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Gonadotropin and Kisspeptin Gene Expression, but not GnRH, are Impaired in cFOS Deficient Mice

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Abstract

cFOS is a pleiotropic transcription factor, which binds to the AP1 site in the promoter of target genes. In the pituitary gonadotropes, cFOS mediates induction of FSHβ and GnRH receptor genes. Herein, we analyzed reproductive function in the cFOS-deficient mice to determine its role *in vivo*. In the pituitary cFOS is necessary for gonadotropin subunit expression, while TSHβ is unaffected. Additionally, cFOS null animals have the same sex-steroid levels, although gametogenesis is impeded. In the brain, cFOS is not necessary for GnRH neuronal migration, axon targeting, cell number, or mRNA levels. Conversely, cFOS nulls, particularly females, have decreased *Kiss1* neuron numbers and lower *Kiss1* mRNA levels. Collectively, our novel findings suggest that cFOS plays a cell-specific role at multiple levels of the hypothalamic-pituitary-gonadal axis, affecting gonadotropes but not thyrotropes in the pituitary, and kisspeptin neurons but not GnRH neurons in the hypothalamus, thereby contributing to the overall control of reproduction.

Keywords

cFOS,
Kisspeptin,
c-Fos / AP-1,
GnRH,
gonadotropin

Introduction

cFOS is a basic leucine-zipper protein which forms a heterodimer with the cJUN isoform, thus forming an AP1 transcription factor that binds the TPA-response element in the promoter of target genes. cFOS is an immediate-early gene that is activated rapidly and transiently in most cell types. It is induced by a variety of growth factors, cytokines, neurotransmitters, and hormonal signals, as well as environmental stimuli. In turn, cFOS controls a diverse array of cellular processes, including cell proliferation, differentiation, survival, and death. Of all of the eclectic variations of cellular functions in which it is involved, the primary role(s) of cFOS, in any given tissue, is dependent on the cell type and stimuli (1, 2). We have shown that although pituitary gonadotropes express receptors for EGF or insulin, which in other cells induce cFOS, in the gonadotrope only GnRH induces cFOS and through it GnRH-target genes (3). Thus, cFOS although versatile, has cell-specific and stimulus-specific function in each cell type.

To study roles of cFOS *in vivo*, two different cFOS deficient mice were created and their phenotypes were analyzed in mouse strains with mixed backgrounds (129/SvJ x C57Black6J). Similar abnormalities were reported for both mouse strains lacking cFOS (4, 5). cFOS nulls are born at the proper Mendelian ratio, demonstrating that cFOS is not necessary for embryonic development. However, after four weeks of age, cFOS null mice exhibit growth retardation, osteopetrosis, and ultimately, hematopoiesis deficiency. Although cFOS null mice exhibit impairments in peripheral organs such as bone and hematopoietic system, the alteration in the central nervous system (CNS) are cell-specific (6-8). For example, adult mice lacking cFOS in the CNS exhibited normal general and emotional behavior but were specifically impaired in hippocampus-dependent spatial and associative learning tasks (8). Johnson et al. reported infertility in both sexes, though the cause was not examined (4). Since cFOS null mice are viable, redundancy poses a question and the potential complementary role of a closely related protein, FOSB, was also examined *in vivo*. Unlike cFOS null mice, FOSB deficient mice were reported to be healthy, viable, fertile, and had a normal life expectancy (9). Thus, cFOS is crucial for fertility, whereas

FOSB is not. Moreover, intact, functional FOSB is unable to substitute for the loss of cFOS with regards to the reproductive system. This necessitates further examination of the roles of cFOS in modulating the hypothalamic-pituitary-gonadal axis.

cFOS is rapidly induced in gonadotrope cells following GnRH treatment, both *in vivo* (10) and in model cell lines (11, 12). cFOS mediates GnRH induction of the FSHβ gonadotropin subunits by binding to the AP1 site in the proximal mouse FSHβ promoter (13). Furthermore, cFOS is involved in synergistic induction of FSHβ by GnRH and activin, which is specific for FSHβ and may play a role in differential expression of gonadotropin subunits (14). Specific decrease of cFOS protein turnover may contribute to the rise in FSHβ transcription during the time of low GnRH pulse frequency (15). Induction of the GnRH receptor by GnRH is also dependent on cFOS binding to both the AP1 site, to mediate GnRH responsiveness (16), and to the GRAS element where, through interaction with SMAD proteins and FOXL2, mediates the synergy between GnRH and activin (17, 18).

In the brain, cFOS serves as a marker of neuronal activation and its expression is increased in GnRH neurons during the preovulatory LH surge and after kisspeptin treatment (19, 20). cFOS expression in kisspeptin neurons also coincides with a preovulatory LH surge (21, 22). However, a role of cFOS in GnRH and kisspeptin neurons is still poorly understood. In the gonads, as well, cFOS is expressed in germ cells and, granulosa and theca cells in females (23), and Sertoli cells in males (24), but its target genes are not known. Thus, a role for cFOS in the testes and ovaries is not elucidated.

The involvement and necessity of cFOS at different levels of the reproductive axis is not well-addressed, and therefore, the underlying cause(s) of infertility in mice lacking cFOS remains unknown. Here, we examined several levels of the hypothalamic-pituitary-gonadal axis in cFOS null mice of both sexes, to ascertain if gene expression impairments exist within the reproductive axis at either the brain, pituitary, and/or gonadal levels.

Materials and Methods

cFOS-null mice

The cFOS-null mice were obtained from Jackson Laboratories, where the Papaioannou laboratory deposited them, and back crossed to C57Bl6J for six generations. Animals were maintained under a 12-hour light, 12-hour dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines using 5 and 6 weeks old animals. Genomic DNA was extracted from toe biopsies and analyzed with PCR according to JAX protocol. Animals of both sexes were studied to determine potential sex differences. At least 5 animals per sex per group (WT and null) were analyzed. Males and females were analyzed separately to determine the effect of genotype alone.

Immunohistochemistry

Tissues from 6-week old animals were fixed in 4% paraformaldehyde overnight at 4°C and dehydrated in ethanol/water washes before embedding in paraffin. Embedded tissues were cut into 14-µm coronal sections with a microtome and floated onto SuperFrost Plus slides (Fisher Scientific, Auburn, Alabama). Slides were incubated at 60°C for 30 minutes, deparaffinized in xylene washes, and rehydrated in ethanol/water washes. Antigen unmasking was performed by heating for 10 minutes in a Tris-EDTA-Tween20 mixture and endogenous peroxidase was quenched by incubating for 10 minutes in 0.3% hydrogen peroxide. After washing in phosphate-buffered saline (PBS), slides were blocked (PBS, 5% goat serum, 0.3% Triton X-100) for 45 minutes and incubated with primary antibodies against GnRH (1:1000, PA1-121, Pierce, Thermo Rockford, IL) overnight at 4°C. After washing, slides were incubated with biotinylated goat anti-rabbit IgG (1:300, Vector Laboratories) for 30 minutes. The Vectastain ABC elite kit (Vector Laboratories) was used per manufacturer's instructions and incubated for 30 minutes. After washing, the VIP peroxidase kit was used for colorimetric staining for 3 minutes. Slides were

dehydrated in an ethyl alcohol series and xylene, and cover-slipped using Vectamount (Vector Laboratories).

qPCR analysis

Tissues from 5-week old animals for were dissected, total RNA extracted 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 1, under the following conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. A standard curve with dilutions of 10 pg/well, 1 pg/well, 100 fg/well, and 10 fg/well of a plasmid containing LHβ, FSHβ, or GAPDH cDNA was generated in each run with the samples. The amount of the gene of interest was calculated by comparing threshold cycle obtained for each sample with the standard curve generated in the same run. Replicates were averaged and divided by the mean value of GAPDH in the same sample. To quantify expression of genes for which cDNA-containing plasmid was unavailable to generate a standard curve, relative gene expression was calculated using $2^{-\Delta\Delta Ct}$. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated. Five animals per group were used and males and females were analyzed separately. Statistical differences in expression between genotypes were determined by Student's T-test, with Tukey-Kramer post hoc HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Serum collection

For serum collection, 5-week old mice were sacrificed by isoflurane inhalation and blood was obtained from the inferior vena cava. The blood was left to coagulate for 15 minutes at room temperature, and then centrifuged at 2000 RCF for 15 minutes for serum separation. Hormone assays were performed by University of Virginia, Ligand Core. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is a fee-for-service core facility and is in part supported by the Eunice

Kennedy Shriver NICHD/NIH (SCCPIR) Grant U54-HD28934. LH was analyzed using a sensitive two-site sandwich immunoassay (25), and mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program) was used as standard. FSH was assayed by RIA using reagents provided by Dr. A.F. Parlow and the National Hormone and Peptide Program, as previously described (26). Mouse FSH reference prep AFP5308D was used for assay standards. Steroid hormone levels were analyzed using validated commercially available assays, information for which can be found on the core's website: http://www.medicine.virginia.edu/research/institutes-and-programs/crr/lab-facilities/assay-methods-page and reported in (27). Limits of detection were 0.24 ng/ml for LH, 2.4 ng/ml for FSH, 3 pg/ml for estradiol, and 10 ng/dL for testosterone. Intra- and inter-assay coefficients of variation were 6.4%/8.0%, 6.9%/7.5%, 6.0%/11.4% and 4.4%/6.4% for the LH, FSH, estrogen (E2) and testosterone (T), respectively. For the assays used for this manuscript, inter-assay coefficients of variation data are the result of 30 assays for LH and FSH, and 60 assays for E2 and T. Five animals per group were used for each hormone analysis and, males and females were analyzed separately to determine differences due to genotype. Statistical differences in hormone levels between wild-type and null groups were determined by Student's T-test, and Tukey-Kramer post hoc HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Ovarian Stimulation and Histology

For harvest of primary mouse granulosa cells, ovaries were dissected from 6-week old female mice in diestrus. Ovarian follicles were punctured with needles to release granulosa cells and oocytes. Cells were separated from debris by filtering through a 100-μm filter and subsequently, oocytes were removed from the granulosa cells by passing the cell suspension through 40-μm nylon mesh cell strainer. Granulosa cells were seeded at 0.2×10^6 cells per well (24-well plate) and cultured in serum-free McCoys 5A culture media containing antibiotics and incubated at 37 ˚C, 5% CO_2 for at least 2 hours, prior to treatment with 50 ng/ml ovine FSH for 1 hour. Ovine FSH was obtained from Dr. A. F. Parlow at the

National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases. After removal of media, cells were lysed, cAMP measured according to the manufacturer's protocol using cAMP- Glo Assay kit (Promega Cat # V1501, Madison, Wisconsin, USA) and concentration was calculated using a standard curve. To stimulate ovulation, 6-week old female mice were injected i.p with 5 IU PMSG, followed by 5 IU hCG i.p. injection 48 hours later (both from Sigma-Aldrich, St. Louis, Missouri). The following day, ovaries were collected for histological analysis and hematoxylin and eosin staining of paraffin sections. The Institutional Animal Care and Use Committee at the University of California, Riverside, approved all animal protocols.

In Situ Hybridization

For *Gnrh* and *Kiss1* gene expression analysis, brains from 6-week old mice were collected at sacrifice, frozen immediately on dry ice, and stored at -80°C. Brains were sectioned on a cryostat into five coronal series of 20 µm sections which were thaw-mounted onto Superfrost-plus slides and stored at -80°C. Single-label *in situ* hybridization was performed as previously described (28, 29). Briefly, slide-mounted brain sections encompassing the entire preoptic area and hypothalamus from one of the 5 sets of serial brain sections were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2X SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanol, and air-dried. Radiolabeled (^{33}P) *Kiss1* or *Gnrh* antisense riboprobe (0.04 pmol/ml) was combined with tRNA, heat-denatured, added to hybridization buffer, and applied to each slide (100 µl/slide). Slides were cover-slipped and placed in a 55°C humidity chamber overnight. The slides were then washed in 4X SSC and placed into RNAse A treatment for 30 min at 37°C, then in RNAse buffer without RNase at 37°C for 30 min. After washing in 2X SSC at room temperature, slides were washed in 0.1X SSC at 62°C for 1 hour, dehydrated in ethanol, and air-dried. Slides were then dipped in Kodak NTB emulsion, air-dried, and stored at 4°C for 4-5 days (depending on the assay) before being developed and cover-slipped.

ISH slides were analyzed with an automated image processing system (Dr. Don Clifton, University of Washington) by a person blinded to the treatment group (30). The software counts the number of silver

grain clusters representing *Kiss1* or *Gnrh* cells, as well as the number of silver grains over each individual cell, which provides a semi-quantitative count of *Kiss1* or *Gnrh* mRNA expressed per cell. Cells were considered *Kiss1* or *Gnrh* positive when the number of silver grains in a cluster exceeded that of background by 3-fold. Neuron numbers and mRNA levels per cell from males and females were analyzed with two-factor ANOVA and Tukey-Kramer HSD post hoc test, with a significance $p<0.05$, using JMP software (SAS Institute; Cary, North Carolina).

Results

Gonadotrope gene expression is lower in cFOS null mice

We determined previously that cFOS is a critical transcription factor through which GnRH induces FSHβ gene in the LβT2 gonadotrope model cell line (13). It is also involved in differential expression of gonadotropin subunits that are necessary for reproductive fitness (14, 15). In this study, we analyzed a role of cFOS in reproduction *in vivo*, using cFOS null animals. As reported before (4), we observed that heterozygous crosses result in the Mendelian ratio of offspring at birth. There was no gene dosage effect, since heterozygous animals were not different from the wild types in any paradigm we examined; thus, we present only wild-type and null results.

Due to our interest in gonadotrope gene expression and a known role for cFOS in FSHβ and GnRH receptor induction by GnRH, we started our analysis of cFOS null animals by assessing gonadotrope gene expression (Fig. 1A-H). In both sexes at postnatal day 35 (p35), cFOS deficient mice had lower *Lhb*, *Fshb*, *Cga* (α-GSU) subunit, and *Gnrhr* mRNA expression than wild-types. *Lhb* was 83% lower in males and 76% lower in females; *Fshb* was 88% lower in males and 93% lower in females; *Cga* was 77% and 80% lower in males and females respectively, and *Gnrhr* was 57% and 80% lower, respectively. Since cFOS deficiency affected gonadotrope specific gene expression, and both gonadotropes and thyrotropes express a common α-GSU which heterodimerizes with the hormone-

specific β subunits, we analyzed the expression of the third glycoprotein hormone β subunit. *Tshb* mRNA levels were the same in wild-type and cFOS deficient animals in both males (Fig. 1I) and females (Fig. 1J). Thus, cFOS is necessary for gonadotrope gene expression, but not for thyrotrope gene expression.

Serum concentrations of circulating gonadotropin hormones were assayed to determine whether reduction in gonadotropin gene expression resulted in lower circulating hormone levels. Difference in serum baseline LH concentration in male animals did not reach statistical significance due to variability, although there was a trend for lower level in the nulls (Fig. 2A). There was no difference in baseline LH levels between wild-type and null females (Fig. 2B). FSH was 85% lower in mutants of both sexes (Fig. 2C, D). To determine pituitary responsiveness to GnRH, we analyzed serum gonadotropin levels 10 minutes following subcutaneous injection of GnRH. Absolute values of GnRH-induced LH were significantly lower in nulls compared to wild-type males (Fig. 2E), due to a different baseline levels. Interestingly, although null male mice started with lower baseline LH levels, both wild-type and null males exhibited a comparable 5-fold increase in LH serum concentration following GnRH injection (compare Figures 2A and 2E). Thus, in null males LH concentration increased in response to GnRH treatment. The response to GnRH in nulls implies sufficient expression of GnRH receptors in null males. In females, wild-types responded to GnRH with increased LH levels (Fig. 2F), while nulls retained the same level as prior to the GnRH treatment (compare Figures 2B and 2F), revealing a significant difference in GnRH-responsiveness between wild-type and null females. This sex difference in GnRH responsiveness may stem from the alterations in GnRH receptor expression that is more severe in null females (Fig. 1H). Although circulating FSH levels were not dramatically increased following GnRH injection, perhaps due to timing of blood collection, null mice of both sexes had 96% lower FSH in the circulation than the wild-types after the GnRH treatment (Fig. 4G, H). Collectively, both pituitary mRNA levels and serum hormone analyses indicate that cFOS is necessary for normal levels of gonadotropins, particularly FSH.

cFOS is <u>not</u> required for steroidogenesis

Given that gonadotropin gene expression was diminished in null animals, we analyzed the effects of lower FSH levels on the gonads. Histological analysis of gonads at 6 weeks of age revealed that cFOS null females exhibited a block in folliculogenesis, reminiscent of a phenotype observed in FSHβ deficient mice (31). Specifically, female nulls lacked antral follicles and corpora lutea, indicating a lack of ovulation, while both antral follicles (AF) and corpora lutea (CL) were present in wild-type littermates (Fig. 3 top panels). Homozygous null ovaries contained normal primordial follicles, a greater number of secondary follicles with normal oocytes, and a thick granulosa cell layer, indicating normal early follicular development and typical granulosa cell proliferation, but no antrum development (Figure 3 top right panel). Testes analysis revealed that most seminiferous tubules in cFOS null males lacked spermatozoa (S1, Fig. 3 bottom panel). Spermatogonia were present (S2), but there was a smaller number of elongated spermatids (S3), indicating potential meiosis problems. Testes also contained a lower number of interstitial Leydig cells (L, Figure 3 bottom panel). Further examination revealed that cFOS null females do not exhibit vaginal opening, an external measure of puberty, by 42 days of age (6 weeks), while wild-type littermates had a mean onset of vaginal opening at p29.2 (data not shown). In the cFOS null males, there was no mature sperm in the epididymis by 6 weeks of age, while wild-type males presented with 4.7 x 10^6/ml sperm count (data not shown). Therefore, cFOS is necessary for late folliculogenesis and spermatogenesis, but not for early germ cell development.

Since gonadal histology differed between the genotypes, we examined sex steroid hormone levels and expression of gonadotropin hormone receptors in the gonads. *Lhr* expression was 62% lower, specifically in female null mice (Fig. 4B), while in the null males there was a trend for a decrease that did not reach statistical significance (Fig. 4A). On the other hand, *Fshr* was significantly higher in null males than wild-types (1.66 fold, Fig. 4C), while null and wild-type females had the same expression of FSH receptor (Fig. 4D). To determine if ovaries can respond to FSH, since FSH treatment induces cFOS in granulosa cells (23, 32), we stimulated primary cultures of granulosa cells with FSH to elicit an increase in intracellular cAMP levels. Granulosa cells from both wild-type and null mice exhibited similar increase

in cAMP following 1-hour treatment with 50 ng/ml FSH, indicating that the FSH receptor are expressed and functional in the granulosa cells from null animals (Fig. 4E). However, stimulation with pregnant mare serum gonadotropins (PMSG) followed by human chorionic gonadotropin (hCG) 48 hours later, did not elicit ovulation in null females, determined by a lack of corpora lutea in the ovarian histological sections (data not shown). We then analyzed concentration of sex-steroid hormones in the circulation. In males, serum testosterone was similar between genotypes (Fig. 4F). Although null males exhibited a trend for lower testosterone levels, the difference did not reach statistical significance, as with the level of LHR expression in males. In females, serum estrogen was the same between genotypes (Fig. 4G). Collectively, these results indicate that cFOS is necessary for gametogenesis, but not for steroidogenesis or gonadotropin hormone receptor expression in the gonads.

GnRH neuron number and migration is not altered due to a lack of cFOS

Given that both gonadotropin hormones, which are synthesized and secreted in response to GnRH, were reduced in null animals, we next examined GnRH expression and the number of GnRH neurons in the hypothalamus in 6 week old animals. Using immunohistochemistry, we elucidated that GnRH neurons are present in both genotypes, in both sexes, in a normal pattern in the preoptic area of the hypothalamus (Fig. 5A), indicating that cFOS is not necessary for developmental migration of GnRH neurons from the olfactory placode to their final adult location. Likewise, the median eminence in both genotypes stained with GnRH, demonstrating that axon targeting of GnRH neurons is not affected by a deletion of cFOS (Fig. 5B). *In situ* hybridization was used to determine GnRH neuron number and mRNA expression levels (Fig. 5C). There was no difference in GnRH neuron number in males, while null females exhibited a 17% decrease compared to wild-types (Fig. 4D). Although statistically significant, this decrease is unlikely to affect fertility, since only 34% of the entire GnRH neuron population is sufficient for full reproductive function (33). Expression levels of *GnRH* mRNA per cell was also the same in both genotypes in both sexes (Fig. 5E), showing that cFOS is not necessary for normal GnRH gene expression. Therefore, although cFOS is activated by a variety of stimuli in GnRH neurons, GnRH

neuron migration, axon targeting, cell number, and gene expression are not altered by the deletion of cFOS.

cFOS is necessary for kisspeptin neurons in female mice

cFOS deficiency resulted in diminished levels of both gonadotropin hormones, but no major alteration was found in GnRH number and mRNA expression levels. Therefore, we examined kisspeptin neurons because kisspeptin is necessary for GnRH secretion. Kisspeptin neuron number and mRNA expression levels in the anteroventral periventricular nucleus (AVPV) were sexually dimorphic, as previously shown, with females having a larger number of *Kiss1* neurons (*in situ* hybridization of female (F) AVPV shown in Fig. 6A). cFOS deficiency resulted in a dramatic 84% decrease in the number of *Kiss1* neurons in females (Fig. 6A, B), despite no genotype difference in circulating estrogen. In males, the decrease did not reach significance. Expression of *Kiss1* mRNA levels per cell in AVPV neurons was 38% lower in males and 80% lower in females (Fig. 6C). In the arcuate nucleus (ARC), again only female null mice demonstrated a significant decrease, with 66% fewer *Kiss1* neurons detected versus wild-types (Fig. 6D, E). Contrary to AVPV neurons, kisspeptin neurons in the ARC of both sexes in both genotypes had the same levels of *Kiss1* mRNA per cell (Fig. 6F). Together, our results indicate that cFOS is necessary for kisspeptin gene expression in both hypothalamic nuclei, with dramatic reductions observed primarily in females.

Discussion

Despite extensive studies on regulation of the gonadotropin subunit genes using cell models and dispersed pituitary cultures, little is known about *in vivo* roles of intermediary immediate early genes, such as cFOS, that are proposed to be involved in GnRH induction of gonadotrope specific genes. Studies using cell lines indicate that cFOS is a direct target of GnRH signaling that, upon induction, activates FSHβ and GnRH receptor gene transcription (13, 16). In the brain, cFOS is also often used as a marker of neuronal activation, in excitation of GnRH neurons and kisspeptin neurons (19, 21). The pleiotropic

transcription factor, cFOS, is induced by a variety of stimuli in various other cell types, but exhibits cell-specific and stimuli-specific function in each tissue examined. Herein, we examined the role of cFOS in hypothalamic-pituitary-gonadal (HPG) axis gene expression *in vivo*, using cFOS-deficient mice, and demonstrate that there is a specificity of cFOS necessity for pituitary gene expression and neuronal gene induction.

In the original report with cFOS null animals, Johnson et al. reported infertility due to impaired gametogenesis (4). Although lethality at two months of age is due to impaired hematopoiesis in adults, at birth, cFOS null mice are viable without gross developmental deficits, indicating that cFOS is not required for embryonic development (4). Several reports analyzing cFOS nulls in the central nervous system identified cell-specific effects, without severe global defects (6-8). Thus, although we cannot discount possible compensatory mechanisms or non-specific defects that impinge on the reproductive axis, our results indicate cell-specific and/or sex-dependent functions of cFOS in the HPG.

As predicted from studies using gonadotrope-derived cell lines (13, 16), we show here *in vivo* that cFOS is necessary for expression of the FSHβ subunit and the GnRH receptor. Additionally, we determined that cFOS is necessary *in vivo* for LHβ and αGSU gene expression. Since LHβ and αGSU gene promoters have been analyzed in detail and cFOS/AP1 response elements have not been identified (34); nor does cFOS overexpression induce their promoters in the cell line (data not shown), the lower levels of these genes in null mice may stem from a decreased expression of the GnRH receptor. Lower FSHβ expression may either stem from a direct effect of cFOS on the FSHβ promoter, as has been shown in cell lines, or due to a decrease in GnRH receptor expression. A decrease in the GnRH receptor was a likely cause of the lack of LH secretion in null females following GnRH stimulus. Although LHβ gene expression and LH levels in response to GnRH are lower in nulls, basal circulatory levels of LH are the same between wild-type and null females. LHβ mRNA has a very long half-life (35), allowing for translational regulation (36) that may be compensatory in knockouts, resulting in the similar levels of LH in circulation. On the other hand, cFOS is <u>not</u> necessary for expression of a third pituitary glycoprotein

hormone, TSHβ. Thus, in the pituitary, cFOS is required for gene expression in the gonadotrope, but not in thyrotrope.

Corresponding to relatively minor effects on gonadotropin hormone receptor gene expression in the gonads, sex steroid levels are the same between wild type and null mice, in males and females. LH receptor was affected in a sex-dependent manner, exhibiting a decrease only in females. Effect only in females would suggest a role for estrogen, however estrogen levels were the same in wild-type and null mice. Furthermore, estrogen receptor α knockout animals and aromatase null mice both exhibit an *increase* in LH receptor expression, pointing to a negative regulation of the LH receptor by estrogen (37, 38). Thus, cFOS may regulate LH receptor expression directly in a sex-specific manner. In contrast to LH receptor, cFOS deficiency did not have an effect on the FSH receptor in females, but FSH receptor expression was increased in null males. This result is surprising, since a cFOS binding site was identified in the FSH receptor promoter and considered to play a role in FSH induction of its own receptor (39). Increased expression in null males point to a repressive role of cFOS for FSH receptor gene. Unexpectedly, sex steroid hormone levels were unchanged, implying that cFOS does not play a critical role in expression of steroidogenic enzymes. Contrary to steroid hormone levels, epididymides of male nulls exhibit absence of mature sperm, and ovaries of female nulls lack antral follicles and do not ovulate, even in response to exogenous gonadotropin treatment. In the testes, cFOS is expressed in spermatogonia, spermatocytes, and Sertoli cells (24). A role for cFOS in germ cells is not known, while in Sertoli cells multiple roles have been postulated, including proliferation and tight junction formation (40, 41). Due to a lack of known cFOS target genes in germ cells, we were not able to delineate the cause of the block in spermatogenesis. In the ovaries, cFOS is present in granulosa cells, theca cells and oocytes (23, 42). In granulosa cells, cFOS is induced by FSH treatment (32). The amount of cFOS decreases with the growth of the follicle and diminishes after luteinization (23). It was therefore postulated, that cFOS plays a role in granulosa cells proliferation. Our results, however, do not agree with a role for cFOS in granulosa cell proliferation, since follicles in null animals had multiple layers of granulosa cells, but indicate a role for cFOS in antrum formation. Due to necessary intra-follicle signaling between theca, granulosa and oocyte,

405 throughout maturation (43), we were not able to explain the cause of anovulation. Estrogen levels in
406 females, and testosterone in males, were the same between genotypes, indicating that although cFOS is
407 necessary for gametogenesis, it is superfluous for steroidogenesis.

408 There was no effect of cFOS deficiency on GnRH neuron migration, number of GnRH neurons,
409 or GnRH transcription. The minor decrease observed in null females is unlikely to be physiologically
410 significant since the number of GnRH neurons can be substantially reduced without impairing fertility
411 (33). The observed minor decrease in cFOS null females may be caused by a reduction in upstream
412 kisspeptin signaling (20, 44), since we detected significant decreases in *Kiss1* neuron number and mRNA
413 levels specifically in females. Although cFOS is activated by numerous neurotransmitters and
414 neuropeptides, and is used as a marker for neuronal activation including in GnRH neurons, GnRH gene
415 expression is not dramatically affected in cFOS nulls. This implies that the GnRH gene is not
416 transcriptionally regulated by any of the signals that activate the neuron through cFOS induction and that
417 cFOS is not necessary for proper GnRH transcription. Lack of major effect on GnRH expression points
418 that the alteration in GnRH secretion rather than transcription is a cause of downstream reproductive
419 consequences. Furthermore, despite a decrease in kisspeptin, absence of an effect on GnRH expression,
420 indicates that kisspeptin does not regulate GnRH gene at the transcriptional level, since lower kisspeptin,
421 particularly in females, is not reflected in dramatically lower GnRH expression at the level of GnRH
422 neuron. Thus, these findings indicate that kisspeptin plays a major role on GnRH secretion or neuronal
423 activation, but not transcription. Thus, this is a likely cause for the observed deficits in pituitary and
424 gametogenesis.

425 The observed decrease in the *Kiss1* gene expression in both the AVPV and ARC, primarily in
426 females, is intriguing. Molecular mechanisms of *Kiss1* gene expression in the hypothalamus have not
427 been elucidated and our results implicate cFOS as an important regulator of *Kiss1* transcription *in vivo*. Li
428 et al. reported that the human kisspeptin promoter contains two cFOS/AP1 sites at positions -1272 and -
429 418 (45), but did not examine whether these sites are functional, i.e. whether kisspeptin expression would
430 be diminished if cFOS/AP1 elements were mutated. It is possible that cFOS binds these elements to

induce *Kiss1* gene expression. By what means these elements respond in a sex-dependent manner or more strikingly in the AVPV, is not clear. We postulate that cFOS is also involved in estrogen regulation of *Kiss1* expression in AVPV. Explicitly, *Kiss1* mRNA levels were decreased only in the AVPV, a brain site where *Kiss1* is strongly upregulated by estradiol as part of estrogen positive feedback during the preovulatory LH surge (46). cFOS involvement in estrogen signaling has been shown in multiple tissues. cFOS is recruited to the estrogen response element in promoters that are induced by estrogen treatment (47). Additionally, estrogen responsive genes that do not contain estrogen responsive elements in their promoters are activated via estrogen receptor recruitment by cFOS to the AP1 site (48, 49). It is entirely possible that similar mechanisms, i.e. estrogen receptor recruitment to the AP1 site, are at play on the kisspeptin promoter in the AVPV. cFOS is very rapidly regulated and involvement of cFOS in the estrogen signaling and upregulation of AVPV *Kiss1* expression may be required for a rapid increase of kisspeptin synthesis prior to the surge.

In the ARC, female nulls also had lower kisspeptin cell numbers. The ARC may be involved in estrogen negative feedback, and estrogen treatment lowers *Kiss1* expression in this region (46). Thus, if cFOS is involved in estrogen signaling in ARC by recruiting estrogen receptor to the AP1 site, it is expected that cFOS nulls would have a higher *Kiss1* expression. More recently, Dubois et al., however, determined that negative feedback does not require estrogen receptor α (50), which would agree with our results. Our data indicate that cFOS plays a role in specification of kisspeptin expressing cell in the ARC, likely due to binding to the putative cFOS/AP1 site in the promoter of the kisspeptin gene. However, to further delineate whether the effect on kisspeptin observed herein is due to direct cFOS binding to the *Kiss1* promoter, promoter analysis using reporter assays or chromatin immunoprecipitation in isolated kisspeptin neurons should be performed.

In summary, our analysis of the cFOS deficient mice reveals several important gene targets of this transcription factor in the hypothalamic-pituitary-gonadal axis, which contribute to impaired gametogenesis and infertility in these mice. We determined that gonadotropin gene expression and

kisspeptin neuron numbers were diminished, while GnRH neuron migration and numbers were unaffected. Interestingly, several targets in gonads, pituitary and hypothalamus, including kisspeptin neurons, exhibited sex-specific effects, which will be a focus of future studies.

Conclusions

We determined that cFOS plays a cell-specific role at multiple levels of the hypothalamic-pituitary-gonadal axis *in vivo*. In the pituitary, cFOS is required for gene expression in the gonadotrope, but not in thyrotrope. Furthermore, cFOS is necessary for spermatogenesis and ovulation, but not for early gametogenesis or sex-steroid hormone synthesis. In the brain, cFOS is essential for kisspeptin expression and kisspeptin neuron number specifically in females, but not for GnRH neuron migration to the hypothalamus, axon targeting to median eminence, or GnRH gene expression. Striking effect on the Kiss1 gene expression in AVPV may implicate cFOS in the estrogen positive feedback, which is necessary for the preovulatory surge.

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477

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Figure Legends

Figure 1. Gonadotrope and thyrotrope gene expression in cFOS null mice. Quantitative PCR of p35 pituitaries shows that gonadotrope gene expression is significantly reduced in cFOS-null mice. Total RNA was purified from 5 male and 5 female mice per WT or KO group, reverse transcribed, and the level of hormone expression assayed by real-time PCR. In each sample, the amount of hormone mRNA, calculated from the standard curve, was compared to the amount of *Gapdh*, and presented as a ratio. Light bars, wild type (WT); black bars, cFOS nulls (KO). Data are presented as the group mean ± SEM; asterisks * indicate significant difference ($p<0.05$) in the expression in the cFOS-null animals from the wild type animals.

Figure 2. Circulatory levels of gonadotropin hormones. A-D, Gonadotropin hormone levels in serum from the p35 day old animals, 5 per group, presented as the mean ± SEM. E-H, Animals were injected subcutaneously with 200 ng/kg GnRH and blood was collected 10 minutes post injection. Asterisks * indicate significant difference, $p<0.05$, in the levels between cFOS-null animals and the wild-type animals.

Figure 3. Histology of cFOS-deficient gonads. Top panels, Hematoxylin and eosin staining of sectioned ovaries collected from wild-type (WT) and cFOS-null mice (KO) at 6 weeks of age demonstrated a lack of corpora lutea (CL) and antral follicles (AF), which were present in wild-type littermates. Bottom panels, Testes from null males at 6 weeks of age revealed reduced Leydig cells (L) and diminished

spermatogenesis, indicated with a lack of spermatozoa (S1) and lower number of spermatids (S3), but a presence of spermatogonia (S2).

Figure 4. Normal sex steroid hormone levels in cFOS null mice. A-D, Total RNA was extracted from 6-week old mice, 5 male and 5 female per WT or KO group, reverse transcribed, and the level of gene expression was assayed by real-time PCR. In each sample, the amount of mRNA of interest was compared to the amount of *Gapdh*, and presented as a ratio. Light bars, wild type (WT); black bars, cFOS nulls (KO). Results are presented as the group mean ± SEM and asterisks * indicate significant difference in the expression in the cFOS-null animals from the wild type animals. E, Primary granulosa cell cultures from 6-week old wild-type (WT) and null female (KO) were treated with 50 ng/ml ovine FSH for 1 hour, to elicit an increase in intracellular cAMP levels. The experiment was repeated 3 times and results, presented as the mean ± SEM, indicate no difference between phenotypes. F-G, Serum estradiol and testosterone levels from 6-week old mice (5 per group) exhibit no difference between phenotypes.

Figure 5. GnRH is not affected with cFOS deficiency. A, Immunohistochemistry of the preoptic area with GnRH antibody was used to identify GnRH neurons in the hypothalami of 6-week old wild-type (WT) and null (KO) mice. B, Median eminence was stained for GnRH. C, *In situ* hybridization was performed to analyze GnRH neurons and quantification of neuron number presented as the group mean ± SEM of 5 animals per group in D, while quantification of the grains per cells to analyze mRNA expression is presented in E. Light bars, wild type; black bars, cFOS nulls in D, E. Results of quantifications were presented as the mean ± SEM and asterisks (*) indicate significant difference ($p<0.05$) in null females in GnRH neuron number, as determined by two-factor ANOVA and Tukey-Kramer HSD post hoc test.

Figure 6. Lower number of Kiss1 neurons in female cFOS null mice determined by in situ hybridization. A, Representative image of the AVPV area in p42 day old female (F) mice. WT, wild

type; KO, cFOS-deficient mice. B, Quantification of *Kiss1* neuron numbers from 5 animals per group shows a decrease specifically in cFOS null female mice compared to wild-type females (Light bars, wild type (WT); black bars, cFOS nulls (KO). C, Expression levels of *Kiss1* mRNA per cell, as determined by grains per cell, is decreased in both cFOS null males and females. Results of quantifications were presented as the mean ± SEM**.** *, indicates a significant decrease in cFOS nulls compared to the wild type within each sex. D, Representative image of the arcuate nucleus (ARC) in females. E, *Kiss1* neuron number in the ARC is lower in female cFOS null mice. F, No genotype difference in *Kiss1* mRNA levels per cell in the ARC in either male or female mice.

TABLE 1

Antibody	**Cat #**	**Dilution**	**Provider**	
GnRH	PA1-121	1:1000	Pierce, Thermo Scientific	
Primers	**Forward**		**Reverse**	
LH-R	AATTCACCAGCCTACTGGTTG		CCACTGAGTTCATTCTCCTCA	
FSH-R	TTTGGAAGAATTGCCTGATGAT		CATGACAAACTTGTCTAGACTA	
LHb	CTGTCAACGCAACTCTGG		ACAGGAGGCAAAGCAGC	
FSHb	GCCGTTTCTGCATAAGC		CAATCTTACGGTCTCGTATACC	
aGSU	ATTCTGGTCATGCTGTCCATGT		CAGCCCATACACTGGTAGATGG	
GnRH-R	GCCCCTTGCTGTACAAAGC		CCGTCTGCTAGGTAGATCATCC	
TSHb	AAGAGCTGGGGTTGTTCAAA		ACAAGCAAGAGCAAAAAGCAC	
GAPDH	TGCACCACCAACTGCTTAG		GGATGCAGGGATGATGTTC	

***Highlights (for review)**

Highlights

1. cFOS is necessary for gonadotropin-beta gene expression *in vivo*
2. cFOS is superfluous for GnRH neuronal migration, cell number, and mRNA levels
3. cFOS is unnecessary for granulosa cell proliferation or steroidogenesis
4. cFOS is essential for antral follicle development and spermiogenesis
5. cFOS is indispensable for normal kisspeptin neuron number in females *in vivo*

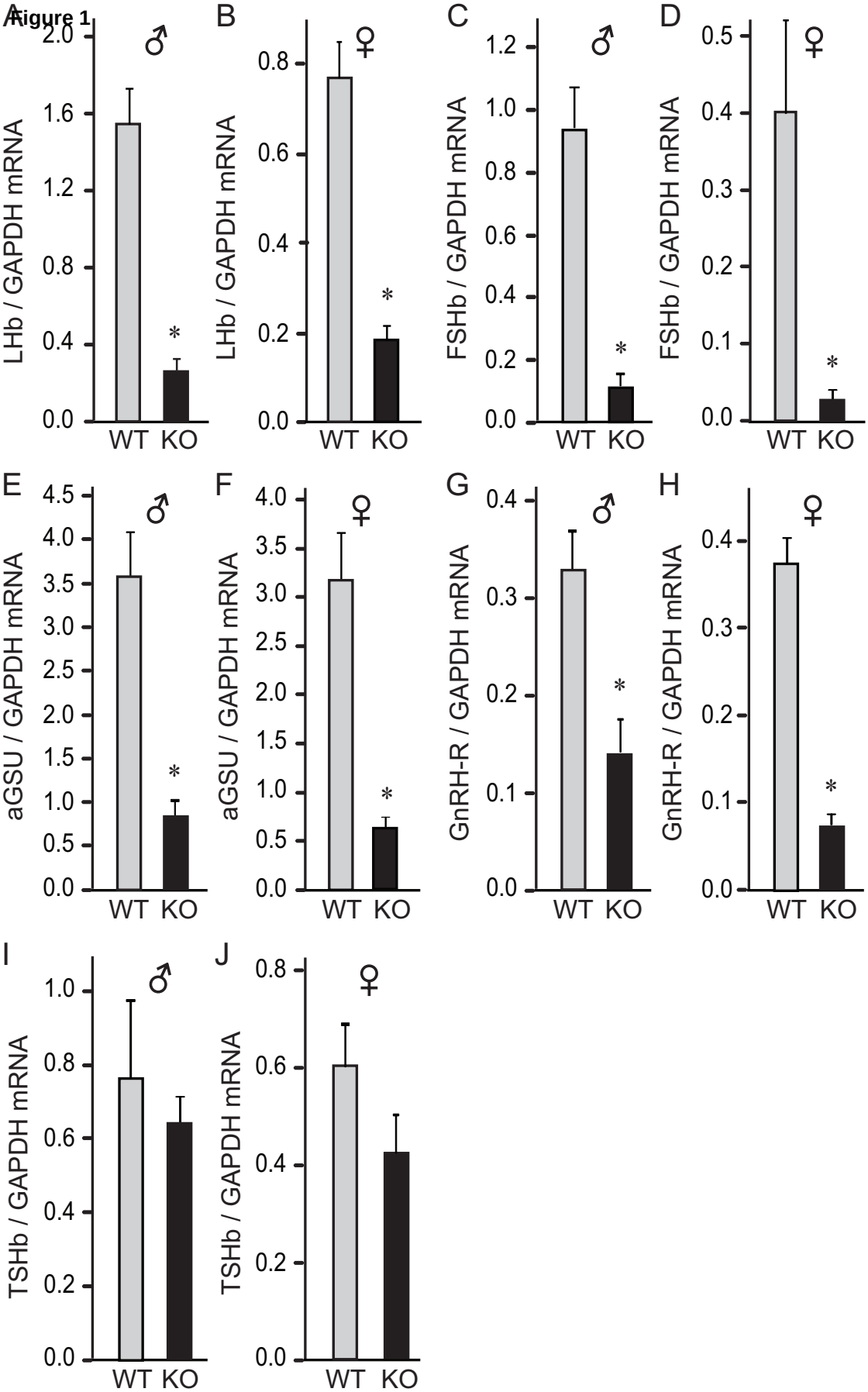

A
♂
LHb / GAPDH mRNA
2.0
1.6
1.2
0.8
0.4
0.0
*
WT KO
B
♀
LHb / GAPDH mRNA
0.8
0.6
0.4
0.2
0.0
*
WT KO
C
♂
FSHb / GAPDH mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
WT KO
D
♀
FSHb / GAPDH mRNA
0.5
0.4
0.3
0.2
0.1
0.0
*
WT KO
E
♂
aGSU / GAPDH mRNA
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
WT KO
F
♀
aGSU / GAPDH mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
WT KO
G
♂
GnRH-R / GAPDH mRNA
0.4
0.3
0.2
0.1
0.0
*
WT KO
H
♀
GnRH-R / GAPDH mRNA
0.4
0.3
0.2
0.1
0.0
*
WT KO
I
♂
TSHb / GAPDH mRNA
1.0
0.8
0.6
0.4
0.2
0.0
WT KO
J
♀
TSHb / GAPDH mRNA
0.8
0.6
0.4
0.2
0.0
WT KO

Figure 1

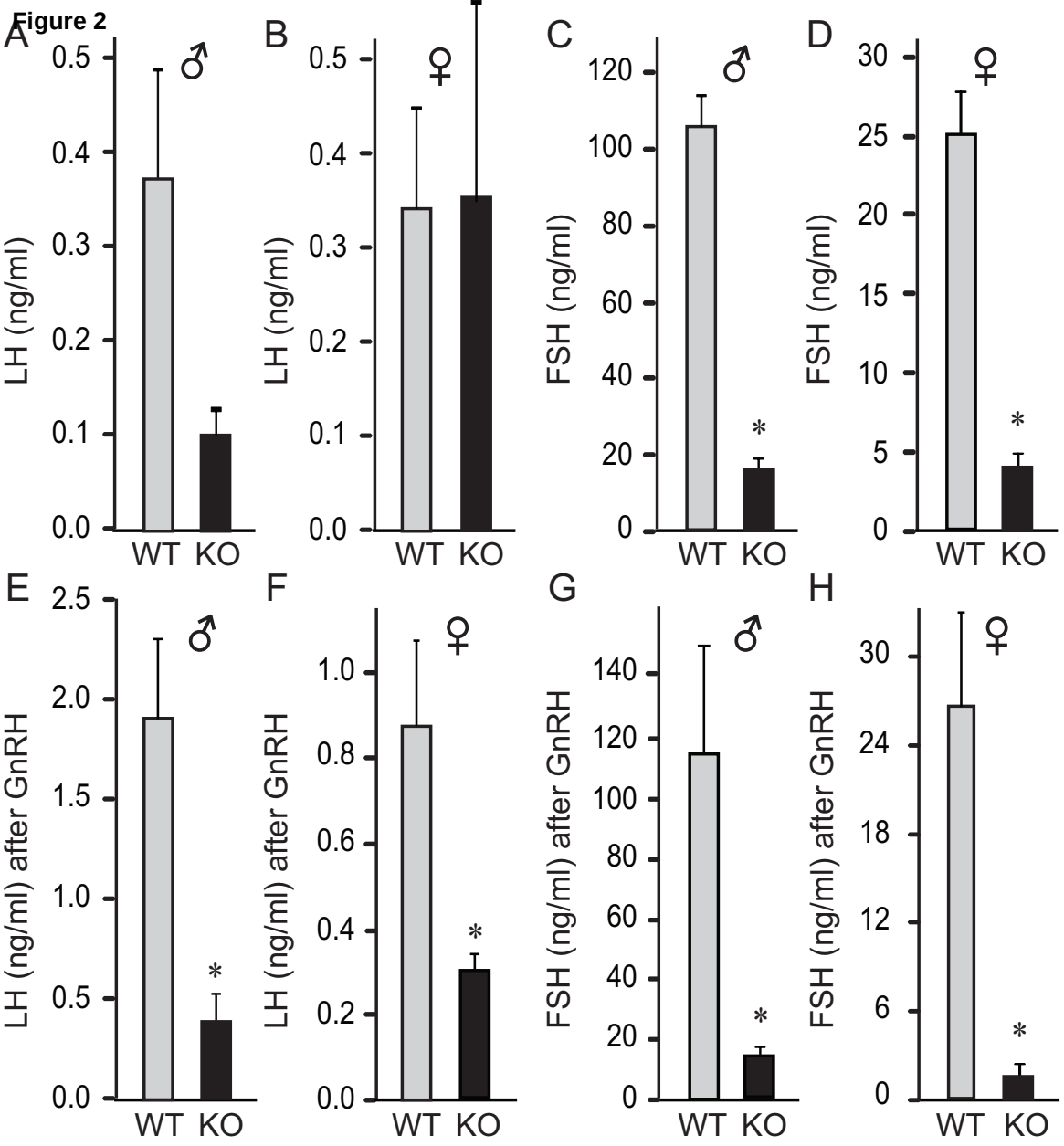


Figure 2
A
B
C
D
E
F
G
H
♂
♀
LH (ng/ml)
FSH (ng/ml)
LH (ng/ml) after GnRH
FSH (ng/ml) after GnRH
WT
KO
*

Figure 2

Figure 3

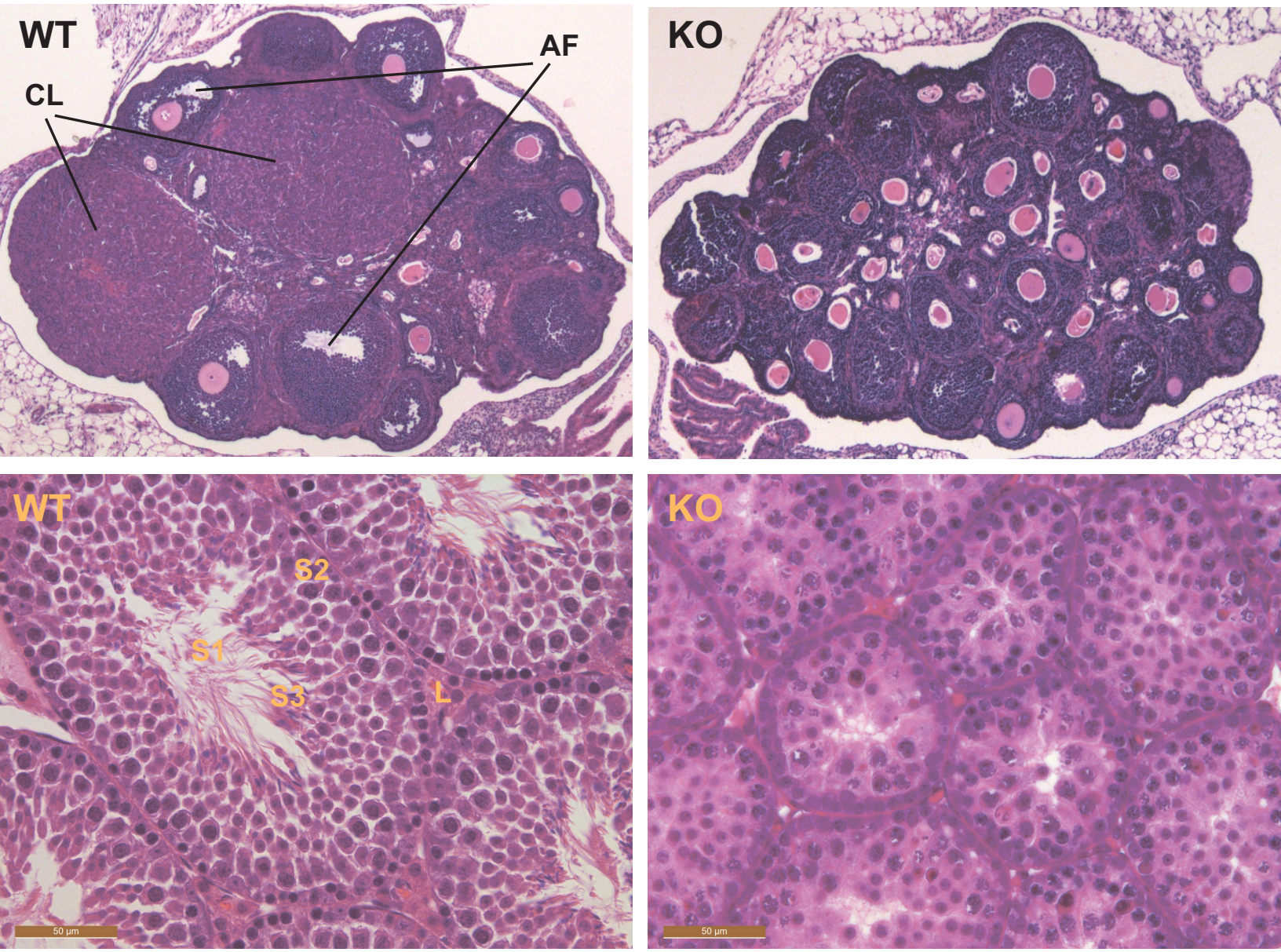
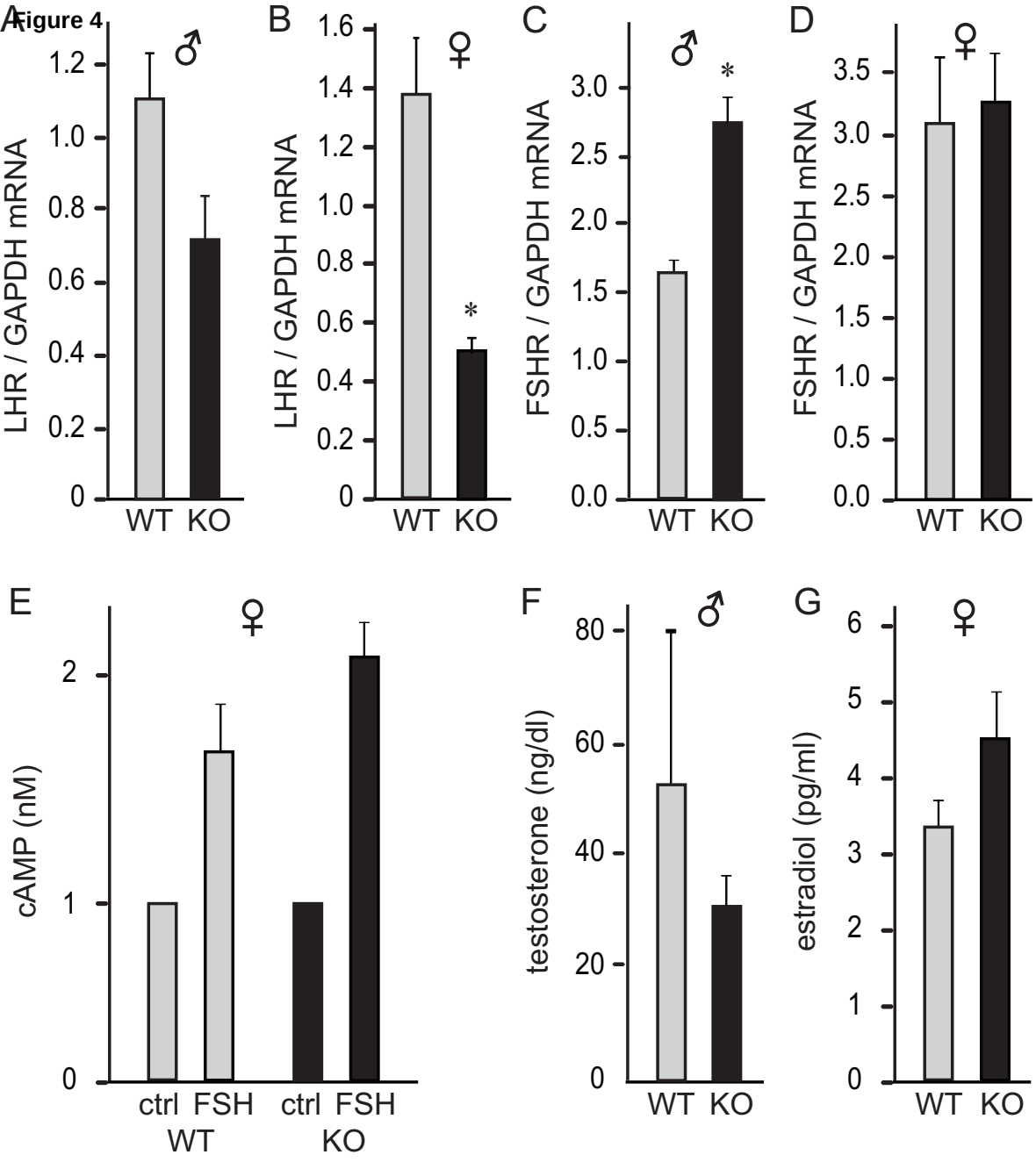


Figure 3


A
LHR / GAPDH mRNA
♂
WT KO
B
LHR / GAPDH mRNA
♀
*
WT KO
C
FSHR / GAPDH mRNA
♂
*
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D
FSHR / GAPDH mRNA
♀
WT KO
E
cAMP (nM)
♀
ctrl FSH ctrl FSH
WT KO
F
testosterone (ng/dl)
♂
WT KO
G
estradiol (pg/ml)
♀
WT KO

Figure 4

Figure 5

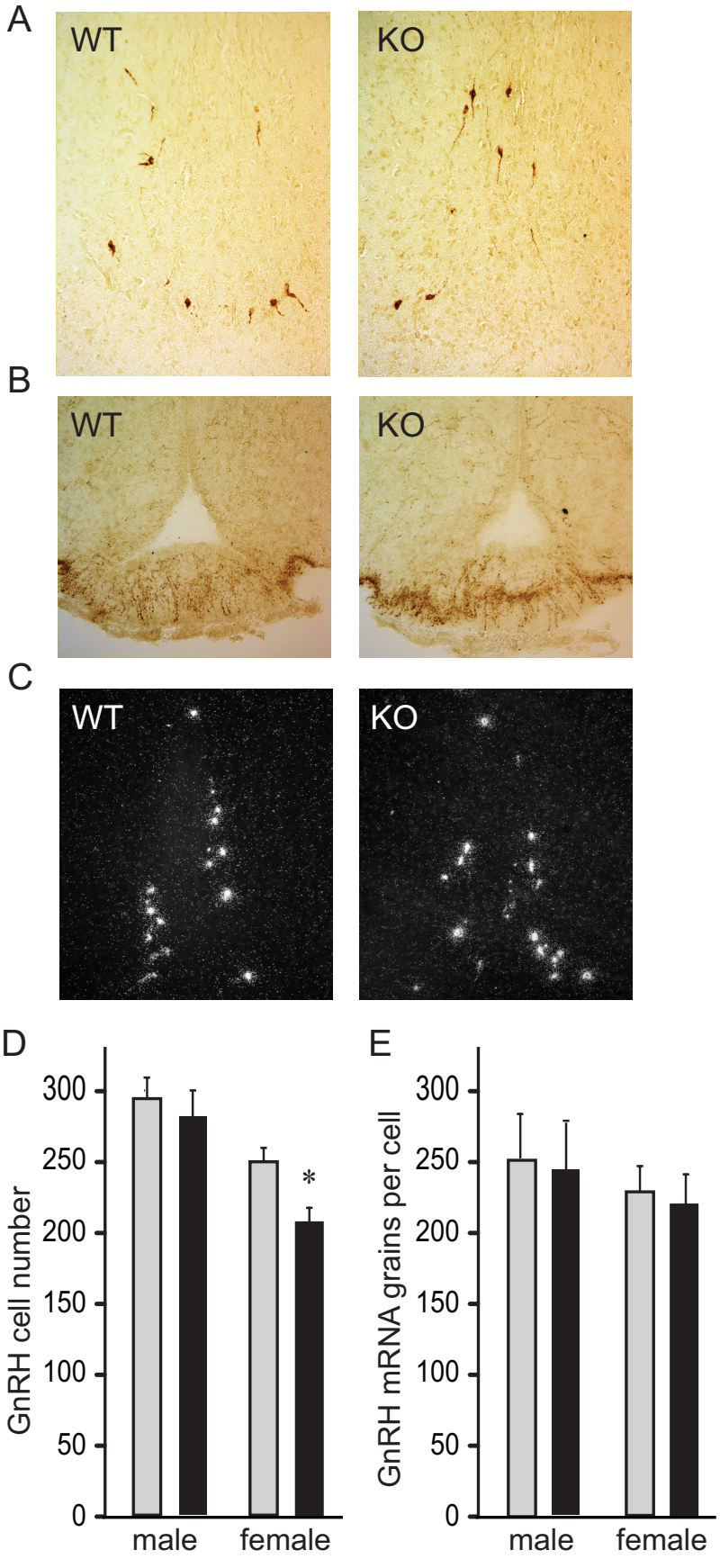

Figure 5

Figure 6a

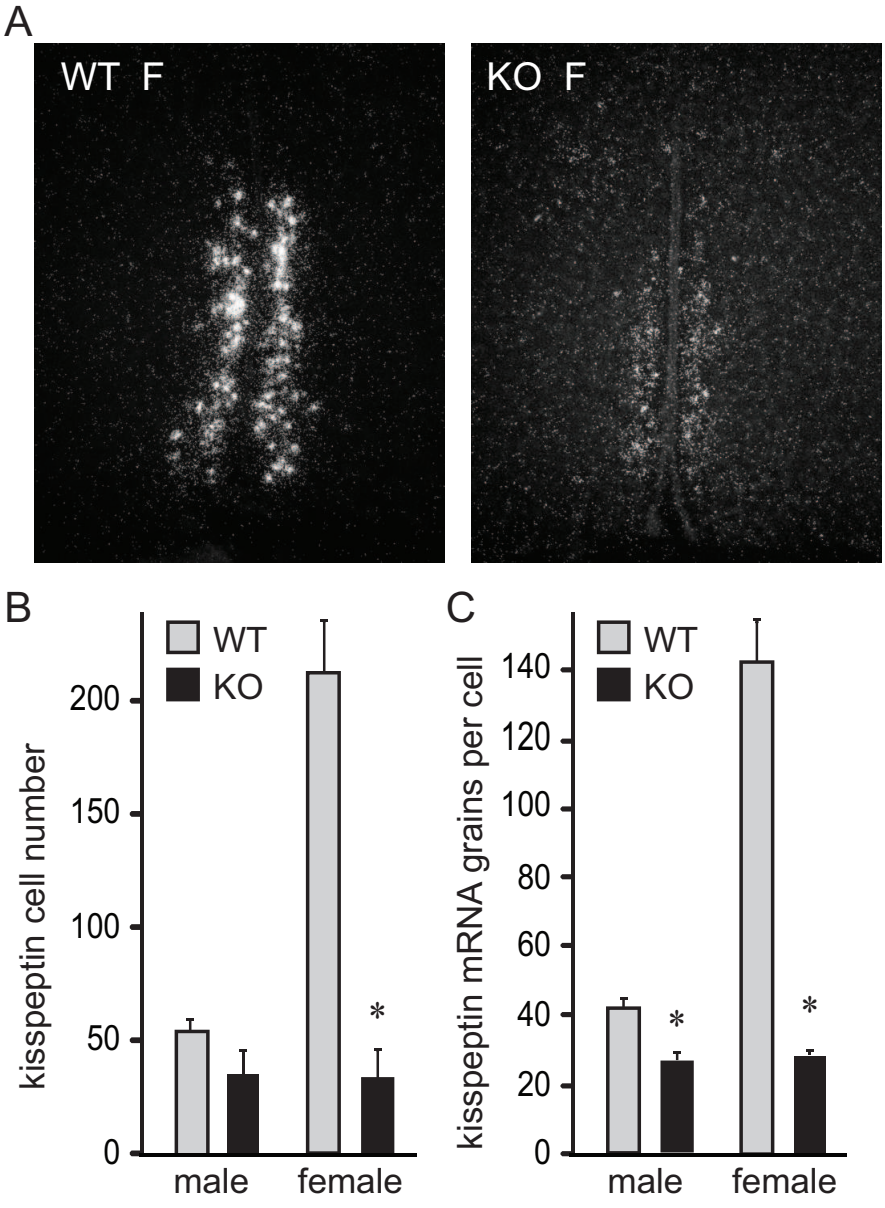

Figure 6 A,B,C

Figure 6d

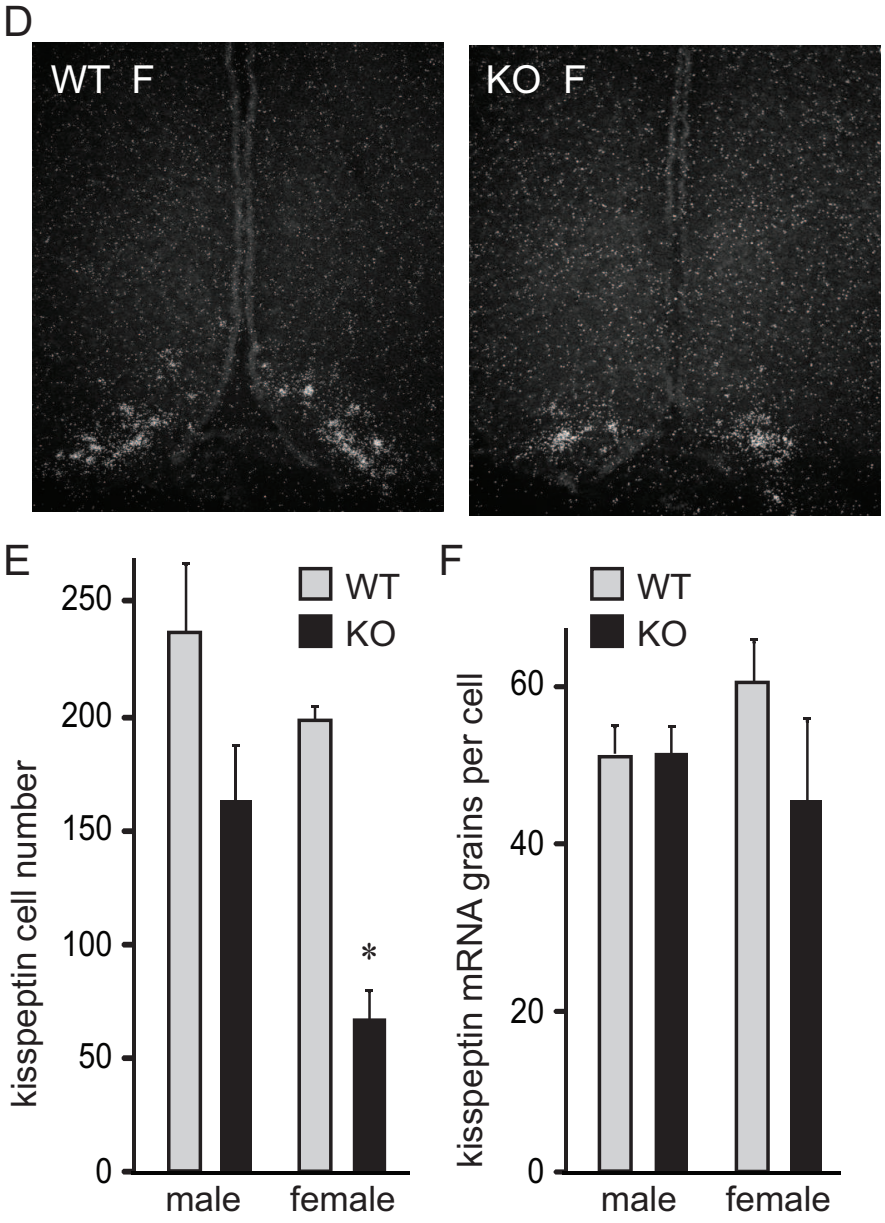

Figure 6 D,E,F