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Leukemia inhibitory factor represses GnRH gene expression via cFOS during inflammation in male mice

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Short title: Neuroinflammation represses GnRH mRNA

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Abstract

Background: The mechanisms whereby neuroinflammation negatively affects neuronal function in the hypothalamus are not clear. Our previous study determined that obesity-mediated chronic inflammation elicits sex-specific impairment in reproductive function via reduction in spine density in GnRH neurons. Neuroinflammation and subsequent decrease in GnRH neuron spine density was specific for male mice, while protection in females was independent of ovarian estrogens.

Methods: To examine if neuroinflammation-induced cytokines can directly regulate GnRH gene expression, herein we examined signaling pathways and mechanisms in males *in vivo* and in GnRH-expressing cell line, GT1-7.

Results: GnRH neurons express cytokine receptors, and chronic or acute neuroinflammation represses GnRH gene expression *in vivo*. Leukemia inhibitory factor (LIF) in particular represses GnRH expression in GT1-7 cells, while other cytokines do not. STAT3 and MAPK pathways are activated following LIF treatment, but only MAPK pathway, specifically p38α, is sufficient to repress GnRH gene. LIF induces cFOS that represses GnRH gene via the -1793 site in the enhancer region. *In vivo*, following high fat diet, cFOS is induced in GnRH neurons and neurons juxtaposed to the leaky blood brain barrier of the organum vasculosum of the lamina terminalis, but not in the neurons further away.

Conclusion: Our results indicate that the increase in LIF due to neuroinflammation induces cFOS and represses GnRH gene. Therefore, in addition to synaptic changes in GnRH neurons, neuroinflammatory cytokines directly regulate gene expression and

reproductive function, and the specificity for neuronal targets may stem from the proximity to the fenestrated capillaries.

Introduction

Gonadotropin releasing-hormone (GnRH) is the final brain output for the regulation of reproduction. GnRH neurons, which are scattered in the hypothalamus, synthesize and secrete GnRH that acts on the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrope cells [1, 2]. LH and FSH act on the gonads to promote steroidogenesis and gametogenesis. GnRH neuronal processes, named “dendrons”, by Herbison group [3], form an interwoven network that receives direct synaptic and neuropeptide input from upstream regulatory neurons, most notably kisspeptin [4, 5]. This GnRH network integrates other signals that impinge on reproduction, such as stress [6, 7], endocrine disruptors [8], circadian rhythms [9, 10], metabolism [11, 12], and acute inflammation during infection [13-15].

Previous studies have implicated acute inflammation, elicited with an injection of lipopolysaccharide (LPS), in the impairment of reproductive function [14, 16, 17]. LPS challenged rodents exhibited reduced levels of LH and GnRH mRNA, diminished release of LH and GnRH, and increased levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the circulation [14, 18-20]. Centrally administered cytokines also provoked reduced LH and GnRH levels, but the mechanism whereby these cytokines mediate their effects is unknown [16, 17, 21-23]. More recently, our group determined that low-grade, chronic inflammation caused by

high fat diet (HFD)-induced obesity may also directly affect GnRH neurons, resulting in reduced levels of LH in circulation and diminished GnRH mRNA levels in the hypothalamus, specifically in male mice [24]. We, and others, have reported that diet-induced obese mice and people exhibited increased levels of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, in circulation [24, 25] and in the hypothalamus, at the mRNA level and protein level [24, 26]. Additionally, we also identified that leukemia inhibitory factor (LIF), a member of the IL-6 family, is increased in the circulation and locally produced in male mice hypothalami in obesity [24]. Interestingly, LIF is increased in a sex-specific manner only in males that exhibit reduction in GnRH mRNA and gonadotropin hormones, but not in females that lack changes in GnRH or gonadotropin hormones. IL-6, a prototypical member of the family, on the other hand, is increased in both sexes. Our previous study postulated that impairment of GnRH neurons stems from reduction in spine density and consequently the connectivity of the GnRH network [24]. However, GnRH neurons express several cytokine receptors [27] and inflammation-induced cytokines may directly regulate intracellular signaling pathways in the GnRH neurons.

Herein, we focus on delineating the mechanisms by which inflammatory cytokines influence GnRH gene expression to provide insight into the etiology of neuroinflammation-induced impairment of reproductive function. TNF-α, IL-1β, and IL-6 are key players in the regulation of immune response and inflammatory processes during infection [28]. In the central nervous system, TNF-α and IL-1β regulate synaptic plasticity, neurodegeneration, learning and memory [29-32]. During infection, both of these cytokines mediate the physiological and behavioral responses in sickness such

as, inducing fever, inhibiting food intake, causing nausea and fatigue [33-35]. TNF-α and IL-1β mediate their effects through activation of downstream signaling molecules: nuclear factor-kappa B (NFκ-B), Janus kinase and signal transducers and activators of transcription pathway (JAK-STAT) and mitogen activated protein kinases (MAPK) [36-38]. Similarly, IL-6 is produced in response to infection and stress, and in turn stimulates various cell populations, also through the JAK-STAT and MAPK pathways [39]. In the brain, IL-6 is involved in degenerative responses. [40, 41]. However, IL-6 is also induced following TNF-α or IL-1β treatment and is involved in the negative feedback that ultimately contributes to the dampening of the immune response and activating tissue repair [42].

LIF is a member of IL-6 family that is induced during inflammatory response [43]. However, its functions are not limited to inflammation: LIF has been demonstrated to play a crucial, non-redundant role in embryo implantation in both mice and humans [44-46]. LIF also maintains stem cells and regulates differentiation of germ cells [47, 48]. In the brain, LIF regulates neuronal function and neuronal response to injury [49-51]. With respect to GnRH neurons, LIF has been shown to regulate the migration of GN11 immature GnRH neuron cell line and regulate the release of GnRH in GT1-7 cells [52-54]. LIF binds its specific receptor, which, similarly to the other members of the IL-6 family, recruits and signals through the GP130 signals transducer, activating JAK-STAT pathway [45].

Signaling pathways involved in the regulation of GnRH gene transcription by any of these cytokines have not been elucidated. GnRH neurons in the rodent hypothalamus are located in the preoptic area surrounding organum vasculosum

laminae terminalis (OVLT) and send long processes to the median eminence (ME) where secretion occurs from the terminals. Both OVLT and ME are areas that contain fenestrated capillaries and a leaky blood-brain barrier [55]. A subpopulation of GnRH neurons extends their processes into the OVLT and across the blood-brain barrier, where they may be able to directly respond to circulating molecules, including cytokines [56]. OVLT and surrounding thermoregulatory neurons are involved in changes in body temperature and inducing fever in response to systemic inflammation. Pyrogenic, pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, produced locally in the hypothalamus, or from the circulation via fenestrated capillaries in the OVLT, stimulate thermoregulatory neurons to increase the body temperature [57-60]. We postulate that these cytokines directly regulate GnRH neurons in the proximity to OVLT.

About 800-1200 GnRH neurons are scattered throughout the forebrain of a mouse [61]. This poses a challenge for molecular studies of GnRH neurons *in vivo*. GT1-7 cells are the only model of mature, terminally differentiated, GnRH-producing neurons, and have been used to identify regulatory elements and transcription factors important for GnRH transcription [62, 63]. GT1-7 cells allow for the investigation of molecular mechanisms and direct effects on GnRH gene expression without confounding variables that may be present in *in vivo* studies. Here, we combine *in vivo* mouse studies and investigate the direct effects of cytokines on GnRH gene expression in GT1-7 cells. Our results delineate the molecular mechanisms and signaling pathways that LIF activates and strongly suggest that LIF directly affects GnRH neurons to regulate GnRH gene expression in infection or obesity induced inflammation of the hypothalamus.

Materials and Methods

Animals

C57BL/6J mice were maintained under a 12-h light, 12-h dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines. C57BL/6J male mice were placed on either a high fat diet (HFD, D12492, 60% kcal from fat; 5.21 kcal/g; protein 20% kcal; fat 60% kcal (lard 0.32 g/g diet, soybean oil 0.03 g/g); carbohydrate 20% kcal; Research Diet, New Brunswick, NJ) or control diet (CTR, D12450J, 10% kcal from fat; matching sucrose levels to HFD; 3.82 kcal/g; protein 20% kcal; fat 10% kcal (lard 0.02 g/g diet, soybean oil 0.025 g/g); carbohydrate 70% kcal; Research Diet, New Brunswick, NJ) from weaning age for 12 weeks. Mice treated with vehicle or LPS were fed standard food pellets (STD, 5053, 4.07 kcal/g; protein 24% kcal; fat 13%; carbohydrates 63%; St. Louis, MO) from weaning. Lipopolysaccharide (LPS, 2.5 mg/kg body weight) from Escherichia coli (catalog # L4391, Sigma, USA) was administered by intraperitoneal injection. Mice were sacrificed 24 h post LPS treatment in parallel with the corresponding control groups. At least 10 animals/diet or treatment were analyzed unless otherwise indicated, and differences from corresponding controls were compared by Student's t test. GnRH-GFP mice were kindly provided by Dr. Suzanne Moenter [64] to facilitate investigation of fluorescently labeled GnRH neurons.

Histological Analysis and Immunohistochemistry

For brain collection, animals were perfused with ice cold phosphate buffer saline (PBS) solution followed by 4% paraformaldehyde solution. Brains were post-fixed in 4% paraformaldehyde 2 h at room temperature and cryopreserved in 30% sucrose/PBS solution for 3 days at 4°C before freezing in OTC. Frozen brains from GnRH-GFP mice were sectioned to 30 µm sections and stained for GFP to visualize GnRH neurons and for GP130 or cFOS. Slides were blocked with 20% goat serum and incubated with primary antibodies against GFP (1:5000 raised in chicken, Table 1) at 4°C for 48 h. After PBS washes, slides were incubated with FITC/Alexa 488 goat anti-chicken IgG (1:300, Molecular Probes, Eugene, OR) for 1 h. Slides were then incubated with primary antibodies against GP130 (1:500, MAB4681, R&D Systems, Minneapolis, MN) or cFOS (1:300, SC-52, Santa Cruz Biotechnology, Inc. Dallas, TX) for 48 h at 4°C followed by Alexa 594 goat-anti-rat IgG (1:300, Molecular Probes, Eugene, OR) or biotinylated goat anti-rabbit IgG (1:300; Vector Laboratories, USA) and Cy5-streptavidin (1:300, 434316, Life Tech. Corp. Eugene, OR) for 1 h each at room temperature, respectively. Sections were mounted and slides covered using VectaSheild mounting media with DAPI (H-1500, Vector Laboratories, USA). Secondary antibody-only controls were performed to determine antibody specificity. Images were obtained using a Leica microscope system.

To quantify the number of cFOS expressing GnRH neurons, coronal sections of the preoptic area in the hypothalamus of GnRH-GFP mice were stained for GFP (green) and cFOS (red). Three hundred GnRH-GFP neurons from each of the four male mice from control and HFD group were counted for the co-labeling with cFOS and results represented as a percent of total GFP labeled neurons. To assess expression of cFOS

in other cells, two 100 μm x 100 μm areas, one area proximal to the organum vasculosum of the lamina terminalis (OVLT) and one more dorsal, in the same section were counted to quantify the number of cFOS expressing cells, where DAPI staining was used to identify cell nuclei. Statistical differences ($p < 0.05$) were determined by Student's T-test.

qPCR Analysis

Hypothalami were dissected, total RNA extracted using MicroRNA kit from Ambion and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA), with primers listed in Table 2, under the following conditions: 95 ºC for 15 min, followed by 40 cycles at 95 ºC for 20 s, 56 ºC for 30 s, and 72 ºC for 30 s. The amount of the gene of interest was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run and normalized to the beta-2-microglobulin (B2M) housekeeping gene in the same sample using $^{\Delta\Delta}$Ct method. Replicates were averaged. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction. Statistical differences ($p < 0.05$) in expression were determined by Student's T-test using JMP software (SAS Institute; Cary, North Carolina).

Cell Culture

GT1-7 cells, kindly provided by Pamela Mellon (University of California, La Jolla, CA), were cultured in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) with 10% FBS.

Leukemia Inhibitory Factor (LIF, 34-8521; eBioscience, USA) and interleukin 6 (IL-6, 216-16, PeproTech, Rocky Hill, NJ) were reconstituted in PBS containing 0.1% BSA and stored in aliquots at -80°C until use. RNA was isolated with TRIzol (Life Tech. Carlsbad, CA) and RT-PCR performed as previously described [65, 66].

Western Blot Analysis

Whole cell lysates were obtained after treatment with 10 ng/mL LIF or vehicle for times indicated, using lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA) with 1% protease inhibitor cocktail (P8340, Sigma, USA) and 1 mM PMSF. Protein content was determined using Bradford reagent (Bio-Rad Laboratories Inc. USA). An equal amount of protein from each sample was resolved on a 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Membranes were blocked in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) with 10% non-fat milk for 2 h room temperature and incubated overnight with specific antibodies for p-STAT3 (1:1000; 9134, Cell Signaling, Danvers, MA), STAT3 (1:1000, 9139, Cell Signaling, Danvers, MA), p-p38 (1:1000, 9211, Cell Signaling Danvers, MA), p38 (1:1000, 9212, Cell Signaling Danvers, MA), p-ERK1/2 (1:1000, 9101, Cell Signaling, Danvers, MA), ERK1/2 (1:1000, 9102, Cell Signaling, Danvers, MA), p-JNK (1:1000, 9255, Cell Signaling, Danvers, MA), JNK (1:1000, 9252, Cell Signaling, Danvers, MA), OCT-1 (1:500, ab66132, Abcam, Cambridge, UK), cFOS (1:1000, SC-52, Santa Cruz Biotechnology, Inc. Dallas, TX), and β-tubulin (1:1000, SC-9104, Santa Cruz Biotechnology, Inc., Dallas, TX). The bands were visualized with horseradish peroxidase (HRP)-linked secondary antibodies and enhanced chemiluminescence

reagent (ECL; Amersham Bioscience), as described by manufacturer. Blots were exposed to autoradiography film (Bioexpress, USA). Each experiment was performed 3 times and representative images are presented.

Transfections

Cells were plated into 12-well plates and transfected using Fugene 6 reagent (Roche Applied Science), as described previously [66-69]. Wells were transfected with 500 ng of reporter plasmid, 100 ng of β-galactosidase reporter plasmid driven by the *Herpesvirus* thymidine kinase promoter, as an internal control for the efficiency of the transfection, and 200 ng of expression vectors or empty vector control, as indicated in the figure legends. 24 h after transfection, cells were switched to serum-free media (DMEM with 0.1% BSA) and treated with either 10 ng/mL LIF, 20 ng/mL IL-6 or vehicle for 24 h. Following treatment, cells were lysed in 0.1 M potassium phosphate buffer, pH 7.8, with 0.2% Triton X-100. Luciferase activity in the lysates was measured with a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA) after injection with 100 μL of luciferase assay buffer (100mM Tris-HCl, pH 7.8, 15 mM $MgSO_4$, 10 mM ATP, and 65 μM luciferin). β-galactosidase activity was measured using the Tropix Galacto-light β-galactosidase assay (Applied Biosystems, Foster City, CA). All experiments were performed three independent times and in triplicates within each experiment. Luciferase values were normalized to β-galactosidase values for each sample. Results are presented as an average of three experiments. Statistical significance, $p < 0.05$, was determined with ANOVA followed by Tukey's post hoc test using JMP software (SAS Institute; Cary, North Carolina).

Plasmids

The reporter plasmids were kindly provided by Pamela Mellon (University of California, La Jolla, CA). The -5 kb rat GnRH (-4984 to +22 relative to the transcription start site); GnRH E/P, which contains GnRH-E1 (-1863 to -1571)/GnRH-P (-173 to +112); and GnRH-P (-173 to +112) luciferase reporters have previously been described [70-73]. Luciferase reporter plasmids containing mutations of AP1 binding site in GnRH enhancer and GnRH promoter, and reporter plasmids with RSV promoter fused to GnRH enhancer (GnRHe/RSVp), have also been previously described [70-73]. The expression vectors for wild type (WT) STAT3 (Stat3 Flag pRC/CMV, #8707, Addgene, Cambridge), dominant negative (DN) STAT3 (Stat3 Y705F Flag pRC/CMV, #8704, Addgene, Cambridge), constitutively active (CA) STAT3 (Stat3-C Flag pRC/CMV, #8722, Addgene, Cambridge), and luciferase reporter containing STAT3 response element (SBR, 4xM67 pTATA TK-Luc, #8688, Addgene, Cambridge, MA) were purchased from Addgene and have previously been described [74]. Expression vectors for constitutively active RAS, MEK1, MEK2, MKK4, MKK7, MKK3, MKK6, and constitutively active isoforms of p38 (p38α, p38β, p38λ, p38δ) were a gift from Peiqing Sun (The Scripps Research Institute, La Jolla, CA) [75]. Expression vector for cFOS has been previously described [65, 76-78].

Results

Inflammation in the hypothalamus induces inflammatory cytokines and represses GnRH.

Inflammation, either acute, caused by an injection of lipopolysaccharide (LPS), or chronic, elicited by high fat diet (HFD), negatively affects hypothalamic neurons and in particular, reproductive function [21, 79]. We and others postulated this impairment is due to repression of the GnRH gene, which is the final brain signal in the control of reproduction. Inflammatory cytokines have previously been proposed to negatively regulate GnRH neurons [16, 18, 80]. We initiated our studies with an analysis of cytokine mRNA levels in the hypothalami of C57BL/6J male mice, induced by LPS injection, a bacterial endotoxin known to elicit an inflammatory response. Our studies revealed that tumor necrosis factor alpha (TNF-α, *Tnf*) mRNA was induced 22-fold, interleukin 1 beta (IL-1β, *Il1b*) was induced 7-fold, IL-6 (*Il6*) 1.6-fold, and leukemia inhibitory factor (LIF, *Lif*) 2.2-fold after LPS injection (Fig. 1A). *Gnrh* mRNA level in mice treated with LPS was reduced by 72% (Fig. 1A).

Obesity is considered a state of chronic inflammation as opposed to acute inflammation elicited by LPS injection [24, 25, 81]. Thus, we exposed male C57BL/6J mice to HFD or to control diet (CTR) for 12 weeks. Diet-induced obesity induced TNF-α 1.6-fold and IL-1β 1.7-fold, although due to variability, it did not reach significance (Fig. 1B). IL-6 was significantly induced 3.1-fold and LIF 1.6-fold, compared to mice fed CTR diet (Fig. 1B). Further analysis revealed significantly reduced *Gnrh* mRNA levels by 46%, compared to the controls (Fig. 1B). Thus, increase in locally produced cytokines, acute and chronic, correlates with repression of GnRH mRNA expression.

LIF represses GnRH

We then determined whether these locally produced, inflammatory cytokines can regulate GnRH gene expression directly. Due to the scarcity and scattered location of GnRH neurons in mice, *in vivo* analysis of molecular mechanisms whereby cytokines regulate GnRH gene is not possible. To analyze direct effects of cytokines on gene expression we employed GT1-7 cells that are an established *Gnrh* expressing and GnRH secreting cell model [62]. We expressed GnRH 5 kb luciferase reporter (5 kb GnRH luc) in GT1-7 cells and treated with inflammatory cytokines. LIF repressed GnRH reporter expression by 45.7% compared to CTR (Fig. 2A, black bar), similar to what was observed *in vivo* with LPS injection and HFD. TNF-α, IL-1β and IL-6 had no effect on GnRH expression. To further analyze the effect of LIF treatment on GnRH gene, RNA was isolated from GT1-7 cells treated with either vehicle or LIF, reverse transcribed and quantitative RT-PCR performed. LIF treatment reduced endogenous GnRH mRNA levels by 39.1% (Fig. 2B). Taken together, these results indicate that LIF represses *Gnrh* gene.

Since LIF and IL-6 belong to the same cytokine family, it was surprising that LIF repressed GnRH while IL-6 did not. These two cytokines share GP130 signaling receptor, but each has a specific receptor for ligand binding. We analyzed the expression of their specific receptors on GnRH neurons to explain why IL-6 had no effect on GnRH gene expression. We first demonstrated the expression of GP130 cytokine signaling receptor (red) on GnRH neurons (green) *in vivo* using immunocytochemistry of hypothalamic slices (Fig. 3A). This suggests that LIF and IL-6 cytokines can act on GnRH neurons in the mouse. Since antibodies for specific LIF

receptor (LIFR) and IL-6 receptor (IL-6R) were not effective in immunohistochemistry, we analyzed mRNA expression of these receptors using RNA isolated from GT1-7 cells. Products of expected size for GP130 (177 bp), and LIFR (452 bp), were present in both GT1-7 cells and spleen, which served as a positive control, but absent in negative control samples lacking reverse transcriptase (Fig. 3B). IL-6R expression (156 bp) was absent in GT1-7 cells, but present in spleen (Fig, 3B). qPCR analysis reveals that the expression of IL-6R is limited in GT1-7 cells, compared to the expression of GP130 and LIFR, while expression levels of these receptors was similar in spleen (Fig. 3C). These results indicate that LIF can directly bind GnRH neurons and affect GnRH gene expression.

LIF functions via GnRH enhancer to repress GnRH gene

The 5 kb GnRH reporter contained two upstream regulatory elements: the 300 bp enhancer (−1863 to −1571) and the evolutionarily conserved promoter (−173 to +1), that confer neuron-specific activation of the GnRH, in culture and *in vivo* [70, 82, 83]. To map the elements necessary for LIF mediated repression of GnRH expression, GT1-7 cells were transiently transfected with a luciferase reporter containing the 5 kb regulatory region upstream of the GnRH transcription start site (5 kb GnRH), a reporter containing the enhancer and promoter without intervening sequences (GnRH E/P) or a reporter containing the promoter (GnRH P). LIF repressed luciferase activity of the 5 kb GnRH reporter and of the reporter containing the enhancer and promoter, GnRH E/P luc, by 45.7% and 42.6% respectively (Fig. 4A and 4B). Luciferase activity of the reporter containing only the promoter, GnRH P luc, did not change in response to LIF

(Fig. 4C). Next, to examine if the enhancer is sufficient for repression, the reporter containing enhancer fused to heterologous RSV promoter was examined (GnRH E/RSVp luc). LIF treatment significantly repressed GnRH E/RSVp luc reporter by 45.2% (Fig. 4D). Given that GnRH E/P and GnRH E/RSVp reporters were repressed to a similar degree by LIF as the full length 5 kb GnRH reporter, these results indicate that the enhancer is sufficient and necessary for repression by LIF.

STAT3 is not necessary for GnRH repression by LIF

LIF signals through GP130 to activate the signal transducer and activator of transcription (STAT) pathway. To analyze signaling pathways activated by LIF, cells were treated with LIF and western blots performed using whole cell lysate. LIF treatment resulted in increased levels of STAT3 phosphorylation (Fig. 5A). To determine whether STAT3 is sufficient to repress GnRH gene expression, GT1-7 cells were co-transfected with a constitutively active STAT3 mutant (CA STAT3) and STAT binding region (SBR), a reporter containing 6 copies of the STAT3 response element that serves as a positive control; or with GnRH E/P reporter. CA STAT3 overexpression induced STAT3-SBR to the similar level as induction by LIF. LIF treatment together with CA STAT3 overexpression did not further increase induction over CA STAT3 alone, indicating that CA STAT3 maximally induces SBR reporter. On the other hand, CA STAT3 overexpression did not affect the expression of the GnRH E/P reporter or its repression by LIF (Fig. 5B). We then analyzed necessity of STAT3 by co-transfecting dominant negative mutant STAT3 (DN STAT3) with SBR or with GnRH E/P. While transfection with DN STAT3 inhibited LIF induction of SBR positive control, DN STAT3

379 did not prevent repression of GnRH E/P by LIF (Fig. 5C). Taken together, these results
380 demonstrate that STAT3 is not necessary for GnRH repression by LIF.
381
382 **LIF activates p38 to repress GnRH**
383 LIF signaling has been shown to activate the mitogen activated protein kinase
384 (MAPK) pathway, which include extracellular signal-regulated protein kinases (ERK1/2),
385 p38, and the c-Jun N-terminal kinases (JNK) in a variety of other cells, including mouse
386 embryonic stem cells, 3T3-L1, and AtT20 pituitary corticotrope cells [84, 85]. LIF
387 treatment of GT1-7 cells resulted in phosphorylation of ERK1/2 and p38, but no
388 changes in JNK phosphorylation were observed (Fig. 6A). To further delineate the
389 necessity of MAPK signaling pathway, co-transfection assays with expression plasmid
390 containing constitutively active RAS (CA RAS), that is upstream to the MAPK pathway,
391 resulted in repression of GnRH expression by 51.4% (Fig. 6B). To identify the MAPK
392 kinase pathway sufficient for repression, co-transfection assays were conducted using
393 constitutively active forms of MEK1 and MEK2 that leads to ERK1/2 activation, MKK3
394 and MKK6 that that activate p38, or MKK4 and MKK7 which activates JNK.
395 Constitutively active MEK1, MEK2, MKK4, MKK7 had no effect on GnRH expression.
396 GnRH expression was repressed with constitutively active MKK3 and MKK6, which lead
397 to activation of p38, by 40.9% and 48.2% respectively (Fig. 6C).
398 The p38 kinases have four isoforms, p38α, p38β, p38γ, and p38σ [86]. To
399 explore the role of these isoforms on GnRH, constitutively active mutants of p38
400 isoforms were analyzed in co-transfection assays. Reporter activity was repressed by
401 31% with constitutively active p38α, while overexpression of constitutively active p38β,

p38γ, and p38σ, had no effect on luciferase activity (Fig. 6D). These results demonstrate that LIF represses GnRH transcriptional activity via the MAPK pathway, specifically p38, and highlights the important role that p38α plays in this repression.

cFOS is induced by LIF in GT1-7 cells and represses GnRH

OCT-1 has been previously identified as an essential regulator of GnRH gene transcription [87]. Western blot analysis revealed that OCT-1 protein level was not affected by LIF treatment in GT1-7 cells, suggesting that OCT-1 may not mediate LIF repression of the GnRH gene. GnRH gene repression by cFOS has previously been shown [72], and thus we next examined cFOS protein levels. Indeed, western blots demonstrated that cFOS was induced by LIF (Fig. 7A). GnRH E/P reporter was repressed by 43.4% with overexpression of cFOS (Fig. 7B). This indicates that induction of cFOS is likely mediating LIF repression of GnRH.

Several cFOS binding sites were previously identified in the GnRH regulatory region [71-73]. To identify the site involved in LIF repression of GnRH, GT1-7 cells were transfected with luciferase reporters containing mutation of the putative cFOS sites at -79 in the promoter region (-79m), -1723 (-1723m), -1782 (-1782m), and -1793 (-1793m) in the enhancer and their expression was compared to wild type GnRH E/P (WT). LIF repressed luciferase activity of all reporters used by 61.0%, 61.8%, 65.9%, and 61.6% respectively, except of the -1793 mutant, indicating that the mutation of this site prevents LIF repression of GnRH (Fig. 7C). Thus, the -1793 site is involved in LIF repression of GnRH. We confirmed the role of this site by transfecting the mutation of the -1793 site in the GnRH E/RSVp reporter and comparing the repression of the

mutant to the wild-type reporter containing the GnRH enhancer linked to the RSV promoter (GnRH E/RSVp). Similar to what was observed in Fig. 7C, LIF repression of GnRH was inhibited with a mutation of the -1793 site (Fig. 7D). Therefore, LIF induces cFOS which represses GnRH gene expression via the -1793 site.

cFOS expression is induced with HFD in mice

Since we determined that cFOS mediates LIF repression of the GnRH gene and we observed reduced GnRH mRNA levels in the hypothalami of mice fed HFD, we explored cFOS expression in the hypothalamus. We concentrated specifically in the sections containing the OVLT where the largest numbers of GnRH neuron soma are found. In the hypothalamus of GnRH-GFP mice fed HFD, a significant increase in the percent of double labeled green GnRH neurons with cFOS (red) was observed compared to control (CTR) (Fig. 8A and 8B.1). In CTR fed mice, 17.9% of GnRH neurons expressed cFOS, while in the HFD mice, 36.3% of GnRH neurons expressed cFOS, suggesting that HFD induces cFOS expression in GnRH neurons. To examine specificity of this increase, we also counted cells that express cFOS in two other areas, one proximal to OVLT and one more dorsal from OVLT, using DAPI to identify cell nuclei. We determined that there is an increase in the percent of cFOS positive neurons in the area proximal to the OVLT of mice fed HFD (45.4%) compared to CTR (30.5%) (Fig. 8A.2, 8B.2). However, in the area dorsal to OVLT, we did not observe a difference in the number of neurons that express cFOS (Fig. 8B.3). Thus, obesity induces cFOS expression in neurons in the proximity to OVLT and in GnRH neurons. Given that cFOS mediates LIF repression of the GnRH gene in GT1-7 cells, and the reduced levels of

GnRH mRNA observed *in vivo* coupled with an increased cFOS expression in GnRH neurons following LIF induction by inflammatory stimuli, our data together demonstrate that LIF represses *Gnrh mRNA in vivo* through cFOS. Furthermore, LIF may mediate repression of the *Gnrh* mRNA and reproductive function caused by inflammation.

Discussion

Both infection and obesity negatively affect hypothalamic function, GnRH neuronal network and reproduction, but the mechanisms are unknown. We postulated that neuroinflammation is a common characteristic between these conditions and that inflammatory cytokines may mediate impairment of reproductive function by both infection and obesity. Classical pro-inflammatory cytokines, TNF-α, IL1β, and IL-6, are induced in the brain during inflammation, when GnRH and LH expression and secretion are diminished, but direct regulation of GnRH neuron function by cytokines remain inconclusive. In our recently published report [24] and herein, we identified a novel cytokine, leukemia inhibitory factor (LIF), which is induced during these inflammatory states that directly affects GnRH gene expression. In this report, we also determined the precise mechanisms of GnRH gene repression and signaling pathways that are necessary for LIF-mediated effects on GnRH neurons. Finally, we demonstrated that the transcription factor induced by LIF to repress GnRH gene is increased in a location specific manner following HFD *in vivo*.

Repression of *Gnrh* mRNA expression is consistently observed during inflammatory states. In agreement with previous reports, acute inflammation, elicited with an injection of LPS and chronic inflammation, caused by high fat diet (HFD)-

induced obesity, suppresses GnRH mRNA in our studies. LPS treatment, resulting in inflammation, represses *Gnrh* in ewes [88], birds [89] and rats [90]. Infusion of the pro-inflammatory IL-1β cytokine into the rodent hypothalamus also represses *Gnrh* expression [16]. We and others reported that obese mice fed HFD, with a low grade chronic inflammation, exhibit lower *Gnrh* mRNA [24, 91, 92]. These reports suggested that repression of *Gnrh* mRNA during inflammation may be a result of increased cytokine concentration. Alternatively, microglia, brain resident immune cells, are involved in the regulation of synaptic transmission and activity-dependent structural remodeling [93]. In neuroinflammation, in response to injury, infection, or disease, microglia engulf damaged synapses [93]. We demonstrated decreased synaptic spine density in GnRH neurons following obesity-mediated neuroinflammation and microglia activation, indicating reduced connectivity [24]. This reduced synaptic activity may regulate *Gnrh* expression in an activity-dependent manner [94, 95]. Some synaptic proteins are regulated in an activity-dependent manner at the transcriptional level [96]. This may be the case with GnRH gene expression as well, since several hypothalamic factors, such as RFamide-related peptide 3 (RFRP-3), a mammalian gonadotropin-inhibitory hormone ortholog; senktide, a neurokinin B receptor agonist; and oxytocin; alter both GnRH secretion and *Gnrh* transcription [97-99]. However, since GnRH neurons express cytokine receptors [27], herein, we investigated if *Gnrh* gene is repressed via activation of cytokine receptor signaling pathways.

We identified LIF as a critical player in the neuroinflammation-induced impairment of GnRH gene expression. In the brain, LIF is involved in neural stem cell maintenance and axonal growth, and in modulation of gene expression [100]. During

496 embryonic development, LIF is expressed in the olfactory placode, which led to the
497 hypothesis that LIF affects GnRH neuron migration [54]. Our results demonstrate that
498 LIF represses GnRH gene expression, which is in agreement with findings that GnRH
499 mRNA is repressed during embryonic GnRH neuron migration [101], in addition to
500 repression during inflammation. To analyze the effects of infection on GnRH, previous
501 studies analyzed classical inflammatory markers, TNF-α, IL-1β and IL-6, but not LIF.
502 Although LPS, or TNF-α or IL-1β administration in the ventricle consistently reduces
503 GnRH secretion *in vivo* [18, 20, 21, 23], some have found that IL-6 treatment of the
504 hypothalamic slices stimulates GnRH secretion [19, 102]. While IL-1β and TNF-α are
505 prototypical pro-inflammatory cytokines, IL-6 may function as either a pro- or anti-
506 inflammatory cytokine depending on timing and stimulus. IL-6 synthesis in the
507 hypothalamus is induced by IL-1β [103], and IL-6 may be engaged in the negative
508 feedback to dampen inflammation [42]. Thus, results may vary due to the differences
509 between *in vivo* and *in vitro* treatments, since initially-induced cytokines may cause a
510 cascade of other cytokines *in vivo*. For this reason, we concentrated on analyzing the
511 effects of LIF in GT1-7 cells. LIF alone repressed *Gnrh* mRNA and GnRH reporter,
512 similarly to HFD-induced and LPS-elicited repression of GnRH mRNA *in vivo*. LIF is
513 induced by IL-1β *in vivo*. Therefore, IL-1β repression of GnRH mRNA, demonstrated in
514 previous studies, may occur via induction of LIF.
515 We determined that LIF induces cFOS which represses GnRH gene expression.
516 GnRH repression by cFOS has been demonstrated previously and several cFOS
517 binding sites have been identified in the GnRH regulatory region [72, 73]. However, a
518 specific site necessary for GnRH repression has not been identified, until now. GnRH

519 gene is inhibited by PKC signaling pathway, which induces cFOS [70, 104]; and PKC
520 activation by TPA treatment represses GnRH gene via the -1793 site [71]. Here we
521 demonstrate that this same site at -1793 is involved in LIF repression of the GnRH
522 gene. This site is juxtaposed to the OCT-1 binding site, and OCT-1 is the critical
523 transcription factor that regulates GnRH gene expression [71, 87, 105, 106]. Thus,
524 although OCT-1 protein levels do not change with LIF treatment, its interaction with
525 cFOS [107], likely modulates that level of GnRH gene expression.

526 Signaling pathways involved in the regulation of GnRH gene transcription by
527 cytokines have not been elucidated. GP130, the common signal transducer for IL-6 and
528 LIF family, is expressed in GnRH neurons *in vivo.* LIF treatment activates STAT3 in
529 GT1-7 cells, a model of mature GnRH neurons, as we show herein, and in GN11 cells,
530 a model of immature, migrating GnRH neurons [54]. However, STAT3 does not play a
531 role in GnRH gene repression. To exert its effect on GnRH gene expression, LIF
532 activates MAPK-p38 pathway, and in particular p38α is critical for repression. This is an
533 important finding, since while other p38 isoforms have normal physiological functions,
534 p38α is involved in inflammatory processes [108]. Moreover, p38α is the target for
535 several potential therapeutics for chronic inflammatory disorders.

536 Leptin is necessary for pubertal transition and normal reproductive function [109-
537 111]. Since leptin is elevated in obese and HFD-fed mice, leptin was previously
538 analyzed as a mediator of obesity-induced impairment of reproductive function [10, 112-
539 114]. Additionally, a mouse model with leptin overexpression exhibits early puberty and
540 lower GnRH at 21 weeks of age [115], which may correlate with precocious puberty in
541 obese girls and lower *Gnrh* mRNA in obese mice in our study. However, previous

studies determined that leptin does not directly affect GnRH neurons in vivo, since deletion of the leptin receptor specifically in GnRH neurons had no effect on fertility [116]. STAT3 signaling molecule that is activated by leptin, is required for leptin's role in energy balance, but is not required for leptin's effect on fertility [117, 118]. STAT3 studies demonstrate separate roles for leptin signaling pathways, involved in metabolic function that require STAT3, and reproductive function that do not. Although leptin receptor is similar to the IL-6 and LIF signaling receptor, GP130, and can activate the same signaling pathways, leptin does not engage GP130 [119]. Given that in vivo studies determined that leptin influences reproductive function via its actions in the brain, but indirectly, via GnRH neuron afferents, it is unlikely that elevated leptin levels in our studies had direct effects on GnRH gene expression.

We demonstrate that cFOS expression is increased specifically in GnRH neurons and in cells located close to the OVLT, but not in cells located more dorsally, following HFD. Others reported increased cFOS following HFD in specific areas and postulated that the location is specific for the function of the neuronal population. For example, cFOS was increased in the reward circuitry following HFD [120] and in the dorsomedial and lateral hypothalamus [121]. cFOS is similarly induced following LPS treatment in the hypothalamus [122] and this induction is location specific [123]. These studies further postulated that the location is dependent on the function of the neurons. We, on the other hand, posit that it is dependent on the proximity to the vasculature. Previous studies also suggested that cFOS was induced following neuron activation, since cFOS is most commonly used marker of neuronal activity. As an immediate early gene, cFOS is induced via activity-dependent transcription in neurons. cFOS expression is increased

in activated GnRH neurons, during the preovulatory LH surge and after kisspeptin treatment [124, 125]. The roles of cFOS as a transcription factor, involved in the GnRH gene regulation, and as a marker of neuronal activity, are not mutually exclusive. Thus, we postulate that increased LIF concentration induces cFOS in GnRH neurons to repress GnRH gene expression. Specificity for GnRH neurons may be due to either their proximity to the OVLT or because GnRH processes extend beyond the blood brain barrier into the OVLT.

Our studies delineate the mechanisms of neuroinflammation impairment of GnRH gene expression, and may explain repression of reproductive function during infection and in obesity. We identified LIF as the cytokine that mediates these effects and p38α as a critical signaling molecule. We also postulate that the proximal location of GnRH neurons to the leaky blood brain barrier, where the local concentration of this cytokine may be the highest, permits for repression of GnRH gene expression via cFOS induction. Future studies will determine *in vivo* role of p38α in neuroinflammation-mediated GnRH repression.

Statements

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Statement of Ethics

All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines.

Disclosure Statement

The authors have no conflicts of interest to declare

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Author Contributions

N. Lainez performed all the experiments and wrote the manuscript. D. Coss conceived the experiment, guided the study and revised the manuscript.

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Figure Legends

1. GnRH expression is repressed when cytokine levels are increased. A, Hypothalami from male mice 24 hours after injection with either vehicle control (CTR, white bars) or lipopolysaccharide (LPS, gray bars). B, Hypothalami from male mice following 12-week feeding with control (CTR, white bars) or high fat diet (HFD, black bars). Cytokine and GnRH (*Gnrh1*) expression was assayed with RT-qPCR. TNF-α, (*Tnf*) tumor necrosis factor alpha; IL-1β, (*Il1b*) interleukin 1 beta; IL-6, (*Il6*) interleukin 6; LIF, (*Lif*) leukemia inhibitory factor. * indicates significant difference ($p<0.05$) determined with t-test.

2. GnRH expression is suppressed by LIF. A, GT1-7 cells transiently transfected with 5 kb GnRH-reporter (5 kb GnRH Luc) treated with vehicle (CTR, white bar), or TNF-α, IL-1β, IL-6, LIF, for 24 hours. Luciferase values were normalized to β-galactosidase values for each sample. Results are presented as an average of three experiments performed in triplicate. Statistical significance ($p < 0.05$) was determined with ANOVA followed by Tukey's post hoc test, and indicated with *. B, RT-qPCR using total RNA from GT1-7 cells treated with LIF, 10 ng/mL for 24h, demonstrates repression of

endogenous GnRH mRNA (*Gnrh1*). * indicates significant difference determined with t-test ($p<0.05$).

3. GP130 is expressed in GnRH neurons *in vivo* and in GT1-7 cells. A, Immunohistochemistry of GnRH neurons genetically labeled with GFP (green), stained for GP130 receptor (red). Bar indicates 25μm. White arrows indicate GnRH neurons that express GP130, while arrowheads indicate GP130 staining in non-GnRH cells. B, RT-PCR demonstrates expression of GP130 (177 bp) and LIFR (452 bp), but not IL-6R (156 bp) in GT1-7 cells (RT+), while all three receptors are expressed in the spleen (S). RT-, GT1-7 cells mRNA used without reverse transcriptase serves as negative controls; L, size ladder. C, RT-qPCR demonstrates relative expression of GP130, LIFR, IL-6R in GT1-7 cells and the spleen.

4. LIF represses GnRH gene expression through the enhancer region. A, 5 kb GnRH reporter containing 5 kb of the GnRH gene regulatory sequence from the transcriptional start site; B, reporter containing GnRH enhancer (E, -1863/-1571) and promoter (P, −173/+1); C, reporter containing GnRH promoter -173/+1 (P) and D, reporter containing GnRH enhancer (E, -1863/-1571) linked to the heterologous RSV (Raus Sarcoma Virus) promoter (GnRH E/RSVp luc); were transfected in GT1-7 cells and cell treated with LIF for 24h. Results demonstrate sufficiency of the enhancer for GnRH repression by LIF. Statistical significance, $p < 0.05$, indicated with *, was determined with a t-test.

5. STAT3 is not sufficient or necessary for GnRH repression by LIF. A, Analysis of STAT3 phosphorylation by western blot of GT1-7 cell lysates after 10 ng/mL LIF treatment, for 10, 30 and 120 minutes. B, Co-transfection of a reporter containing 6 copies of the STAT3 response element (SBR) or GnRH E/P with a constitutively active (CA) STAT3 demonstrates that CA-STAT3 is sufficient to induce SBR, but not GnRH E/P. C, Co-transfection of a dominant negative (DN) STAT3 inhibits LIF induction of SBR-luciferase, but does not prevent LIF repression of the GnRH E/P reporter. Statistical significance (*, $p < 0.05$) was determined with ANOVA followed by Tukey's post hoc test.

6. p38α is sufficient to repress GnRH gene expression. A, LIF treatment activates ERK1/2, p38, but not JNK, demonstrated by western blots using whole cells lysate of GT1-7 cells treated with LIF for times indicated above each lane. GnRH-reporter is repressed by co-transfection with B, constitutively active (CA) RAS; C, CA MKK3 and CA MKK6, upstream activators of p38; and D, CA p38α. Statistical significance, $p < 0.05$, indicated by *, was determined with ANOVA followed by Tukey's post hoc test. .

7. cFOS mediates LIF repression of the GnRH gene. A, LIF treatment of GT1-7 cells induces cFOS, but not OCT-1, demonstrated by western blots of nuclear extracts. B, Overexpression of cFOS, by co-transfection of cFOS expression vector, is sufficient to repress GnRH E/P reporter. * indicates significant difference ($p<0.05$) determined with t-test. C, Mutation of the cFOS binding site at -1793 abolishes LIF repression of GnRH E/P reporter, demonstrated by transient transfections of reporters containing the

mutations of the putative cFOS binding sites in GT1-7 cells. D, the same mutation was created in the GnRH E/RSVp reporter and also abrogates the repression by LIF. Statistical significance, $p < 0.05$ indicated by *, was determined with ANOVA followed by Tukey's post hoc test.

8. More GnRH neurons express cFOS following high fat diet (HFD). A, Coronal sections of the preoptic area in the hypothalamus of GnRH-GFP mice following HFD stained for GFP (green) and cFOS (red). Scale bar represents 100 μm, white arrows indicate GnRH neurons labeled with GFP and cFOS. Numbered squares correspond to enlarged areas below; 1, GnRH neurons green, cFOS red; Arrows indicate GnRH neurons that express cFOS, an arrowhead points to the GnRH neuron without cFOS; 2-3, DAPI channel is included to facilitate cell count; nuclei blue, cFOS magenta. Arrows indicate cells labeled with cFOS and DAPI. B, quantification of neurons expressing cFOS in control (CTR, white bars) and HFD male mice (black bars): 1, increase in the percent of GnRH neurons with cFOS is observed in HFD compared to control. 2, quantification of the neurons that express cFOS, proximal to the OVLT delineated with #2 square; 3, quantification of the neurons that express cFOS, dorsally from the OVLT delineated with #3 square following control and HFD. Three hundred GnRH neurons were counted in each mouse. Statistical significance, $p < 0.05$ indicated by *, was determined with t-test.

1079 **Table 1.**

Antibody	Species	Dilution	Provider, cat #
GFP	chicken	1:5000	Abcam, ab1397
GP130	rat	1:300	R&D Systems, MAB4681; clone#125623
STAT3	mouse	1:1000	Cell Signaling, 9139
p-STAT3	rabbit	1:1000	Cell Signaling, 9145
p38	rabbit	1:1000	Cell Signaling, 9212
p-p38	rabbit	1:1000	Cell Signaling, 9211
ERK 1/2	rabbit	1:1000	Cell Signaling, 9102
p-ERK 1/2	rabbit	1:1000	Cell Signaling, 9101
JNK	rabbit	1:1000	Cell Signaling, 9252
p-JNK	mouse	1:1000	Cell Signaling, 9255
OCT-1	rabbit	1:500	Abcam, ab66132
cFOS	rabbit	1:300, 1:1000	Santa Cruz Biotechnology, sc-52
b-tubulin	rabbit	1:1000	Santa Cruz Biotechnology, sc-9104

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1082 **Table 2.**
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Primers	Forward	Reverse
Gnrh (GnRH)	CTACTGCTGACTGTGTGTTTG	CATCTTCTTCTGCCTGGCTTC
Il6 (IL-6)	TTCTCTGGGAAATCGTGGAAAT	TCCAGTTTGGTAGCATCCATCA
Tnfa (TNF-α)	ATGTCTCAGCCTCTTCTCATTCC	GCTTGTCACTCGAATTTTGAGAA
Il1β (IL1β)	GCAACTGTTCCTGAACTCAACTG	CACAGCCACAATGAGTGATACTG
Lif (LIF)	ATGTGCGCCTAACATGACAG	TATGCGACCATCCGATACAG
B2m (beta-2-microglobulin	TGACCGGCCTGTATGCTATCCA	CAGTGTGAGCCAGGATATAGAAAGAC
LIFR	TCAGTTTCAGCCAGGAGTAA	GCAATAATCAATCCCACAGA
IL6R	AAGCAGCAGGCAATGTTACC	CATAAATAGTCCCCAGTGTCG
GP130	GCGTACACAGATGAAGGTGGGAAAGA	GCTGACTGCAGTTCTGCTTGA

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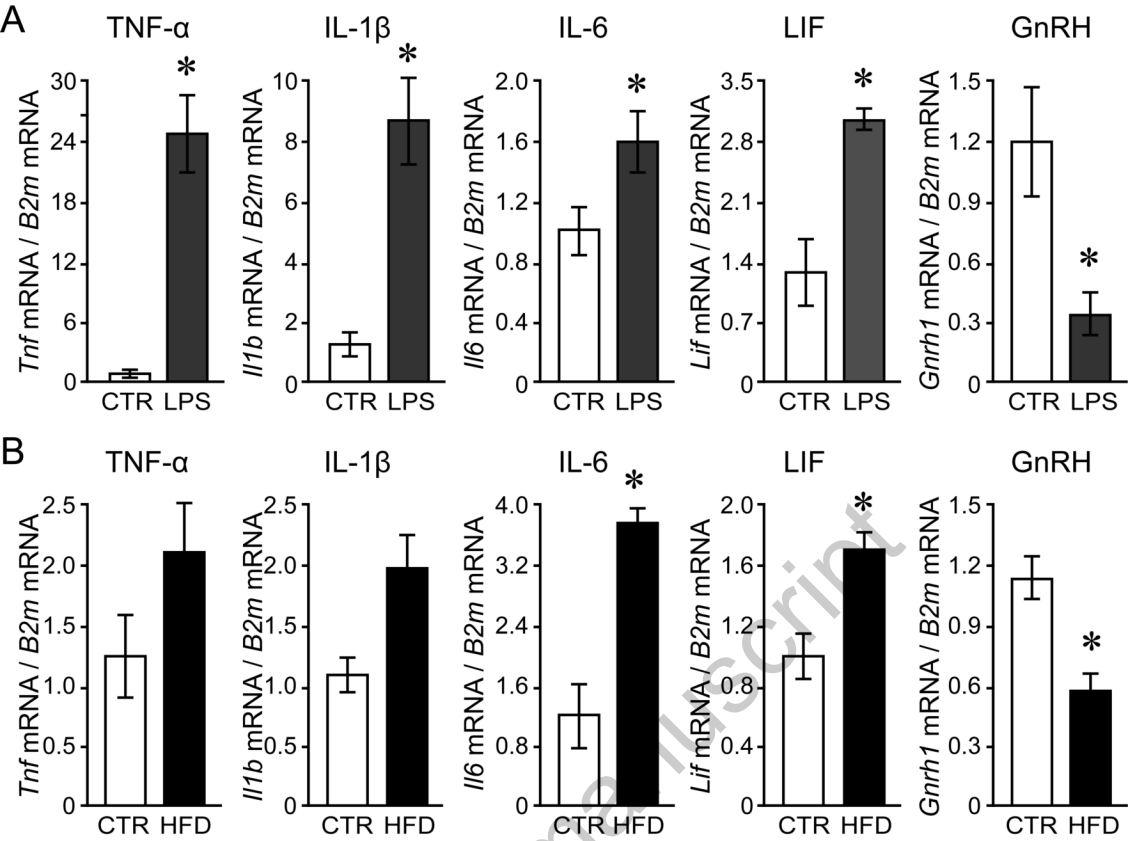


Figure 1

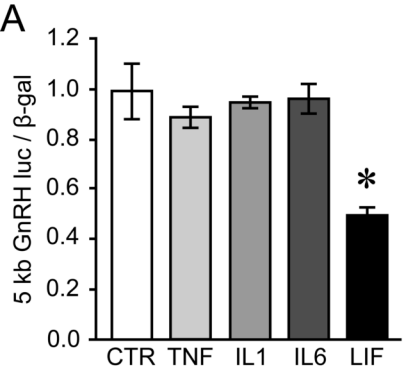


Figure 2

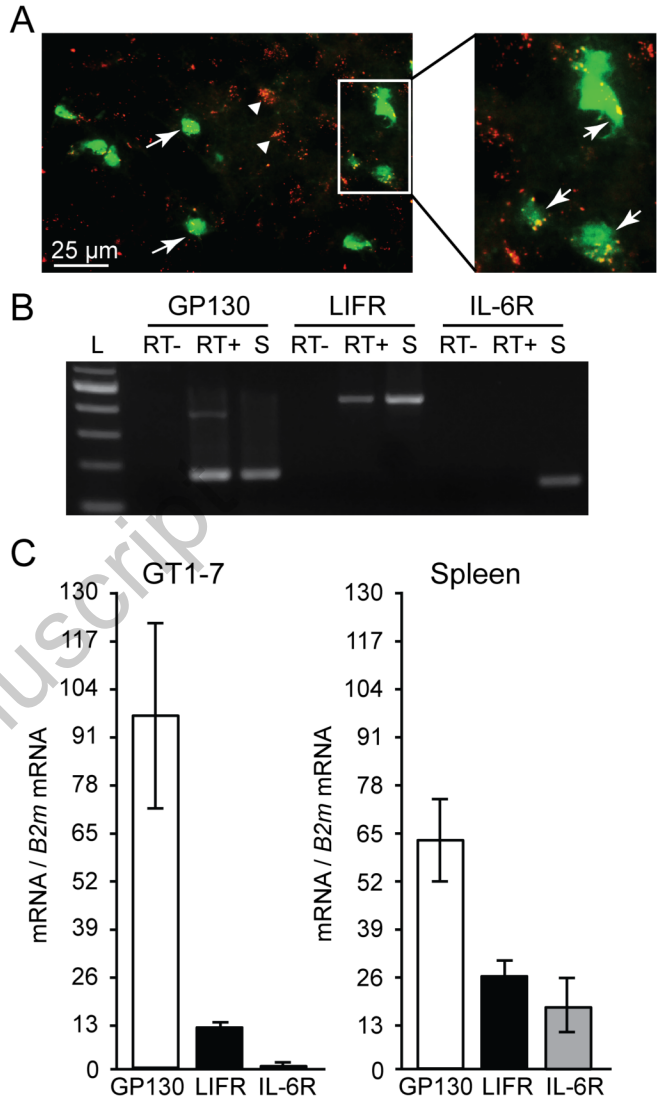


Figure 3

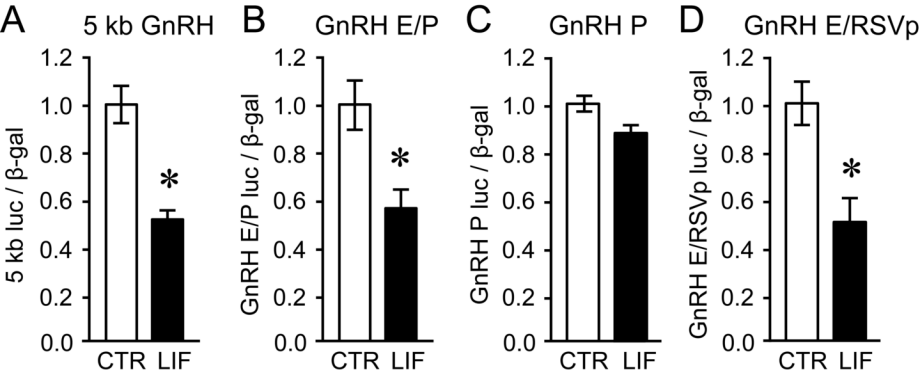
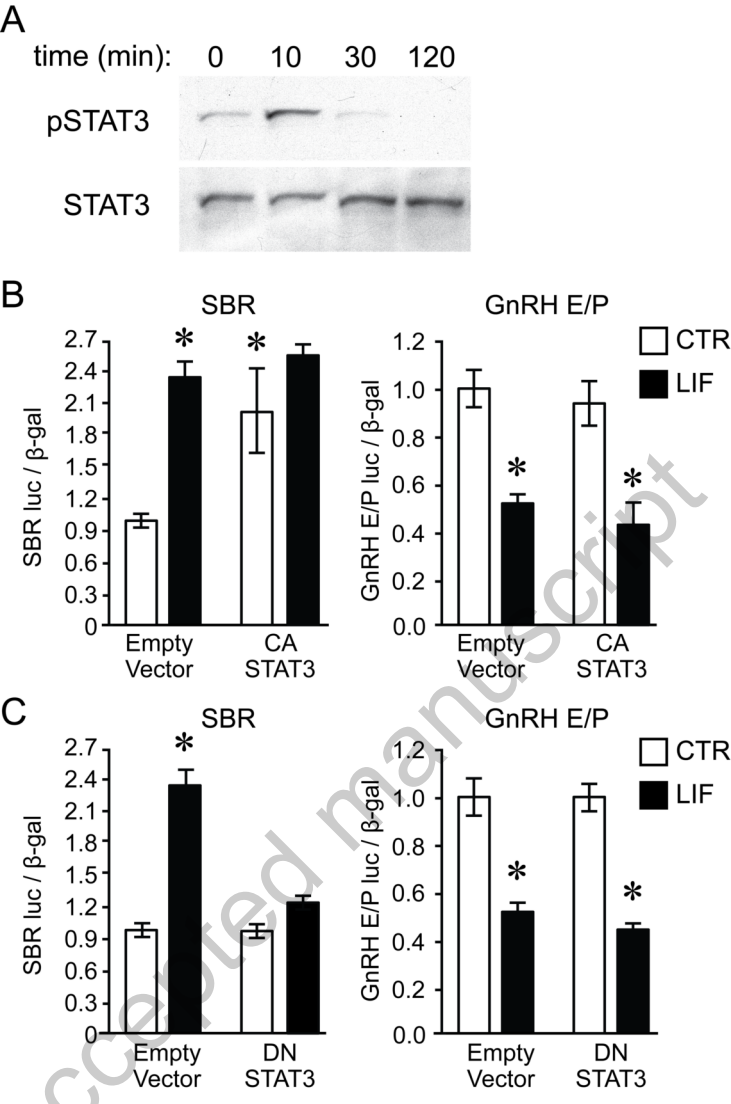


Figure 4



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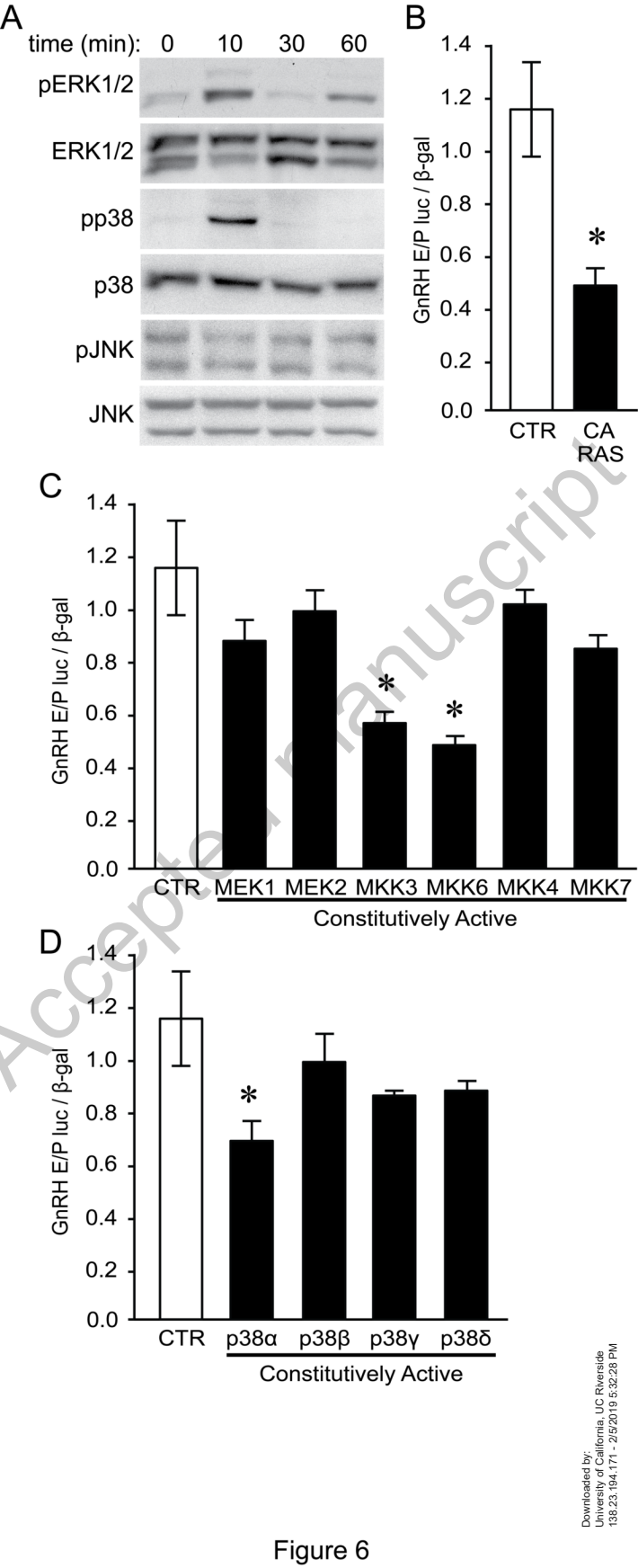
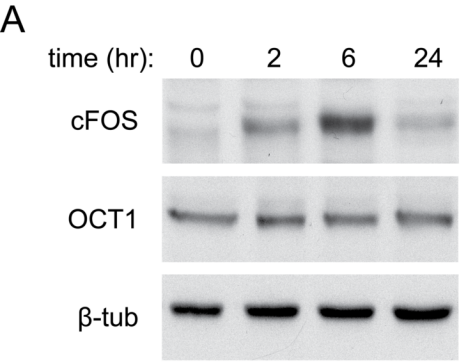
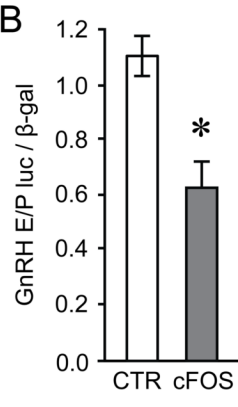
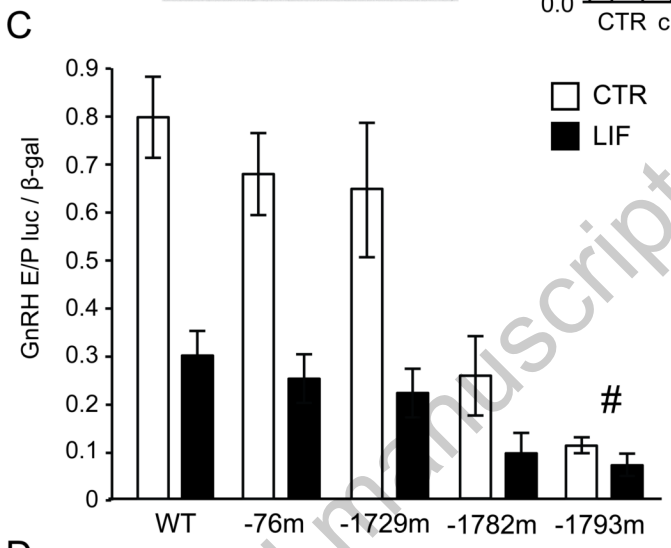


Figure 6







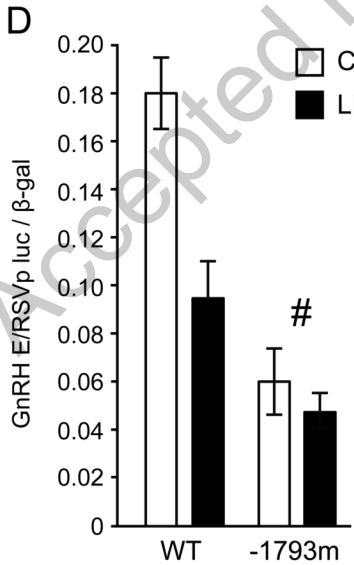
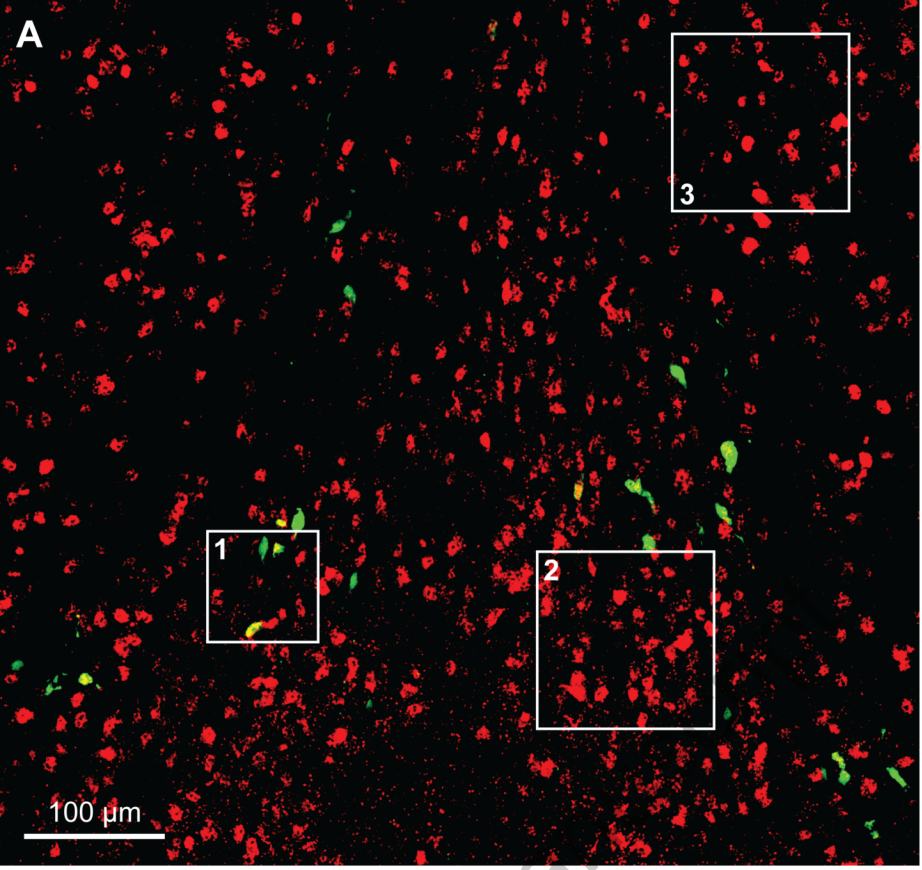
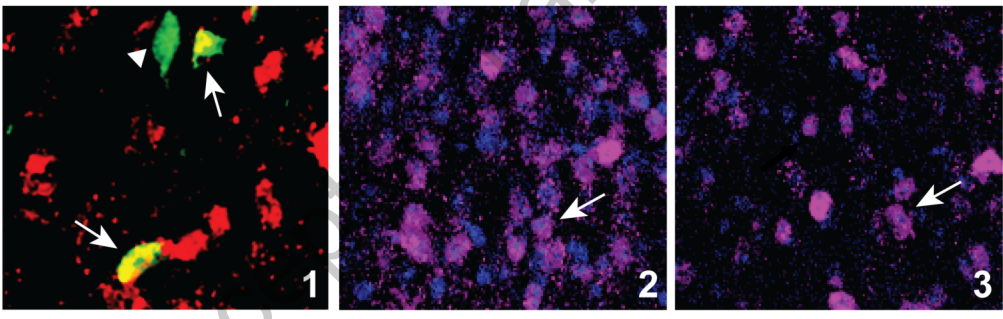


Figure 7





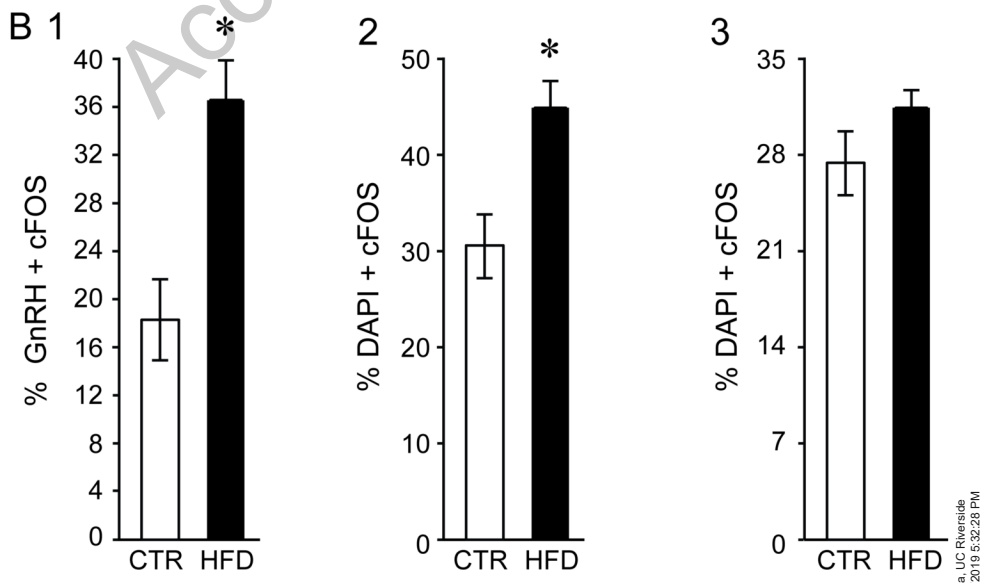


Figure 8