPS treatment in

GPR55$^{-/-}$ animals further reduced the number of immature neurons (DCX+ and DCX+/BrdU+) as compared to saline treated animals. The number of Ki67+ and BrdU+ cells were also diminished yet these results were not significant. It may be that GPR55$^{-/-}$ animals had such low rates of NSC proliferation and immature neuron formation that, although LPS did have a trend in reduction, significance could not be achieved. Administration of O-1602 had no effect in GPR55$^{-/-}$ animals, either under control or inflammatory conditions, further demonstrating that the neuroprotective results seen in C57BL/6 mice were due to GPR55. These results demonstrate for the first time that activation of GPR55 elicits neuroprotective effects within the SGZ of the hippocampus during chronic neuroinflammatory conditions. It should be noted that O-1602 was administered at the same time as LPS suggesting that pre-treatment is therapeutic under inflammatory conditions which may be beneficial as a therapy for people predisposed to inflammatory diseases or at high risk for systemic inflammation. It is therefore necessary to determine if GPR55 is an effective therapeutic target after inflammatory conditions are already present.

Since GPR55 is expressed by primary microglia, it is possible that the neuroprotective properties of O-1602 *in vivo* during inflammatory insult may not have a direct NSC effect (Pietr et al. 2009). Indeed, activation of GPR55 by its endogenous ligand LPI shows neuroprotective effects in hippocampal slice cultures after glutamate induced excitotoxic lesion and on CA1 and CA3 hippocampal neurons after ischemic stroke (Kallendrusch et al. 2013; Blondeau et al. 2002). Protective effects in hippocampal slice cultures were found to be microglia-dependent, suggesting that targeting GPR55 reduces microglial activation under inflammatory conditions. Here we found that chronic LPS administration at a low dose induces microglial activation in both C57BL/6 and GPR55$^{-/-}$ mice as determined by increases in microglial number as well as increases in the volume percentage of the microglial marker IBA-1 and CD68, a protein found in microglial/macrophage lysosomes and endosomes commonly used as a marker for phagocytic microglia (Hoeijmakers et al. 2017; Hoeijmakers et al. 2018). It is known that during inflammatory insult microglia numbers increase as does the expression of CD68 by microglia, especially when these cells are engaging in phagocytosis (Nimmerjahn, Kirchhoff, and Helmchen 2005; Neumann, Kotter, and Franklin 2009; Schafer et al. 2012). O-1602 had no effect on the number of microglia present within the dentate gyrus, nor on increases in IBA-1 volume, CD68 volume, or the percentage of IBA-1 positive signal colocalized with CD68 suggesting that the neuroprotective outcomes seen on NSCs were not mediated by reductions in microglial activation. Moreover, we did not detect any differences in microglial number or marker expression between C57BL/6 and GPR55$^{-/-}$ animals. The lack of effect on microglia may not necessarily be due to lack of GPR55 signaling but may be due to the agonist used. LPI was seen to reduce microglial activation in the study by Kallendrusch et al., but LPI and O-1602 have different chemical structures and therefore may not act similarly on microglia. Another recent study by McHugh et al. describes an increase of microglial migration due to O-1602 on BV-2 microglia *in vitro,* but this action was mediated through another candidate cannabinoid receptor, GPR18 (McHugh et al. 2012). This other receptor may be present on microglia in the C57BL/6 and GPR55$^{-/-}$ CNS, but we do not believe that O-1602 is having any significant effect through activation of GPR18 because, again, we did not see any

differences in microglial activation between LPS-treated animals that received either vehicle or O-1602.

In summary, our findings support the hypothesis that activation of GPR55 protects against deficits in neurogenesis induced by inflammatory insult both *in vitro* and *in vivo* through direct targeting of NSCs. Better understanding of the mechanisms by which GPR55 provides neuroprotection is critically necessary and may provide a more directed target for future therapeutics.

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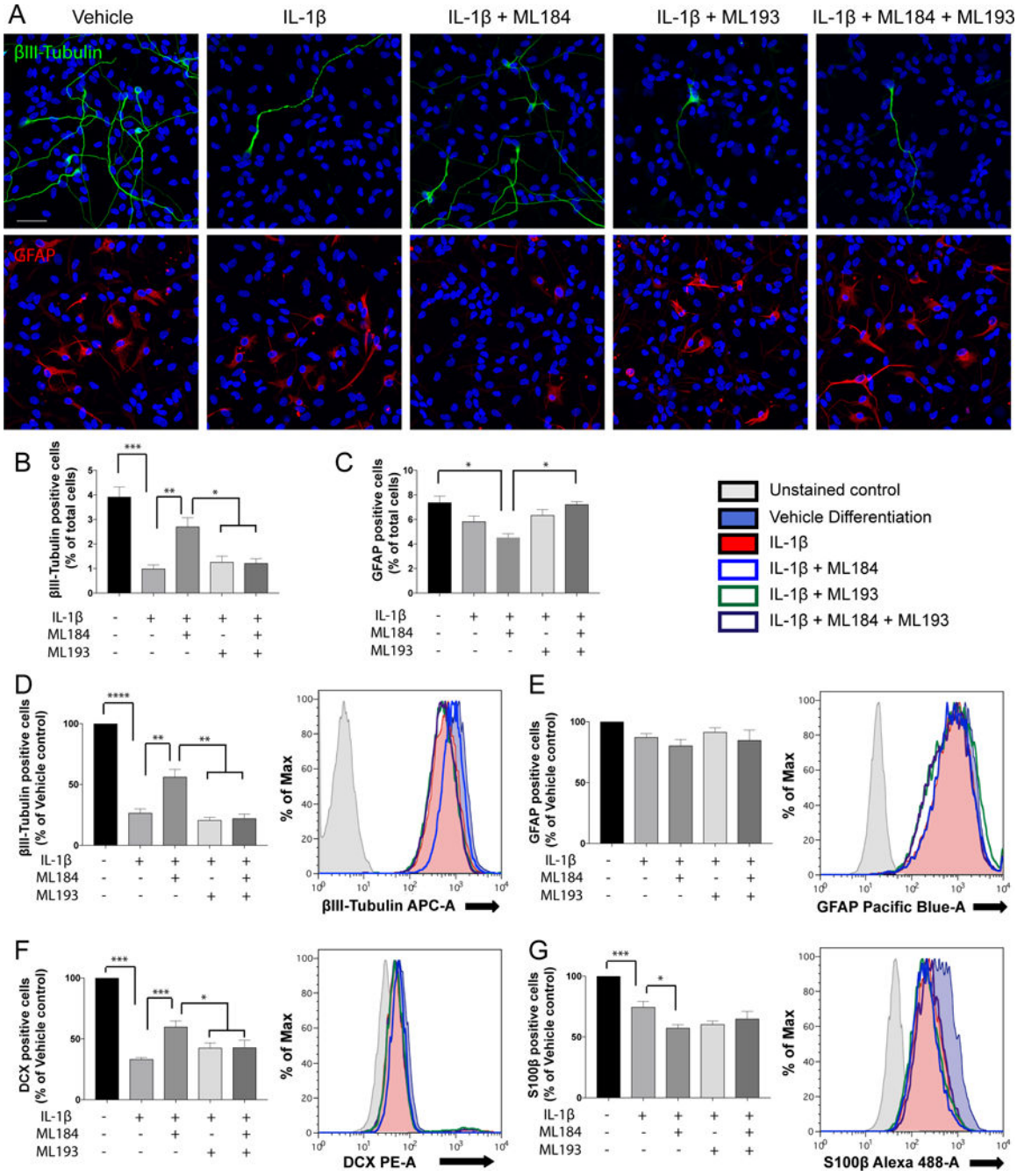

Figure 1: Activation of GPR55 on human neural stem cells is protective against reduced neuron formation due to IL-1β *in vitro*.

hNSCs (ReNcell VM) were cultured under differentiating conditions (removal of bFGF, EGF) in 8-well chamber slides for 10 days. Cells were treated with IL-1β (10 ng/mL) and either the GPR55 agonist ML184 (1 μM), GPR55 antagonist ML193 (5 μM), or both. Antagonist was added 30 minutes prior to agonist, and agonist was added 30 minutes prior to IL-1β. Half media was replaced every other day. Cells were then stained for neuronal (βIII-Tubulin; green) and glial (GFAP; Red) differentiation. Representative imaging is presented in A. Quantification of neurons and astrocytes derived from hNSC cultures are

shown in B and C, respectively. For flow cytometry studies, hNSCs were cultured in T-75 flasks as outlined above. After 10 days, cells were collected and stained for βIII-Tubulin (APC), DCX (PE), GFAP (Pacific Blue), and S100β (Alexa-488). Cytometric acquisition was performed using a BD FACS Canto II flow cytometer and analyzed with FlowJo software. Quantification of positive cells and representative histograms for βIII-Tubulin (D), GFAP (E), DCX (F), and S100β (G) are displayed. Bars represent mean ±SEM. Analysis was One-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; $n=5$ for IFA studies, $n=6$ for flow cytometry studies. Scale bar = 50 μM.

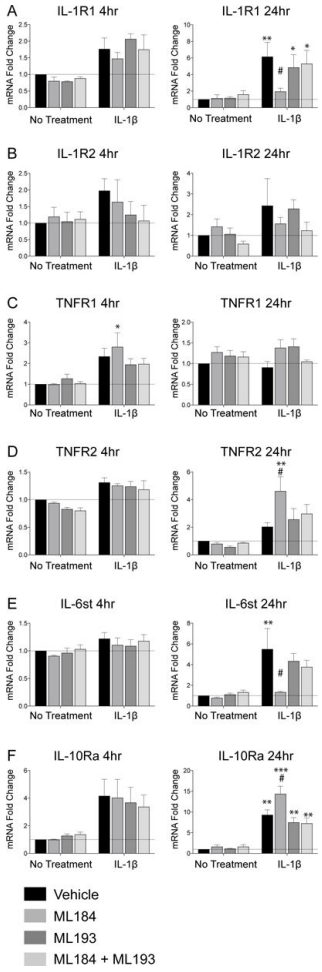

Figure 2: hNSC cytokine receptor expression is altered by GPR55 activation after inflammatory insult with IL-1β.

hNSCs were treated with GPR55 agonist ML184 (1 μM), GPR55 antagonist ML193 (5 μM) or both 30 minutes prior to inflammatory insult with IL-1β (100 ng/mL) under proliferative conditions. Cells were collected after 4 or 24 hours exposure to IL-1β. Expression of cytokine receptor IL-1RI (A), IL-1RII (B), TNFR1 (C), TNFR2 (D), IL-6st (E), and IL-10Rα (F) mRNA was assessed by qPCR. The data are represented as fold difference (mean ± SEM) of expression where expression of specific receptors on vehicle-treated cells was assigned a value of 1. Statistics were performed on ΔC_T values, Two-way ANOVA followed by Tukey's post hoc test; *p<0.05, ** p<0.01, *** p<0.001 as compared to vehicle treated control; #p<0.05 as compared to IL-1β treated samples; n=3 independent experiments.

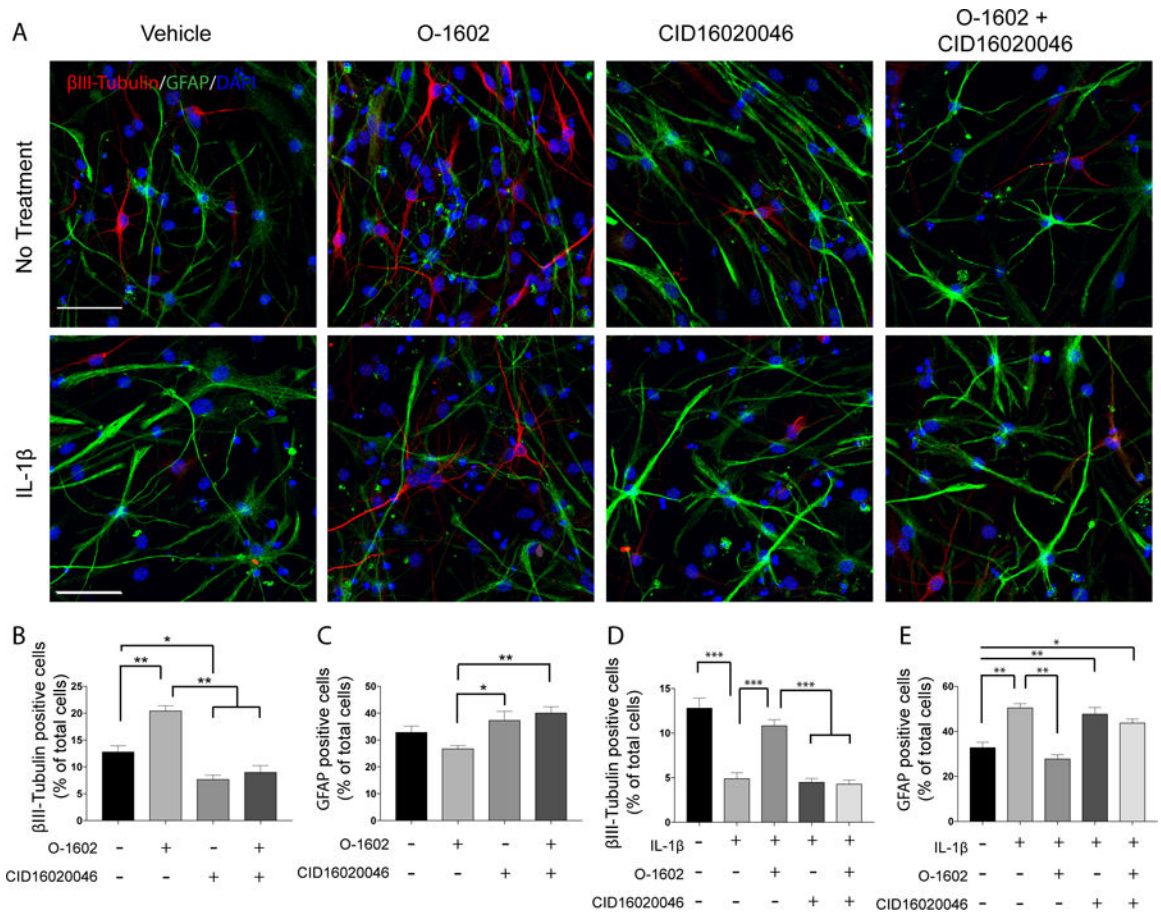

Figure 3: Activation of GPR55 on murine neural stem cells increases neurogenesis and is protective against reduced neuron formation due to IL-1β *in vitro*.

Primary neural stem cells were harvested from the hippocampus of C57BL/6 mice and cultured under proliferative conditions as an adherent monolayer. To determine rates of neurogenesis, cells were plated in 8 well chamber slides under differentiating conditions (removal of bFGF, EGF, addition of N2 supplement) and treated with vehicle (0.01% DMSO in medium), the GPR55 agonist O-1602 (2 μM), GPR55 antagonist CID16020046 (10 μM), or both. For inflammatory conditions, murine IL-1β (10 ng/mL) was added. Antagonist was added 30 minutes prior to agonist, and agonist was added 30 minutes prior to IL-1β. Half media was replaced every other day for a total of 5 days of differentiation. Cells were then stained for neuronal (βIII-Tubulin; red) and glial (GFAP; green) differentiation. Representative imaging is presented in (A). Quantification of neurons and astrocytes derived from mNSC cultures under homeostatic conditions is shown in B and C, respectively. Quantification of neurons and astrocytes derived from mNSC cultures under inflammatory conditions is shown in D and E, respectively. Bars represent mean ±SEM. One-way ANOVA followed by Tukey's post hoc test; *p<0.05, ** p<0.01, *** p<0.001 as compared to vehicle treated control; n=4 independent experiments. Scale bar = 50 μM.

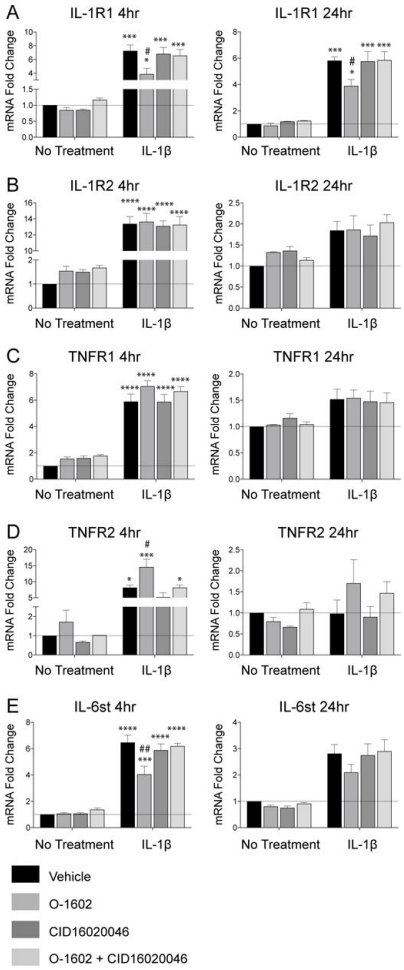

Figure 4: mNSC cytokine receptor expression is altered by GPR55 activation after inflammatory insult with IL-1β.

mNSCs were treated with GPR55 agonist O-1602 (2 μM), GPR55 antagonist CID16020046 (10 μM) or both 30 minutes prior to inflammatory insult with IL-1β (100 ng/mL) under proliferative conditions as an adherent monolayer. Cells were collected after 4 or 24 hours of exposure to IL-1β. Expression of cytokine receptor IL-1RI (A), IL-1RII (B), TNFR1 (C), TNFR2 (D), and IL-6st (E) mRNA was assessed by qPCR. The data are represented as fold difference (mean ± SEM) of expression where expression of specific receptors on vehicle-treated cells was assigned a value of 1. Statistics were performed on ΔC_T values; Two-way ANOVA followed by Tukey's post hoc test; *p<0.05, *** p<0.001, **** p<0.0001 as compared to vehicle treated control; #p<0.05, ##p<0.01 as compared to IL-1β treated samples; n=3 independent experiments.

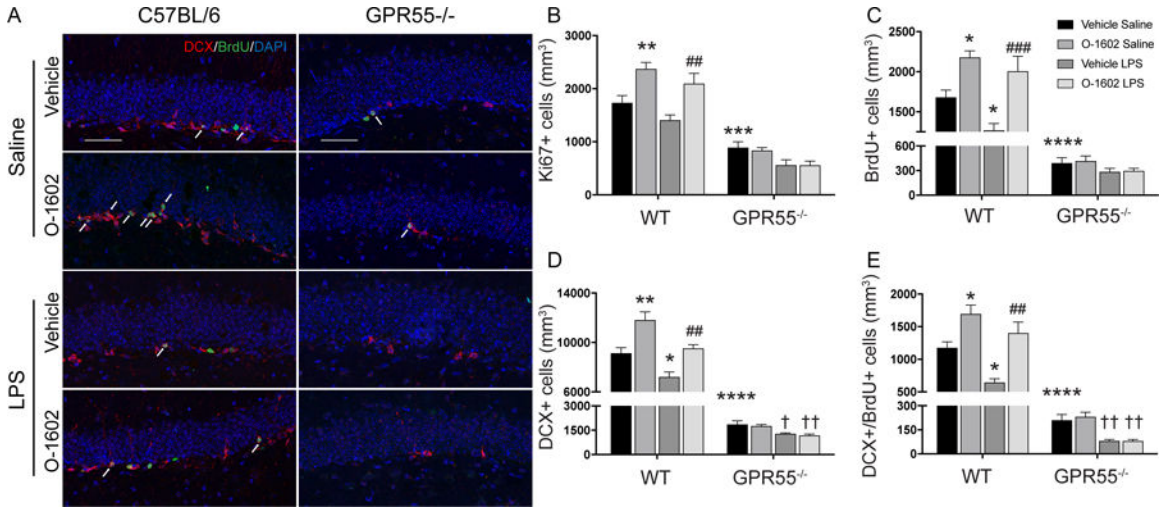

Figure 5: Direct intrahippocampal infusion of GPR55 agonist O-1602 is neuroprotective during chronic inflammation *in vivo*.

C57BL/6(WT) and GPR55$^{-/-}$ mice had osmotic pumps (Alzet) implanted sub-dermally for a continuous dispersion time of 14 days. Two pumps were implanted. The first pump contained vehicle or O-1602 and was connected to a stainless-steel cannula which was implanted into the left hippocampus. The second pump contained saline or LPS (0.2 mg/kg/day) and was implanted for systemic administration. (A) Representative images are shown depicting the dentate gyrus from 30 μm brain sections of 12–15-week-old WT and GPR55$^{-/-}$ mice immunostained with anti-BrdU(green) and anti-DCX antibodies(red). Scale bar = 50 μm. Quantitative analysis of Ki67 (B), BrdU (C), DCX (D), and DCX/BrdU (E) total positive cells per dentate gyrus per mouse. Bars represent mean ±SEM, n=6–8 mice per group. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 as compared to WT vehicle saline group; ##, p<0.01; ### p<0.001 as compared to WT vehicle LPS group; †, p<0,05; ††, p<0.01as compared to GPR55$^{-/-}$ vehicle saline group by Two-way ANOVA followed by Tukey's post hoc test.

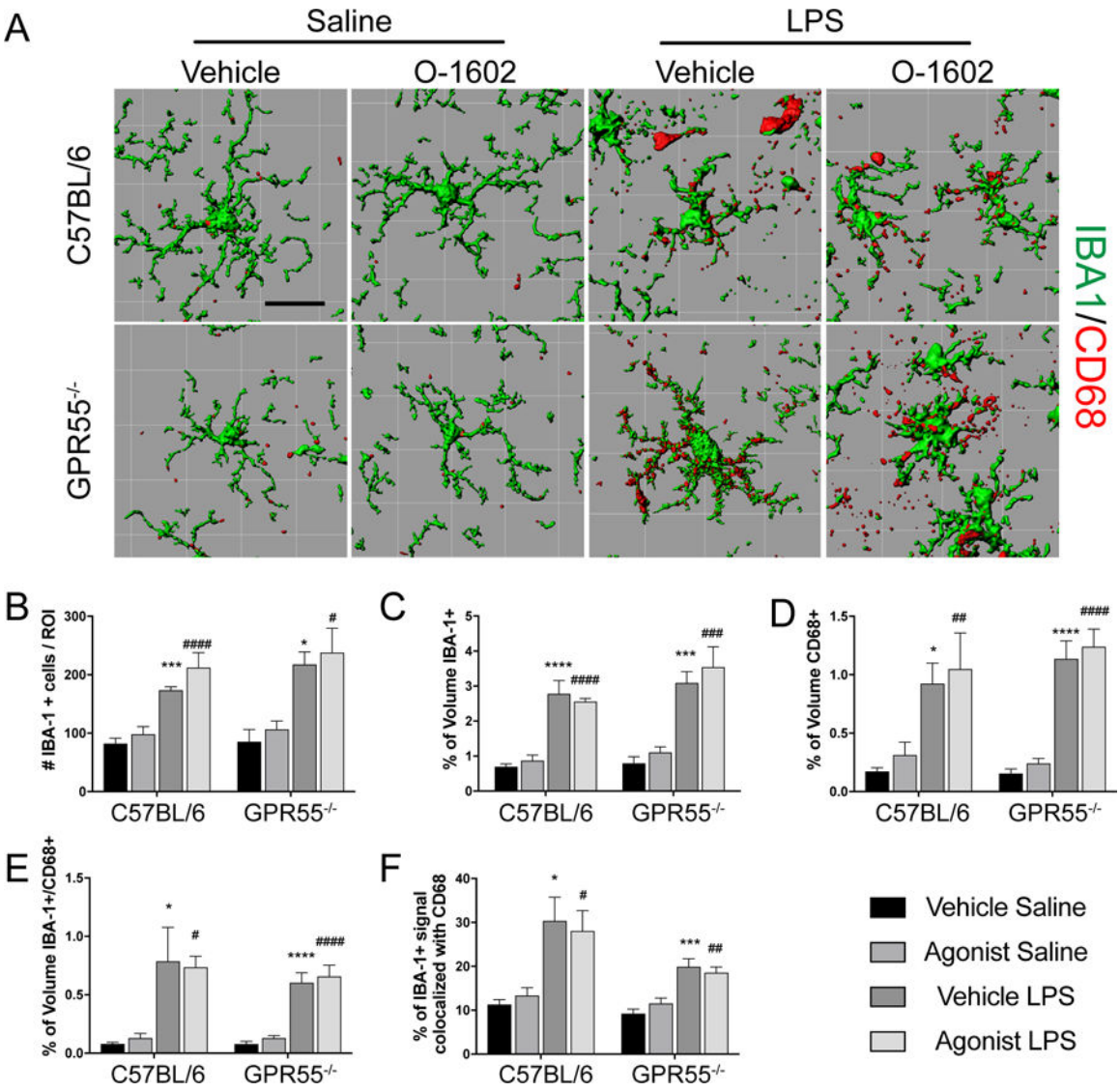

Figure 6: Microglial activation is not attenuated by intrahippocampal administration of GPR55 agonist O-1602 during chronic inflammation *in vivo*.

C57BL/6(WT) and GPR55$^{-/-}$ mice had osmotic pumps (Alzet) implanted sub-dermally for a continuous dispersion time of 14 days. Two pumps were implanted. The first pump contained vehicle or O-1602 and was connected to a stainless-steel cannula which was implanted into the left hippocampus. The second pump contained saline or LPS (0.2 mg/kg/day) and was implanted for systemic administration. (A) Representative images depicting the hippocampal area from 30 μm brain sections of 12–15-week-old WT and GPR55$^{-/-}$ mice immunostained with anti-IBA-1(green). Scale bar = 100 μm. Quantitative morphometric analysis of microglial volume (B) and surface area (C) depicting activated state of resident microglia due to systemic inflammation. Morphology was assessed using Imaris software. 3D surfaces were created on positive immunostained microglia to create representative whole cells. Insets in upper right quadrant of close up images are examples of single cell 3D surface renderings from Imaris software and are located in the white box of image. Volume of cells and surface area were determined from 3D representations. Bars

represent mean ±SEM. *, p<0.05; **, p<0.01 as compared to vehicle saline animals; #, p<0.05; ##, p<0.01 as compared to agonist saline animals within genotype by Two-way ANOVA followed by Tukey's post hoc test. n= 4 animals per group and analyzing a minimum of 300 positive cells per animal.

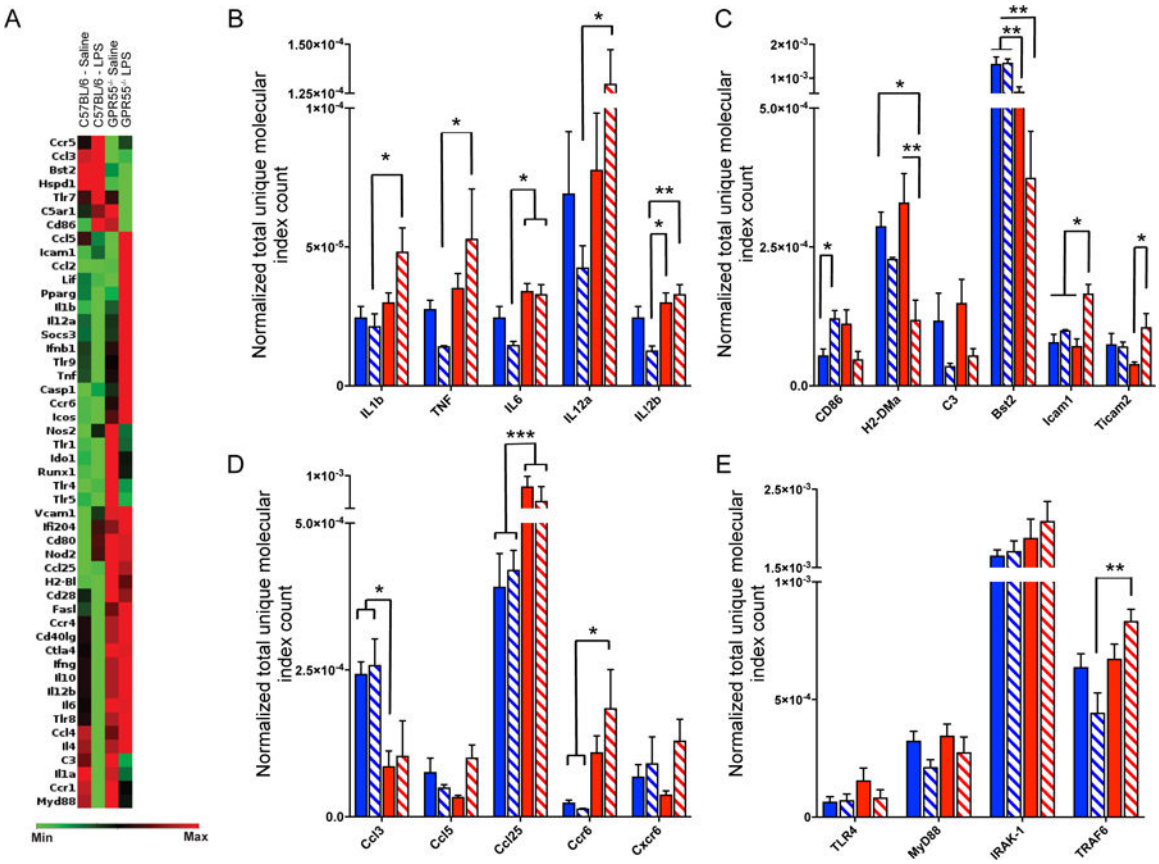

Figure 7: Lack of GPR55 induces altered inflammatory responses after 14 days of chronic, low-level systemic inflammation *in vivo*.

C57BL/6 and GPR55$^{-/-}$ mice were chronically treated with LPS (0.2 mg/kg/day) or saline (C57BL/6 + saline, n=5; C57BL/6 + LPS, n=4; GPR55$^{-/-}$ + saline, n=5; GPR55$^{-/-}$ + LPS, n=4) for 14 days. After harvest, the hippocampi were removed and processed for RNA. The expression of inflammation-related genes in the hippocampus was analyzed using the Qiagen mouse Inflammation and Immunity Transcriptome array. (A) Heat map analysis of inflammatory array data showing differentially expressed genes between groups. Normalized Total Unique Molecular Index Count for each gene was compared between groups. Results are displayed as pro-inflammatory cytokines (B), co-stimulators (C), chemokines (D), and LPS related signaling molecules (E). Bars represent mean ±SEM. $p<0.05$ was considered significant, represented as *$p<0.05$; ** $p<0.01$; ***$p<0.001$. Analysis was by Two-way ANOVA followed by Tukey's post hoc test.

Table 1:

Human qPCR primer/probe sets

Target	Assay ID	Amplicon Length
IL-10Rα	Hs00155485_m1	72
IL-1R1	Hs00991002_m1	152
IL-1R2	Hs01030384_m1	77
IL-6st	Hs00174360_m1	72
GAPDH	Hs02786624_g1	157
TNFR1	Hs01042313_m1	150
TNFR2	Hs00961749_m1	82

Table 2:

Mouse qPCR primer/probe sets

Target	Assay ID	Amplicon Length
IL-1R1	Mm00434237_m1	63
IL-1R2	Mm00439629_m1	65
IL-6st	Mm00439665_m1	80
TNFR1	Mm00441883_g1	82
TNFR2	Mm00441889_m1	64
Rn18s	Mm04277571_s1	115

Table 3:

Statistics for human NSC inflammatory response (Figure 2)

Two-way ANOVA	Interaction (Pharmacological treatment vs Inflammatory treatment) F(3,16)	Pharmacological treatment (vehicle vs agonist) F(3,16)	Inflammatory treatment (saline vs IL-1β) F(1,16)
IL-1R1 4 hr	F = 0.0495, p=0.9849	F = 0.0484, p=0.9854	F = 0.9355, p=0.3478
IL-1R1 24 hr	F = 3.414, p=0.0431	F = 2.735, p=0.0779	F = 24.98, p<0.0001
IL-1R2 4 hr	F = 0.5996, p=0.6245	F = 0.7969, p=0.5135	F = 7.8, p=0.0130
IL-1R2 24 hr	F = 0.4889, p=0.6948	F = 0.0349, p=0.9909	F = 2.494, p=0.1339
TNFR1 4 hr	F = 0.729, p=0.5495	F = 0.7627, p=0.5314	F = 12.04, p=0.0032
TNFR1 24 hr	F = 0.8552, p=0.8552	F = 1.041, p=0.4014	F = 0.0045, p=0.9475
TNFR2 4 hr	F = 0.1745, p=0.9121	F = 0.694, p=0.5690	F = 7.684, p=0.0136
TNFR2 24 hr	F = 2.729, p=0.0783	F = 4.28, p=0.0213	F = 26.03, p<0.0001
IL-6st 4 hr	F = 0.5328, p=0.6663	F = 0.6336, p=0.6041	F = 0.0026, p=0.9599
IL-6st 24 hr	F = 2.113, p=0.1387	F = 3.405, p=0.0434	F = 27.52, p<0.0001
IL-10Rα 4 hr	F = 0.1841, p=0.9056	F = 0.0784, p=0.9708	F = 45.07, p<0.0001
IL-10Rα 24 hr	F = 3.807, p=0.0311	F = 5.21, p=0.0106	F = 80.09, p=<0.0001

Table 4:

Statistics for mouse NSC inflammatory response (Figure 4)

Two-way ANOVA	Interaction (Pharmacological treatment vs Inflammatory treatment) F(3,16)	Pharmacological treatment (vehicle, agonist, antagonist) F(3,16)	Inflammatory treatment (saline vs IL-1β) F(1,16)
IL-1R1 4 hr	F = 2.769, p=0.0755	F = 3.304, p=0.0473	F = 136.6, p<0.0001
IL-1R1 24 hr	F = 1.027, p=0.3957	F = 1.757, p=0.1782	F = 105.9, p<0.0001
IL-1R2 4 hr	F = 0.1844, p=0.9054	F = 0.1499, p=0.9282	F = 666.1, p<0.0001
IL-1R2 24 hr	F = 0.4742, p=0.7027	F = 0.1797, p=0.9092	F = 12.55, p=0.0014
TNFR1 4 hr	F = 1.033, p=0.4043	F = 2.748, p=0.0770	F = 395.5, p<0.0001
TNFR1 24 hr	F = 0.1306, p=0.9411	F = 0.0552, p=0.9826	F = 10.93, p=0.0026
TNFR2 4 hr	F = 4.107, p=0.0297	F = 6.422, p=0.0067	F = 68.76, p<0.0001
TNFR2 24 hr	F = 1.11, p=0.3904	F = 1.607, p=0.2490	F = 4.315, p=0.0645
IL-6st 4 hr	F = 4.673, p=0.0158	F = 5.296, p=0.0100	F = 333.6, p<0.0001
IL-6st 24 hr	F = 0.346, p=0.7923	F = 0.5819, p=0.6318	F = 40.25, p<0.0001

Table 5:

Statistics for *in vivo* hippocampal inflammatory transcriptome analysis (Figure 7)

Two-way ANOVA F(1,14)	Interaction (Genotype vs Inflammatory treatment)	Genotype (C57BL/6 vs GPR55$^{-/-}$)	Inflammatory treatment (saline vs LPS)
IL1B	F = 4.435, p=0.0537	F = 10.11, p=0.0067	F = 2.226, p=0.1579
TNF	F = 3.307, p=0.0904	F = 7.342, p=0.0169	F = 0.063, p=0.8060
IL6	F = 2.367, p=0.1462	F = 21.8, p=0.0004	F = 3.582, p=0.0793
IL12A	F = 4.301, p=0.0570	F = 6.363, p=0.0244	F = 0.4474, p=0.5144
IL12B	F = 4.287, p=0.0574	F = 14.13, p=0.0021	F = 1.327, p=0.2686
CD86	F = 14.56, p=0.0019	F = 0.5926, p=0.4542	F = 0.0928, p=0.7651
H2-DMa	F = 4.42, p=0.0541	F = 0.8652, p=0.3680	F = 13.93, p=0.0022
C3	F = 0.0284, p=0.8686	F = 0.467, p=0.5055	F = 5.616, p=0.0327
BST2	F = 0.7985, p=0.3867	F = 41.06, p<0.0001	F = 0.4318, p=0.5217
ICAM1	F = 8.191, p=0.0125	F = 5.344, p=0.0365	F = 20.43, p=0.0005
TICAM2	F = 4.997, p=0.0422	F = 0.0014, p=0.9705	F = 3.966, p=0.0663
CCL3	F = 0.0014, p=0.9711	F = 17.48, p=0.0009	F = 0.192, p=0.6680
CCL5	F = 8.035, p=0.0132	F = 0.0722, p=0.7921	F = 1.543, p=0.2346
CCL25	F = 0.8894, p=0.3616	F = 61.16, p<0.0001	F = 0.2272, p=0.6410
CCR6	F = 1.698, p=0.2135	F = 15.31, p=0.0016	F = 0.9721, p=0.3409
CXCR6	F = 1.54, p=0.2351	F = 0.0212, p=0.8864	F = 4.253, p=0.0582
TLR4	F = 1.176, p=0.2965	F = 1.992, p=0.1800	F = 0.8237, p=0.3795
MYD88	F = 0.1872, p=0.6719	F = 0.7739, p=0.3939	F = 3.634, p=0.0773
IRAK1	F = 0.1718, p=0.6848	F = 2.633, p=0.1270	F = 0.5287, p=0.4792
TRAF6	F = 7.701, p=0.0149	F = 11.11, p=0.0049	F = 0.067, p=0.7993