UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of Metabolic and Reproductive Effects of Peroxisome Proliferator-Activated Receptor Gamma Knockout in Astrocytes

A Thesis submitted in partial satisfaction of the requirements
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in

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by

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The Thesis of Katherine Hsueh is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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2014

I dedicate this thesis to my friends and family for their continual advice and support, and to all my professors for the knowledge they have provided me which has been so essential for me to accomplish my goals.

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LIST OF ABBREVIATIONS

AKO	Astrocyte Knockout
CNS	Central Nervous System
FFA	Free Fatty Acids
FSH	Follicle Stimulating Hormone
GFAP	Glial Fibrillary Acidic Protein
GnRH	Gonadotropin-Releasing Hormone
GTT	Glucose Tolerance Test
HFD	High-Fat Diet
HPG	Hypothalamic-Pituitary-Gonadal
ITT	Insulin Tolerance Test
LFD	Low-Fat Diet
LH	Luteining Hormone
PCOS	Polycystic Ovarian Syndrome
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
qPCR	Quantitative Polymerase Chain Reaction
TAM	Tamoxifen
TZD	Thiazolidinedione
WT	Wild Type

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ABSTRACT OF THE THESIS

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Thiazolidinediones are PPAR-γ agonist drugs used to treat type 2 diabetes and improve symptoms of infertility in polycystic ovarian syndrome (PCOS) patients. Because both disorders correlate with obesity, which increases inflammation in the central nervous system, we hypothesized PPAR-γ in astrocytes may be involved in regulating metabolic and

reproductive functions centrally. We therefore generated astrocyte-specific PPAR-γ knockout (AKO) mice using Cre-*loxP* technology to study these effects.

On normal chow, AKO mice had reduced numbers of estrous cycles but normal pituitary response to GnRH suggesting a central defect. Loss of PPAR-γ did not cause astrocyte activation, as *Gfap* expression in the brain was unchanged. When maintained on low-fat diet (LFD), they showed decreased food intake which was not further suppressed by leptin. Body weights were normal, but they displayed slight glucose intolerance, faster recovery from insulin-induced hypoglycemia, and increased liver expression of *G6pase*, *Cidec,* and *Gfap*. At the reproductive level, AKO mice had decreased estrous cycles, lower diestrus LH levels, abolished FSH response to kisspeptin, and increased hypothalamic expression of *Gnrh1, Kiss1, Npvf,* and *Hcrt*.

AKO mice challenged with a high-fat diet (HFD) showed significantly increased glucose tolerance which was associated with their decreased *G6pase* expression but increased *Cidec* and *Gfap* expression in the liver. Food intake and leptin response were normal. They also had significantly more estrous cycles than AKO mice on LFD, but still had suppressed diestrus LH levels and impaired gonadotropin response to kisspeptin. *Gfap* expression in the brain was also significantly increased suggesting astrocyte activation by HFD.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of a class of nuclear receptor proteins that act as transcription factors and regulate genes involved in metabolism, cellular differentiation, and development. PPARs heterodimerize with the retinoid X receptor (RXR) and are constitutively nuclear. In the absence of ligand, the PPAR/RXR dimer binds and represses transcription of genes containing a peroxisome proliferator hormone response element (PPRE) through the action of transcriptional co-repressors (NCORs). Upon the binding of a ligand, the co-repressors dissociate from the PPAR complex and are replaced by transcriptional co-activators (NCoAs). The complex then recruits the transcriptional machinery and polymerase complex to the gene promoter allowing transcription of various genes.[1]

PPAR-γ is a PPAR subtype that not only regulates lipid and glucose metabolism, but also has anti-inflammatory qualities.[2] Pioglitazone is a thiazolidinedione (TZD) drug that is a synthetic PPAR-γ agonist currently used as drug treatment for patients with type 2 diabetes. Through the activation of PPAR-γ in fat cells,[3] muscle cells, and macrophages,[4] TZDs increase overall insulin sensitivity in patients. Moreover, through such insulin sensitizing effects, TZDs have reportedly improved reproductive functions in women suffering from polycystic ovarian syndrome (PCOS), a reproductive problem characterized by insulin resistance and infertility due to hormonal imbalances. After TZD treatment, PCOS patients experienced increased ovulation, decreased serum androgen levels, normalized LH levels, and improved insulin sensitivity.[5]

Both type 2 diabetes and PCOS are strongly correlated with obesity, which is quickly becoming a worldwide issue.[6] The underlying cellular mechanisms that link such health problems to obesity are still not fully understood. However, it has been shown that obesity activates immune cells which then increase expression of chemokines such as IL-6, TNF-α, and nitric oxide, leading to insulin resistance.[7] This increased inflammatory tone is suppressed by TZD treatment in parallel with increases in insulin sensitivity, suggesting a causal connection. Recently, much interest has focused on the role of the central nervous system (CNS) as selected neuronal populations have been shown to regulate feeding, metabolism,[8] and insulin resistance.[9]

The CNS also regulates reproductive development and normal reproductive function. The hypothalamic-pituitary-gonadal (HPG) axis is the reproductive hormone signaling cascade through which the brain controls the reproductive system in the periphery.[10] Hypothalamic neurons secrete gonadotropin-releasing hormone (GnRH) to the anterior pituitary in a pulsatile manner approximately every 30-120 minutes.[11] The gonadotrope cells in the pituitary respond by secreting both luteinizing hormone (LH) and follicle stimulating hormone (FSH), collectively known as gonadotropins, into the bloodstream. Gonadotropins circulate to and act on the gonads, testes in males and ovaries in females, to stimulate production of sex hormones that promote gametogenesis and sexual development.[12] In women, surges in LH and FSH hormones in the late-follicular phase of the menstrual cycle induce ovulation. Abnormal levels of reproductive hormones often result in reproductive dysfunctions such as PCOS[13] or functional hypothalamic amenorrhea.[14]

Inflammation in the CNS has also been connected to reproductive abnormalities through suppression of GnRH neurons.[15]

Previous studies performed in our lab showed PPAR-γ in neurons played a role in metabolic and reproductive functions in mice, which concur with the improvements seen in diabetic and PCOS patients given TZD treatment. The obesity-inflammation connection suggested that inflammatory cells in the brain may also regulate the HPG axis.[16] Glia cells are known to be the "immune system" of the CNS due to their functions in mediating inflammation and repairing injured cells in the CNS. Astrocytes are the major subtype of glia cell and are the most abundant cell type in the CNS providing support and energy for neurons.[17] Astrocytes are known to respond to inflammatory signals and increase levels of glial fibrillary acidic protein (GFAP) expression when activated.[18] As PPAR-γ has anti-inflammatory effects, we wanted to assess whether PPAR-γ in astrocytes might modulate the inflammatory tone in the brain and hence indirectly regulate metabolism and reproduction. Our study looks into the role of astrocyte PPAR-γ in metabolism and reproduction *in vivo*, as well as its anti-inflammatory effects in diet-induced obesity.

MATERIALS AND METHODS

Generation and Maintenance of Transgenic Mice

Astrocyte-specific PPAR-γ knockout mice were generated using Cre-*loxP* recombination. Astrocyte-specific *GCTF-CRE* mice were a generous gift from Dr. Frank Kirchhoff (Max Planck Institute, Gottingen, Germany). These mice express a Cre fusion protein under control of the human glial fibrillary acidic protein (GFAP) promoter that is expressed in all glial cells. The Cre fusion protein contains a mutant estrogen receptor that does not bind estrogen but binds tamoxifen. On addition of tamoxifen, the Cre-ER fusion translocates to the nucleus to allow recombination.[19] The *Pparg*-floxed mice were from the Olefsky Lab (UC San Diego, La Jolla, CA), and have *loxP* sites flanking exon 1 and exon 2 of *Pparg*. Upon Cre-mediated deletion, PPAR-γ1 protein will be absent and PPAR-γ2 will be a non-functional protein. *GCTF-CRE* mice were crossed with *PPAR-*$\gamma^{f/f}$ mice in the F_0 generation. F_1 generation *GCTF-CRE*$^{+}$*: PPAR-*$\gamma^{f/+}$ female offspring were back-crossed against a *PPAR-*$\gamma^{f/f}$ male to generate the desired F_2 generation *GCTF-CRE*$^{+}$*: PPAR-*$\gamma^{f/f}$ progeny. Further experimental mice were maintained by breeding *GCTF-CRE*$^{+}$*: PPAR-*$\gamma^{f/f}$ female with *PPAR-*$\gamma^{f/f}$ male to produce 1:1 ratio of *GCTF-CRE*$^{+}$*: PPAR-*$\gamma^{f/f}$ (Cre+, inducible AKO) and *PPAR-*$\gamma^{f/f}$ (Cre-, WT) pups.[20]

Genotyping of the mice were performed by PCR of DNA from a tail sample using sequence-specific primers for the upstream *loxP* site (*Pparg-flox* forward 5'-CTAGTGAAGTATACTATACACTGTGCAGCC-3', *Pparg-flox* reverse 5'-GTGTCATAATAAACATGGGAGCATAGAAGC-3'); and *Cre* transgene (*Cre* forward 5'-

GCATTACCGGTCGTAGCAACGAGTG-3', *Cre* reverse 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3'). All mice used were of a hybrid C57Bl/6:Balb/c background.

Mice were group-housed in a controlled environment with a 12L:12D light cycle at 22°C room temperature and *ad libitum* access to food and water. Experiments were performed on mice after 8 weeks of age, upon which they reach developmental and sexual maturity.[21] All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego and in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Inducing Astrocyte-Specific PPAR-γ Knockout with Tamoxifen Diet

Mice were fed a Tamoxifen diet (TAM) (Harlan Laboratories, Madison, WI) for 2 weeks starting at 11 weeks of age to induce the knockout in Cre+ mice (AKO). Cre- mice were also treated with the same diet and used as the control group (WT). Cre+ mice that had not been treated with TAM were also used as controls. Mice were then returned to a normal chow diet for another 2 weeks to allow for the metabolism and excretion of TAM remnants before proceeding with experiments. To confirm astrocyte specificity of the PPAR-γ knockout, genomic DNA was extracted from various AKO and WT tissues in lysis buffer (Allele Biotechnology, San Diego, CA). These samples were then amplified by PCR using sequence-specific primers for recombinant PPAR-γ: *Pparg flx FP1* forward 5'-CTAGTGAAGTATACTATACACTGTGCAGCC-3', *Pparg flx RP2* reverse 5'-CTTATACCTGGGACAGCATATCCC-3'. The PCR protocol followed initial activation of DNA polymerase at 94°C for 2 minutes, then 35 cycles of denaturing at 94°C for 30 seconds,

annealing at 60°C for 50 seconds, and synthesis at 72°C for 40 seconds. This primer pair gave a 230 base pair product which was detected by gel electrophoresis.

Diet Induced Obesity

TAM-treated female mice were randomly assigned to be fed a 60% high-fat diet (HFD) to induce obesity or matched 10% low-fat diet (LFD) (Research Diets Inc., New Brunswick, NJ) for the remainder of the study. Body weight and food intake data were recorded weekly. Mice that did not gain the expected weight on HFD were excluded from the study.

Estrous Cycle Analysis by Vaginal Smear Cytology

Monitoring of female mouse estrous cycles was used as a method of fertility assessment. Vaginal secretion samples were collected in the morning from female mice with 100 µL water by pipet for 21 consecutive days. Samples were then transferred onto microscope slides, and cytologic examination of the unstained vaginal smears were performed under light microscopy. Estrous cycle stages were identified by the presence of certain cell types: Diestrus stage (D) by an abundance of leukocytes, proestrus stage (P) by nucleated epithelial cells, estrus stage (E) by cornified squamous epithelial cells, metestrus stage (M) by a mixture of leukocytes with some nucleated epithelial and cornified squamous epithelial cells.[22] Diestrus and metestrus stages were combined in the estrous cycle graphs. One estrous cycle was defined as cycling from diestrus through proestrus to estrus. Intervals of 21-day sampling were performed before TAM treatment, 2 weeks after

TAM treatment, after 6 weeks on LFD/HFD, and after 12 weeks on LFD/HFD to assess changes in fertility over the course of the experiment.

Hormone Measurements

All blood samples were collected from tail vein into microcapillary tubes and centrifuged using Adams MHCT II Microhematocrit centrifuge. Plasma was collected and stored at -80°C for gonadotropin level measurement using Luminex assay kit (Millipore Corporation, Billerica, MA).

To analyze gonadotropin levels during specific estrous cycle stages, blood samples were collected in the morning of diestrus and evening of proestrus. All female mice were sacrificed at the end of the experiment in the morning of diestrus. For GnRH or kisspeptin stimulation tests, basal blood gonadotropin levels were first taken prior to hormone administration. GnRH (1 ug/kg; Sigma, St. Louis, MO) or kisspeptin (30 nmoles; Tocris, Bristol, UK) was administered via intraperitoneal (IP) injection and a second blood sample was collected again after injection. The second blood sample was taken after 10 minutes in the GnRH stimulation test, and after 20 minutes in the kisspeptin stimulation test.

Metabolic Studies

Mice were fasted for 6 hours prior to the glucose tolerance test (GTT) and insulin tolerance test (ITT). Basal blood glucose was sampled by tail vein bleeding and measured using a glucose meter (OneTouch®; LifeScan, Inc., Milpitas, CA) . Glucose (1,000 mg/kg; Hospira, Lakewood, IL) or insulin (0.4 units/kg; Novolin R, Novo-Nordisk, Copenhagen) was

then administered to mice via IP injection followed by additional blood glucose sampling 15, 30, 45, 60, 90, and 120 minutes after injection.

Mice were single-housed for the leptin suppression test starting one week prior to experimentation to allow for acclimation. Body weight and food intake were monitored to screen for irregularities in feeding pattern, in which case the mouse was excluded from the study. Upon the start of the experiment mice were administered leptin via IP injections (0.5 mg/kg for LFD, 1 mg/kg for HFD; A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center) at 6 A.M. and 6 P.M. for 2 consecutive days, for a total of 4 injections. A control experiment was then performed injecting the same animals PBS vehicle (100 μl, pH 7.3) in the same manner 1 week later. Body weight and food weight were recorded throughout the leptin suppression test. Food intake was calculated by taking the difference of daily food weight in the morning.

Quantitative PCR and Measurement of Relative Gene Expression

Tissues were collected at the time of sacrifice and stored at -80°C until RNA extraction. Total RNA was extracted with RNA-Bee (Tel-Test, Friendswood, TX) following the manufacturer's protocol. 1 μg RNA was synthesized into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed with 20 μL sample reactions using VeriQuest SYBR Green qPCR Master Mix (2X) (Affymetrix, Santa Clara, CA) on a MJ Research Chromo4 instrument, or using BioMark™ HD System (Fluidigm, San Francisco, CA). Sequence-specific primers were used for the qPCR

reactions and designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The qPCR protocol followed initial activation of DNA polymerase at 95°C for 3 minutes, then 41 cycles of denaturing at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and synthesis at 72°C for 20 seconds. Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with normalization to housekeeping genes GAPDH or RPII, and graphed as relative fold changes in respect to wildtype mice on low-fat diet.

Statistical Analysis

Data are presented as mean ± SEM and graphed using the program Prism (Version 6.4, GraphPad Software). Analysis by unpaired t-test was used for experiments comparing two groups, and analysis by one-way or two-way ANOVA was used for experiments comparing three or more groups. A *P* value of less than 0.05 to be considered statistically significant.

RESULTS

Tamoxifen Treatment Effects on Body Weight and Food Intake

GCTF-CRE^{+}: PPAR-$\gamma^{f/f}$ female and *PPAR-$\gamma^{f/f}$* male breeding pair were used to generate 50% Cre+ pups and 50% Cre- pups used for the study. To induce the knockout in the Cre+ mice, a tamoxifen diet was fed to the mice for 2 weeks. Tamoxifen is an estrogen receptor antagonist and a common drug treatment for patients with estrogen receptor positive breast cancer.[23] In this study, tamoxifen (TAM) was used as the chemical signal to activate the Cre enzyme present only in Cre+ mice that recombines floxed PPAR-γ allele.[19] Both Cre+ and Cre- mice were fed a TAM diet at 11 weeks of age to allow mice to reach sexual maturity before TAM treatment, as tamoxifen might interfere with pubertal development. After the first week of TAM diet, Cre+ mice lost 6.28% in body weight and Cre- mice lost 5.27% in body weight but recovered to normal relative to TAM-untreated mice by the second week of returning to normal chow diet (Fig. 1). There was no difference in body weight between Cre+ and Cre- at any point during and after the TAM treatment. Mice were allowed to recover from the TAM treatment for a minimum of 2 weeks to allow tamoxifen effects to dissipate before the study.

Confirmation of PPAR-γ Knockout in Astrocytes

To confirm the knockout, various tissues (brain, liver, spleen, pancreas, and testes) were collected from AKO and WT mice for PCR amplification of recombinant *Pparg* (Fig. 2). *RP2/FP1* primer pair was used to amplify a region present in only recombinant *Pparg* (Fig. 2b). *Flox* primer was run as positive control and respective WT tissues were used as

negative control for the PCR (Fig. 2a). Gel electrophoresis showed the presence of the recombined *Pparg* allele in only AKO brain. No bands were seen in any WT tissue and non-brain AKO tissue, confirming the specificity of the knockout.

Reproductive and Metabolic Changes in AKO Mice

Several experiments were performed to identify any reproductive and metabolic changes due to the PPAR-γ knockout. Analysis of estrous cycles by vaginal cytology showed that AKO had a statistically significant decrease in number of cycles compared to WT, and a decrease in number of days spent in proestrus stage (Fig. 3a,b). Because the defect could be in the level of the hypothalamus or pituitary, we tested pituitary response to GnRH to further investigate the underlying mechanisms of the reproductive changes. The GnRH stimulation test showed no changes in gonadotropin response to GnRH in AKO mice (Fig. 4), indicating the pituitary response was normal. There were also no differences in metabolism as shown by glucose tolerance test (GTT) (Fig. 5a) and insulin tolerance test (ITT) (Fig. 6a) at 17-19 weeks of age, and no differences in body weight between WT and AKO mice. Assessment of astrocyte activation by qPCR of GFAP in the brain showed no changes in activation due to PPAR-γ deletion (Fig. 7a), which was consistent with the minimal metabolic and reproductive changes.

Body Weight and Food Intake Changes in Mice on High-Fat Diet

Female AKO and WT mice were randomly assorted to be fed high-fat diet (HFD) or low-fat diet (LFD) to observe the effects of diet-induced obesity in PPAR-γ AKO. Both AKO

and WT mice on LFD maintained a body weight below 30 grams throughout the study (Fig. 8). Mice on HFD continually gained a significant amount of body weight throughout the study up to 50 grams.

Food was weighed and refilled every week and food intake measurements were calculated as the difference in food weight from week to week. WT/LFD mice ate significantly more food than WT/HFD mice (Fig. 9a). Due to caloric differences of the diets, caloric intake in mice on HFD was significantly higher (Fig. 9b). AKO mice had a decreased absolute food intake on LFD that was comparable to that of AKO/HFD mice (Fig. 9a), however the caloric intake of AKO/HFD was higher.

Metabolic Changes Due to Knockout and Diet Induced Obesity

GTTs were performed after 4 weeks and after 10 weeks on diets to assess metabolic changes in the mice. It is expected that mice on HFD will become glucose intolerant and insulin insensitive over time due their increased body weight and continual exposure to high-calorie foods.[24]

The 4 week diet GTT showed that WT/LFD mice had blood glucose levels peak to 216.5 ± 17.4 mg/dL (15 min) then gradually decrease back to basal levels (Fig. 5b). However, WT mice on HFD had blood glucose levels spike significantly higher to 312.1 ± 12.4 mg/dL (15 min) and remain elevated throughout the 15-90 min time points relative to WT/LFD levels, indicating increased glucose intolerance due to HFD. Although AKO mice on LFD had higher blood glucose levels compared to their WT counterparts, AKO/HFD mice surprisingly

did not display further increased blood glucose levels, and were generally more glucose tolerant than WT/HFD mice.

While the GTT response in WT/LFD mice was unchanged in the 10 week diet GTT, the WT/HFD glucose intolerance became even more apparent, as WT/HFD mice showed a slightly elevated basal glucose level, which then spiked to 332.0 ± 30.7 mg/dL (15 min) and remained significantly heightened compared to that of WT/LFD mice (Fig. 5c). AKO mice on LFD still maintained slightly higher blood glucose levels than levels in WT/LFD mice. Although blood glucose levels in AKO/HFD mice spiked higher in the 10 week diet GTT, AKO/HFD mice were still able to lower blood glucose levels quickly and return to basal glucose levels by the 120 min time point. AKO/HFD mice were also still significantly more glucose tolerant than WT/AKO mice.

To assess if differences in insulin sensitivity could be contributing to the changes in glucose tolerance, an ITT was performed after 11 weeks on diets. WT/LFD mice exhibited the expected ITT response in which blood glucose levels initially drop to a minimum of approximately 48.1 ± 4.8 mg/dL (45 min) and then gradually increase to recovery (Fig. 6b). AKO mice on LFD had a normal insulin response in which blood glucose levels were also able to decrease to 64.0 ± 13.3 mg/dL (30 min). However, AKO/LFD mice recovered in blood glucose levels much more rapidly, resulting in a significantly higher blood glucose level at the 90 and 120 min time points in AKO/LFD mice compared to WT/LFD mice.

As expected, WT mice on HFD were slightly more insulin resistant, as their initial decrease in blood glucose in response to insulin was not as steep as that seen in WT/LFD mice, and exhibited a minimum glucose concentration of 85.0 ± 30.4 mg/dL (45 min)(Fig. 6c).

AKO/HFD mice not show a difference in glucose levels, but had faster glucose recovery compared to WT/HFD mice as well.

To further investigate the ability of AKO mice to recover glucose levels more quickly in the ITT, we analyzed liver genes by qPCR for changes in gluconeogenic gene expression. WT liver expression of *G6pase* was significantly increased on HFD compared to LFD (Fig. 10). *G6pase* encodes for the glucose 6-phosphatase enzyme, which catalyzes the final step of gluconeogenesis in which the phosphate group is cleaved from glucose-6-phosphate to produce glucose.[25] Compared to their WT counterparts, AKO/LFD mice had slightly elevated *G6pase* expression on LFD which was not further elevated by HFD. This data is supportive of the GTT and ITT in which relative glucose intolerance was seen in AKO/LFD mice but not in AKO/HFD mice compared to WT mice. In addition, HFD elevated expression of *Cidec* and *Gfap* in the liver of WT mice, and AKO mice had even more elevated expression of these genes. Both genes are known to be associated with lipid droplet accumulation in the liver.[26,27] Although there were no significant changes in *Pepck* and *Pparg* expression in the liver, HFD slightly increased *Pepck* expression and slightly decreased *Pparg* in both WT and AKO mice.

Changes in Sensitivity to Leptin Hormone

After 17 weeks on diet, a leptin sensitivity test was performed in which leptin was injected and food intake was recorded as a measurement of response to leptin. Leptin is an adipokine that acts on the hypothalamus to suppress appetite.[29] Food intake is therefore expected to decrease significantly after leptin injections as seen in WT mice. Although

AKO/HFD animals were equally responsive to leptin relative to their WT counterparts, AKO/LFD mice were generally less responsive to the leptin injections and did not display a significant decrease in food intake (Fig. 11).

Reproductive Changes Due to PPAR-γ Knockout and Diet Induced Obesity

Estrous cycle analysis by vaginal smear cytology was used to assess reproductive changes in the mice on HFD versus animals on LFD. Cycle changes due to HFD were monitored after 6 weeks on diets and again after 12 weeks on diets. After 6 weeks on diets, there were no changes in the number of days spend in each stage of the estrous cycle (Fig. 3c). However, WT mice exhibited a significant decrease in number of cycles when put on HFD (Fig. 3d). Although AKO/LFD mice had slightly fewer cycles compared to WT/LFD mice, but AKO/HFD mice had significantly more cycles than WT/HFD mice.

Results during reproductive assessment after 12 weeks on diets were similar. There were still no changes in number of days spent in each stage (Fig. 3e), but WT/LFD mice exhibited a slightly decreased number of cycles that was comparable to that of WT/HFD mice (Fig. 3f). While AKO/LFD mice maintained fewer cycles than WT/LFD mice, AKO mice on HFD interestingly maintained their increased cyclicity, and had significantly higher number of cycles compared to WT/HFD mice as well as AKO/LFD mice.

We then measured gonadotropin levels during diestrus and proestrus stages to investigate differences in hormone surges. Gonadotropin levels are expected to be relatively lower during diestrus and higher during proestrus.[22] Luminex assay showed that WT/LFD mice had significantly higher FSH levels in proestrus than in diestrus, and that this

difference was not statistically significant in WT/HFD mice (Fig. 12a). Conversely, AKO/LFD mice had no difference in FSH levels between the stages, but AKO/HFD mice had significantly higher FSH levels in proestrus than in diestrus.

Overall, WT mice had significantly higher LH levels than AKO mice, as there were no diet differences in LH measurements (Fig. 12b). In addition, there was a noticeably greater variability in diestrus LH measurements in WT mice than in AKO mice. Although only a fraction of mice displayed an LH surge in proestrus, there were no differences in the average LH levels or in the number of mice that surged in proestrus.

Changes in Expression of Genes Involved in Reproductive Functions

To investigate potential reproductive differences in the hypothalamus, we performed a qPCR on hypothalamic RNA to look for changes in expression of genes known to play a role in GnRH regulation. There were no differences in gene expression between WT/LFD mice and WT/HFD mice. However, AKO/LFD mice had a significantly increased expression of the *Gnrh1*, *Kiss1*, *Npvf*, and *Hcrt* genes (Fig. 13). The proteins expressed from the *Npvf* and *Hcrt* genes were previously found to have inhibitory effects on the activity of GnRH neurons,[30,31] which is supportive of decreased reproductive functions in AKO/LFD mice from estrous cycle analysis. AKO mice on HFD exhibited significantly decreased expression of the *Kiss1* and *Npvf* genes relative to AKO/LFD expression.

Kiss1 encodes for the kisspeptin protein, which is a hormone known to stimulate GnRH neurons to secrete GnRH and thereby induce LH and FSH secretion.[32] The elevated *Kiss1* expression is thus incongruent with the other data. We therefore examined changes in

response to kisspeptin by administering a kisspeptin analog and measuring gonadotropin levels. All mice exhibited a normal LH increase in response to kisspeptin, with the exception of AKO/HFD mice, which displayed a significantly diminished LH response to kisspeptin compared to AKO/LFD mice (Fig. 15a). In addition, FSH secretion was unresponsive to kisspeptin in AKO mice; AKO/LFD maintained lower FSH levels and AKO/HFD maintained elevated FSH levels (Fig. 15b). This suggested that despite increased *Kiss1* expression in AKO/LFD mice, irregular response to kisspeptin in AKO mice may be contributing to reproductive irregularities. Abnormal AKO FSH response to kisspeptin was inconsistent with previous data that showed improved reproductive functions in AKO/HFD mice.

Analysis of Neuroinflammation

To assess glial activation, *Gfap* expression in the brain[18] was measured by qPCR and there was a significantly elevated *Gfap* expression in AKO/HFD mice compared to WT/HFD mice (Fig. 7b). This is indicative of increased glial activation in AKO mice on HFD, which was not previously seen in AKO mice on normal chow. Gene expression of various pro-inflammatory genes in the hypothalamus showed AKO/LFD mice had significantly increased levels of *Il12b* compared to WT/LFD mice, but there were no significant changes in inflammatory gene expression overall (Fig. 14).

DISCUSSION

According to the data presented in this study, astrocyte PPAR-γ plays a significant role in both metabolic and reproductive regulation, particularly in diet-induced obesity.

The glucose tolerance tests consistently showed slight glucose intolerance in AKO mice on the low-fat diet. Although it is well-known that high-fat diets and obesity worsen metabolic functions,[24] metabolic impairments in the mice were not exacerbated by a high-fat diet, in striking contrast to the WT mice. This suggests that deletion of PPAR-γ in the astrocytes may have protective effects against metabolic dysfunction in diet-induced obesity. This slight glucose intolerance may be explained by the qPCR of glucose 6-phosphatase (*G6Pase*) expression levels in AKO mice. The increased expression of G6Pase may contribute to the slight glucose intolerance as the enzyme function produces more glucose in the blood.[25] The HFD increases *G6pase* expression in WT mice but has no effect on AKO mice, which mirrors the patterns of glucose tolerance. In the insulin tolerance test, although AKO mice responded to insulin similarly as their WT counterparts, AKO mice generally displayed faster recovery in glucose levels. The data suggests that knockout of astrocyte PPAR-γ probably does not have a significant role in altering insulin resistance, but may remove inhibitory signals affecting gluconeogenic pathways.

In the leptin suppression test, AKO mice on the low-fat diet was the only group that did not display a statistically significant decrease in food intake after leptin administration. This lack of change in food intake is indicative of leptin insensitivity.[24] However, this finding may also be attributed to the fact that AKO/LFD mice were already eating less according to weekly food intake data, and leptin may not be able to further suppress their already lower

food intake. Other homeostatic functions or increased expression of other feeding signals such as ghrelin[34] may also be involved to counteract the effects of leptin. Interestingly, AKO/HFD mice were able to respond similarly to WT/HFD mice. However, the suppression in the HFD can not be compared to that in the LFD as higher dosages of leptin were used on HFD mice. Obese animals on HFD tend to become insensitive to leptin over time, and therefore more leptin was administered to generate a measureable response.[35] Therefore, no direct comparisons can be made between LFD and HFD mice.

Similar trends were seen in the AKO reproductive assessments in which reproductive functions were slightly impaired in AKO/LFD mice but significantly improved in AKO/HFD mice, with an increased number of cycles. This finding was consistent with previous studies conducted in our lab on neuronal knockout PPAR-γ (BKO) mice, in which BKO/HFD mice exhibited increased cyclicity compared to the WT/HFD mice.[35] Despite all these improvements seen in AKO/HFD mice, the kisspeptin stimulation test showed that they actually had an impaired response to kisspeptin. AKO/HFD secreted the least amount of LH and did not have changes in FSH levels in response to kisspeptin. In AKO/LFD mice, although LH responded well to kisspeptin, FSH levels were also unresponsive and remained low, possibly because of elevated *Npvf* and *Hcrt* expression in these mice. This data shows the disconnection between the secretion of the gonadotropins LH and FSH in the astrocyte PPAR-γ knockout, even though they are both under the control of GnRH. GnRH has different signaling pathways for LH and FSH,[36] and an important PPAR-γ function may therefore be maintaining the integrity of normal signaling of FSH secretion. Despite these irregular responses to kisspeptin stimulation, the AKO mice are still able to have reproductive

functionality, albeit slightly decreased in AKO/LFD mice. Thus, their abnormal responses are not detrimental and the negative physiological effects are most likely attenuated by other reproductive hormones to maintain estrous cycles.

Assessment of astrocyte inflammation in the brain prior to diets showed no changes in GFAP expression due to the deletion of PPAR-γ in astrocytes. However, GFAP expression significantly increased in AKO mice on a high-fat diet. This was very surprising since increased inflammation is correlated with insulin resistance and reproductive dysregulation.[1] Because AKO/HFD mice actually displayed improved metabolic and reproductive functions regardless of the increased astrocyte activation, this suggests PPAR-γ in the astrocytes may actually be mediating the HFD effects on metabolism. Therefore, knocking out PPAR-γ results in loss of the ability for the body to respond negatively to the inflammation induced by HFD.

In both metabolic and reproductive assessments, there is a trend in data in which functionalities are slightly impaired in AKO mice on LFD but not worsened in AKO mice on HFD. This is intriguing because WT mice on HFD become severely compromised in these physiological aspects, which also worsen over time. We therefore hypothesize that these abnormalities seen in WT/HFD mice may be because free fatty acids (FFA) of the HFD act as a ligand for astrocyte PPAR-γ,[1] which mediates the onset of such dysfunctions. Therefore, the lack of PPAR-γ in AKO mice prevents the FFA signaling via PPAR-γ, and attenuates such negative physiological outcomes. Despite the protective effects of AKO seen in HFD mice, the mechanism behind the recurring pattern of slightly impaired metabolic and reproductive functions in AKO/LFD mice relative to WT/LFD mice is unclear. Astrocyte PPAR-

γ therefore probably also has some beneficial effects that are lost in the knockout, although it seems that much larger-scale negative outcomes are attributed to the gene. Another possibility is that an effect of the AKO may also be altered communication with neurons that control these processes in the periphery[17] resulting in diminished functionalities.

Although astrocytes have been historically thought to have minimal contribution to the CNS compared to neurons,[37] this study shows that astrocytes clearly have a major role in important physiological functions and inflammation signaling. Research in astrocyte PPAR-γ is promising, and further studies investigating the exact signaling pathways and mechanisms of PPAR-γ in the CNS may finally unveil the connection between obesity and health problems, and possibly lead to new targeted treatments for such obesity-related diseases.

FIGURES

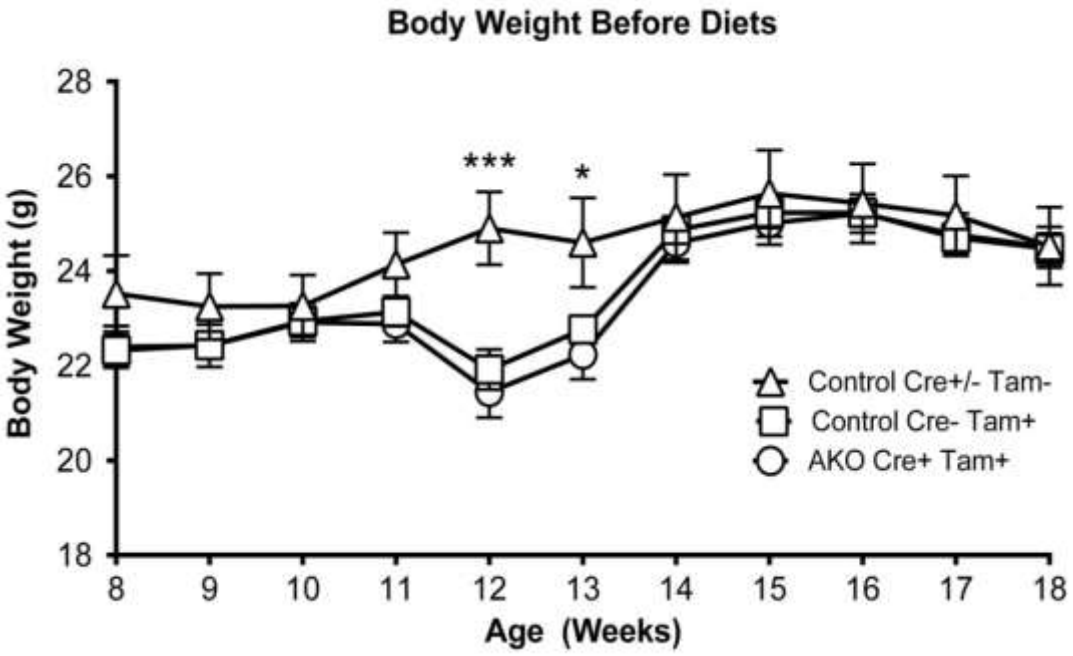

Figure 1. Changes in body weight throughout tamoxifen treatment. Mice were fed a tamoxifen (TAM) diet for 2 weeks to induce the knockout in Cre+ mice. TAM treatment began at 11 weeks of age and ended at 13 weeks of age.

* $P < 0.05$, *** $P < 0.001$.

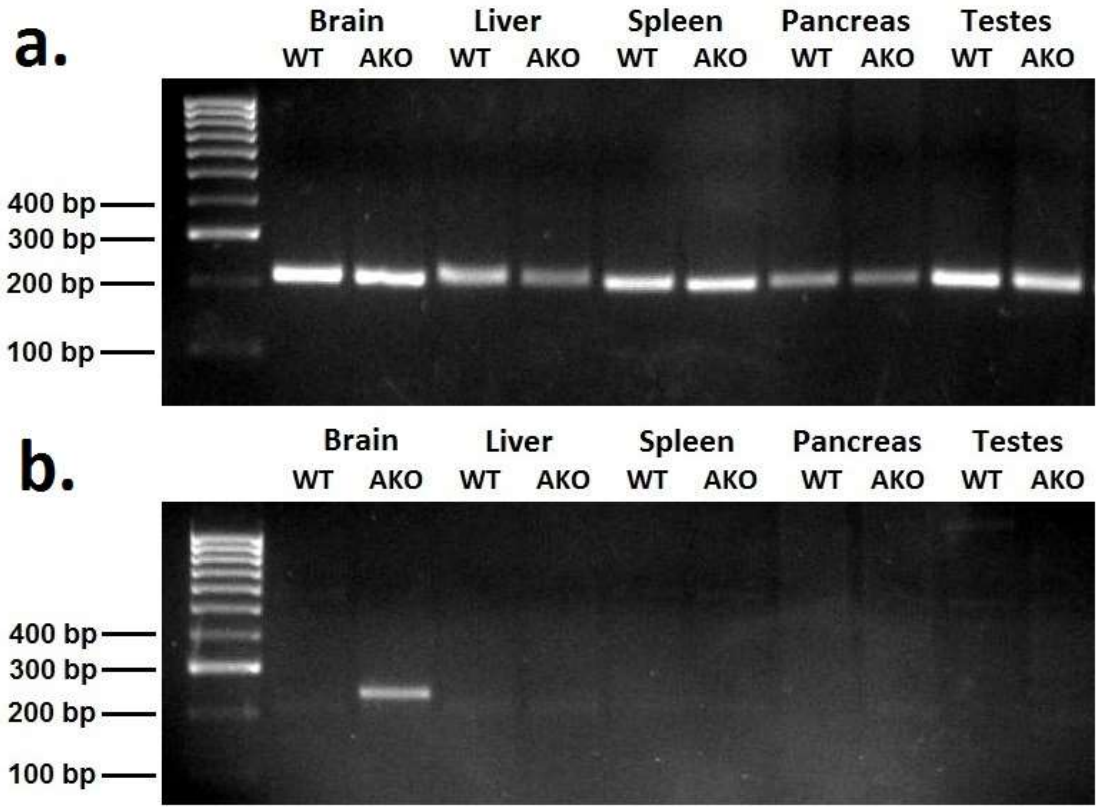

Figure 2. Confirmation of astrocyte-specific knockout of PPAR-γ. Tissues were collected upon sacrifice of WT and AKO mice and put in lysis buffer overnight. Samples were then amplified by PCR for the recombined *Pparg* allele, then analyzed by gel electrophoresis. **(a)** The amplification of the upstream *loxP* site as a positive control for the PCR experiment. **(b)** PCR amplification of recombinant *Pparg* gives a 230 base pair product.

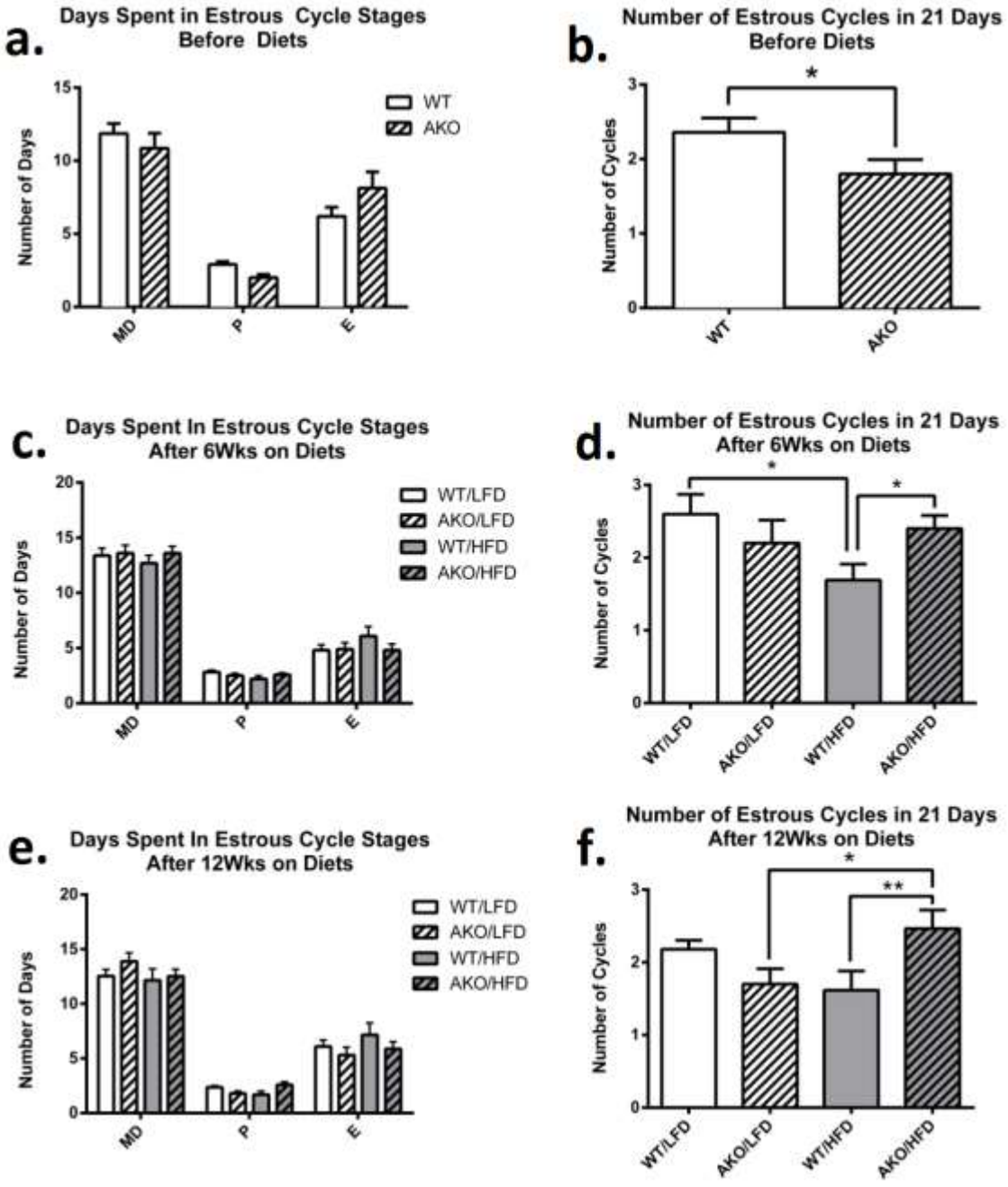

Figure 3. Estrous cycle analysis. Vaginal secretion sample was collected for 21 days for each assessment period.

* $P < 0.05$, ** $P < 0.01$.

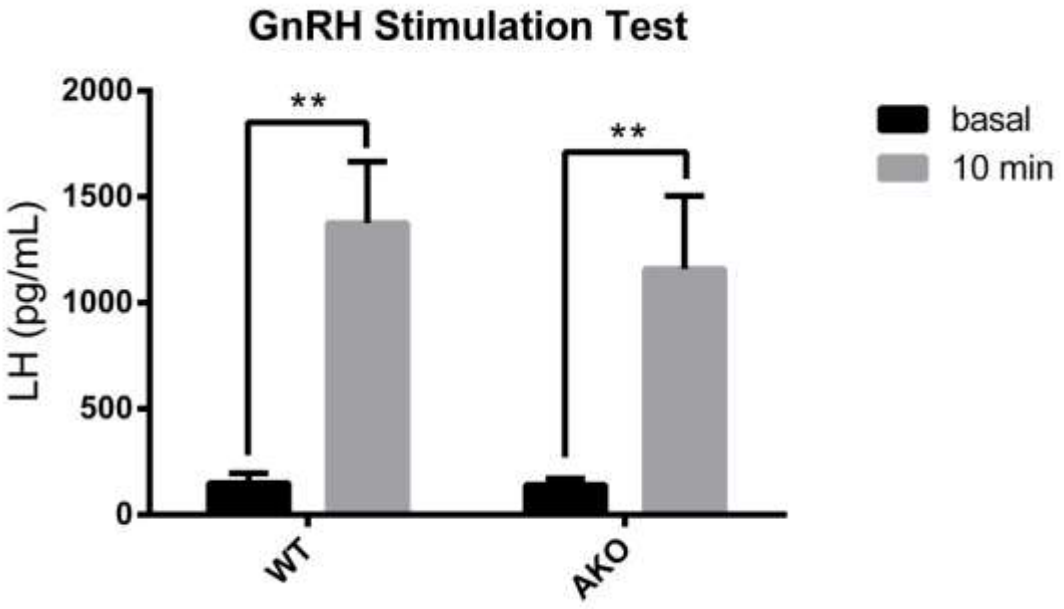

Figure 4. GnRH stimulation test in mice. GnRH was administered via IP injections and plasma LH levels were measured to assess changes in GnRH sensitivity.

** $P < 0.05$.

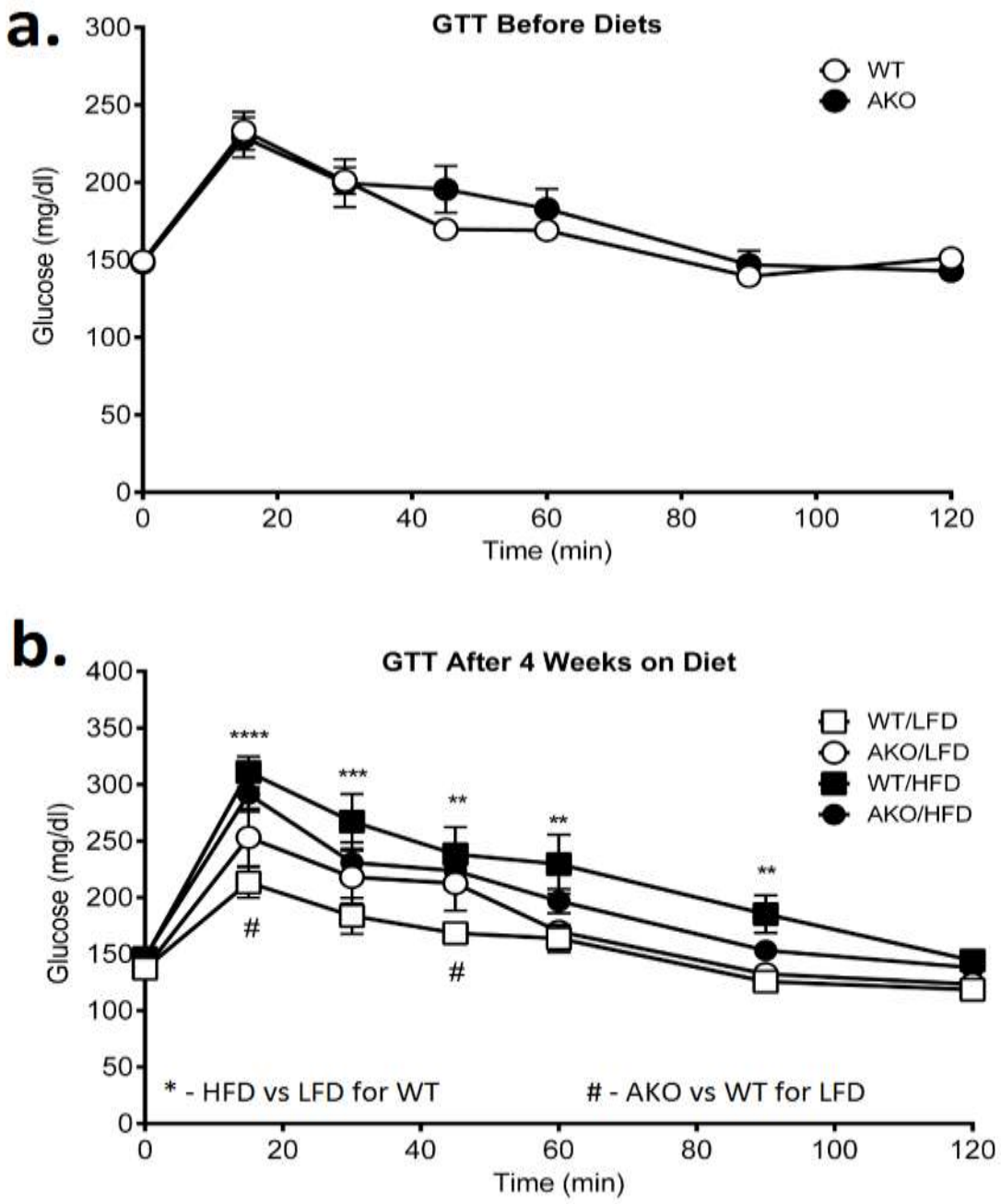

Figure 5. Metabolic assessment by glucose tolerance test (GTT). Mice were fasted for 6 hours prior to the GTT. Glucose was administered via IP injections and blood glucose concentration was measured over time.

*&#$ $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

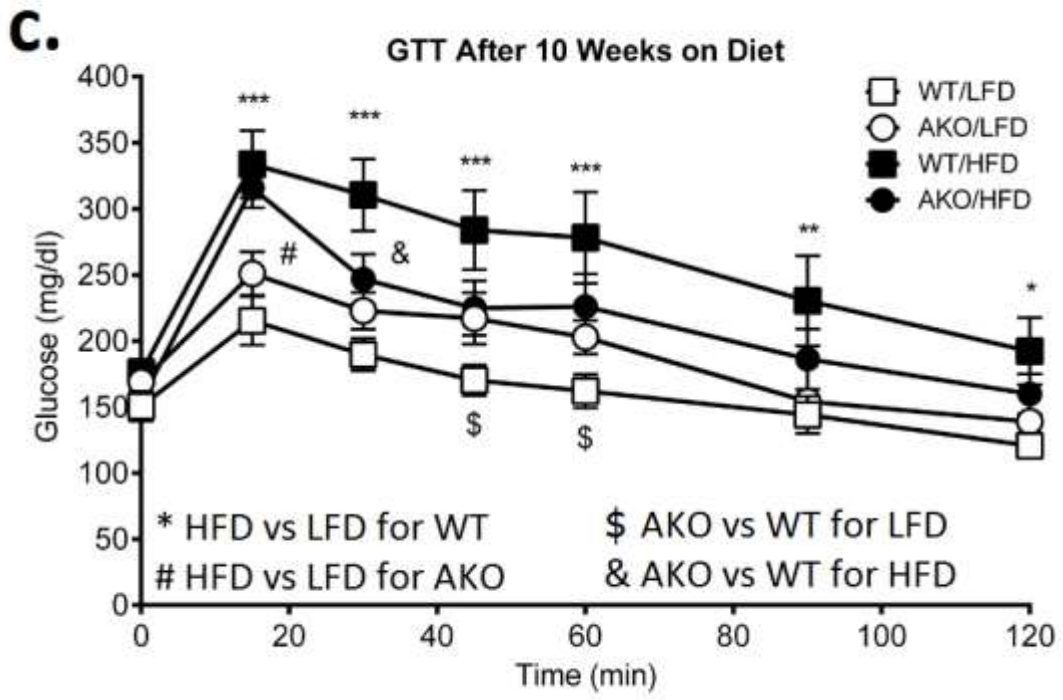

Figure 5 (continued). Metabolic assessment by glucose tolerance test (GTT). Mice were fasted for 6 hours prior to the GTT. Glucose was administered via IP injections and blood glucose concentration was measured over time.

*&#$ $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

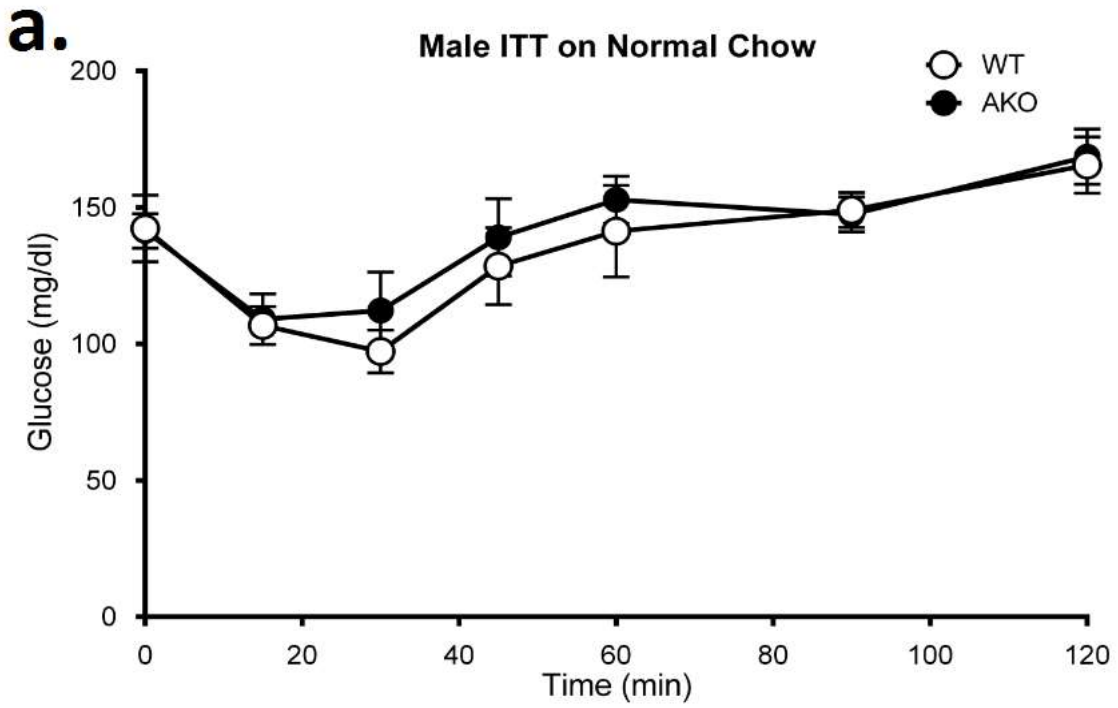

Figure 6. Metabolic assessment by insulin tolerance test (ITT). Mice were fasted for 6 hours prior to the ITT. Insulin was administered via IP injections and blood glucose concentration was measured over time.

* $P < 0.05$.

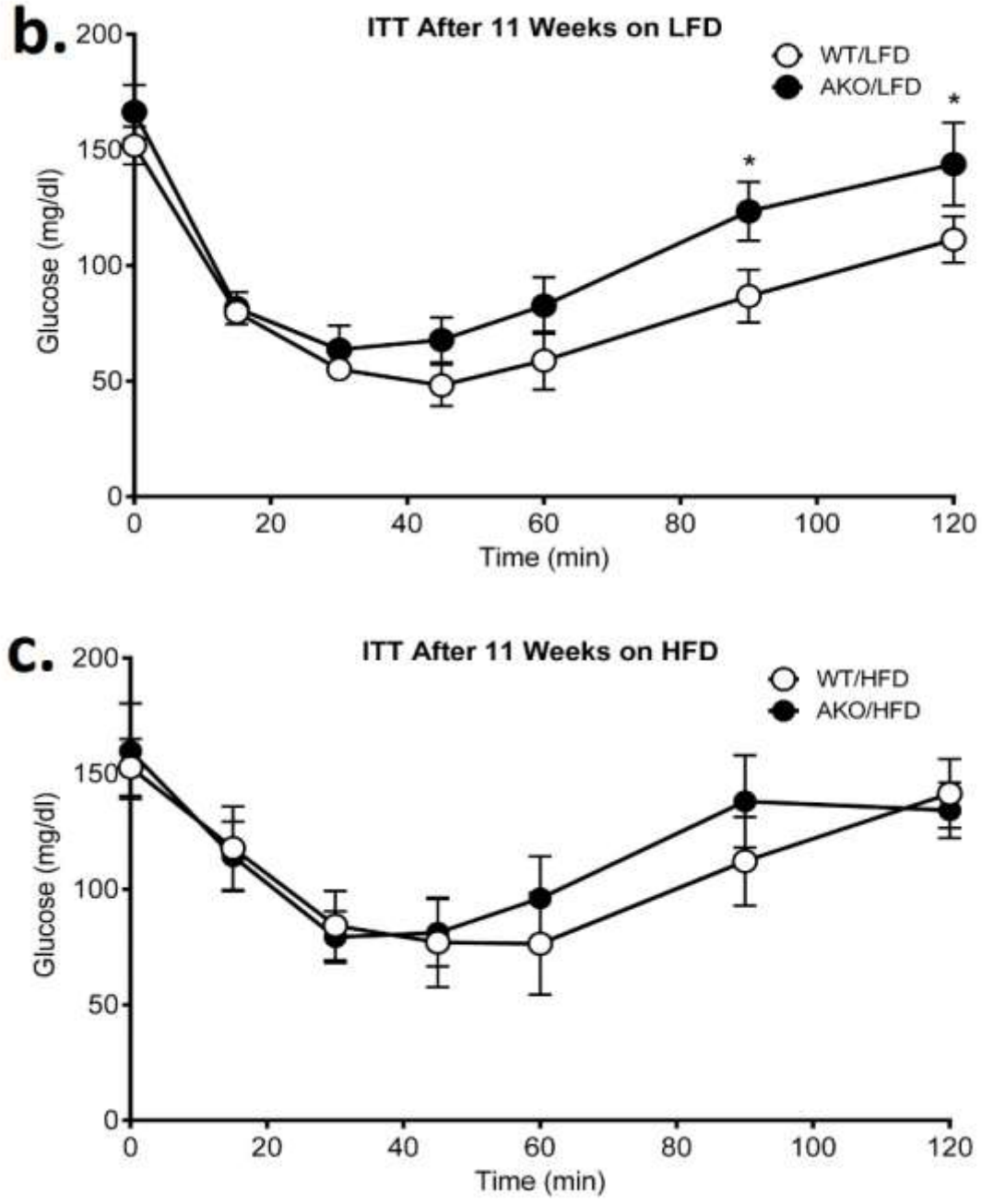

Figure 6 (continued). Metabolic assessment by insulin tolerance test (ITT). Mice were fasted for 6 hours prior to the ITT. Insulin was administered via IP injections and blood glucose concentration was measured over time.

* $P < 0.05$.

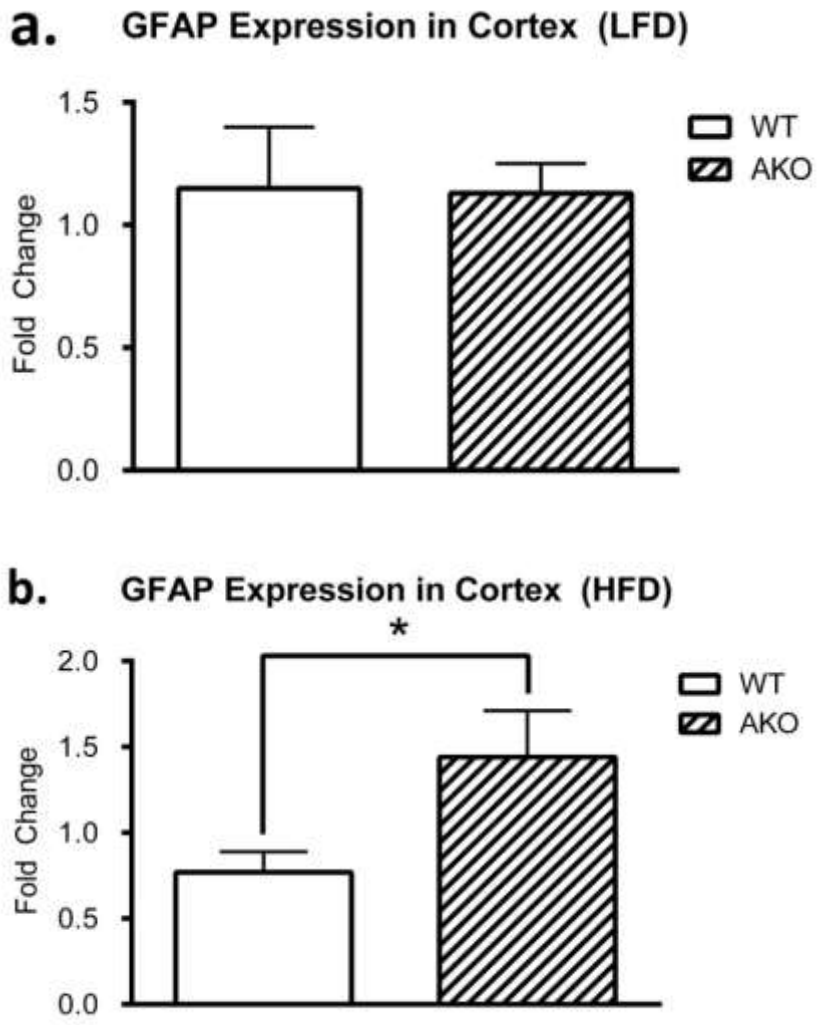

Figure 7. Expression of *Gfap* in cortex. Quantitative PCR analysis of cortex tissue was used to assess inflammation and activation of astrocytes.

* $P < 0.05$.

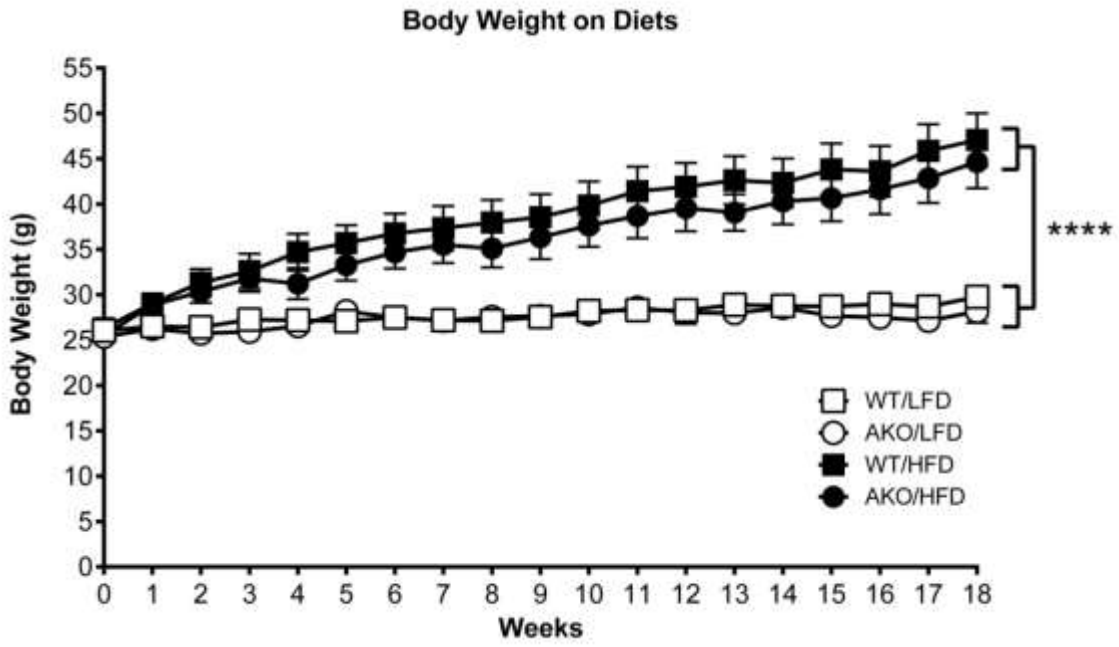

Figure 8. Weekly body weight measurements on diets. All mice on HFD continually gained a significant amount of body weight throughout the study.

**** $P < 0.05$.

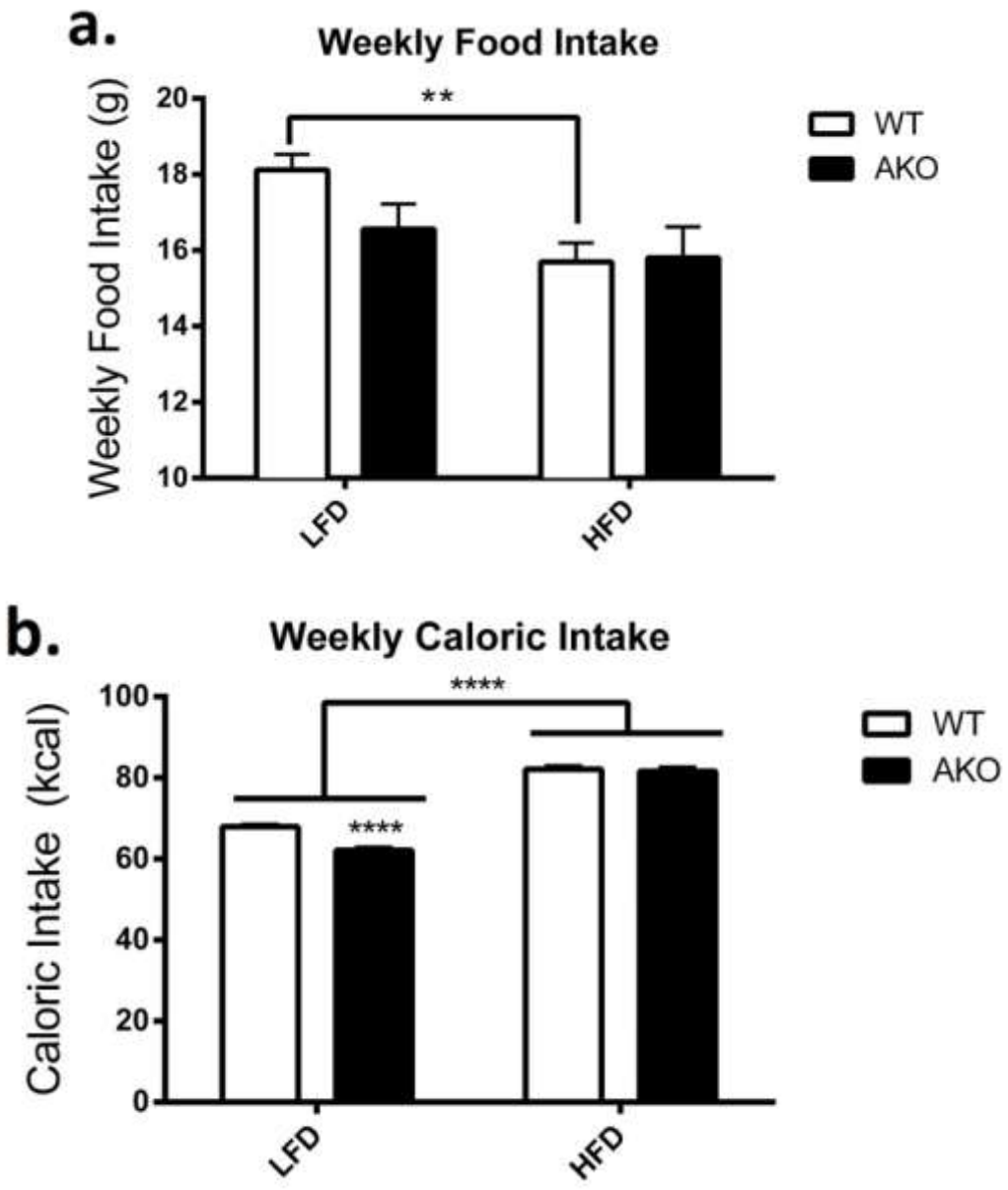

Figure 9. Measurement of weekly food and caloric intake on diets. Food intake was recorded every week for 16 weeks throughout the study. **(a)** Average weekly intake by mass of food. **(b)** Total caloric intake was calculated based on nutritional information according to the manufacturer (LFD 3.83 kcal/gm, HFD 5.24 kcal/gm).

** $P < 0.01$.

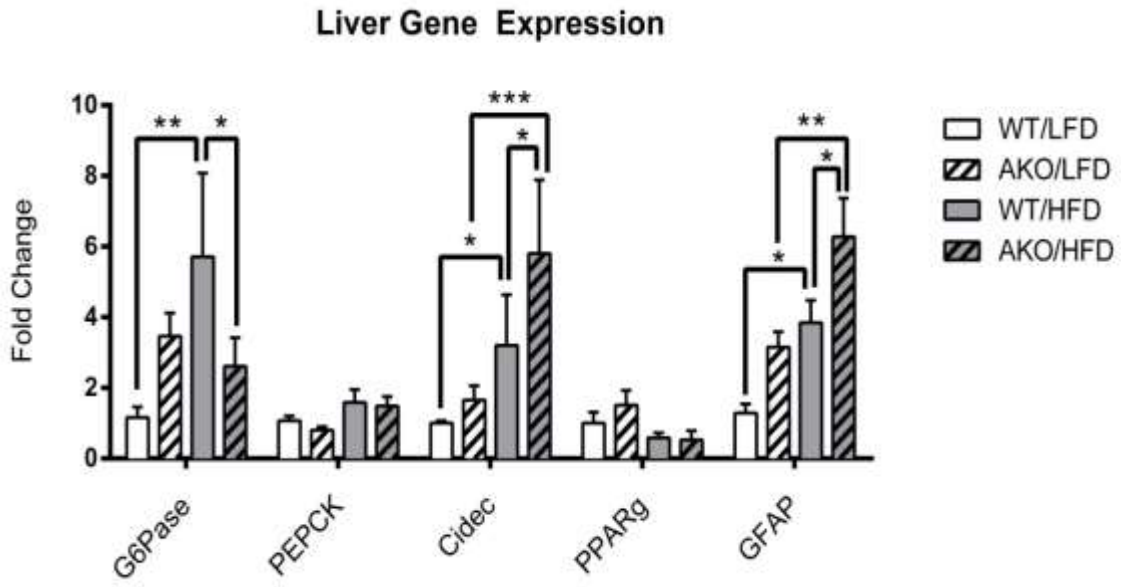

Figure 10. Measurement of gene expression in the liver.

* $P < 0.05$, ** $P < 0.01$.

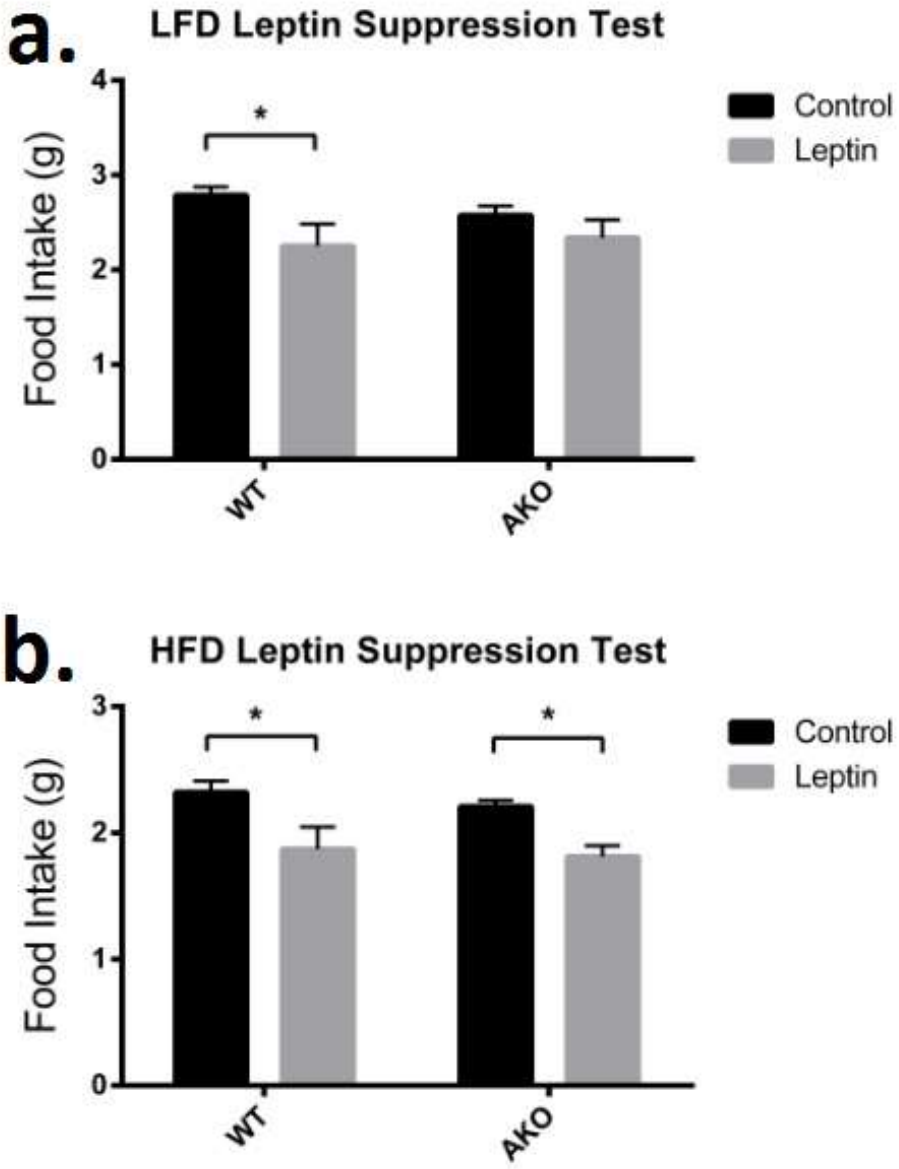

Figure 11. Food intake changes in leptin suppression test. Leptin was administered to mice via IP injection and food intake was monitored.

* $P < 0.05$.

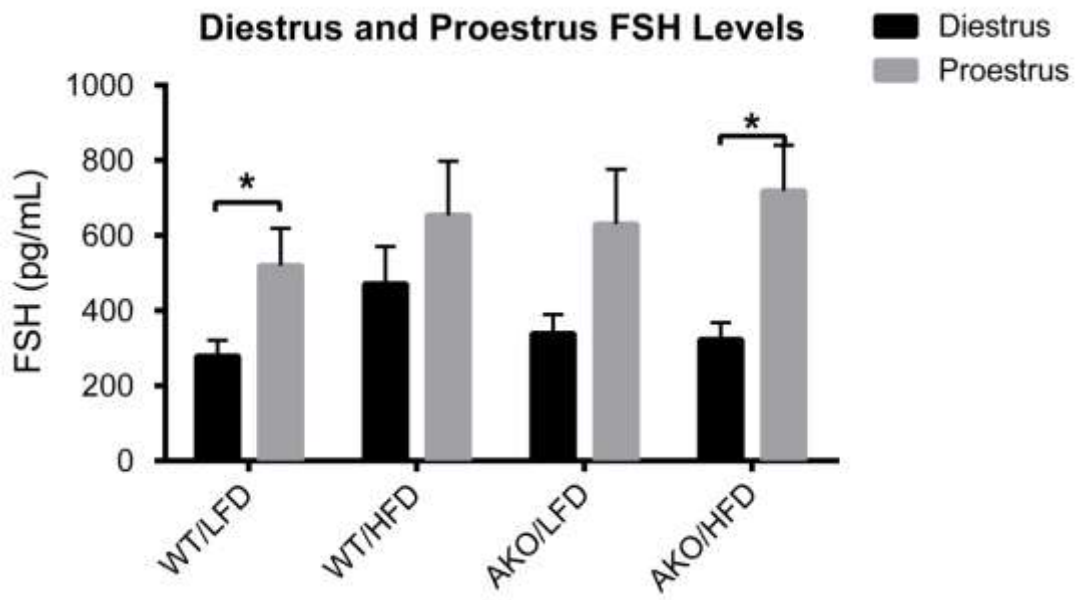

Figure 12. Plasma gonadotropin measurements in diestrus and proestrus stages. Blood samples were collected during the diestrus (morning) and proestrus stages (evening) of the estrous cycle, and LH and FSH plasma levels were measured.

* $P < 0.05$.

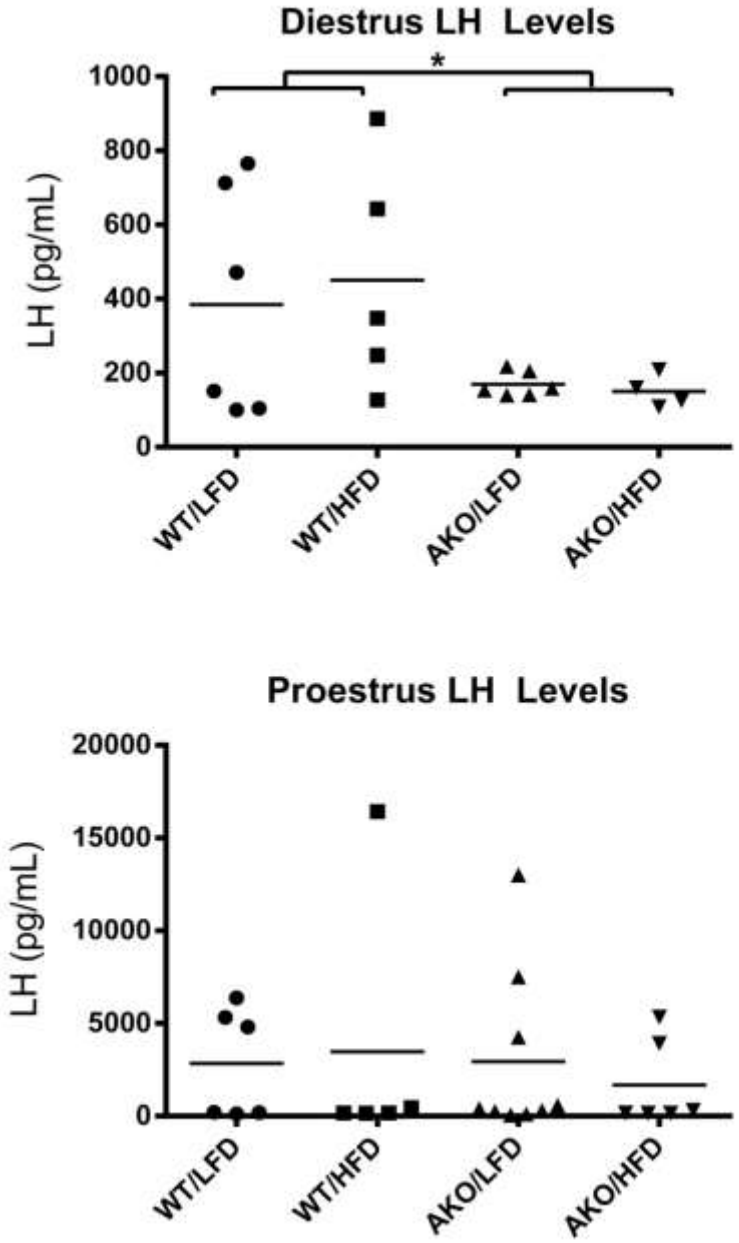

Figure 12 (continued). Plasma gonadotropin measurements in diestrus and proestrus stages. Blood samples were collected during the diestrus (morning) and proestrus stages (evening) of the estrous cycle, and LH and FSH plasma levels were measured.

* $P < 0.05$.

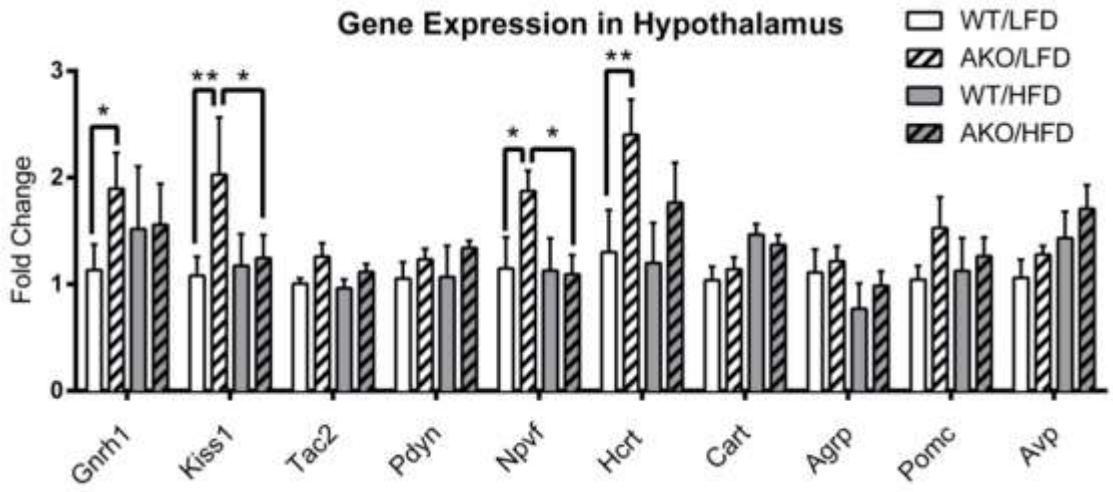

Figure 13. Analysis of hypothalamic genes involved in reproductive regulation.

* $P < 0.05$, ** $P < 0.01$.

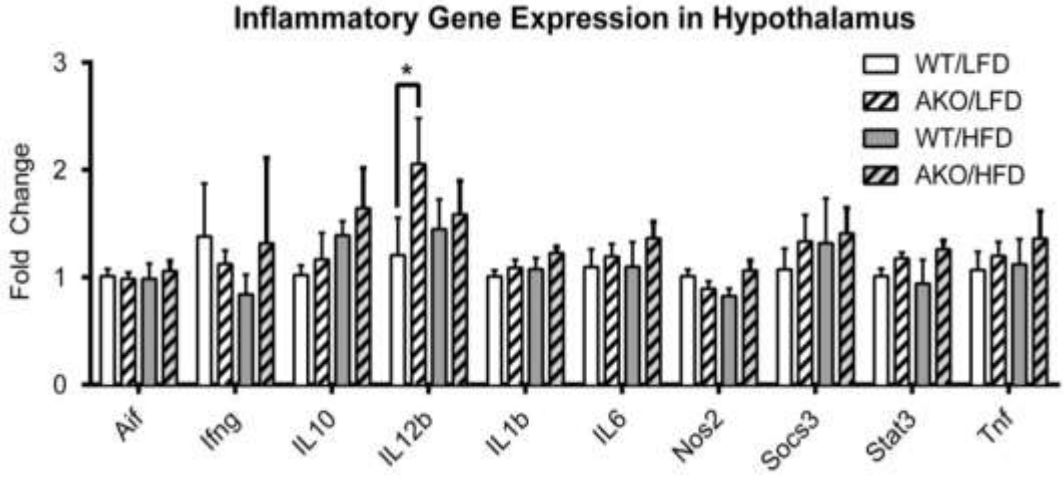

Figure 14. Measurement of inflammatory gene expression in the hypothalamus.

* $P < 0.05$.

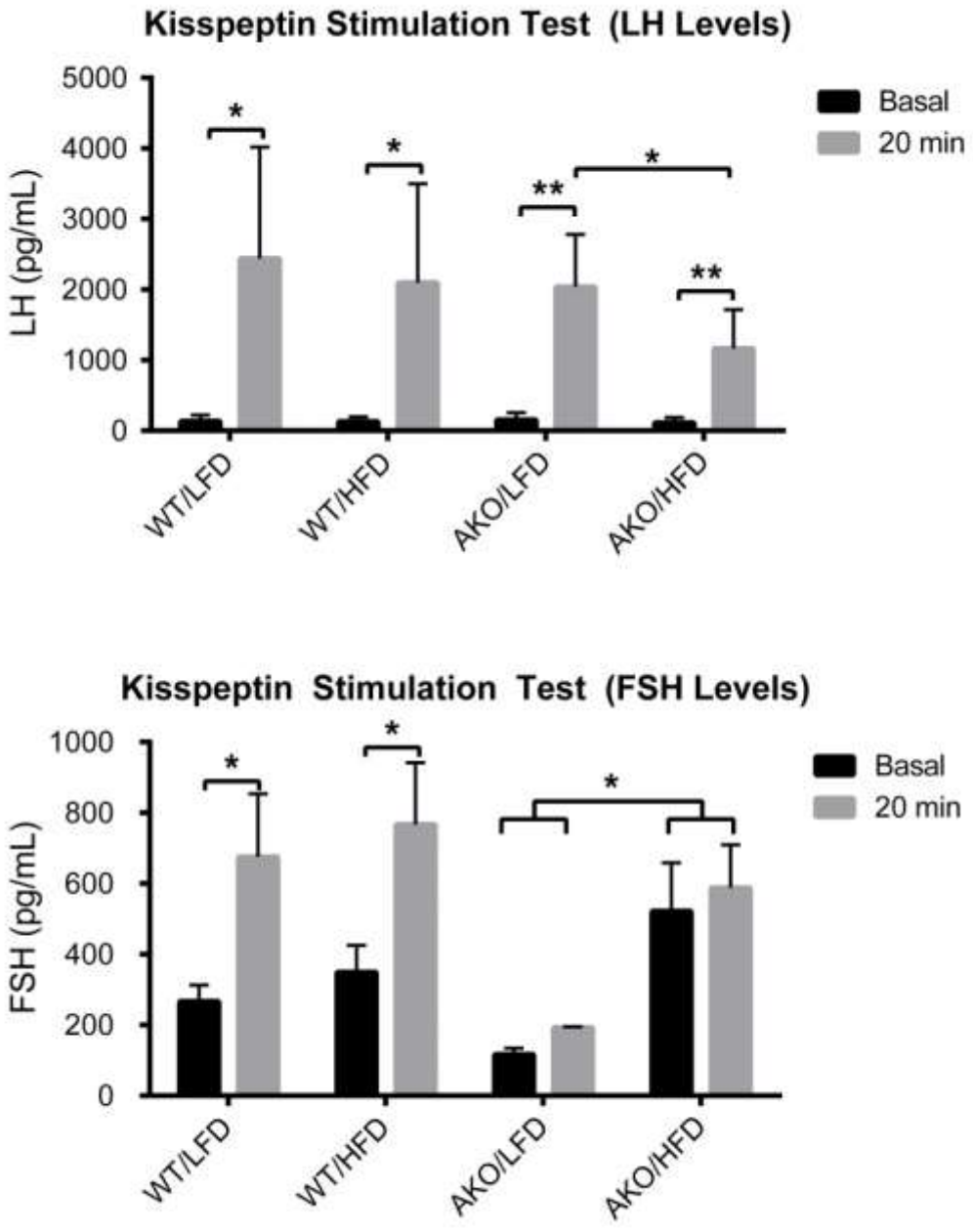

Figure 15. Gonadotropin measurements after kisspeptin administration. Mice were administered a kisspeptin analog via IP injection. Blood sample was collected 20 minutes after administration for LH and FSH measurements.

* $P < 0.05$, ** $P < 0.01$

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