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Kiss1 is differentially regulated in male and female mice by the homeodomain transcription factor VAX1

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Abstract

Regulation of *Kiss1* transcription is crucial to the development and function of the reproductive axis. The homeodomain transcription factor, ventral anterior homeobox 1 (VAX1), has been implicated as a potential regulator of *Kiss1* transcription. However, it is unknown whether VAX1 directly mediates transcription within kisspeptin neurons or works indirectly by acting upstream of kisspeptin neuron populations. This study tested the hypothesis that VAX1 within kisspeptin neurons regulates *Kiss1* gene expression. We found that VAX1 acts as a repressor of *Kiss1 in vitro* and within the male arcuate nucleus *in vivo.* In female mice, we found that the loss of VAX1 caused a reduction in *Kiss1* expression and *Kiss1*-containing neurons in the anteroventral periventricular nucleus at the time of the preovulatory luteinizing hormone surge, but was compensated by an increase in *Kiss1-cFos* colocalization. Despite changes in *Kiss1* transcription, gonadotropin levels were unaffected and there were no impairments to fertility.

Keywords

Kiss1; Kisspeptin; VAX1; gene regulation; hypothalamus

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CRediT Author Statement

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1. Introduction

Kisspeptin, encoded by the *Kiss1* gene, is a critical peptide hormone in the reproductive neuroendocrine axis. Kisspeptin, released from neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC), directly stimulates gonadotropin-releasing hormone (GnRH) release by binding to its receptor (Kiss1R) located on GnRH neurons (Gottsch et al., 2004; Han et al., 2005; Irwig et al., 2004; Matsuda et al., 2019; Messager et al., 2005). GnRH, in turn, stimulates the gonadotrope cells of the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to regulate ovulation, folliculogenesis, and the synthesis of gonadal sex steroids (Kaprara and Huhtaniemi, 2018; Plant, 2015; Stamatiades and Kaiser, 2017). Disruptions to kisspeptin signaling by mutations in the *Kiss1* and *Kiss1R* genes, in both mice and humans, lead to impairments in sexual maturation, gonadotropin and sex steroid secretion, and fertility (Gottsch et al., 2006; Kauffman, 2010; Topaloglu, 2017; Topaloglu et al., 2012).

Kiss1 expression in the AVPV and ARC is differentially regulated by sex steroid feedback, leading to divergent roles in reproductive function. Many ARC kisspeptin neurons coexpress the stimulatory peptide, neurokinin B, and the inhibitory peptide, dynorphin, and are thought to be responsible for stimulating basal pulsatile GnRH secretion (Clarkson et al., 2017; Goodman et al., 2007; Matsuda et al., 2019; Moore et al., 2018; Navarro et al., 2009; Wakabayashi et al., 2010). Sex steroids, such as androgens, estradiol (E2), and progesterone, are detected by sex steroid receptors on kisspeptin neurons in the ARC and produce a negative feedback response resulting in decreased *Kiss1* expression and decreased LH pulse frequency (McQuillan et al., 2019; Smith et al., 2005a; Smith et al., 2005b). Conversely, AVPV kisspeptin neurons, which are sexually dimorphic and primarily found in females, increase *Kiss1* expression in response to E2 (Smith et al., 2005a). This positive feedback, along with increased neuronal activity, is necessary for increased GnRH secretion, leading to the induction of the LH surge that prompts ovulation (Dror et al., 2013; Dungan et al., 2007).

Recently, several homeodomain transcription factors have emerged as critical regulators of the reproductive axis, yet little is known of their influence on kisspeptin signaling (Hoffmann et al., 2021; Hoffmann et al., 2019; Hoffmann et al., 2014; Pandolfi et al., 2020; Pandolfi et al., 2018; Pandolfi et al., 2019). Ventral anterior homeobox 1 (VAX1) is a highly conserved homeodomain transcription factor, with the amino acid sequence of the homeodomain binding domain identical between mouse and human, and was first recognized for its importance in the development of the rostral and ventral forebrain (Hallonet et al., 1998). In mice, *Vax1* is expressed in the olfactory placode, hypothalamus, pituitary, and the testis, but not the ovary (Hoffmann et al., 2014). VAX1 has recently been implicated as a crucial regulator of reproduction. The loss of a single *Vax1* allele in the *Vax1* heterozygous animal reduces the number of GnRH-expressing neurons by half, and results in reproductive deficiencies, such as a reduction in total sperm and sperm motility in males and increased estrous cycle length in females (Hoffmann et al., 2014; Hoffmann et al., 2016). Complete loss of *Vax1* results in no detectable GnRH-expressing cells, demonstrating a gene-dose dependent role of VAX1 (Hoffmann et al., 2016). Selective deletion of VAX1 from GnRH neurons abolishes GnRH expression, resulting in hypogonadism and complete

infertility in both male and female mice (Hoffmann et al., 2016; Pandolfi et al., 2020), while VAX1 in the suprachiasmatic nucleus (SCN) regulates the LH surge (Hoffmann et al., 2021). However, these additional studies, do not fully explain the reproductive phenotypes observed in *Vax1* heterozygous females. While the importance of *Vax1* expression within GnRH neurons and the SCN has been demonstrated (Hoffmann et al., 2021; Hoffmann and Mellon, 2016; Pandolfi et al., 2020), the role of *Vax1* in other regions and cell types within the hypothalamus has largely been unexplored.

Haploinsufficiency of *Vax1* in heterozygous females leads to a robust increase in *Kiss1* mRNA levels in the AVPV and a corresponding increase in circulating LH and E2 (Hoffmann et al., 2014), which was not observed in mice with selective deletion of *Vax1* in GnRH neurons (Hoffmann et al., 2016). Several regions of both mouse and human *Kiss1/KISS1* promoters contain the VAX1 consensus sequence (ATTA), yet it is unknown whether the increased *Kiss1* transcription is due to a direct effect of VAX1 at the level of the kisspeptin neurons, or an indirect effect through altered steroid feedback to kisspeptin neurons. Additionally, it is unknown whether a homozygous loss of *Vax1* within ARC kisspeptin neurons would lead to a change in *Kiss1* gene expression. Our current study aims to test the hypothesis that VAX1 contributes to the regulation of *Kiss1* transcription in ARC and AVPV kisspeptin populations and consequently regulates gonadotropin secretion. To address our hypothesis, we used immortalized kisspeptin cell lines, as well as transgenic mouse models with VAX1 deleted from *Kiss1*-expressing cells. We found that VAX1 regulates the human *KISS1* promoter *in vitro* and *Kiss1* transcription *in vivo,* but the loss of VAX1 from *Kiss1*-expressing cells *in vivo* does not alter fertility.

2. Materials and Methods

2.1. Animals

All animal procedures were performed in accordance with the University of California, San Diego Institutional Animal Care and Use Committee regulations. To produce mice lacking VAX1 in kisspeptin cells, we crossed $Vax1^{Flox/Flox}$ mice (RRID:MGI:6500817) (Hoffmann et al., 2016) with $Kiss1^{Cre}$ mice (RRID:IMSR_JAX:023426) (Cravo et al., 2011). Offspring were backcrossed to generate $Vax1^{Flox/Flox}$:$Kiss1^{Cre}$ conditional knockouts ($Vax1^{KissCre}$) or $Vax1^{Flox/Flox}$:$Kiss1^{WT}$ ($Vax1_{WT}$). $Kiss1^{Cre}$ reporter mice were generated by crossing $Kiss1^{Cre}$ mice with Ai9 Rosa-tdTomato mice (RRID:MGI:104735) (Madisen et al., 2010) to create mice in which $Kiss1^{Cre}$ expressing cells were identifiable by tdTomato expression. All mice were on a C57BL/6 background, group housed, and maintained on a 12-hour light, 12-hour dark cycle, with *ad libitum* chow and water. Animals were randomly assigned to experimental groups. Mice were sacrificed with CO_2 or isoflurane overdose followed by rapid decapitation. All animals were sacrificed between zeitgeber time (ZT) 4-7, unless otherwise stated. Female mice were sacrificed during diestrus unless otherwise stated.

For genotyping, genomic DNA was extracted from hypothalamus, pituitary, testis, ovary, and tail tip using DNeasy Kit (Qiagen) according to manufacturer's instructions. To differentiate between a Vax1 flox allele and wildtype allele we used the following primers: VaxFlox-Forward: 5'-GCCGGAACCGAAGTTCCTA-3', VaxWT-Forward: 5'-CCAGTAAGAGCCCCTTTGGG-3', and Vax-Reverse: 5'-CGGATAGAC

CCCTTGGCATC-3'. To detect *Vax1* recombination, the following primers were used: VaxRec-Forward: 5'-GCAGTGGCCTAGAGAGATCG-3' and VaxRec-Reverse: 5-GCACTGTGTAGTGCTCCTAT-3'. CRE genotyping was performed using CRE-Forward: 5'-GCATTACCGGTCGTAGCAACGAGTG-3' and CRE-Reverse: 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3'. tdTomato mice were genotyped using tdtF 1: 5'-GGCATTAAAGCAGCGTATCC-3', tdtR1: 5;- CTGTTCCTGTACGGCATGG-3, tdtF2: 5'-CCGAAAATCTGTGGGAAGTC-3', tdtR2: 5'-AAGGGAGCTGCAGTGGAGTA-3'. Mice that were positive for germline recombination of VAX1 were excluded from the study.

2.2. Immunohistochemistry

$Kiss1^{Cre}$-$tdTomato^{+/-}$ mice were euthanized at ZT6. Brains were quickly dissected and submerged in 4% PFA overnight. The following day, brains were transferred to 30% sucrose until they sank. 40 pm sections containing the AVPV were sectioned using a cryostat and sections transferred to PBS. On the day of staining, sections underwent heated antigen retrieval in 1X Citra buffer (Biogenex). Sections were washed and processed with a Mouse-on-Mouse ABC kit (Vector Labs) according to manufacturer's instruction. VAX1 antibody (RRID:AB_2723772) (Clone OTI4E5, Origene) was used at 1:100. After ABC processing for 30 minutes, sections were washed and incubated in 1:250 biotinylated tyramide (Akoya Biosciences) with 0.003% H_2O_2 for ten minutes. Sections were washed again and incubated in 1:200 Streptavidin DyLight 488 (Invitrogen) for 30 minutes. Sections were washed, mounted, and coverslipped with ProLong with DAPI (Invitrogen).

2.3. RNA Isolation and qPCR

Brains were collected on dry ice and immediately stored at −80°C. Two mm AVPV and ARC micropunches were collected on a cryostat from 0.3 mm thick sections as previously described (Di Giorgio et al., 2013; Tonsfeldt et al., 2019). RNA from micropunches was isolated with RNeasy-Micro Kit (Qiagen) according to manufacturer's instructions. Purified RNA was converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). cDNA products were detected on a Bio-Rad CFX Connect quantitative real-time PCR system (Bio-Rad laboratories) using SYBR Green Supermix (Bio-Rad Laboratories). Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), by normalizing the gene of interest to *Gapdh*, and represented as mean fold change compared to $Vax1^{WT}$±SEM. The following primers were used: Kiss1-Forward: 5'-TGCTGCTTCTCCTCTGT-3', Kiss1-Reverse: 5'-ACCGCGATTCCTTTTGC-3' (Gottsch et al., 2011), Pdyn-Forward: 5'-GTGTGCAGTGAGGATTCAGG-3', Pdyn-Reverse: 5'-AGTCATCCTTGCCACGGAGC-3' (Schoeller et al., 2016), Tac2-Forward: 5'-CTGCTTCGGAGACT CT ACG-3', Tac2-Reverse: 5'-GGTTGGCTGTTCCTCTTGC-3' (Schoeller et al., 2016), Gapdh-Forward: 5'-TGCACCACCAACTGCTTAG-3', Gapdh-Reverse: 5'-GGATGCAGGGATGATGTTC-3'.

2.4. Cell Culture

KTaR-1 (RRID:CVCL_VS93) and KTaV-3 (RRID:CVCL_VS94) cell lines (kindly provided by Patrick E. Chappell, Oregon State University) (Jacobs et al., 2016) were maintained in complete medium consisting of DM EM (Corning) containing 10% fetal bovine serum

(FBS) (Omega Scientific) and 1% penicillin/streptomycin (HyClone) and incubated at 37°C with 5% CO_2.

2.5. Hormones

17β-estradiol (E2) was obtained from Sigma-Aldrich (St. Louis, MO). For cell culture, E2 was dissolved in absolute ethanol and diluted to a 1 nM stock. Immediately prior to hormone treatment, 1 nM E2 stock was diluted 1:1000 in charcoal-stripped DMEM to a final concentration of 1 pM E2. For E2 pellets, E2 was dissolved in sesame oil to a concentration of 25 μg/ml. Methyltrienolone (R1881) was obtained from NEN Life Sciences (Boston, MA) and diluted to a 10 mM R1881 stock in absolute ethanol. Immediately prior to hormone treatments, 10 mM stock was diluted 1:1000 in charcoal-stripped DMEM to a final concentration of 10 μM R1881.

2.6 Site-Directed Mutagenesis

Site-directed mutagenesis of three regions of the −1313/+27 human-Kiss-Luc in a pGL2 backbone (hKiss-Luc) (Mueller et al., 2011), kindly provided by Alejandro Lomniczi and Sergio Ojeda, (Oregon National Primate Research Center), was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to manufacturer's instructions. The μ-1211-1193 sequence was mutated at −1211 to −1193 base pairs (bp) upstream of the transcriptional start site (TSS) from **TAAT**GGGTGTGA**TAATAAT** to **CGGC**GGGTGTGA**CGGCGGC.** The μ-1111-1108 sequence was mutated at −1111 to −1108 bp upstream of the TSS from **ATTA** to **CGGC.** The μ-362-359 sequence was mutated at −362 to −359 bp upstream TSS from **ATTA** to **CGGC.**

2.7. Transient Transfections and Luciferase Assays

One day prior to transfections, KTAR-1 and KTAV-3 cells were seeded at 3×10^4 cells per well in 12-well plates with DMEM containing 10% FBS. Transient transfections were performed using Polyjet In Vitro DNA Transfection Reagent (SignaGen Laboratories), following manufacturer's instructions. For all experiments, each well was transfected with 500 ng of reporter plasmid, −1313/+27 human-Kiss-Luc in a pGL2 backbone (hKiss-Luc) (Mueller et al., 2011) or reporter backbone, pGL2, and co-transfected with VAX1-CMV6 (VAX1) (Hoffmann et al., 2016) or CMV6-empty vector (EV) (TrueClone, Origene). 100 ng of a reporter plasmid containing β-galactosidase driven by the herpes virus thymidine kinase promoter (TK-βgal) was co-transfected to serve as an internal control for transfection efficiency. To determine optimal VAX1 concentration, 0 to 50 ng VAX1 or EV was co-transfected with reporter plasmids. To determine if VAX1 regulated the *KISS1* promoter through regions containing ATTA sites, we co-transfected hKiss-Luc, μ-1211-1193, μ-1111-1108, and μ-362-359 or pGL2, with 50 ng VAX1 or EV. Polyjet/DNA complex-containing medium was removed after 24 hours and replaced with complete medium. Cells were harvested 48 hours from start of transfection.

For transfections involving hormone treatments, cells were seeded in charcoal-stripped FBS. 50 ng VAX1 or EV was transfected along with 100 ng ratAR-pSG5 (Ikonen et al., 1998) or 50 ng ERα-pcDNA3.1 to ensure adequate expression of steroid receptors. Polyjet/DNA complex-containing medium was replaced after 24 hours with charcoal-stripped medium

containing 10 μM R1881 (synthetic androgen), 1 pM E2, or vehicle (100% ethanol). Cells were harvested 48 hours following hormone treatment. To harvest cells, medium was aspirated, cells were washed with 1X PBS and then lysed with 0.1 M K-phosphate buffer, pH 7.8, containing 0.2% Triton X-100. Luciferase and β-galactosidase assays were performed as previously described (Givens et al., 2005). Transfections were performed in triplicate. Within each well, luciferase values were normalized to TK-βgal values. Luciferase/TK-βgal triplicate values were averaged and then hKiss-Luc, μ-1211-1193, μ-1111-1108, and μ-362-359 values were normalized to pGL2 values. A minimum of three independent replicates per experiment were performed.

2.8. Hormone Analysis

Blood samples were collected at time of euthanasia, allowed to clot at room temperature for 1 hour, centrifuged at 2000 ×g for 15 minutes, and then serum was collected and stored at −20°C until assayed. Serum LH, FSH, estradiol, and testosterone were measured by The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. LH was measured using LH RIA with a reportable range between 0.02 – 75.0 ng/mL (Intra-assay CV=5.5%, Inter-assay CV=8.4%). FSH was measured using FSH RIA with a reportable range between 3.0 – 75.0 ng/mL (Intra-assay CV=6.7%, Inter-assay CV=8.7%). Estradiol was measured using an ELISA with reportable range between 3.0 – 300.0 pg/mL (Intra-assay CV=7.5%, Inter-assay CV=10.1%). Testosterone was measured using an ELISA with reportable range between 10.0 – 1600.0 ng/dL (Intra-assay CV=6.0%, Inter-assay CV=9.3%).

2.9. LH Surge

Between ZT 2 - 5, female mice (10-16 weeks old, weighing between 18-28 grams) were ovariectomized and a pellet containing 0.75 μg of 17-β estradiol dissolved in sesame oil subcutaneously implanted to mimic proestrus levels of E2 (Dror et al., 2013). Two days after surgery, mice were sacrificed either in the morning (AM), between ZT 4-5, or at the time of lights off (PM), between ZT 12-13. Blood and brains were collected at sacrifice. An LH surge was conservatively defined as LH values that were 3 standard deviations above the AM average (Dungan et al., 2007).

2.10. Fluorescent In Situ Hybridization (ISH)

20 μm serial coronal sections were collected from fresh frozen brains, spanning the length of the AVPV or ARC (Di Giorgio et al., 2013; Tonsfeldt et al., 2019). Sections were fixed in chilled 4% PFA, washed two times with 1X PBS, and dehydrated through a series of ethanol washes ranging from 50%-100% ethanol. RNAscope Multiplex Fluorescent v2 Assay (Advanced Cell Diagnostic, 323100) was performed according to manufacturer's instructions with the following probes: Mm-Kiss1 (500141) and Mm-Fos-C2 (316921-C2). Sections were counterstained with DAPI and coverslipped with ProLong Gold (Invitrogen).

2.11. Microscopy and Image Analysis

Fluorescent microscopy was performed at the Nikon Imaging Core (UCSD) using a Nikon Eclipse Ti2-E microscope with Plan Apo objectives. Samples were excited by the Lumencor

SpectraX and acquired with a DS-Qi2 CMOS camera using NIS-Elements software. Lighting was determined by using the minimum LED intensity and exposure time for each channel using positive and negative control slides, and then used for all subsequent acquisition. Images were imported into FIJI (NIH ImageJ) (Schindelin et al., 2012). To determine the number of *Kiss1*-positive cells in the AVPV, a defined 0.7 mm^2 region encompassing both sides of the third ventricle was set. *Kiss1*-positive and *cFos*-positive cells were counted manually, using FIJI Cell Counter tool. Counts were performed by two independent experimenters who were both blinded to genotype.

2.12. Puberty, First Estrus, Estrous Cyclicity, and Fertility Assessment

Beginning at weaning (21 days of age), mice were checked daily for pubertal onset. Date of pubertal onset was marked by the occurrence of vaginal opening in females and by preputial separation in males. Following female pubertal onset, vaginal lavages were taken daily until the first occurrence of estrus was observed. Beginning at 12 weeks of age, vaginal smears were taken for 16 consecutive days to assess estrous cyclicity. The slides used for vaginal lavages were stained with 0.1% methylene blue stain and stage of cycle was determined by the composition of cell types present (Byers et al., 2012). To assess fertility, 12-15-week-old virgin $Vax1^{KissCre}$ or $Vax1^{WT}$ mice were paired with a $Vax1^{WT}$ breeder for 90 days. The number of offspring, latency to first litter, and total number of litters were recorded.

2.13. Sperm Motility and Total Sperm Count

Cauda epididymides were dissected and placed in M2 media (Sigma-Aldrich) at room temperature. The right epididymis was cut in half and forceps were used to manually expel sperm into M2 media and left undisturbed for 15 minutes. Motile sperm were counted on a hemocytometer and then placed on a 55°C heat block for 5 minutes to immobilize all sperm. Following immobilization, all intact sperm cells on the hemocytometer were counted. Motile sperm counts were divided by the total counts and multiplied by 100 to determine the percent motility. The left epididymis was minced, filtered through a 70 μM filter (Falcon), and diluted in water. Using a hemocytometer, all sperm heads were counted to determine total sperm count.

2.14. Statistical Analysis

Students t-test, Welch's t-test, one-way ANOVA, and two-way ANOVA were used to determine differences between groups as indicated in the figure legends. For one-way and two-way ANOVA, significant effects were followed by Tukey's Honest Significant Difference test. Graphpad PRISM 9 was used for statistical analysis, with $p<0.05$ indicating significance.

3. Results

3.1. Generation of $VAX1^{Flox/Flox}$:$Kiss1^{Cre}$ mice

The loss of a single allele in *Vax1* heterozygous mice has been shown to increase *Kiss1* mRNA in diestrus females (Hoffmann et al., 2014). To test the hypothesis that the increase of *Kiss1* is mediated by a direct effect of VAX1 within kisspeptin neurons, we first established whether *Kiss1* and VAX1 were colocalized. Using a newly-available VAX1

monoclonal antibody, we confirmed VAX1 is expressed in the SCN (Fig. 1A), an area of high *Vax1* mRNA expression in adulthood according to the Allen Brain Atlas (http://mouse.brain-map.org)(Allen Institute for Brain Science, 2011) (Fig. 1B). We also detected VAX1 protein in tdTomato-containing neurons from $Kiss^{Cre}$-tdTomato mice (Fig. 1C), establishing the presence of VAX1 in *Kiss1*-expressing neurons in adulthood. Then, we selectively deleted *Vax1* from *Kiss1*-expressing cells in mice by generating $Vax1^{Flox/Flox}$:$Kiss1^{Cre}$ ($Vax1^{KissCre}$) and $Vax1^{Flox/Flox}$:$Kiss1^{WT}$ ($Vax1^{WT}$) mice (Fig. 1D). Exons 2 and 3 of the *Vax1* gene, containing the homeodomain coding region, are flanked by LoxP sites, allowing for the functional protein to be present in $Vax1^{WT}$ mice and excised in $Vax1^{KissCre}$ mice. We collected genomic DNA and verified that recombination of the $Vax1^{Flox}$ allele occurred in tissues known to express *Kiss1*, such as the hypothalamus, pituitary, testis, and ovary (Ikeda et al., 2017; Merhi et al., 2016; Salehi et al., 2015; Smith et al., 2005a; Smith et al., 2005b), while recombination was absent from male pituitary and tail (Fig. 1E). There was no recombination detected in any tissues from $Vax1^{WT}$ mice (Fig. 1E), signifying intact *Vax1* expression. These findings indicate the successful recombination of the $Vax1^{Flox}$ allele in areas known to express *Kiss1.*

3.2. VAX1 regulates Kiss1 and Tac2 mRNA in the ARC of male mice

Using $Vax1^{KissCre}$ mice, we tested whether VAX1 plays a role in regulating *Kiss1* gene expression in ARC kisspeptin neurons *in vivo.* We took micropunches of the ARC from $Vax1^{WT}$ and $Vax1^{KissCre}$ intact male and intact diestrus female mice. We found that *Kiss1* was increased 2-fold in $Vax1^{KissCre}$ male mice compared to $Vax1^{WT}$, while levels in diestrus female mice were unchanged (Fig. 2A, B). We also measured levels of *Tac2* and *Pdyn*, which are coexpressed with *Kiss1* in approximately 90% of ARC kisspeptin neurons (Navarro et al., 2009). We found that *Tac2* mRNA was increased 1.4-fold in $Vax1^{KissCre}$ males, but unchanged in females (Fig. 2C, D). We found no change in *Pdyn* in mutants of either sex (Fig. 2E, F). We did not identify a sex difference in *Vax1* mRNA expression in the ARC of intact male and intact diestrus-staged or OVX + E2-treated females. However, we did observe a significant reduction of *Vax1* levels in OVX + E2-treated females compared to diestrus-staged females (Fig. 2G). These findings show that *Kiss1* is differentially regulated by VAX1 in males and females *in vivo.*

3.3. VAX1 regulates the KISS1 promoter in vitro

We next used a human −1313/+27 *KISS1*-Luciferase reporter (hKiss-Luc) (Mueller et al., 2011), which contains three ATTA sites located at −1211 to −1193, one ATTA site located at −1111 to −1108, and one ATTA site located at −362 to −359 bp upstream of the *KISS1* transcriptional start site (TSS) (Fig. 3A), to examined whether VAX1 can regulate the kisspeptin promoter *in vitro*. We co-transfected hKiss-Luc with various concentrations of a VAX1 expression vector or an empty vector (EV) control into the immortalized mouse ARC (KTaR-1) kisspeptin cell line (Jacobs et al., 2016). We found that overexpression of VAX1 represses hKiss-Luc transcription in KTaR-1 cells in a dose-dependent manner (Fig. 3B). To determine if VAX1 mediated *KISS1* repression through any of the three regions on the promoter that contain ATTA sites, we used site-directed mutagenesis to create cis-mutations at −1211 to −1193 bp (μ-1211-1193), −1111 to −1108 bp (μ-1111-1108), and −362 to −359 bp (μ-362-359) on the hKiss-Luc promoter (3A). We found that VAX1 was not able to

significantly repress transcription on μ-1211-1193 and μ-362-359 as compared to the control (hKiss-Luc), while μ-1111-1108 was able to repress transcription to a similar degree as hKiss-Luc (Fig. 3C). These findings indicate that, within immortalized kisspeptin neurons derived from the female ARC, VAX1 acts upon two regions of the *KISS1* promoter to mediate repression and are consistent with the effects seen in the male ARC *in vivo*.

To better understand the differential regulation of *Kiss1* expression observed between males and females *in vivo*, we determined whether sex steroids influence the effects of VAX1 on *KISS1* promoter activity. We co-transfected KTaR-1 cells with 50 ng of VAX1 and 100 ng of androgen receptor (AR) in the presence or absence of 10 μM R1881, a synthetic androgen. We found that the combination of VAX1 overexpression and androgen-containing medium caused a significant reduction in hKiss-Luc transcription compared to VAX1 or R1881 alone, and that there was a significant interaction between VAX1 and R1881 ($p<0.05$) (Fig. 3D). To approximate the female hormonal milieu *in vitro*, we transfected KTaR-1 cells with 50 ng of VAX1 and 50 ng of estrogen receptor alpha (ERα), in the presence or absence of 1 pM E2. We show that E2, along with ERα can repress *hKiss-Luc* transcription (Fig. 3E). We found that in the presence of E2, the effects of VAX1 on the *KISS1* promoter were masked and that the effects of the combination of E2 and VAX1 were not different than the effects of E2 or VAX1 alone. However we did observe a significant interaction between VAX1 and E2 ($p<0.05$) (Fig. 3E). These findings support our *in vivo* results, in which we saw an upregulation of *Kiss1* following the loss of VAX1 in males, but not in females.

3.4. Basal serum gonadotropin levels are not altered by loss of Vax1 from Kiss1 cells

Because we observed that overexpression of VAX1 represses *Kiss1* transcription *in vitro* and the loss of VAX1 *in vivo* led to increased *Kiss1* transcription in males, we next wanted to determine whether the loss of VAX1 *in vivo* led to altered levels of circulating LH and FSH. We found that LH levels in $Vax1^{WT}$ and $Vax1^{KissCre}$ males were not significantly different (Fig. 4A). However, they had significantly different variances (F test, $p = 0.005$). We found that circulating levels of FSH (Fig. 4B) between $Vax1^{WT}$ and $Vax1^{KissCre}$ males were not different. We also detected no change in testosterone levels ($Vax1^{WT}$: T = 318.3 ± 133.1 ng/dL, $Vax1^{KissCre}$: T = 135.3 ± 67.5 ng/dL, N=10-13, $p > 0.05$), but found a significant difference in variance (F test, $p = 0.021$). We found no difference among LH and FSH levels (Fig. 4C, D) or E2 ($Vax1^{WT}$: E2 = 3.2 ± 0.2 pg/mL, $Vax1^{KissCre}$: E2 = 3.3 ± 0.2 pg/mL, N=7-8, $p > 0.05$, level of detection = 3.0 pg/mL, all values below level of detection were set to 3.0 pg/mL) between female $Vax1^{KissCre}$ and $Vax1^{WT}$ mice.

3.5. VAX1 regulates Kiss1 in the AVPV of females during induced proestrus

After our data suggested that the effect of VAX1 on *Kiss1* was steroid-dependent in the ARC, we next determined whether VAX1 has a role in the regulation of *Kiss1* in the AVPV of female mice. We transfected immortalized mouse AVPV kisspeptin cells (KTaV-3) (Jacobs et al., 2016) with VAX1 and found that overexpression of VAX1 repressed the hKiss-Luc promotor, similar to what was observed in KTaR-1 cells (Fig. 5A). We then measured *Kiss1* mRNA in micropunches of the AVPV from intact diestrus female mice, when E2 and AVPV *Kiss1* levels are low (Marraudino et al., 2017; Wang et al., 2016). We

found no change in AVPV *Kiss1* levels between $Vax1^{WT}$ and $Vax1^{KissCre}$ females, differing from our *in vitro* findings (Fig. 5B).

Next, we examined the role of VAX1 in the AVPV during high E2 conditions. We hypothesized that the loss of a repressive element would lead to higher *Kiss1* expression. We induced an LH surge in $Vax1^{WT}$ and $Vax1^{KissCre}$ mice via ovariectomy (OVX) and implantation of an E2 pellet to mimic proestrus levels of circulating E2. In this model, *Kiss1* mRNA and LH are low in the morning, and high in the evening, at the time of the expected LH surge. We performed qRT-PCR on punches from OVX+E2 treated females harvested in the evening of the induced proestrus. Unexpectedly, we found that AVPV *Kiss1* mRNA was significantly reduced in $Vax1^{KissCre}$ mice compared to $Vax1^{WT}$ mice (Fig. 5C). Because we only observed a difference in *Kiss1* gene regulation in OVX + E2-treated females, we wanted to determine if *Vax1* was regulated by different E2 states. We found that AVPV *Vax1* levels were comparable between diestrus-staged and OVX + E2-treated $Vax1^{WT}$ females, when collected between ZT 4-7. We then assessed circulating LH levels to determine if the changes in *Kiss1* mRNA in the AVPV caused a physiological change in the ability of $Vax1^{KissCre}$ females to induce an LH surge. As expected, AM $Vax1^{WT}$ females maintained low LH levels, while PM $Vax1^{WT}$ females had significantly Increased levels of LH (Fig. 5E). Despite having reduced AVPV *Kiss1* levels, the LH levels in PM $Vax1^{KissCre}$ females were not significantly different from PM $Vax1^{WT}$ mice (Fig. 5D). The proportion of $Vax1^{WT}$ (6 out of 8) and $Vax1^{KissCre}$ (7 out of 12) mice meeting the surge criteria was independent of genotype (X^2= 0.586, df =1).

To understand the mechanisms enabling $Vax1^{KissCre}$ mice to show an LH surge despite reduced *Kiss1* expression, we performed fluorescent *in situ* hybridization to assess *Kiss1* and *cFos* expression (Fig. 6A). Only mice that met the surge criteria were assayed. We measured the overall number of *Kiss1*-containing neurons, as well as the number of *Kiss1* neurons which colocalized with the immediate early gene *cFos* to assess neural activity. We found fewer *Kiss1*-positive neurons in the AVPV at the time of the induced surge in $Vax1^{KissCre}$ mice compared to $Vax1^{WT}$ mice (Fig. 6B). Despite a reduction in the number of overall AVPV *Kiss1* cells, there was an increase in the percent of kisspeptin neurons that colocalized with *cFos* in $Vax1^{KissCre}$ mice compared to $Vax1^{WT}$ (Fig. 6C). These findings suggest that, despite having lower *Kiss1* mRNA and fewer overall *Kiss1*-positive cells, $Vax1^{KissCre}$ mice have a larger proportion of activated AVPV kisspeptin neurons during the LH surge, which may compensate and result in surge-level LH concentrations.

3.6. VAX1 in Kiss1-expressing cells is not required for reproductive function

To investigate whether deletion of Vax1 within kisspeptin neurons would impact gross reproductive function, we measured several parameters, including pubertal onset, estrous cyclicity, spermatogenesis, and fecundity. We found that female and male $Vax1^{KissCre}$ mice have normal timing of pubertal onset (Fig. 7A, B) and females also have normal timing of first estrus ($Vax1^{WT}$: 41.6 ±1.4 days, $Vax1^{KissCre}$: 42.5 ± 1.3 days, N=11-14, $p > 0.05$), suggesting that *Kiss1*-specific VAX1 does not alter sexual maturation in mice. Weights at pubertal onset were comparable between $Vax1^{WT}$ and $Vax1^{KissCre}$ mice for both females ($Vax1^{WT}$: 12.24 ± 0.23 g, $Vax1^{KissCre}$: 12.39 ± 0.26 g) and males ($Vax1^{WT}$: 14.21 ± 0.28 g,

Vax1^{KissCre}: 14.77 ± 0. 35g). We observed no disruptions to estrous cyclicity in Vax1^{KissCre} females as measured by cycle length (Fig. 7C) and time spent in each stage of estrous (Fig. 7D). However, male Vax1^{KissCre} mice have significantly reduced total sperm counts compared to Vax1^{WT} (Fig. 7E), but no difference in sperm motility (Fig. 7F). To measure fecundity, we performed a 120-day fertility assessment, in which we paired Vax1^{KissCre} and Vax1^{WT} mice with a male or female wildtype mouse. We found that both female and male Vax1^{KissCre} mice had comparable number of litters to Vax1^{WT} mice (Fig. 7G) and the time to produce the first litter was not different between groups (Fig. 7H). There were no significant differences in the number of pups born in each litter (Fig. 7I). Given these assessments, we found that the loss of VAX1 from kisspeptin cells does not impact gross fertility measures in either sex.

4. Discussion

While the importance of kisspeptin signaling within the reproductive axis is unquestioned, the dynamics of *Kiss1* gene regulation are not well established. We previously demonstrated that the heterozygous loss of the homeodomain transcription factor VAX1 leads to increased *Kiss1* expression in the AVPV of female mice (Hoffmann et al., 2014). Using our Vax1^{KissCre} mouse model and immortalized kisspeptin cell lines, we have established that VAX1 is a regulator of *Kiss1 in vitro* and *in vivo* and this regulation is dependent on brain region and hormone conditions. We found that VAX1 regulates *Kiss1* expression in the male, but not female ARC, and in the female AVPV at the time of the LH surge, but these changes were not sufficient to significantly affect fertility.

Kiss^{Cre}-tdTomato mice were utilized to show that adult kisspeptin neurons express VAX1. We then selectively deleted *Vax1* from *Kiss1*-expressing cells by creating Vax1^{KissCre} mice and verified that the *Vax1* allele was recombined in areas that are known to express *Kiss1*. Although *Vax1* was recombined in the female pituitary of the Vax1^{KissCre} mice, it is not expressed in αGSU-expressing cells, such as the gonadotrope cells (Hoffmann and Mellon, 2016). Correspondence with authors from Ho, et al., 2020, informed us that *Vax1* is mainly constrained to melanotroph cells and was not detected in 8-week-old mouse gonadotropes, according to their single-cell RNA-seq analysis (Ho et al., 2020). Thus, the recombination detected in the pituitary should not impact gonadotrope function.

The loss of VAX1 alters expression of *Kiss1* and *Tac2*, the gene that encodes for the stimulatory neuropeptide neurokinin B, in the male arcuate. Future studies would be needed to determine if the change in *Tac2* is VAX1-dependent, or a compensatory response to the increase in *Kiss1*. Although we observed increased gene expression of *Kiss1*, we found no significant change in LH and FSH secretion in male Vax1^{KissCre} mice. It is possible that the increase in *Kiss1* was not sufficient to cause a physiologic change in LH secretion. However, while the difference in LH levels was not significantly different in male Vax1^{KissCre} versus Vax1^{WT} mice, we did find a significant difference in the variation between these groups. It is important to note that these LH values were taken from a single time point, and did not evaluate LH pulses. One possibility is that increased *Kiss1* could increase LH pulse amplitude or pulse frequency, which would lead to a higher probability of detecting a pulse in a one-time measurement. However, profiling LH pulses in intact male mice is difficult due

to pulses occurring only once every two to three hours (Steyn et al., 2013). Further exploration is needed to establish whether or not LH profiles are altered; however, our findings suggest any changes are insufficient to affect overall fertility.

We also found that Vax1^{KissCre} males had a reduction in total sperm production, which is consistent with a phenotype observed in *Vax1* heterozygous mice (Hoffmann et al., 2014). We observed a less severe sperm reduction in Vax1^{KissCre} compared to that reported in the *Vax1* heterozygotes, and we reported no impairments in reproductive capacity in Vax1^{KissCre} males. Several studies have reported that male mice are able to maintain fertility and fecundity despite large reductions in total sperm. For example, *Arl4*-null mice had a 60% reduction and FSH-deficient mice had a 75% reduction in total sperm counts without impairments to litter size or frequency (Kumar et al., 1997; Schurmann et al., 2002). We cannot conclude whether the sperm reduction in our Vax1^{KissCre} mice is due to a loss of *Vax1* in kisspeptin neurons or if it is caused within the testis, as *Vax1* and *Kiss1* both are expressed therein (Hoffmann et al., 2014). Within the mouse testis, *Kiss1* mRNA and kisspeptin protein have been reported in Leydig cells and elongated spermatids, and may play roles in sperm capacitation and spermatogenesis (Anjum et al., 2012; Hsu et al., 2014; Salehi et al., 2015; Sharma et al., 2020; Toolee et al., 2019). However, it remains to be determined which cell types in the testis express *Vax1* as no *Vax1* enrichment was detected in a microarray analysis from an HA-pulldown from either Leydig cell-specific or Sertoli cell-specific RiboTag mice (Sanz et al., 2013). Sperm production could also be affected by changes to upstream hormone secretion. LH binds to receptors on Leydig cells to promote the synthesis of testosterone, driving spermatogenesis (Ge et al., 2009; Zirkin and Papadopoulos, 2018). Correct levels of circulating LH are important for maintaining these processes as, in humans, increased LH levels are associated with decreased sperm counts (Andersson et al., 2004; Liu et al., 2017). While this association has not been investigated in mice, it leads us to postulate that an increase in LH levels or pulse frequency, driven by increased *Kiss1*, could lead to a reduction in total sperm production.

Using immortalized kisspeptin cell lines, we demonstrate that VAX1 acts as a repressor of the *KISS1* promoter *in vitro*. VAX1 can regulate transcription through binding at ATTA consensus sites (Hoffmann et al., 2021; Hoffmann et al., 2016; Pandolfi et al., 2019; Slavotinek et al., 2012). The human *KISS1* promoter contains 5 ATTA sites within the 1313 bp upstream of exon 1 (http://genome.ucsc.edu/) (Kent et al., 2002), presenting potential sites for VAX1 to act as a direct regulator of *KISS1* transcription. Indeed, we found that two regions containing ATTA sites on the *KISS1* promoter regulated VAX1-mediated repression of the promoter. The most distal −1211 to −1193 bp region, containing three ATTA sites, and the most proximal −362 to −359 bp region were both necessary for repression by VAX1. Although homology between the mouse and human *Kiss1/KISS1* promoters is not high (~55% homology according to Needleman-Wunsch alignment between two sequences) (Altschul et al., 1997), the mouse promoter contains 8 ATTA sites within the 1313 bp upstream of the first exon, with multiple regions containing overlapping ATTA sites (http://genome.ucsc.edu/) (Kent et al., 2002), which could potentially facilitate a similar function.

Intriguingly, we observed that *Kiss1* was differentially regulated by VAX1 in female mice compared to males *in vivo*. We postulate that this sex difference could be due to the

interaction of VAX1 with androgens or estrogens. Both androgens and E2 inhibit ARC *Kiss1* mRNA *in vivo* (Smith et al., 2005a; Smith et al., 2005b) and E2 represses *Kiss1* mRNA in the ARC derived cell line (KTaR-1) *in vitro* (Jacobs et al., 2016). We showed, for the first time, that the synthetic androgen, R1881, and E2 can regulate transcription by repressing *KISS1* promoter activity in KTaR-1 cells. Additionally, we observed that the combination of VAX1 and R1881 was able to repress the *KISS1* promoter to a greater degree than either of the two independently, and that there was a significant interaction. The combined effects of VAX1 and E2, on the other hand, could not be differentiated. It is likely that R1881 and E2 are mediating their actions through their steroid receptors, AR and ERα, respectively. It is possible that binding of AR or ERα to the *KISS1* promoter alters VAX1 binding efficiency, or conversely, that binding of VAX1 alters AR or ERα binding. Another possibility is that R1881 or E2 treatments could alter the expression of other genes that are endogenously expressed in the KTaR-1 cell line that could potentially modulate the effects of VAX1. One limitation of the study is that KTaR-1 cells were derived from a female mouse and the effects of androgens on kisspeptin neurons derived from male ARC could produce a different response (Jacobs et al., 2016). Further studies will be required to determine more specifically how steroid hormones acting via nuclear receptors might be interacting with VAX1 on the *KISS1* promoter.

To understand the influence of VAX1 on female AVPV *Kiss1* regulation, we assessed gene expression during diestrus, when we expect AVPV kisspeptin activity to remain low (Marraudino et al., 2017), as well as during the preovulatory LH surge, when there is active *Kiss1* mRNA transcription (Robertson et al., 2009). Previously, it was observed that diestrus-staged *Vax1* heterozygous females had elevated AVPV *Kiss1*, serum LH, and E2 levels compared to WT mice. In our study, we found that diestrus $Vax1^{KissCre}$ mice had normal AVPV *Kiss1*, E2, and LH levels. The differences in our data lead us to postulate that the increased AVPV *Kiss1* seen in *Vax1* heterozygotes could be due to positive feedback from the high E2 levels. The source of elevated E2 levels observed in the *Vax1* heterozygous mice is unknown, however, as we did not observe a difference in E2 levels in our $Vax1^{KissCre}$ females, the increase in *Kiss1* in *Vax1* heterozygous mice is likely driven by the loss of *Vax1* from cell populations outside of kisspeptin neurons. *Vax1* heterozygous mice have a copy of *Vax1* deleted from all *Vax1*-expressing cells in the body, including all of those in the hypothalamus, while the homozygous deletion of *Vax1* in $Vax1^{KissCre}$ mice is confined to kisspeptin neurons. The differences in our data could also be due to a developmental effect, resulting from the timing of $Kiss1^{Cre}$ onset of expression. While the *Vax1* haploinsufficiency is present throughout development (Bertuzzi et al., 1999), the $Kiss1^{Cre}$ is not expressed in AVPV kisspeptin neurons until *Kiss1* expression begins postnatally (P10-16) (Semaan et al., 2010), potentially allowing $Vax1^{KissCre}$ females to overcome developmental modulation of the kisspeptin neurons that occurs prior to *Kiss1* expression and thus, VAX1 deletion.

To examine *Kiss1* levels during heightened AVPV kisspeptin activity, we used a validated E2-induced LH surge paradigm (Christian et al., 2005). The presence of both E2 and a circadian signal is required for the induction of an appropriately timed LH surge. We found that *Kiss1* mRNA levels in $Vax1^{KissCre}$ mice were significantly lower than $Vax1^{WT}$ mice when collected at the time of lights off. These findings deviate from our *in vitro* findings, and suggest a complex relationship between VAX1, E2, and circadian regulation. Despite

changes in *Kiss1* gene expression, Vax1^{KissCre} mice were still capable of inducing an LH surge. Upon closer examination of the molecular changes occurring at the time of the surge, we found that Vax1^{KissCre} females had fewer *Kiss1*-positive cells in the AVPV than Vax1^{WT} mice. We were unable to determine if this reduction was a true reduction in the number of kisspeptin cells, or a reflection of decreased *Kiss1* mRNA that led to fewer cells being detected. Future experiments using lineage tracing will need to be performed to determine if VAX1 is necessary for the development or survival of kisspeptin neurons or for the maintenance of *Kiss1* gene expression in adulthood. Although there was an overall reduction in the number of *Kiss1*-positive cells, there were approximately 10% more *Kiss1*-positive cells that colocalized with *cFos* in Vax1^{KissCre} mice than in Vax1^{WT} mice. This indicates that a higher proportion of Kiss1 neurons are active (*cFos* is expressed in activated neurons), potentially compensating for the overall reduction in *Kiss1* neurons. These findings are consistent with the ability of Vax1^{KissCre} females to produce an LH surge and maintain fertility.

This study shows for the first time that the homeodomain transcription factor VAX1 is a transcriptional repressor of *Kiss1* transcription. We show that VAX1 differentially regulates ARC *Kiss1* expression in males and females *in vivo* and VAX1 differentially regulates the *KISS1* promoter in the presence of androgens and estrogens *in vitro*. We confirmed a previous association between loss of *Vax1* and reduced sperm production, although it is insufficient to alter fecundity. In addition, we found that *Kiss1* mRNA levels and the number of *Kiss1*-expressing neurons in the AVPV were reduced at the time of an E2-induced LH surge. The changes in *Kiss1* expression did not significantly impact fertility, hormone levels, or the ability to mount an LH surge. Overall, these findings increase our understanding of the impacts of homeodomain transcription factors on *Kiss1* gene regulation.

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Highlights

- VAX1 differentially regulates *Kiss1* gene expression in the ARC of male and female mice.
- VAX1 represses the human *KISS1* promoter under basal conditions and in the presence of androgens *in vitro*.
- The loss of VAX1 reduces the number of *Kiss1*-expressing cells in the AVPV at the time of an induced-LH surge in female mice.

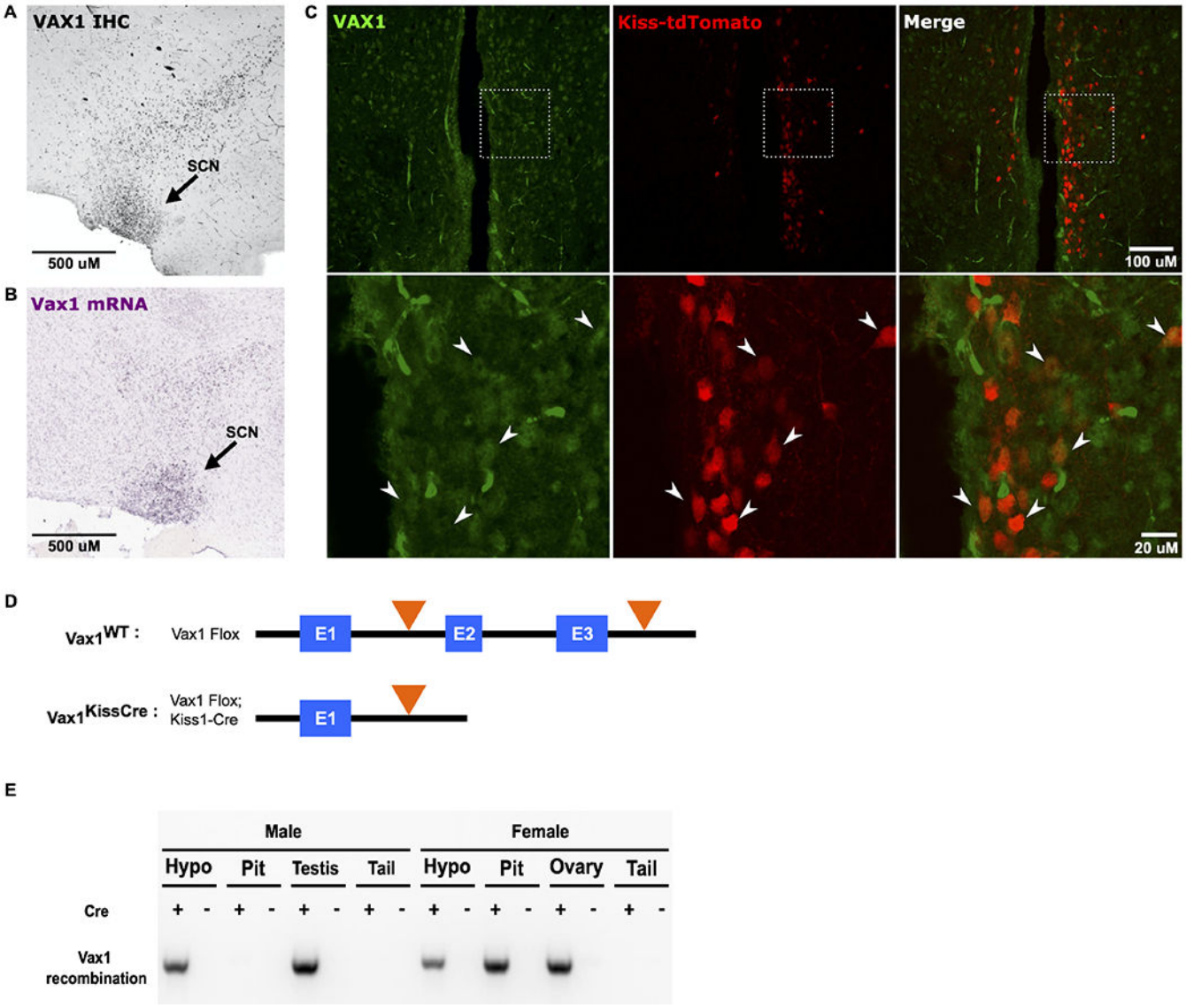

Fig. 1. VAX1 is expressed in *Kiss1* neurons and recombined in *Kiss1*-Cre-expressing tissues.

(A) Representative sagittal image of VAX1 immunoreactivity in the suprachiasmatic nucleus (SCN) and surrounding area in an adult WT mouse brain. **(B)** *Vax1 in situ* hybridization from adult male mouse, Allen Brain Atlas, image 18, Vax1 - RP_090303_04_G09 – sagittal, https://mouse.brain-map.org/gene/show/22083. **(C)** Fluorescent imaging showing VAX1 (green) and tdTomato (red) in $Kiss1^{Cre}$-tdTomato mice. Arrows show colocalization of tdTomato-positive neurons and VAX1. **(D)** Schematic of the *Vax1* gene in $Vax1^{WT}$ and $Vax1^{KissCre}$ mice. Black line depicts intronic sequence, blue boxes represent exons, and orange triangles depict LoxP sites. **(E)** Representative PCR to show recombination of the $Vax1^{Flox}$ allele from genomic DNA, collected from tissues known to either express *Kiss1* (hypothalamus, pituitary, testis, ovary) or not express *Kiss1* (tail) in $Vax1^{KissCre}$ (Cre+) and $Vax1^{WT}$ (Cre−) mice.

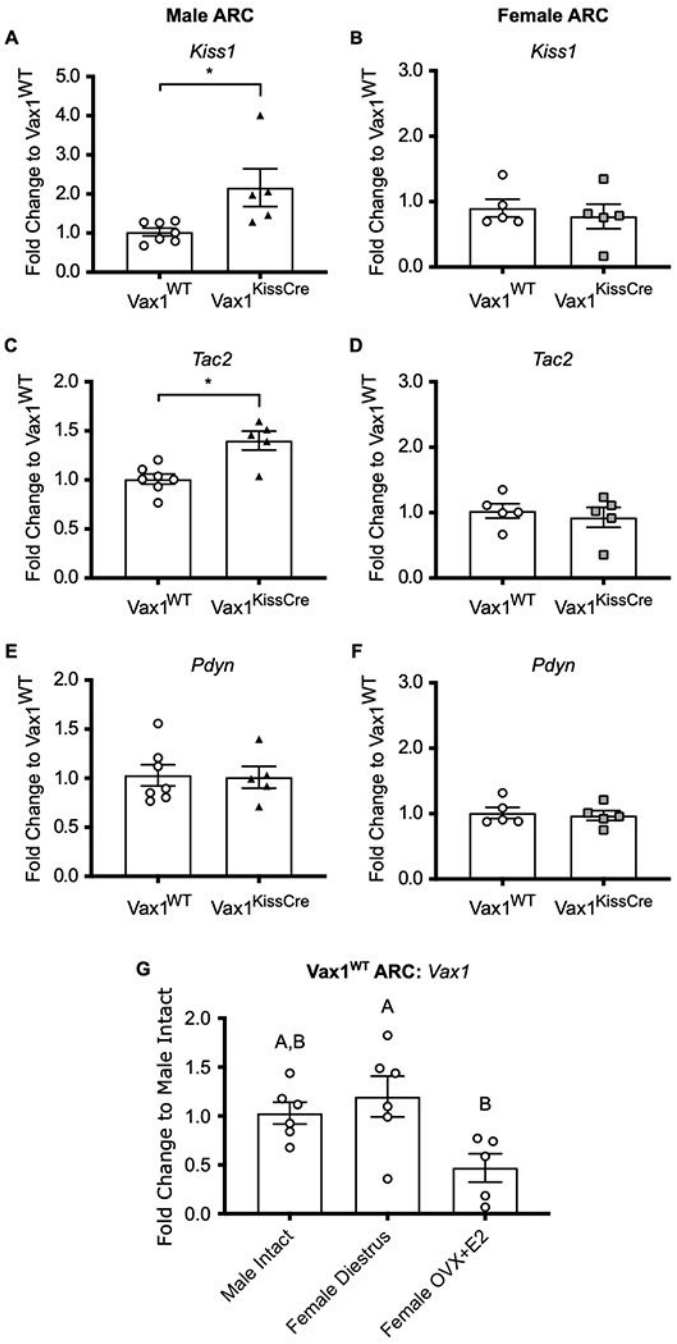

Fig. 2. VAX1 is required for *Kiss1* and *Tac2* expression in male ARC.

RT-qPCR profiles of **(A,B)** *Kiss1*, **(C,D)** *Tac2*, and **(E,F)** *Pdyn* in the ARC of intact males and intact diestrus females. Data were analyzed by the 2^-delta delta Ct method and represented as fold change compared to $Vax1^{WT}$ ± SEM. **(G)** RT-qPCR of *Vax1* in ARC of intact $Vax1^{WT}$ males, diestrus-staged $Vax1^{WT}$ females, and OVX+E2 $Vax1^{WT}$ females. Data were analyzed by the 2^-delta delta Ct method and represented as fold change compared to Male Intact ± SEM. All samples were collected between ZT 4-7. All data were analyzed

using (A-F) Student's t-test or (G) One-way ANOVA. * or different letters indicate significance of $p<0.05$. N=5-7.

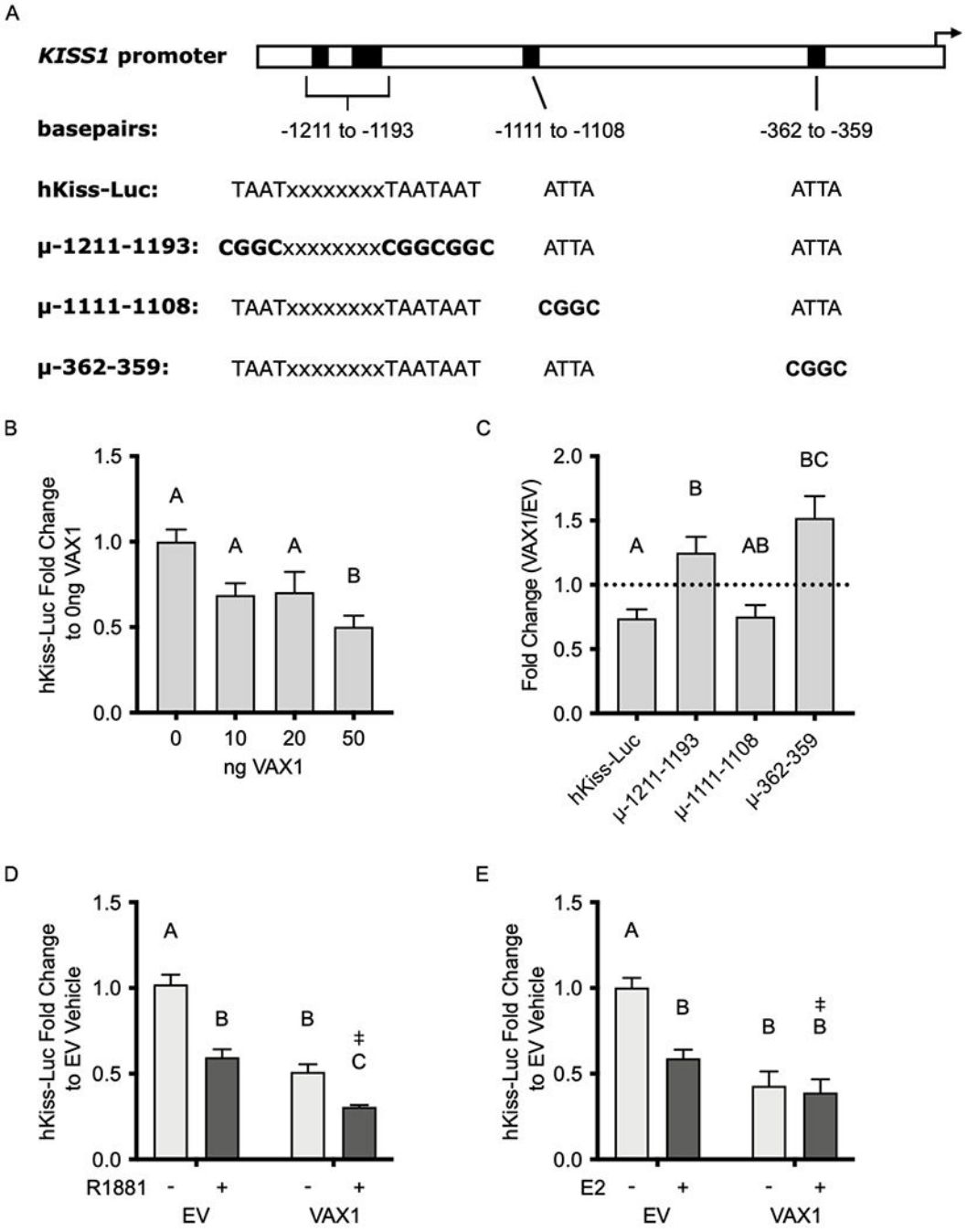

Fig. 3. VAX1 regulates transcription from the *KISS1* promoter in KTaR-3 cells.

(A) Schematic of ATTA binding sites (black rectangles) on the *KISS1* promoter and the sequence of these sites on the hKiss-Luc reporter or hKiss-Luc constructs containing cis-mutations at the indicated positions upstream of the transcriptional start site (black arrow). Bolded sequences indicate base pairs that were changed via site-directed mutagenesis. **(B)** hKiss-Luc was co-transfected with increasing concentrations of VAX1 expression vector into KTaR-1 cells. Data are represented as fold change as compared to 0 ng VAX1. **(C)** hKiss-Luc, μ-1211-1193, μ-1111-1108, and μ-362-359 were co-transfected into KTaR-1

cells with 50 ng VAX1 or EV. Data is represented as fold change of VAX1/EV. Dotted line indicates EV/EV. **(D)** KTaR-1 cells were transfected with hKiss-Luc or pGL2, 50 ng VAX1 or empty vector (EV), and 100 ng AR plasmids, and then treated with 10 μM R1881 (+) or vehicle (−). Data are represented as fold change as compared to EV vehicle. **(E)** KTaR-1 cells were transfected with hKiss-Luc or pGL2, 50 ng VAX1 or EV, and 50 ng ERα plasmids, and then treated with 1 pM E2 (+) or vehicle (−). Data are represented as fold change to EV vehicle. For all experiments, values represent means ± SEM. Data were analyzed using (B, C) One- or (D, E) Two-way ANOVA. Different letters denote significance, $p<0.05$. ‡ denotes a significant two-way factor interaction, $p<0.05$. N=3-4.

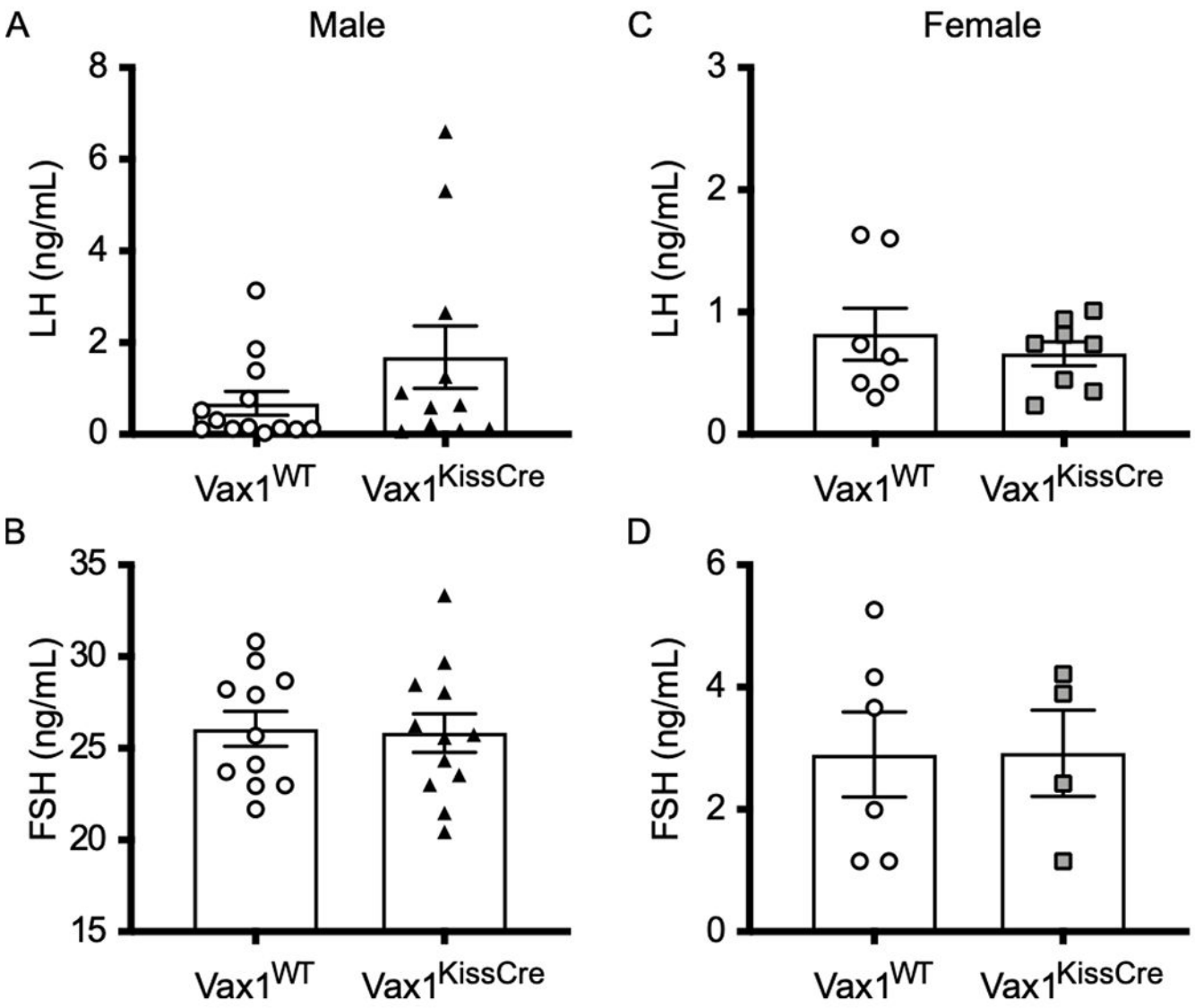

Fig. 4. Circulating hormone levels are comparable between intact male and female Vax1^{WT} and Vax1^{KissCre} mice.

(A) Intact male LH levels. **(B)** Intact male FSH levels. **(C)** Diestrus-staged female LH levels. **(D)** Diestrus-staged female FSH levels. Each symbol represents a biological replicate. Bars represent mean ± SEM. Data were analyzed using (A) Welch's t-test or (B-D) Student's t-test. N=4-7.

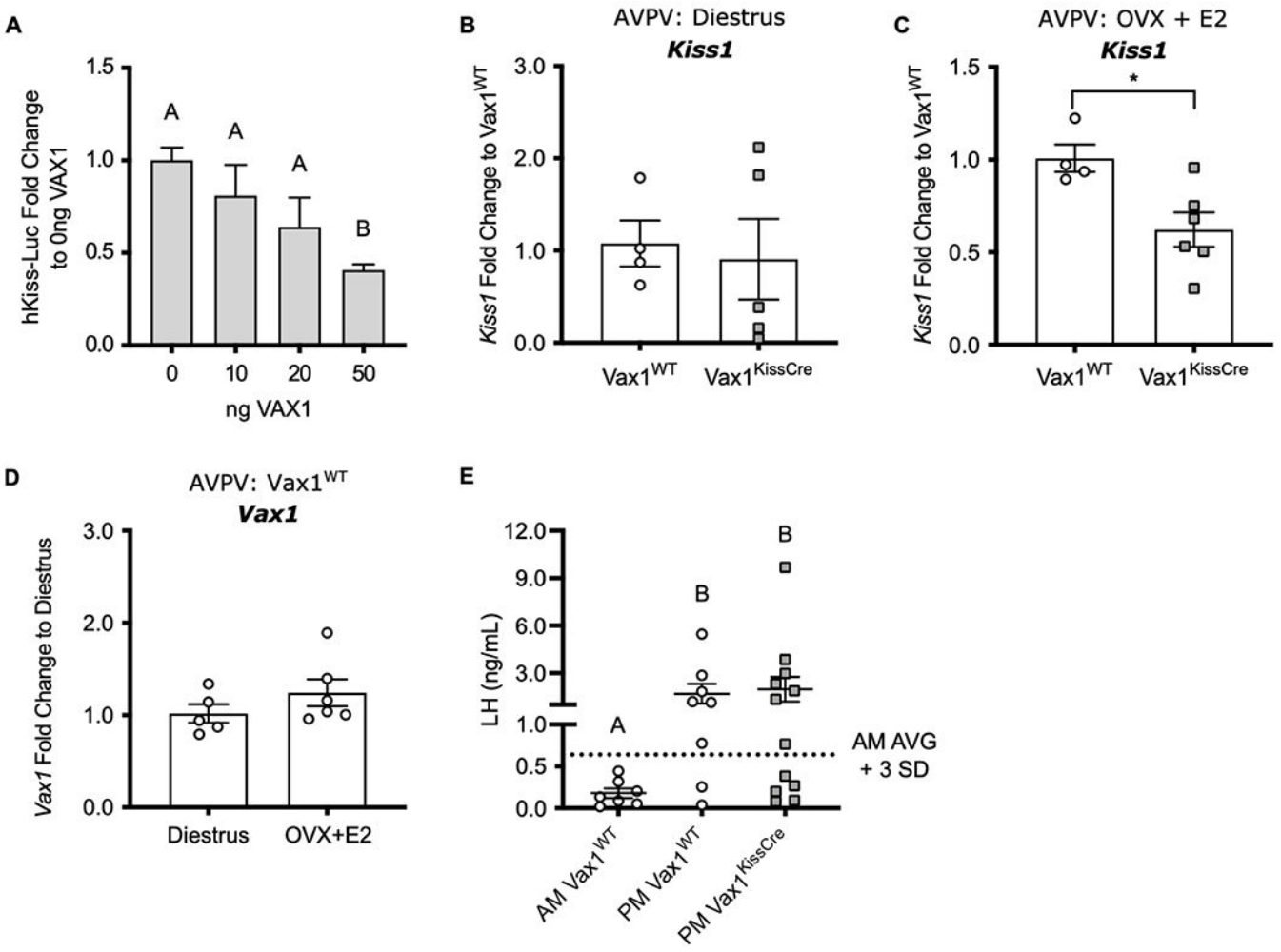

Fig. 5. *Kiss1* expression is reduced in the AVPV of E2-treated Vax1^{KissCre} females.

(A) hKiss-Luc or pGL2 was co-transfected with increasing concentrations of VAX1 expression vector into KTaV-3 cells. hKiss-Luc values were normalized to pGL2 values and represented as fold change compared to 0 ng VAX1. Values represent means ± SEM. N=4. RT-qPCR of *Kiss1* in the AVPV of **(B)** diestrus-staged female mice collected at ZT 4-7 and **(C)** OVX + E2 treated females collected at ZT 12-13. **(D)** RT-qPCR of *Vax1* from the AVPV of intact diestrus-staged Vax1^{WT} collected at ZT 4-7 or OVX + E2-treated Vax1^{WT} females collected at ZT 4-5. qPCR data were analyzed by the 2^-delta delta Ct method and represented as fold change compared to (B, C) Vax1^{WT} or (D) Diestrus ± SEM. **(E)** Serum LH levels from OVX+E2 treated females, collected at ZT 4-5 (AM) or at ZT 12-13 (PM). Dotted line indicates surge threshold (AM average + 3 SD = 0.64 ng/mL), N=7-12. Data were analyzed using (A, E) One-way ANOVA or (B-D) Student's t-test. Different letters or * indicate significance of $p<0.05$.

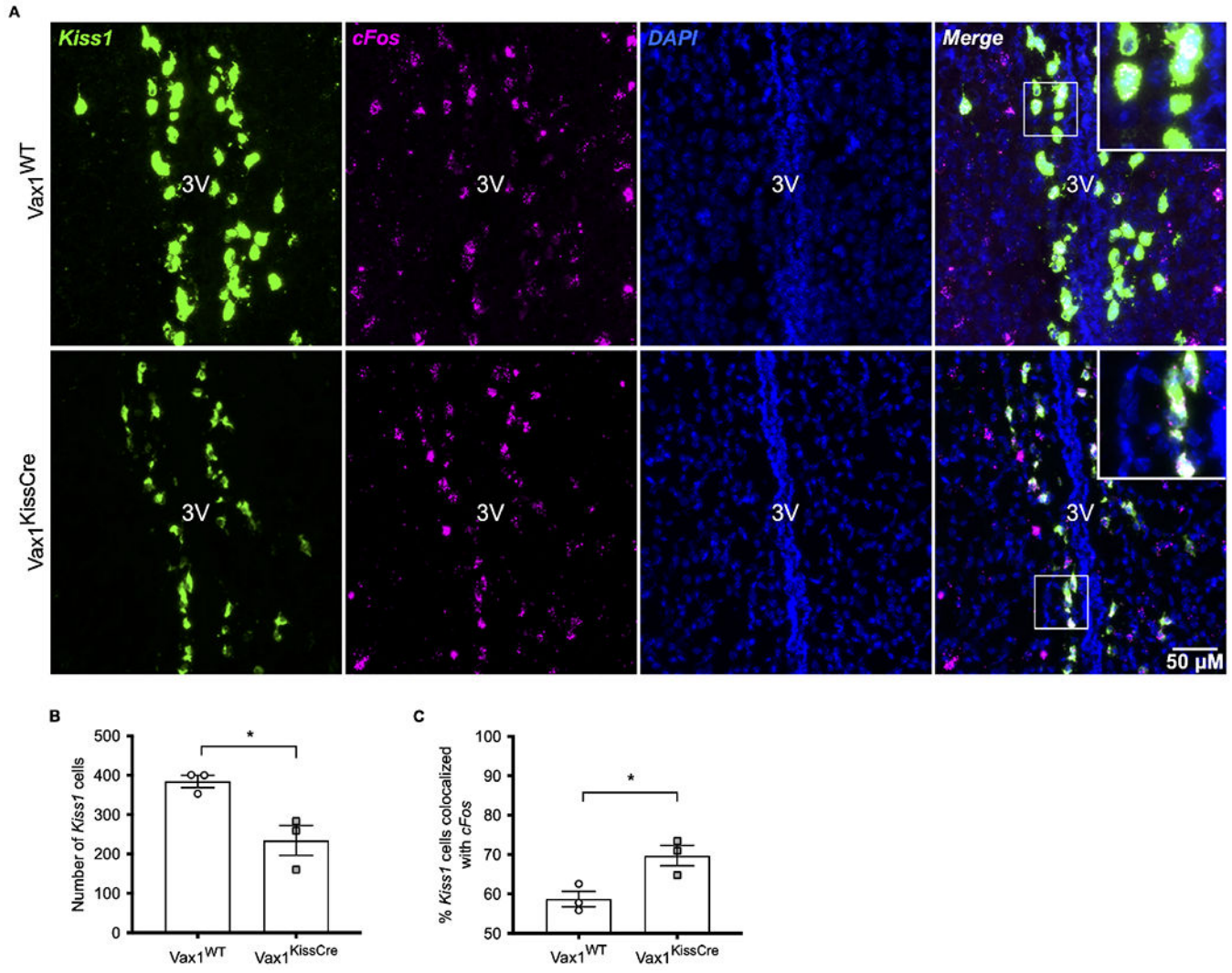

Fig. 6. Vax1^{KissCre} females have fewer *Kiss1* neurons and increased *cFos* colocalization during an E2-induced LH surge.

(A) Representative images of fluorescent *in situ* hybridization to detect *Kiss1* (green) and *cFos* (magenta) in the AVPV of OVX+E2 treated females collected at ZT 12-13. Colocalization of *Kiss1* and *cFos* are visualized in white. Sections were counterstained with DAPI (blue) for visualization of nuclei. **(B)** Quantification of the number of *Kiss1*-positive cells, N=3 per group. **(C)** Percentage of *Kiss1* cells colocalized with *cFos*, N=3 per group. **(B,C)** Data were analyzed using Student's t-test. *, $p<0.05$.

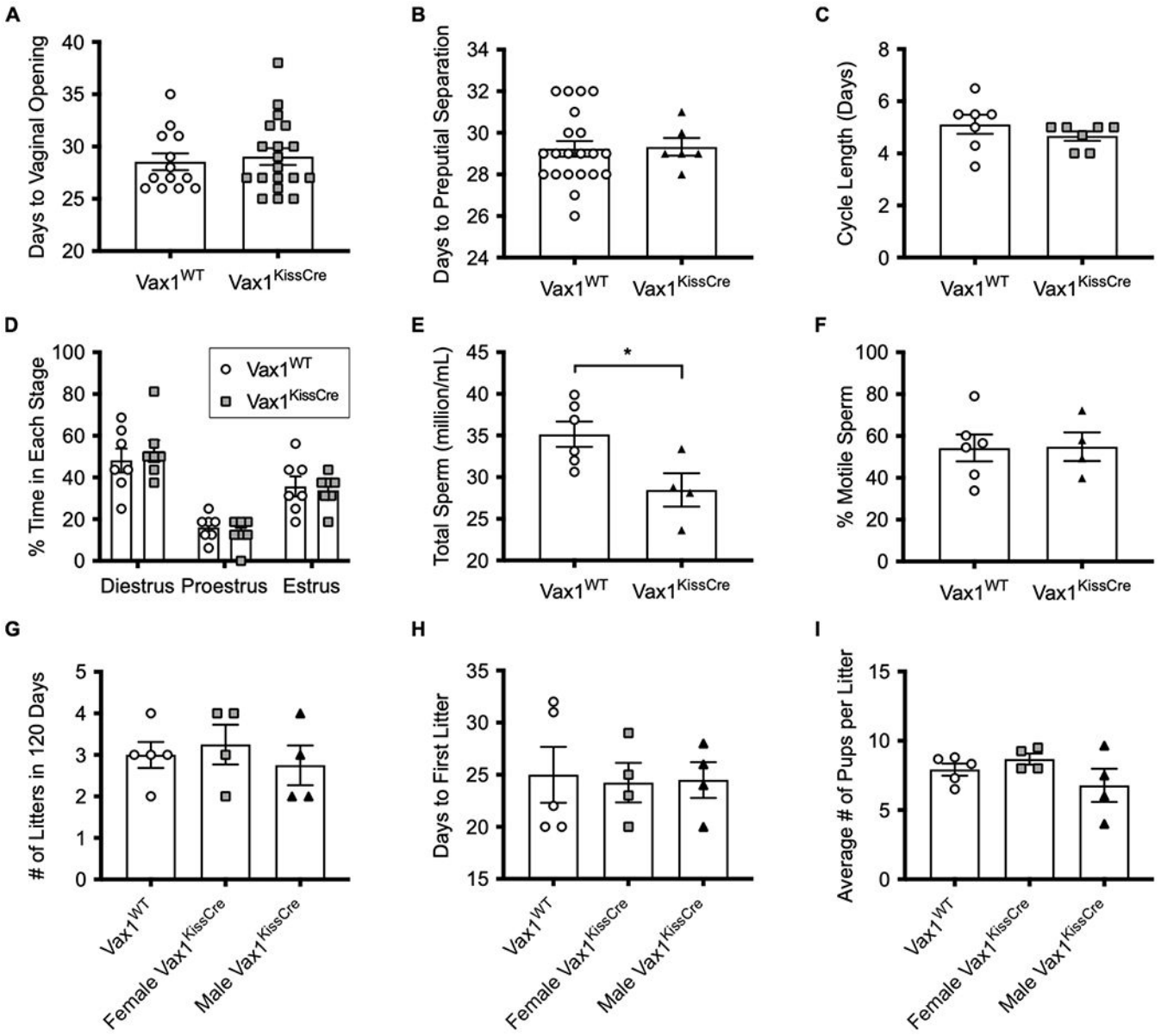

Fig. 7. Deletion of VAX1 from kisspeptin cells does not alter reproductive function.

(A) Time to pubertal onset in females determined by the number of days to reach vaginal opening. N=13-18. **(B)** Time to pubertal onset in males determined by the number of days to preputial separation. N=6-21. **(C)** Average length in days to complete one estrous cycle in female mice. N=7. **(D)** Time spent in each stage of the estrous cycle within a 16-day period in female mice. N=7. **(E)** Total sperm concentration per epididymis. N=4-6. **(F)** Percentage of motile sperm. N=4-6. **(G)** Number of litters produced in 120 days. N=4. **(H)** Number of days until first litter was born. N=4. **(I)** Average number of pups per litter. N=4. For all experiments, values represent means ± SEM. Data were analyzed using One-way ANOVA or Student's t-test. *, $p<0.05$.