Pituitary mechanisms regulating gonadotropin production and fertility *in vivo*

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ABSTRACT

Despite major advances in assisted reproductive technologies, infertility remains a major societal burden, with 1 in 6 couples unable to conceive a child naturally. Mammalian reproduction is regulated by the brain, the pituitary gland, and the gonads (testes or ovaries). In the brain, the hypothalamus produces and secretes gonadotropin-releasing hormone (GnRH). GnRH is released in the median eminence, where it enters the pituitary portal vessels and binds its receptor on gonadotrope cells. In response, pituitary gonadotropes secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), though the mechanisms through which GnRH stimulates FSH production are unclear. FSH is also regulated by activins, transforming growth factor β (TGFβ) ligands produced within the pituitary gland, acting in an autocrine and paracrine manner. The TGFβ-induced intracellular signaling cascade has been well described in gonadotropes, but the receptors through which these ligands act *in vivo* are not fully described. LH and FSH enter the general circulation, where they bind their respective receptors in the gonads to regulate gametogenesis and steroidogenesis. Sex steroids can negatively feedback to the hypothalamus and pituitary gland to decrease GnRH, LH, and FSH secretion. One of these steroids, progesterone, has clear effects on GnRH production in the brain, but its function in gonadotropes remains undescribed. The gonads also produce inhibins, which are endogenous antagonists of activins, and inhibit FSH production. The exact mechanisms through which these different factors are integrated to provide gonadotropes the unique ability to produce FSH will be explored in this thesis. I first tested the hypothesis that GnRH induces FSH production by alleviating chromatin occupancy of the FSHβ subunit (*Fshb*) promoter by histone deacetylases (HDACs). In contrast, I showed that HDACs play a permissive, rather than inhibitory role in FSH production *in vitro*. Second, I explored the cell autonomous role of the progesterone receptor (PR) in gonadotrope cells. Using genetically-engineered mice, I showed that PR is dispensable for LH and FSH production in males and females, though its absence decreases the amplitude of the pre-ovulatory LH surge in females. Third, I interrogated the identity of the receptors mediating activin, or activin-like, signaling in gonadotrope cells *in vivo*. I successfully showed that ACVR2A and ACVR2B are the relevant type II receptors required for FSH production. Finally, I used genetically-engineered mice to assess whether a transcription factor, GATA2, contributes to the

unique ability of gonadotropes to produce FSH. While I showed that GATA2 is required for quantitatively normal FSH production, it became evident that this protein has pleiotropic roles in gonadotropin production. The loss of GATA2 mainly affected males, and not females. I discovered that GATA2 is required for gremlin-1 expression. Gremlin-1 is a secreted factor that bioneutralizes bone morphogenetic proteins (BMPs), a sub-group of TGFβ ligands. These novel findings significantly contribute to our understanding of LH and FSH regulation, and may provide insights into designing new therapeutic strategies to inhibit or stimulate gonadotropin production.

RÉSUMÉ

Malgré les avancées fondamentales dans les technologies de procréation assistée, l'infertilité reste un fardeau important dans notre société. En effet, un couple sur six est incapable de concevoir un enfant naturellement. La reproduction des mammifères est régulée par le cerveau, l'hypophyse et les gonades (testicules ou ovaires). Dans le cerveau, une région spécifique appelée l'hypothalamus produit l'hormone de libération des gonadotropines hypophysaires (ou gonadolibérine, GnRH). La GnRH est sécrétée dans l'éminence médiane, où elle circule dans les vaisseaux de l'hypophyse et stimule son récepteur sur les cellules gonadotropes. En réponse, ces cellules sécrètent l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH); cependant, les mécanismes impliqués dans la production de FSH par la GnRH sont peu compris. La production de FSH est également contrôlée par les activines, membres de la famille des facteurs de croissance transformants (TGFβ), produites au sein de l'hypophyse, agissant de manière autocrine et paracrine. Les changements intracellulaires causés par les TGFβ sont bien compris, mais les récepteurs par lesquels ces ligands agissent *in vivo* sont incomplètement décrits. La LH et la FSH entrent dans la circulation sanguine générale, pour atteindre leurs récepteurs respectifs dans les gonades et réguler la gamétogenèse et la stéroïdogenèse. Les stéroïdes sexuels peuvent circuler jusqu'à l'hypothalamus et l'hypophyse afin d'inhiber la sécrétion de GnRH, LH et FSH. Un de ces stéroïdes, la progestérone, a des effets evidents au niveau de l'hypothalamus, mais sa fonction au niveau des gonadotropes n'a pas été évaluée directement. Les gonades produisent également des inhibines, qui s'opposent aux activines hypophysaires et diminuent spécifiquement la production de FSH. Les mécanismes, au travers desquels ces facteurs sont intégrés pour réguler la production de FSH, seront explorés dans cette thèse. J'ai tout d'abord contesté l'idée selon laquelle la GnRH induit la production de FSH en réduisant la présence d'histones désacétylases (HDACs) sur la chromatine. J'ai montré *in vitro* que les HDACs sont nécessaires à la production de FSH. Ensuite, j'ai exploré le rôle direct de la progestérone au niveau des cellules gonadotropes. En utilisant des souris génétiquement modifiées, j'ai montré que le récepteur de la progestérone (PR) n'est pas nécessaire pour la production des gonadotropines chez les souris tant mâles que femelles, bien qu'il joue un rôle

dans l'amplitude de la poussée pré-ovulatoire de LH chez les femelles. En outre, j'ai identifié les récepteurs induisant la signalisation TGFβ dans les cellules gonadotropes *in vivo*. J'ai démontré avec succès que ACVR2A et ACVR2B sont les récepteurs nécessaires à la production de FSH par les cellules gonadotropes. Enfin, j'ai utilisé des souris génétiquement modifiées pour déterminer si un facteur de transcription, GATA2, permet d'expliquer la capacité, que seuls possèdent les gonadotropes, à produire la FSH. Bien que GATA2 soit nécessaire pour la production normale de FSH, il est évident que cette protéine joue un rôle pléiotrope dans la production de gonadotropines. Notamment, GATA2 semble avoir des effets spécifiques chez les souris mâles, et non chez les femelles. J'ai découvert que GATA2 est indispensable à l'expression du gêne *Grem1* (gremlin-1). Gremlin-1 est un facteur qui bioneutralise les protéines morphogénétiques osseuses (BMPs), un sous-groupe de la famille des facteurs TGFβ. Ces nouvelles découvertes contribuent de manière significative à notre compréhension de la régulation de la LH et de la FSH, et pourraient servir à la conception de nouveaux traitements visant à inhiber ou à stimuler la production de gonadotropines.

This thesis was assembled following McGill University's Guidelines of Thesis Preparation. It is a manuscript-based thesis and comprises four manuscripts in Chapters 2 through 5. Two of these manuscripts have been published (Chapters 2 and 3). Two manuscripts are in preparation for publication (Chapters 4 and 5).

Contribution to original knowledge

A) Main findings and contribution of authors

Chapter 2

HDAC inhibitors impair *Fshb* subunit expression in murine gonadotrope cells

Schang G., Toufaily C., Bernard DJ.

Journal of Molecular Endocrinology. 2019 Feb 1;62(2):67-78

Main finding: Histone deacetylases are required for *Fshb* expression *in vitro*.

Contribution of authors: I was responsible for conducting most of the experiments and analyses. Dr. Chirine Toufaily conducted the experiments involving GnRH treatment (Figs. 2.1 A-C). All the work was performed under the supervision of Dr. Daniel J. Bernard. I wrote the first draft of the manuscript and worked with Dr. Daniel J. Bernard to prepare the final version. All authors approved the final version of the manuscript before submission.

Chapter 3

Impaired LH surge amplitude in gonadotrope-specific progesterone receptor knockout mice

Toufaily C.*, **Schang G.***, Zhou X., Wartenberg P., Boehm U., Lydon J., Roelfsema F., Bernard DJ. *Journal of Endocrinology*. 2020 Jan 244(1):111-122

* Contributed equally to the work

Main findings: The progesterone receptor (PR) is dispensable for gonadotropin production in mice. PR in gonadotropes plays a role in the amplitude of the pre-ovulatory LH surge in females, independently of GnRH self-priming, but is not required for fertility.

Contribution of authors: Dr. Chirine Toufaily and I were co-first authors on this publication. We shared equal workloads in terms of experimental design, data collection and analysis, and manuscript preparation. Xiang Zhou provided assistance with serial blood collections (Figs. 6 and 7). Philip Wartenberg generated the immunofluorescence images in Fig. 9 under the supervision of Dr. Ulrich Boehm. Dr. Boehm provided the GRIC mouse strain. Dr. John Lydon provided the floxed *Pgr* mouse strain. Dr. Ferdinand Roelfsema conducted the statistical analyses in Figs. 3.6 (Table 3.2) and 3.8. All the work (except for Fig. 3.9) was performed under the supervision of Dr. Daniel J. Bernard. Dr. Chirine Toufaily and I wrote the first draft of the manuscript and worked with Dr. Daniel J. Bernard to prepare the final version. All authors approved the final version of the manuscript before submission.

Chapter 4

Murine FSH production depends on the activin type II receptors ACVR2A and ACVR2B

Schang G., Ongaro L., Schultz H., Wang Y., Zhou X., Brûlé E., Boehm U., Lee SJ., Bernard DJ. *Endocrinology,* manuscript in revision

Main findings: The activin type II receptors ACVR2A and ACVR2B are required for FSH production *in vivo*.

Contribution of authors: I was responsible for conducting most of the experiments and analyses. Dr. Luisina Ongaro and Xiang Zhou provided assistance with organ and blood collections, as well as with mouse colony management and data analysis. Hailey Schultz conducted the ovarian

follicle counting (Figs. 4.2F-G and 4.4F-G). Ying Wang conducted the Luminex assays to measure serum FSH levels. Émilie Brûlé generated the immunofluorescence images. Dr. Ulrich Boehm provided the GRIC mouse strain. Dr. Se-Jin Lee provided the floxed *Acvr2a* and *Acvr2b* strains. All the work was performed under the supervision of Dr. Daniel J. Bernard. I wrote the first draft of the manuscript with feedback from Dr. Luisina Ongaro, and worked with Dr. Daniel J. Bernard to prepare the final version. Author consent will be obtrained prior to submission for publication.

Chapter 5

GATA2 regulation of FSH production in male mice may depend on the BMP antagonist gremlin-1

Schang G., Ongaro L., Brûlé E., Zhou X., Wang Y., Boehm B., Ruf-Zamojski F., Seenarine N., Amper MA., Nair V., Ge Y., Sealfon SC., Bernard DJ.

Manuscript in preparation

Main findings: GATA2 is necessary in gonad-intact males, but not females, for normal FSH production in gonadotropes. GATA2 is required in male gonadotropes for gremlin-1 expression, a bioneutralizing protein of certain BMPs.

Contribution of authors: I was responsible for conducting most of the experiments and analyses. Luisina Ongaro, Emilie Brûlé, and Xiang Zhou provided assistance with organ and blood collections. Ying Wang conducted the Luminex assays to measure serum FSH levels. Dr. Ulrich Boehm provided the GRIC mouse strain. Most of the work was performed under the supervision of Dr. Daniel J. Bernard. Drs. Frederique Ruf-Zamojski, Nitish Seenarine, Mary Ann Amper, Venugopalan Nair, and Yongchao Ge were all involved in RNA-seq data generation and analyses, under the supervision of Dr. Stuart Sealfon. I wrote the first draft of the manuscript, and worked with Dr. Bernard to prepare the final version. Author consent will be obtrained prior to submission for publication.

B) Other publications: published journal articles

Ongaro O., **Schang G.**, Kumar TR., Treier M., Deng Cx., Boehm U., Bernard DJ. (2020) Human follicle-stimulating hormone β subunit expression depends on FOXL2 and SMAD4 in gonadotropes of transgenic mice. *Endocrinology*, doi: 10.1210/endocr/bqaa045. [Epub ahead of print].

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* Contributed equally to the work

Li Y., **Schang G.**, Wang Y., Zhou X., Levasseur A., Boyer A., Deng CX., Treier M., Boehm U., Boerboom D., Bernard DJ. (2018) Conditional deletion of FOXL2 and SMAD4 in gonadotropes of adult mice causes isolated FSH deficiency. *Endocrinology* 159(7): 2641-2655.

Walker R.G., Czepnik M., Goebel EJ., McCoy JC., Vujic A., Cho M., Oh J., Aykul S., Walton KL., **Schang G.**, Bernard DJ., Hinck AP., Harrison CA., Martinez-Hackert E., Wagers AJ., Lee RT., Thompson TB. (2017) Structural basis for potency differences between GDF8 and GDF11. *BMC Biology* 15(1): 1-22.

Li Y., **Schang G.**, Boehm U., Deng CX., Graff J., Bernard DJ. (2016) SMAD3 regulates follicle-stimulating hormone (FSH) synthesis by pituitary gonadotropes *in vivo*. *Journal of Biological Chemistry* 292(6): 2301-2314.

Schang G., Robaire B., Hales BF. (2016) Organophosphate flame retardants act as endocrine-disrupting chemicals in MA-10 mouse tumor Leydig cells. *Toxicological Sciences* 1250(2): 499-509.

C) Other publications: journal articles in preparation

Ongaro O., Zhou X., Wang Y., **Schang G.**, Thompson TB., Boehm U., Su G., Bernard DJ. Mice lacking ALK4 and ALK5 in gonadotropes are FSH-deficient and hypogonadal.

Brûlé E., Li Y., **Schang G.**, Wang Y., Zhou X., Bernard DJ. IGSF1 does not regulate FSH synthesis or secretion.

Schang G.*, Schultz H.*, Boehm U., Bernard DJ. Absence of steroidogenic factor 1 expression in murine gonadotropes leads to loss of gonadotropins in males and females.
*Contributed equally to the work

D) Other publications: book chapters

Bernard, D., Li, Y., Toufaily, C., **Schang, G.** (2019) Regulation of gonadotropins. In *Oxford Research Encyclopedia of Neuroscience.* Oxford University Press.

Bernard DJ., **Schang G.**, Li Y., Ongaro L., Thompson, TB. (2018) Activins and Inhibins in Female Reproduction. In M. K. Skinner (Ed.), *Encyclopedia of Reproduction* vol. 2, pp. 202–210. Academic Press: Elsevier.

E) Other publications: newsletters

Schang G., Fernandez-Fuertes B, Lean SC, Nguyen AL, Pascottini OB. (2017) Infinity sperm storage: the gift that keeps on giving. *Mol Reprod Dev* 84(8): 667

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List of abbreviations

3βHSD: 3β-hydroxysteroid dehydrogenase

Ab: antibody

ABP: androgen-binding protein

Actb: encodes beta-actin

ACVR1A: activin receptor type 1A, also called ALK2

ACVR1B: activin receptor type 1B, also called ALK4

ACVR1C: activin receptor type 1C, also called ALK7

ACVR2A: activin receptor type 2A

ACVR2B: activin receptor type 2B

ACVRL1: activin receptor-like 1, also called ALK1

ADH: antidiuretic hormone

AIS: anti-inhibin serum

ALK1: activin receptor-like kinase 1, also called ACVRL1

ALK2: activin receptor-like kinase 2, also called ACVR1A

ALK3: activin receptor-like kinase 3, also called BMPR1A

ALK4: activin receptor-like kinase 4, called ACVR1B

ALK5: activin receptor-like kinase 5, also called TGFBR1

ALK6: activin receptor-like kinase 6, also called BMPR1B

ALK7: activin receptor-like kinase 7, also called ACVR1C

AMH: anti-Müllerian hormone

AMHR2: anti-Müllerian hormone receptor type 2

α-MSH: melanocyte-stimulating hormone alpha

ANOVA: analysis of variance

AP-1: activator protein 1

AR: androgen receptor

ATAC-seq: assay for transposase-accessible chromatin using sequencing

BMP: bone morphogenetic protein

BMPR1A: bone morphogenetic protein receptor type 1A, also called ALK3

BMPR1B: bone morphogenetic protein receptor type 1B, also called ALK6

BMPR2: bone morphogenetic protein receptor type 2

BPES: blepharophimosis, ptosis, and epicanthus inversus syndrome

BSA: bovine serum albumin

CaMK: calmodulin kinase

cAMP: cyclic adenosine monophosphate

CBP: CREB binding protein

CC: clomiphene citrate

CCND1: cyclin D1

CCND2: cyclin D2

CGA: chorionic gonadotropin alpha (also called gonadotropin subunit alpha)

cKO: conditional knockout

CL: corpus luteum

COC: cumulus-oocyte complex

CREB: cAMP response element binding protein

CYP: cytochrome P450

CYP19A1: aromatase

DAG: diacylglycerol

dcKO: double conditional knockout

DM: differentiation medium

DMSO: dimethyl sulfoxide

Dyn: dynorphin

ED: embryonic day

EDTA: ethylenediaminetetraacetic acid

EGFP: enhanced green fluorescent protein

EGR1: early growth response 1

ELISA: enzyme-linked immunosorbent assay

ELK1: ETS-like 1

ERK1/2: extracellular signal-regulated kinases type 1 and 2

ERα: estrogen receptor alpha

Esr1: encodes estrogen receptor α (ERα)

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FGF: fibroblast growth factor

FGFR1: fibroblast growth factor receptor 1

Floxed: flanked by loxP sites

Fos: encodes cFos

FOXL2: forkhead box L2

FSH: follicle-stimulating hormone

FSHβ: follicle-stimulating hormone β subunit

Fshb/*FSHB*: encodes follicle-stimulating hormone β subunit (FSHβ)

FSHR: follicle-stimulating hormone receptor

FST: follistatin

FSTL3: follistatin-like 3

GATA2: GATA binding protein 2

GATA3: GATA-binding protein 3

GDF: growth differentiation factor

GDNF: glial cell-line derived neurotrophic factors

GDX: gonadectomized

GFP: green fluorescent protein

GH: growth hormone

GM: growth medium

GnRH: gonadotropin-releasing hormone

Gnrh1/*GNRH1*: encodes gonadotropin-releasing hormone (GnRH)

GnRHR: gonadotropin-releasing hormone receptor

GPCR: G protein-coupled receptor

GPR54: G protein-coupled receptor 54, now called KISS1R

GR: glucocorticoid receptor

Grem1: encodes gremlin-1

$G\alpha_i$: G protein alpha subunit type i

$G\alpha_{q/11}$: G protein alpha subunit types q and 11

$G\alpha_s$: G protein alpha subunit type s

H4K12: histone H4, lysine 12

HAT: histone acetyltransferase

hCG: human chorionic gonadotropin

HDAC: histone deacetylase

HEK293: human embryonic kidney 293

HIF2α: hypoxia-inducible factor 2 alpha

HPG: hypothalamic-pituitary-gonadal

hpg: hypogonadal

HS: horse serum

HSP90: heat-shock protein 90

IC_{50}: half maximal inhibitory concentration

ICSI: intracytoplasmic sperm injection

ID1: inhibitor of DNA binding 1

ID2: inhibitor of DNA binding 2

ID3: inhibitor of DNA binding 3

Inha: encodes inhibin α subunit

Inhba: encodes inhibin βA subunit

Inhbb: encodes inhibin βB subunit

IP3: inositol 1,4,5-trisphosphate

IVF: *in vitro* fertilization

JDP2: c-JUN dimerization protein 2

KAL1: encodes anosmin

Kiss1/*KISS1*: encodes kisspeptin

KISS1R: kisspeptin receptor (used to be called GPR54)

KS: Kallman's syndrome

LH: luteinizing hormone

LHβ: luteinizing hormone β subunit

Lhb/*LHB*: encodes luteinizing hormone β subunit (LHβ)

LHR: luteinizing hormone receptor

LHX3: LIM homeobox protein 3

Luc: luciferase

MAP2K: mitogen-activated protein kinase kinase

MAP3K: mitogen-activated protein kinase kinase kinase

MAPK: mitogen-activated protein kinase

MH1: Mad homology domain 1

MH2: Mad homology domain 2

MMLV RT: Moloney murine leukemia virus reverse transcriptase

MS-275: entinostat

Myc: encodes c-Myc

NELF: negative elongation factor

nHH: normosmic hypogonadotropic hypogonadism

NKB: neurokinin B

Nr3c1: encodes glucocorticoid receptor (GR)

Nr5a1: encodes steroidogenic factor 1 (SF1)

OCT: optimal cutting temperature

OHSS: ovarian hyperstimulation syndrome

OMM: outer mitochondrial membrane

OVX: ovariectomized

PBS: phosphate-buffered saline

PCOS: polycystic ovary syndrome

PCR: polymerase chain reaction

PEI: polyethylenimine

PIP2: phosphatidylinositol 4,5-bisphosphate

PIT1: POU domain, class 1, transcription factor 1

pitKO: pituitary knockout

PITX1: *paired*-like homeobox 1

PITX2: *paired*-like homeobox 2

PKA: protein kinase A

PKC: protein kinase C

PLB: passive lysis buffer

PLC: phospholipase C

PMSG: pregnant mare serum gonadotropin

POA: preoptic area

Pol II: RNA polymerase II

POMC: pro-opiomelanocortin

PR: progesterone receptor

PRL: prolactin

PROP1: paired-like homeobox 1

qPCR: quantitative polymerase chain reaction

Rpl19: ribosomal protein L19

rPOA: rostral preoptic area

R-SMAD: receptor-regulated SMAD protein

RT-qPCR: reverse transcription quantitative polymerase chain reaction

SDS: sodium dodecyl sulfate

SEMA3A: semaphorin 3A

SEMA3E: semaphorin 3E

SERM: selective estrogen receptor modulator

SF1: steroidogenic factor 1

SHH: sonic hedgehog

SMAD: homolog of *Drosophila* mothers against decapentaplegic

StAR: steroidogenic acute regulatory protein

SV-40: simian virus-40

TAM: tamoxifen

TBS: tris-buffered saline

TBX19: T-box 19, also called Tpit

TGFBR1: transforming growth factor beta receptor type 1, also called ALK5

TGFBR2: transforming growth factor beta receptor type 2

TGFBR3: transforming growth factor beta receptor type 3, also called betaglycan

TGFβ: transforming growth factor beta

TSA: trichostatin A

TSH: thyroid-stimulating hormone

YFP: yellow fluorescent protein

τGFP: tau green fluorescent green protein

Chapter 1: General introduction

Reproduction is necessary for species survival. In vertebrates, this is tightly regulated by the coordination between three major endocrine organs: the hypothalamus, the pituitary gland, and the gonads (ovaries in females, testes in males). Together, they form the hypothalamic-pituitary-gonadal axis (or HPG axis, see Fig. 1.1). Understanding the mechanisms through which these organs crosstalk and function holds several benefits. First, given that 1 in 6 couples is infertile, it may lead to novel and better therapeutic strategies to assist these patients. Second, research in reproductive endocrinology will advance our knowledge of the aetiology of syndromic disorders, like polycystic ovary syndrome (PCOS), the most common endocrine disorder in women of reproductive age. Third, such research is necessary for conservation, notably for species facing extinction.

Gonadotropin-releasing hormone (GnRH) is one of the main secreted factors from the hypothalamus targeting the pituitary gland (Fig. 1.1). GnRH binds its receptor on pituitary gonadotrope cells, stimulating the production of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH is also regulated by intra-pituitary transforming growth factor beta (TGFβ) ligands called activins (Fig. 1.1). LH and FSH enter the general blood circulation to reach the gonads where they regulate the production of gametes and sex steroids. Sex steroids (progestagens [e.g. progesterone], androgens [e.g. testosterone], and estrogens [e.g. estradiol]) negatively feedback to the hypothalamus and pituitary to inhibit further production and/or release of GnRH, LH, and FSH (Fig. 1.1). This intricate and elaborate crosstalk is essential for reproductive capacity. In Chapter 1 of my thesis, I will describe what is currently known about the HPG axis. I will indicate gaps in knowledge that I addressed in my thesis research, and that are described in Chapter 2 through Chapter 5.

1.1 Pathology and physiology of the reproductive system

Reproductive capacity is dependent on the proper functioning and crosstalk between the hypothalamus, the pituitary gland, and the gonads. Several factors leading to hypo- or hyperactivity of one or more of these organs have now been identified, ultimately resulting in subfertility or infertility.

1.1.1 Causes of infertility

Given the complexity of the regulation of the HPG axis, a plethora of factors can cause infertility in both men and women. Infertility in humans is defined by the World Health Organization as the inability of a couple to achieve pregnancy after a year of unprotected intercourse. Around 1 in 6 couples is classified as infertile, with male and female factors equally likely to be the cause[1,2].

Male-factor infertility is less studied and characterized, due to the focus of infertility evaluations on women and societal norms that make men reluctant to seek fertility aid[3]. As such, it is challenging to obtain complete statistics in men, but common causes of infertility include azoospermia (or severe oligozoospermia), impaired sperm motility, epididymal blockage, erectile dysfunction, and age[3-5]. Female infertility can be caused by fallopian tube blockage, age (diminished ovarian reserve), endometriosis, and uterine factors[6,7]. The most common cause of female infertility is anovulation, affecting ~30% of infertile women[8].

In both men and women, infertility can be sub-classified into three categories: hypogonadotropic hypogonadism (decreased gonadal function due to impaired gonadotropin production), normogonadotropic normogonadism (normal or slightly hyperactive gonadal function with no major impairment in gonadotropin production; usually idiopathic), and hypergonadotropic hypogonadism (compensatory increase in gonadotropin production due to the failure of gonads to respond to hormones)[9].

Hypogonadotropic hypogonadism can be hypophyseal or hypothalamic in origin, is rarely seen in the population, and can be broadly subclassified into normosmic hypogonadotropic hypogonadism (nHH) and Kallman's syndrome (KS)[10-13]. At the level of the pituitary gland, nHH is caused by mutations in genes encoding gonadotropin subunits (FSH beta, encoded by *FSHB*[14-16],

and LH beta, encoded by *LHB*[17,18]) or the GnRH receptor (GnRHR, encoded by *GNRHR*[19]). At the level of the hypothalamus, mutations in genes encoding *GNRH1*[20] or other factors necessary for GnRH production (such as kisspeptin, encoded by *KISS1*[21], and kisspeptin receptor, encoded by *KISS1R*[22-24]) cause nHH. These mutations result in a failure to produce one or both gonadotropins, causing a loss of gonadal functions (see Fig. 1.1).

KS[10] is characterized by defects in the migration of GnRH neurons[25-27]. GnRH neurons emerge in the olfactory placode during embryonic development[28-31], and require guidance cues to migrate into the hypothalamus. In instances where these signals are abnormal or absent[32-37], the entire olfactory bulb is affected, leading to migration defects of other neuronal populations, namely those involved in smell. This explains why KS patients also typically show anosmia. The first genetic cause of KS was linked to the *KAL1* gene, which encodes anosmin[38,39]. Anosmin's function is to interact with the fibroblast growth factor (FGF) receptor 1 (FGFR1)[40-42], which is critical for GnRH neuron migration out of the olfactory placode. Mutations in FGFs or FGFR1 can lead to either nHH or KS, depending on the severity of the mutation[12,32,33]. Mutations in other genes involved in GnRH neuron migration that cause KS are those encoding semaphorins (*SEMA3A*, *SEMA3E*)[35-37]; a group of secreted or membrane-bound proteins, which act as neuronal guidance cues throughout the nervous system[37].

Mutations leading to nHH or KS are rare in the general population. The most common type of infertility observed in both men and women is normogonadotropic normogonadism. Although men in this category typically have idiopathic infertility, most women have PCOS. PCOS[43] is the most common endocrine disorder affecting women of reproductive age (~10%) and is the single most common cause of anovulation[44]. Though the aetiology of this pathology remains unclear, GnRH neurons tend to be hyperactive in these women[45]. This leads to excess androgen production by the ovary (see Fig. 1.1), causing the typical PCOS symptoms of hirsutism, irregular menstruations, and anovulation[46-48].

Finally, hypergonadotropic hypogonadism, or primary hypogonadism, is caused by the inability or reduced ability of the gonads to respond to gonadotropins. The main genetic causes are mutations in the receptors for LH[49,50] and FSH[51,52] (LHR and FSHR, encoded by *LHR* and *FSHR*, respectively), though some men with *FSHR* mutations are still fertile[53]. Other mutations have

been linked to premature ovarian failure in women, which describes an early-onset ovarian loss of function[54]. Acquired causes may involve torsion, trauma, inflammation, and drugs[55]. As a consequence, the gonads produce less sex steroids, leading to a loss of negative feedback at the level of the pituitary and hypothalamus, causing elevated gonadotropin levels (see Fig. 1.1).

A common way to assist infertile couples is through *in vitro* fertilization (IVF), usually involving controlled ovarian hyperstimulation (COH)[56]. This consists of sequential injections of exogenous gonadotropins to mimic critical phases of the female menstrual cycle. In order to better understand the underlying procedure of IVF and COH (and other therapeutic avenues), I will explain the physiological changes that take place throughout the menstrual cycle.

1.1.2 Overview of the female reproductive cycle

1.1.2.1 The human menstrual cycle

The menstrual cycle can be divided into four stages: menstruation, the follicular phase, ovulation, and the luteal phase. In women, a menstrual cycle lasts around 28 days, and spans from the first day of menses (or menstrual bleeding) to the onset of menses of the following cycle[57,58].

1.1.2.1.1 Follicular phase

The follicle is the functional unit of the ovary (Fig. 1.2), composed of an oocyte (the female gamete) and surrounding granulosa cells (supportive somatic cells)[59]. Through a still unclear mechanism, several primordial follicles are recruited to undergo folliculogenesis[59,60]. Folliculogenesis is the description of the maturation events of these follicles. Upon recruitment, primordial follicles, which contain a single layer of granulosa cells, will grow in size through granulosa cell proliferation[59] (Fig. 1.2). They become primary, then secondary follicles. This process of growth is considered to be gonadotropin-independent[61-63].

During the first half of the follicular phase, ovarian hormone levels are low (Fig. 1.3; see estradiol and progesterone). As a consequence, there is a lack of negative feedback to the hypothalamus and pituitary gland, causing gonadotropin levels to increase (Fig. 1.3)[64,65]. In parallel, secondary follicles recruit a new layer of somatic cells, called theca cells[60] (Fig. 1.2). This

new population expresses the LH receptor and produces androgens in response to LH stimulation[66-68]. At that point, folliculogenesis becomes gonadotropin-dependent.

The theca-derived androgens stimulate granulosa cells to express the FSH receptor[69-71]. Those follicles that are mature enough will be selected by the high levels of FSH at the midpoint of the follicular phase[72]. FSH stimulation of granulosa cells leads to: 1) estradiol production, 2) granulosa cell proliferation, and 3) expression of the LH receptor. As multiple follicles produce estradiol, this inhibits hypothalamic and hypophyseal activities, leading to a progressive decrease in gonadotropin production (Fig. 1.3)[64,65]. In parallel, granulosa cells also produce a TGFβ ligand, inhibin B, that feeds-back to the pituitary gland to inhibit FSH production (Fig. 1.3)[64,73,74]. At that point, FSH and LH levels remain sufficiently high to support the growth of a single follicle (generally), called the dominant follicle[75,76]. The decreasing FSH levels are insufficient to support the growth of the other follicles, which will undergo atresia, a process of degeneration[77-79].

The dominant follicle will continue to grow in size, due to granulosa cell proliferation and the formation of a cavity around the oocyte, called the antrum (Fig. 1.2). Within this antral follicle (or Graafian follicle) the granulosa cells in close interaction with the oocyte are called cumulus cells, while those around the antrum are called mural granulosa cells[80,81]. The antral follicle progressively becomes a preovulatory follicle, at which point estradiol production increases dramatically (Fig. 1.3). Estradiol now positively feeds-back to the hypothalamus and pituitary gland, causing an LH surge.

1.1.2.1.2 Ovulation

The LH surge is required for the final maturation events of the preovulatory follicle[64,65]. First, it is necessary for resumption of meiosis by the oocyte[82,83]. Since embryonic development, female gametes are paused at the prophase of the first round of meiosis (prophase I), on their way to becoming haploid[84]. Following the LH surge, meiosis resumes, until metaphase of the second round of meiosis (metaphase II).

Second, an event termed cumulus cell expansion occurs. This involves the production of a viscoelastic matrix by the cumulus cells, partly composed of hyaluronic acid[85,86]. Third, the LH surge will cause luteinization of mural granulosa cells (which are now sensitive to LH, due to FSH

stimulation) and theca cells[65,83,87] (see Fig. 1.2). As a consequence, the cells enlarge and will become the future corpus luteum (CL)[88]. Finally, the follicle ruptures, liberating the oocyte surrounded by cumulus cells (forming a cumulus-oocyte complex, or COC) into the oviduct and fallopian tube[89-91]. This is thought to release leftover inhibin B within the follicular fluid, leading to a peri-ovulatory peak of inhibin B levels in circulation (Fig. 1.3)[74].

Following release of the COC, the matrix produced by cumulus cells during cumulus expansion is critical for efficient capture and movement of the COC inside the oviduct, and to protect the oocyte from mechanical stress[92-94]. Hyaluronic acid in the COC is also critical for sperm motility and contributes to the success of fertilization[95-97].

1.1.2.1.3 Luteal phase

The residual luteinized mural granulosa cells and theca cells form the CL, a transient structure that secretes high levels of estradiol and progesterone (Figs. 1.2 and 1.3). The primary function of the CL is to prepare the endometrium for implantation of the embryo[98,99]. More specifically, progesterone increases vascularization of the endometrial lining and prepares the uterus for implantation of the future embryo[100,101]. The CL also secretes high levels of inhibin A, which, similarly to inhibin B, maintains FSH levels low[64,73,102] (Fig. 1.3). The function and survival of the CL is dependent upon LH or LH-like signaling[99,103]. In the event that fertilization takes place, following implantation, the embryo secretes human chorionic gonadotropin (hCG)[104,105]. hCG is structurally similar to LH, and signals through the LH receptor in the CL[106-108]. This is necessary for continued production of estradiol and progesterone.

Should fertilization not occur, hCG will not be produced, and the CL will eventually undergo luteolysis after about 2 weeks[109-111]. As a consequence, estradiol and progesterone levels decline (Fig. 1.3), leading to shedding of the endometrial wall, causing menstrual bleeding, and the cycle repeats itself. Specifically, given the progressive loss of inhibin A through the luteal phase, this provides a permissive endocrine environment for FSH to increase in the subsequent cycle (Fig. 1.3).

1.1.2.2 The rodent estrous cycle

Throughout this thesis, mice are used as models to better understand human reproduction. Although similar, both species exhibit significant differences, which I will describe in this section. Rodents display estrous cycles, with similar hormonal profiles and dynamics as described above (Fig. 1.3). However, rodents do not show menstrual bleeding; the endometrial wall is resorbed, rather than shed[112]. Also, the estrous cycle is divided into four phases: metestrus, diestrus, proestrus, and estrus, typically lasting 4-6 days in rodents[113].

Proestrus is characterized by a rise in estradiol (coming from multiple dominant follicles; see Fig. 1.3), the LH surge, resumption of meiosis, cumulus cell expansion, and luteinization of theca and mural granulosa cells[64,114].

This is closely followed by estrus, at which point follicular rupture, ovulation, and mating occur. "Estrus" is derived from the Latin *oestrus* (or "frenzy") and is described by behavioral displays of sexual receptivity in females ("heat period"). On the morning of estrus, rodents will exhibit a secondary FSH surge, which is dependent on activin, rather than GnRH, signaling in the pituitary gland[64,115-117]. This peak of FSH is critical for the subsequent wave of folliculogenesis[118], and is generated as a consequence of the drop in both inhibins A and B (Fig. 1.3). Of note, contrary to humans, inhibin B levels are high throughout most of the reproductive cycle in rodents, except on the morning of estrus (Fig. 1.3). Moreover, inhibin A production steadily increases until the pre-ovulatory LH surge in rodents, while it peaks during the mid-luteal phase in humans (Fig. 1.3).

The following days (metestrus/diestrus) are characterized by elevated progesterone levels (gradually decreasing; Fig. 1.3), due to the activity of the CLs[64,119]. Unlike in humans (or non-human primates and horses), CLs in species exhibiting estrous cycles do not require stimulation from an embryonic LH-like hormone (i.e., hCG in humans). CLs can last up to 3 consecutive cycles[120], and further maintenance of the CLs requires prolactin (PRL) secretion by the pituitary gland, which is induced by cervical stimuli (i.e., mating)[121-123]. PRL acts in the CL to downregulate enzymes involved in progesterone catabolism[124].

1.1.3 Treatments of infertility

Now that I reviewed the regulation of folliculogenesis and reproductive cycles, I will describe the strategies used to treat infertile couples. As mentioned in section 1.1.1, a common way to treat infertile couples is through IVF. With its development and success in 1978 with the birth of Louise Brown[125], it appeared that infertility would be "cured". Since then, IVF protocols have been refined and made more affordable.

This technology usually relies on COH, designed to induce follicular growth and ovulation-related events using exogenous gonadotropins. The general paradigm of COH involves daily exogenous FSH injections to stimulate folliculogenesis (i.e., to mimic the follicular phase)[126]. When estradiol levels reach a certain level, GnRH signaling is blocked to prevent a premature endogenous LH surge. Indeed, the goal of COH is to retrieve as many preovulatory follicles as possible[127]. Once a sufficient number of follicles reach the appropriate size (16-18 mm in diameter), an exogenous hCG stimulus is given, which triggers ovulation-related events (resumption of meiosis, cumulus cell expansion, luteinization)[126]. hCG is used due to its better pharmacokinetic profile relative to LH (namely longer half-life)[128,129]. Before follicular rupture occurs, the follicular fluid (containing the oocyte) is aspirated, the oocyte denuded (i.e., the granulosa cells are removed) and can either be cryopreserved or undergo IVF or, more commonly nowadays, intracytoplasmic sperm injection (ICSI)[126,130,131]. The fertilized eggs are cultured for several days, until the blastocyst stage, following which the most promising embryos are selected and either frozen or transferred to the woman's uterus for implantation[132-134].

While revolutionary, IVF technologies remain expensive and can lack efficiency, with only about 25% of cycles resulting in a live birth[135,136]. The hormone injections involved can also cause severe side effects, such as ovarian hyperstimulation syndrome (OHSS)[137,138], and can be cumbersome to perform. As such, there is a need for other pharmacological strategies that can assist couples in achieving pregnancy.

Clomiphene citrate (CC) is one of the most successful fertility drugs developed, leading to 30-40% pregnancy rates in anovulatory women[139-142]. It is orally available, inexpensive, and well tolerated[143]. CC is a selective estrogen receptor modulator (SERM), with weak anti-estrogenic activity, thereby blocking estradiol's negative feedback in the brain and pituitary gland,

promoting gonadotropin production[144-147]. A significant proportion of women do not respond to CC[148,149], in which case aromatase inhibitors, such as letrozole, can be administered [142,150-152]. Letrozole inhibits the conversion of androgens to estrogens in the ovary, leading to decreased negative feedback to the brain and pituitary gland, and increased gonadotropin production[152-154]. Letrozole can be used in combination with CC to diminish the amount of exogenous gonadotropins required, thereby reducing costs and side effects[155,156].

Still, there is no clinically available drug that allows direct modulation of endogenous FSH, which is the principal gonadotropin required for follicle growth. Thus, there is a need for alternative treatment avenues that would allow the direct stimulation of FSH production from gonadotrope cells, without the need to rely on expensive exogenous injections. In order to design new therapeutic strategies to modulate FSH, we first need to understand the precise regulatory mechanisms that control FSH production. In section 1.2, I will describe the function of each organ within the HPG axis, and in section 1.3, I will outline the most important hypothalamic and hypophyseal pathways that regulate gonadotropin production.

1.2 The hypothalamic-pituitary-gonadal axis and regulation of fertility

1.2.1 The hypothalamus

The hypothalamus, one of the smallest areas of the brain, integrates neuronal and hormonal inputs in virtually all aspects of endocrinology. The hypothalamus can generally be divided into three parts[157,158]. The most rostral part, or preoptic area (POA), is involved in thermoregulation, circadian rhythms, and sexual behavior. The POA also contains a small cluster of cells called the anteroventral periventricular nucleus (AVPV). Moving caudally, the middle section, or tubular hypothalamus, is involved in feeding and sexual behavior, as well as autonomic and endocrine responses. The tubular hypothalamus contains the arcuate nucleus (ARC). Finally, the most caudal part of the hypothalamus, or posterior hypothalamus, is involved in stress and arousal.

1.2.1.1 GnRH neurons

The idea that a hypothalamic factor is responsible for regulating fertility was first described in the late 1930s. Seminal work showed that electrically stimulating the hypothalamus could induce ovulation in rabbits[159]. It then took nearly 30 years for hypothalamic regulation of pituitary function to be revisited. Indeed, hypothalamic extracts triggered gonadotropin release in pituitary cultures[160,161]. This was caused by a hypothalamic peptide, later to be called GnRH, which was successfully purified by two independent groups in 1971[162-165]. Shortly thereafter, the *hpg* mouse model was described and established; these mice have a natural inactivating mutation in the *Gnrh1* gene (encoding GnRH), rendering homozygous mutant males and females sterile[166,167]. Since then, loss-of-function mutations causing sterility have also been described in the human *GNRH1* and *GNRHR* (encoding the GnRH receptor, GnRHR) genes[19,22,168].

GnRH is produced by specialized neurons that have rather unique properties. First, they do not form a distinct nucleus in a specific area of the hypothalamus; rather they are scattered in the rostral POA (rPOA) in rodents, and more dorsally in humans[169,170]. Second, although their function is critical to fertility, their population size is small, with only 800 to 2000 neurons in humans[171-175]. Third, GnRH neurons do not originate in the brain. Rather, as mentioned above, these cells migrate from the olfactory placode (in the olfactory bulb) into the hypothalamus along olfactory axon fibers between embryonic days (ED) 12.5 and 15.5 in mice[29,176]. Several factors have been hypothesized to direct this migration, including semaphorins[35,36], FGFs[32,33] , and more recently, anti-Müllerian hormone (AMH)[34]. Failure or defects in this migration cause infertility and hypogonadism and can be associated with anosmia and Kallmann's syndrome (as described in section 1.1.1.).

Once established in the forebrain, GnRH neurons send projections to the median eminence, where a vast network of permeable and fenestrated capillaries acts as an interface to transmit hypothalamic outputs to the pituitary gland[177-179]. Postnatally, until puberty, GnRH neuronal activity can be seldom detected[180,181]. At the outset of puberty, GnRH neurons become more active, and exhibit rhythmic activation[182-184]. More specifically, GnRH is secreted as pulses, and this episodic secretion is critical for proper pituitary function. The idea of such a synchronized network is remarkable since, as described above, the GnRH neuronal population is scattered. One

important feature of these neurons is that their nerve terminals cluster at the level of the median eminence. From there, it is hypothesized that a pulse generator outside of the GnRH neuronal network can control these neurons' activity, perhaps at the level of the GnRH terminals.

1.2.1.2 Kisspeptin neurons

It had been hypothesized since the 70's that a population of neurons, namely in the ARC, controlled GnRH pulsatility, as lesions of the ARC abolished LH pulses[185]. A clue on the identity of these pulse generator cells was provided following the first association between infertility and a loss-of-function mutation in the *GPR54* (now called *KISS1R*) gene[22,23], encoding the kisspeptin receptor (KISS1R). Kisspeptins are a family of small peptides produced from a differentially cleaved pre-proprotein[186-188]. Different kisspeptins are named based on their number of amino acids. Kisspeptin-54 (hereafter called kisspeptin) is the most abundant kisspeptin in humans, and had been described since the 1990s as an anti-metastatic agent *in vitro*[189]. It was shown to bind KISS1R[186,188,190], and it became clear that kisspeptin was pleiotropic across physiology[22,23,191]. Rapidly, the kisspeptin neurons were described in the hypothalamus and were shown to innervate GnRH neurons[192-194]. GnRH neurons express *Kiss1r*/*KISS1R* and are potently stimulated by kisspeptin[195-199].

Two different kisspeptin neuronal populations have been described (Fig. 1.4): one located in the ARC and the other in the POA. This has been described in several mammalian species, including mice[192,194,200], monkeys[201-205], and humans[201,206]. While the spatial and biochemical signatures of the ARC population appear consistent across species, those of the POA population vary. In rodents, preoptic kisspeptin neurons are located in the AVPV[192,207], while humans and non-human primates have a more scattered population in the rostral POA[203,206]; whether they are homologous remains to be elucidated.

Despite these species differences, kisspeptin is necessary for GnRH and, by extension, gonadotropin production. Indeed, *Kiss1* and *Kiss1r* global knockout mice and rats are sterile[191,208,209], and humans with loss-of-function mutations in these genes fail to go through puberty[21-23]. Moreover, rodents, ewes, sheep, monkeys, and humans injected with exogenous kisspeptin exhibit increased gonadotropin and/or GnRH production[202,210-216]. These data however

do not indicate that kisspeptins directly activate GnRH neurons. *In vitro*, kisspeptin can stimulate *Gnrh1* expression and GnRH secretion in GT1-7 cells[199,217,218], an immortalized GnRH neuron-like cell line[219,220]. More importantly, the sterility of *Kiss1r*-mutant mice can be rescued by specific re-introduction of KISS1R in GnRH neurons[221].

These data indicate that kisspeptin acts as a GnRH secretagogue by directly activating GnRH neurons (Fig. 1.4) and is necessary across mammalian species for reproduction.

1.2.2 The pituitary gland

The pituitary gland is located just below the brain and consists of three distinct morphological areas: the anterior, intermediate, and posterior lobes. The anterior lobe contains gonadotropes (LH- and FSH-producing, the target cells of GnRH), thyrotropes (thyroid-stimulating hormone [TSH]-producing), corticotropes (adrenocorticotropic hormone [ACTH]-producing), somatotropes (growth hormone [GH]-producing), and lactotropes (PRL-producing). Somatotropes and lactotropes are the most abundant cell types, representing ~40% and ~30% of the anterior lobe, respectively[222-224]. Corticotropes represent ~15% of the population, while thyrotropes and gonadotropes each comprise of 5-10% of the anterior lobe cell population[222-224].

The intermediate lobe contains melanotropes (melanocyte-stimulating hormone alpha [α-MSH]-producing). The posterior lobe contains the terminals of magnocellular neurons (antidiuretic hormone [ADH]- and oxytocin-producing).

1.2.2.1 Pituitary development

1.2.2.1.1 Organogenesis and lineage specification

Organogenesis of the pituitary gland is a tightly controlled process, regulated by timely activation of various transcription factors (Fig. 1.5), as well as gradients of soluble factors. Two important structures are involved in early embryonic development: the oral ectoderm (the roof of the mouth), which will give rise to the anterior and intermediate lobes, and the neural ectoderm (or ventral diencephalon, the bottom of the hypothalamus), from which the posterior lobe will arise[225-227]. The following section focuses on mouse pituitary morphogenesis, as it has

been the most studied. However, similar mechanisms have been described in chicks[228,229] and humans[230,231] (though the timelines differ between species).

At embryonic day (ED) 8.5, the oral ectoderm invaginates upward, forming a rudimentary pouch (Rathke's pouch) by ED 9.5[232,233]. This is dependent on WNT family 5A (WNT5A), bone morphogenetic protein 4 (BMP4), FGF8, and FGF10 secretion from the neural ectoderm[225], and BMP2 and sonic hedgehog (SHH) secretion from the oral ectoderm[234-236]. Between ED 9.5 and 12.5, cells within Rathke's pouch proliferate upwards (forming a definitive pouch) while the ventral diencephalon extends downwards, becoming a structure called the infundibulum[227,233].

By ED 12.5, Rathke's pouch is detached from the oral cavity to become the future anterior and intermediate lobes of the pituitary gland[233,237,238]. The infundibulum will become the posterior lobe[237]. At that stage, the pouch is characterized by highly proliferative progenitor cells, which progressively become quiescent (most cells no longer proliferate by ED 14.5)[239]. Differentiation of the hormone-producing cells of the anterior lobe occurs on different days, and requires the sequential activation of transcription factors[240-242] and signaling by secreted factors[229,234,243,244].

First, between ED 11.5 to ED 13.5, nearly all pituitary cells require expression of *paired*-like homeodomain transcription factors 1 and 2 (PITX1 and PITX2)[245-248], which induces LIM-homeodomain transcription factor 3 (LHX3, encoded by *Lhx3*)[249-251] (Fig. 1.5). PITX1 and PITX2 mostly control expansion of the pouch, and contribute to cell lineage specification later in development[245,247]. LHX3 is critical for maintenance of the rudimentary pouch, and *Lhx3* knockout mice show no pituitary development following ED 10.5 due to apoptosis[252,253].

Another transcription factor activated by PITX1 is PROP1 (*paired*-like homeobox 1, encoded by *Prop1*/*PROP1*), which is highly expressed in most pituitary cells starting at ED 11.5[236,254,255]. *Prop1* knockout mice have small pituitaries, with deficiencies in GH, TSH, PRL, and gonadotropins[255]. In humans, mutations in *PROP1* lead to a more progressive loss of hormone production, along with a failure to go through puberty[256,257]. The main function of PROP1 is to induce the expression of POU domain, class 1, transcription factor 1 (PIT1, also known as POU1F1, encoded by *Pou1f1*/*POU1F1*)[255]. However, BMP2 produced by the oral ectoderm creates a ventral-to-dorsal gradient, which induces expression of the GATA-binding factor 2 (GATA2,

encoded by *Gata2*) around ED 10.5, thereby inhibiting PIT1 activity[258]. This leads to the formation of GATA2+ cells (gonadotropes), GATA2+/PIT1+ cells (thyrotropes), and PIT1+ cells (somatotropes and lactotropes)[225] (Fig. 1.5). Though PROP1 is required for gonadotrope differentiation, its expression seems transient and it is unclear how gonadotropes become PROP1-deficient during pituitary development[258-260].

These patterns of expression are necessary for the development of all cell types within the anterior lobe, except for corticotropes. This lineage depends on T box factor 19 (TBX19 or Tpit) activation[261,262], which in turn induces pro-opiomelanocortin (POMC) expression, a characteristic product of corticotropes[262]. This is a rather early event during pituitary organogenesis, and corticotropes are the first hormone-producing cells to reach terminal differentiation, around ED 12.5[242] (Fig. 1.5). This is followed by TSH-positive thyrotropes at ED 14.5, GH-positive somatotropes at ED 15.5, prolactin-positive lactotropes at ED 16.5, and LH- and FSH-positive gonadotropes at ED 17.5 (though LH can be detected as early as ED 16.5)[230,242] (Fig. 1.5).

1.2.2.1.2 Control of gonadotrope differentiation

Though several transcription factors have been implicated in the specification of the gonadotrope cell lineage, no single protein controlling this process has been identified.

A transcription factor called steroidogenic factor 1 (SF1, encoded by *Nr5a1*) is first detectable at E13.5. It is critical for gonadotrope function, as demonstrated by the absence of gonadotropin production in global *Nr5a1* knockout mice[263,264], or in mice lacking *Nr5a1* expression specifically in the pituitary gland[265,266]. In light of these results, along with the observation that *Nr5a1* is unique to gonadotrope cells within the pituitary gland[267-271], SF1 was a promising candidate for controlling gonadotrope differentiation. However, exogenous GnRH injections in *Nr5a1*-null animals resulted in LH and FSH production, indicating that gonadotropes: 1) are still terminally differentiated in the absence of SF1, 2) can still respond to GnRH, and 3) have the necessary transcription factors to express gonadotropin subunit genes[272]. Moreover, a female patient with a *NR5A1* loss-of-function mutation failed to develop reproductive organs, but her gonadotropin levels increased following a GnRH injection[273]. This patient also had

elevated FSH levels in circulation, based on human reference levels[274,275]. In sum, these data indicate that SF1 is required for normal gonadotrope function, but is not necessary for gonadotropin subunit expression.

GATA2 is another transcription factor hypothesized to contribute to gonadotrope development. *Gata2* mRNA can be detected in the ventral area of Rathke's pouch as early as ED 10.5[258]. Mice that overexpress GATA2 in cells normally destined to express PIT1 show a dramatic expansion of the gonadotrope population[258]. Moreover, mice which express a dominant-negative GATA2 mutant show a reduction in gonadotrope numbers[258]. These data suggest that GATA2 is actively involved in gonadotrope cell commitment. However, mice in which *Gata2* was knocked out in gonadotropes and thyrotropes still produced FSH (LH levels were not measured)[276]. This mouse model was generated using the Cre-lox strategy[277-279]. In these mice, the *Gata2* locus contained loxP sites; specifically, the GATA2 DNA-binding domain sequence was flanked by loxP sites (floxed)[276]. These animals were crossed to mice expressing a Cre recombinase transgene under the control of the *Cga* promoter[280]. *Cga* encodes the chorionic gonadotropin alpha, or gonadotropin subunit alpha, and its promoter is active in gonadotropes and thyrotropes in the pituitary gland at ED 11.5[267,271,281]. The mouse cross resulted in animals lacking *Gata2* expression in gonadotropes and thyrotropes (called "*Gata2* pitKO")[276]. Given the decreased FSH production in these *Gata2* pitKO animals, GATA2 does appear to be necessary for gonadotrope function.

However, several caveats should be noted in the examination of *Gata2* pitKO animals. First, Cre-mediated recombination was not specific to gonadotropes within the pituitary gland[280], precluding an assessment of the cell autonomous role(s) of GATA2 in gonadotropes. Second, the Cre-encoding transgene in these animals displayed leaky expression (in ovaries and cardiac and skeletal muscle, and some expression in testes and brain)[280,282], indicating that *Gata2* deletion may have occurred outside of the pituitary gland. Third, only males were analyzed[276], and it is unclear if *Gata2* pitKO females also displayed impaired FSH production. To mitigate these caveats and to better characterize the role of GATA2, I developed a mouse model in which *Gata2* was specifically deleted in gonadotropes. This was achieved using mice that specifically express Cre recombinase in this cell population, so-called GRIC mice[283]. These mice express Cre recombinase from the endogenous *Gnrhr* locus[283] (see more details in section 1.3.2.1). Crossing these mice to

floxed *Gata2* mice[276] generated gonadotrope-specific *Gata2* conditional knockout (cKO) animals. I describe the phenotype of these animals in Chapter 5 of this thesis and address the role of GATA2 in male and female gonadotrope development and function.

In sum, the transcription factor(s) critical for gonadotrope function and/or mediating gonadotrope differentiation remain to be fully characterized. GATA2 stands as a promising candidate involved in these processes (see Chapter 5).

1.2.2.2 Overview of gonadotropin structure and secretion

Though the precise molecular mechanisms of gonadotrope differentiation are unclear, the functions of this cell population on the other hand have been well-characterized. Gonadotropes are unique in their ability to produce LH and FSH, which are essential for fertility. In this section, I will describe how both gonadotropins are synthesized, packaged, and secreted into the general circulation.

1.2.2.2.1 LH

LH is a dimeric hormone composed of an alpha subunit (CGA) and a beta subunit (LHβ, encoded by *Lhb/LHB*). CGA is expressed and produced in excess, suggesting that LHβ production is the rate-limiting step in the production of the dimeric hormone[284-287]. Following translation in the endoplasmic reticulum, disulfide bridges form between cysteine residues within each subunit (forming characteristic cystine-knot structures)[288,289], and the alpha and beta subunits non-covalently dimerize[290-292]. Then, in the Golgi, each subunit undergoes specific post-translational modifications[287,293,294].

The alpha subunit contains two asparagine (Asn) residues that become N-linked glycosylated, while the LHβ subunit has one Asn residue that gets modified with sulfate/N-acetyl-galactosamine[295-299]. These modifications explain the relatively short half-life of LH (~20 min in the general circulation)[300-302]. Indeed, these modifications enable LH to be recognized by hepatocytes, underlying the rapid clearance of LH from circulation[303]. Moreover, mice that lack the sulfotransferase responsible for this modification on the LHβ subunit show elevated LH levels in circulation (due to decreased clearance)[304].

Following dimerization between CGA and LHβ, LH is stored in small, dense granules within the regulated secretory pathway, as opposed to the constitutive secretory pathway[305-308]. This specific packaging is thought to be caused by a hydrophobic heptapeptide sequence at the C-terminus of the LHβ subunit (Leucine-Serine-Glycine-Leucine-Leucine-Phenylalanine-Leucine)[309,310]. As a consequence, LH-containing vesicles reside in proximity to the plasma membrane of gonadotropes, ready to be released upon GnRH signaling[307,311]. When LHβ is engineered to lack the heptapeptide sequence, LH gets packaged in less dense granules and is released constitutively[312].

1.2.2.2.2 FSH

FSH is composed of the same alpha subunit as LH, non-covalently linked to FSHβ (encoded by *Fshb*/*FSHB*). FSH goes through a similar maturation and packaging process as LH, with some key differences.

First, FSHβ contains two (as opposed to one for LHβ) Asn residues that can be post-translationally modified[296,297]. These residues are modified with galactose/sialic acid (rather than sulfate/N-acetyl-galactosamine on LHβ)[313], which greatly extends the hormone's half-life (3-4 hours)[314]. FSH exists as different glycoforms, with different levels of glycosylation depending on which FSHβ Asn residue is glycosylated. Hypoglycosylated FSH refers to FSH whose β subunit lacks one (or both) Asn glycosylation, and hyperglycosylated FSH refers to FSH whose β subunit is fully glycosylated[315,316]. The different functions of these glycoforms have been well-investigated in humans. Hypoglycosylated human FSH (hFSH) is more bioactive[317,318], but is cleared faster from circulation than hyperglycosylated hFSH[314].

Second, FSHβ does not contain the C-terminal hydrophobic heptapeptide sequence observed in LHβ and, as a consequence, FSH is sorted into the constitutive secretory pathway[312]. As a proof of concept, mice that produce a mutant FSHβ containing the LHβ heptapeptide sequence possess FSH vesicles within the regulated secretory pathway[319].

1.2.2.2.3 GnRH pulses and gonadotropin production and secretion

While LH and FSH are processed differently, both are dependent on GnRH signaling. However, the dynamics of GnRH secretion matter as much as its presence. This was made clear through seminal work done in the late 1970s. Administration of exogenous GnRH as pulses (one per hour) led to the restoration of LH and FSH secretion in rhesus monkeys with hypothalamic lesions[320]. However, continuous infusion of GnRH in these animals led to a loss of both gonadotropins, which could be rescued by pulsatile GnRH administration[320]. This necessity for episodic, rather than constant, GnRH stimulation was confirmed across species[321-325]. Given the relative half-lives of LH and FSH, GnRH pulses stimulate LH pulses whereas FSH levels appear to be relatively stable.

In addition, the frequency of GnRH pulses dictates how gonadotrope cells produce and/or secrete LH and FSH. High pulse frequencies of GnRH (1 pulse every 30 minutes or 1 h) preferentially stimulate *Lhb* expression and LH release, while lower pulse frequencies (1 pulse every 2-4 hours) preferentially stimulate *Fshb* expression and FSH secretion[326-328], at least in rodents and primates (including humans).

1.2.3 The gonads

Following the description of the hypothalamic (section 1.2.1) and hypophyseal (section 1.2.2) functions, I now describe the effects of gonadotropins in the gonads (testes in males, ovaries in females).

1.2.3.1 Testicular physiology

1.2.3.1.1 Actions of LH on Leydig cells

The testis is the main source of androgens[329,330]. In the testis, androgens are produced and secreted by Leydig cells, residing in the interstitial space (Fig. 1.6). Two different Leydig cell populations have been described: fetal Leydig cells (FLCs) and adult Leydic cells (ALCs)[331]. While both populations produce androgens, their function and regulation differ between species.

FLCs in both rodents and humans are necessary for the production of androgens that will be converted to dihydrotestosterone (DHT) via 5α-reductase activity in the skin[331]. This is

essential for masculinization of the external male genitalia[332]. Androgen production by FLCs is dependent on LHR activation in humans[333,334], as demonstrated by the lack of masculinized external genitalia in men with *LHR* inactivating mutations[333]. In contrast, *Lhr* knockout mice have normal masculinization at birth[335], suggesting that FLC activity in mice (and rats) is dependent on other factors, likely originating from other cells in the fetal testis[331].

On the other hand, steroidogenesis in ALCs is dependent on LHR activation, at least in rodents and humans[336,337]. LHR is a G protein-coupled receptor (GPCR) that signals through the $G\alpha_s$ pathway[338,339]. As such, LH binding to its receptor leads to stimulation of adenylyl cyclase, increasing intracellular levels of cyclic adenosine monophosphate (cAMP), and activating protein kinase A (PKA)[340]. PKA then has acute and long-term effects on steroidogenesis.

PKA directly promotes the translocation of cholesterol from the plasma membrane to the outer mitochondrial membrane (OMM) by phosphorylating and activating hormone sensitive lipases that hydrolyze cholesterol esters into free cholesterol[341-343]. At the OMM, cholesterol is imported inside the mitochondrial matrix through the transduceosome[344], a large complex composed of several mitochondrial and cytoplasmic proteins, one of which is steroidogenic acute regulatory protein (STAR, encoded by *Star*/*STAR*)[345]. STAR phosphorylation by PKA is required to activate the transduceosome[346-348]. STAR-mediated cholesterol transport is typically considered the rate-limiting step in steroidogenesis[344,349-351]. In the mitochondria, cholesterol is converted into pregnenolone by cytochrome P450 11A1 (CYP11A1). Pregnenolone is exported out of the mitochondria into the smooth endoplasmic reticulum and undergoes sequential enzymatic reactions to produce progesterone (by 3β-hydroxysteroid dehydrogenase 2, or 3β-HSD2), androstenedione (by CYP17A1), and lastly testosterone (by 17β -HSD3)[352,353]. Testosterone is then either released into the general circulation or is concentrated in the testis by binding to androgen binding protein (ABP)[354,355].

In a parallel pathway, PKA also phosphorylates and activates ryanodine receptors (RyRs) at the surface of the endoplasmic reticulum[356,357]. Upon activation, RyRs act as calcium channels, leading to an increase in intracellular calcium levels. In turn, calcium activates calmodulin kinase I (CamKI), which is necessary for quantitatively normal responsiveness of ALCs to LH[358,359].

Both PKA and CamKI phosphorylate and activate transcription factors, such as cAMP responsive element binding protein (CREB), GATA4, and cJUN[360,361], and increase the expression of others, such as NUR77, NURR1, and NOR1[362]. The coordinated and sequential activities of these transcription factors are necessary for maximal LH-induced steroidogenesis in ALCs[362-365].

1.2.3.1.2 Spermatogenesis and actions of FSH on Sertoli cells

Beyond Leydig cells, the testis is mainly composed of seminiferous tubules, in which spermatogenesis takes place (Fig. 1.6). There, spermatogonial stem cells sequentially differentiate into spermatogonia, spermatocytes (after one round of meiosis, or meiosis I), spermatids (after a second round of meiosis, or meiosis II), and ultimately, spermatozoa[366-369] (Fig. 1.6). These tubules are protected by the blood-testis barrier, which is composed of Sertoli cells (also called nurse cells)[370,371]. Sertoli cells express the FSH receptor (FSHR, encoded by *Fshr*/*FSHR*), a GPCR coupled to $G\alpha_s$[372]. FSH binding to its receptor leads to elevated cAMP levels in Sertoli cells, activation of PKA, and CREB phosphorylation[372-375]. This signaling pathway is critical during neonatal life, as CREB induces the transcription of key factors involved in cell proliferation, such as c-Myc (encoded by *Myc/MYC*) and hypoxia-inducible factor 2α (HIF2α)[376,377]. In turn, HIF2α further upregulates *Myc* expression, along with *Ccnd1* (encoding cyclin D1), which are also required for the proliferation of immature Sertoli cells[377]. Failure of Sertoli cells to proliferate ultimately decreases sperm production, as the total number of Sertoli cells determines the upper limit of spermatogenesis[378,379].

The Sertoli-specific genes that are regulated by FSH beyond the neonatal period are not consistent across studies[380]. Two factors are generally considered to be potently induced by FSH. The first is inhibin B, a member of the TGFβ superfamily, critical to provide negative feedback at the level of the pituitary to inhibit FSH production[381-383]. Another important FSH-target gene may be *Abp* (encoding androgen binding protein, ABP)[355,384]. ABP binds androgens produced by Leydig cells and concentrates them in the seminiferous tubules[355]. Intratesticular testosterone can reach 50 to 100 times the levels found in the general circulation, depending on the species[385-387]. Though such high concentrations promote sperm production, they are not essential, as rats with

70% lower intratesticular testosterone relative to controls show quantitatively and qualitatively normal spermatogenesis[388].

Additionally, the necessity for FSHR signaling in spermatogenesis across species is debatable. FSH- and FSHR-deficient mice are fertile, though oligozoospermic[62,389]. However, male mice in which FSH is lost during adulthood show no impairment in spermatogenesis or testicular function[390]. This indicates that FSH in rodents is required for normal testicular development early postnatally[391], but plays little to no role in adult spermatogenesis. However, men with loss-of-function mutations in *FSHB* and *FSHR* are azoospermic and oligozoospermic, respectively[14,53], indicating a necessity for FSH in spermatogenesis in humans. Monkeys in which only one testis was removed displayed decreased inhibin B and increased FSH levels, leading to hypertrophy and increased spermatogenesis in the remaining testis[392,393]. The same effects were observed in unilaterally orchidectomized men[394]. This suggests that FSH can induce spermatogenesis in adult men and monkeys. However, FSH bioneutralization in adult monkeys led to inconsistent decreases in spermatogenesis[395-398]. Overall, these indicate that FSH is more critical in establishing spermatogenesis during testicular development in humans and non-human primates relative to rodents. Furthermore, FSH is necessary for quantitatively normal spermatogenesis during adulthood in humans and non-human primates, but not in rodents.

In sum, Leydig and Sertoli cells in the testis respond to LH and FSH, respectively, in a coordinated fashion to produce sex steroids and regulate spermatogenesis, the extent to which varies across species.

1.2.3.2 Ovarian physiology

The ovary, just like the testis, is an organ in which germ cell maturation continuously takes place during the reproductive lifespan. However, unlike the testis, the ovary does not contain a stem cell population to renew these gametes[399,400]. This means that females are born with a given number of gametes, and once this reserve is exhausted, it marks the end of the reproductive period (e.g., menopause in women).

During embryonic life, the female germ cells, or oocytes, form clusters called germ cell cysts (or nests) surrounded by supporting somatic cells in the prenatal ovary[401-404]. In mice, these

nests break down within a few days after birth, and each individual oocyte becomes surrounded by granulosa cells, forming primordial follicles[401,405,406] (Fig. 1.2).

The process of folliculogenesis was described in section 1.1.2. As a reminder, the initial stages of folliculogenesis (from primordial to secondary follicles) are gonadotropin-independent. This is well demonstrated by the fact that ovaries from LH, FSH, and GnRH-deficient mice still contain secondary follicles[61,62,166,407]. In this section, I will focus on gonadotropin-dependent follicle growth, and the effects of LH and FSH in the ovary.

Theca cells express the LHR and produce androgens in response to LH signaling[68,408]. As in male Leydig cells, the LH receptor couples to $G\alpha_s$, ultimately activating PKA and inducing transcription of steroidogenic genes, such as *Star*. The principal androgen produced by theca cells is androstenedione, which has weaker activity relative to testosterone[409]. Theca-cell derived androgens stimulate the expression of *Fshr* in granulosa cells[69-71].

Granulosa cell-sensitivity to FSH is critical to allow further follicle growth and maturation. FSHR activation in granulosa cells leads to different outcomes relative to those observed in male Sertoli cells. Mainly, FSH induces the expression of three key granulosa cell transcripts: *Cyp19a1* (encoding aromatase), *Lhr*, and *Ccnd2* (encoding cyclin D2)[410-416]. Aromatase is essential for the conversion of thecal androgens to estrogens[417]. Expression of LHR in mural granulosa cells is critical for their luteinization following the preovulatory LH surge (see section 1.1.2). Cyclin D2 mediates FSH-induced granulosa cell proliferation beyond the secondary follicle stage[418].

However, granulosa cells and Sertoli cells share the ability to produce inhibin B in response to FSH, which negatively feeds back to pituitary gonadotropes to decrease FSH production[419-421]. While the testis only produces inhibin B in most species, the ovary can produce both inhibin A and inhibin B (Fig. 1.3), which both inhibit FSH production[419]. The specific differences between inhibins A and B will be discussed below, in section 1.3.4.

In sum, contrary to males, females show incredible sensitivity to changes in gonadotropin levels, and LH and FSH stimulate theca and granulosa cells, respectively. The coordinated and cooperative actions of these two cell populations within the follicle are essential for proper folliculogenesis, leading to the generation of fertilizable gametes.

1.3 Regulation of gonadotropin production and secretion

Now that I described the physiological roles of the hypothalamus, pituitary gland, and gonads, I will focus on the specific regulation of LH and FSH production by gonadotrope cells. Given the major role of GnRH in regulating both gonadotropins, I will first describe the hypothalamic regulation of GnRH secretion. Throughout, I will emphasize those areas in which research is still needed, some of which are the focus of this thesis (Chapter 2 through Chapter 5).

1.3.1 Kisspeptin and GnRH neurons

Kisspeptin, produced by kisspeptin neurons, is a potent GnRH secretagogue. Recall that GnRH neurons are dispersed in the POA, and likely require an external signal to coordinate the secretion of discrete GnRH pulses. This external signal is hypothesized to be produced by a GnRH pulse generator. Also, at the time of the pre-ovulatory LH surge, estradiol's effect on GnRH release switches from inhibitory to stimulatory. This also suggests the existence of a GnRH surge generator, separate from the pulse generator.

As discussed in section 1.2.1.2, there are two populations of kisspeptin neurons in rodents; one in the AVPV and one in the ARC (Fig. 1.4). The differential role(s) of these two spatially distinct populations, and whether one may act as the GnRH pulse generator and the other as the GnRH surge generator, has been under intensive investigation over the past decade.

1.3.1.1 GnRH pulse generator

Kisspeptin neurons within the ARC project to the nerve terminals of the GnRH neurons (in addition to the cell bodies), near the median eminence[422-424] (Fig. 1.4). Given the widespread distribution of GnRH neurons, this could enable ARC kisspeptin neurons to control GnRH release at that site (Fig. 1.4).

An important characteristic of ARC kisspeptin neurons is that they express neurokinin B (NKB) and dynorphin (Dyn)[205,425-434] (hence why these neurons are now called "KNDy neurons"). Importantly, KNDy neurons also express NKB and Dyn receptors, making these cells responsive to the ligands they produce. Indeed, NKB stimulates[429,432,435-440] and Dyn inhibits[432,440,441] kisspeptin release from KNDy neurons[442]. Given that these cells project to one another[427,428,443],

this provides a mechanism through which KNDy neurons can synchronize their activities, through autocrine/paracrine signaling. Finally, these cells project to GnRH neuron nerve terminals, at the level of the median eminence (Fig. 1.4), indicating that their synchronized activity can cause a GnRH pulse, with a subsequent LH pulse[440]. Importantly, using optogenetics, pulsatile activation of ARC KNDy neurons causes LH pulses, while inhibition of these neurons prevents LH release[444].

Given this role in the generation of GnRH (and LH) pulses, ARC KNDy neurons are described to mediate sex steroids' negative feedback. Indeed, loss of ovarian hormones following ovariectomy causes an increase in KISS1-positive neurons in the ARC, which can be reversed by estradiol injections[445]. Similarly, estradiol and androgens enhanced the inhibitory effects of dynorphin on kisspeptin release[446]. This supports the idea that KNDy neurons are negatively modulated by sex steroids and suggests that increases or reductions in ARC kisspeptin production directly translate into changes in GnRH release.

Overall, these data support the hypothesis that ARC KNDy neurons act as the steroid-sensitive GnRH pulse generator. Of importance, these observations are consistent in both males and females[442,447-449], which is essential as both sexes exhibit GnRH and LH pulses and are subject to steroid-mediated negative feedback.

1.3.1.2 GnRH surge generator

Based on these data and observations, it was intriguing to consider whether the second population of kisspeptin neurons in the POA (specifically in the AVPV in rodents) could act as the GnRH surge generator. Contrary to the pulse generator, this would have to be female-specific, as males are typically incapable of mounting LH surges. Indeed, in rodents, the AVPV (but not the ARC) is sexually dimorphic, with females exhibiting far more *Kiss1*-positive neurons compared to males[194,450]. Furthermore, these kisspeptin neurons are positively regulated by estradiol. As a reminder, the preovulatory GnRH and LH surges are dependent on high estradiol levels in circulation (see Fig. 1.3). Loss of ovarian hormone production (following ovariectomy) causes a decrease in the number of KISS1-positive neurons in the AVPV[445]. Also, Cre-mediated ablation of the estrogen receptor α (ERα, encoded by *Esr1*, which mediates many of estradiol's effects in

cells) specifically in the AVPV leads to dramatically dampened LH surges relative to wild-type animals[451]. These data confirm that estradiol stimulates kisspeptin release in the AVPV.

This description of two separate kisspeptin populations acting as a GnRH pulse and a GnRH surge generator holds true in rodents. Though the presence of an ARC KNDy neuronal population has been confirmed in goats[432], cows[431], and non-human primates[205], the model that ARC kisspeptin neurons act as the GnRH pulse generator has been challenged over the years[425,432,437,452-457]. Also, given that kisspeptin neurons are more spread-out throughout the POA in non-rodent species, it has been debated whether this population acts as the GnRH surge generator[458].

While more research is needed to determine the relative roles of the ARC and POA kisspeptin populations across species, kisspeptin remains a potent GnRH secretagogue, and is necessary for GnRH pulsatility and fertility.

1.3.1.3 Kisspeptin regulation of GnRH secretion

The mechanisms through which kisspeptin activates GnRH neurons have been well described. Kisspeptin binds its receptor, KISS1R, on the GnRH neuron cell surface. KISS1R is a GPCR coupled to $G\alpha_{q/11}$[197,459,460], leading to phospholipase C beta (PLCβ) activation, which cleaves intracellular, membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG)[461]. IP_3 diffuses through the cytoplasm to bind its receptor, IP_3R, on the surface of the endoplasmic reticulum. IP_3R is a calcium channel, which upon activation exports calcium from the endoplasmic reticulum, increasing cytoplasmic calcium concentrations. DAG remains membrane-bound and is necessary to activate protein kinase C (PKC). PKC stimulates a cascade of phosphorylation events, involving the sequential activation of mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K), MAPK kinase (MAP2K), and extracellular signal-regulated kinases 1/2 (ERK1/2)[461,462].

Intracellular calcium mobilization and ERK1/2 activation events are critical for GnRH neuron depolarization and/or GnRH secretion. Indeed, rat hypothalamic cultures treated with calcium, PLC, or MAP2K inhibitors fail to secrete GnRH in response to exogenous kisspeptin[463]. On the other hand, blocking adenylyl cyclase has no effect on kisspeptin-mediated GnRH

release[463], indicating that the $G\alpha_s$-pathway is not required. These data confirm that kisspeptin-induced $G\alpha_{q/11}$ signaling is necessary for GnRH secretion.

1.3.2 GnRH and gonadotrope cells

GnRH binds its receptor, GnRHR, on the gonadotrope cell surface. GnRHR is a GPCR that primarily couples to $G\alpha_{q/11}$ and $G\alpha_s$[464], though coupling to $G\alpha_i$ has been occasionally reported *in vitro* when GnRH is at high concentrations[465].

GnRH concentration and pulse frequency determine the preferential production of LH or FSH. As described in section 1.2.2.2.3, high GnRH pulse frequencies favor *Lhb* expression and LH secretion, while low pulse frequencies favor *Fshb* expression and FSH production. How gonadotrope cells, using a single receptor (GnRHR), decode GnRH pulses has been and remains an enigma. One potential mechanism is that the GnRHR preferentially couples to $G\alpha_{q/11}$ (leading to *Lhb* expression) at high pulse frequencies and to $G\alpha_s$ at low pulse frequencies (leading to *Fshb* expression)[466]. Study of this phenomenon has been made possible by the generation of gonadotrope-like cell lines, namely αT3-1[467] and LβT2[468,469] cells.

1.3.2.1 Models to study gonadotrope function

Both the αT3-1 and LβT2 cell lines will be refered to throughout this thesis. The establishment of both models was instrumental in elucidating the signaling cascades regulating gonadotropin subunit expression. Indeed, prior to their characterization, primary pituitary cultures were the only tool available. αT3-1 and LβT2 cell lines were isolated from murine pituitary tumors in which the simian virus-40 (SV-40) large T antigen was expressed under the control of the human *CGA* promoter (αT3-1 cells)[467] or the rat *Lhb* promoter (LβT2 cells)[468,470]. Of note, both cell lines express *Nr5a1*, *Cga*, and *Gnrhr*, while only LβT2 cells express *Lhb*[467,470]. *Fshb* is not expressed in αT3-1 cells and has low basal *Fshb* expression in LβT2 cells, but can be induced by GnRH and activins in the latter[469,471]. Of note, the LβT2 cells remain the only homologous cell line available to date to study endogenous *Lhb* and *Fshb* expression.

In order to validate and/or extend findings made in αT3-1 and LβT2 cells, several mouse strains have been characterized in the past decade, facilitating the study of gonadotrope function

in vivo[283,472-474]. In this thesis, the so-called GRIC model is used extensively. As briefly described in section 1.2.2.1.2, these knock-in mice were genetically engineered to express Cre recombinase from the *Gnrhr* locus[283,475]. More specifically, these mice produce a bicistronic mRNA that encodes both *Gnrhr* and Cre recombinase. It is well established that Cre activity in the GRIC model is present in male and female gonadotropes, as well as in the male germline (at the round spermatid stage, and beyond)[283,475]. Though the relevance of *Gnrhr* expression in male germ cells is unclear, one way to circumvent Cre activity in these cells is to breed the GRIC allele through females, as they do not exhibit Cre activity in the ovary[283,475].

Overall, the αT3-1 and LβT2 cell lines, along with GRIC mice, have been instrumental in advancing our understanding of gonadotrope function and regulation, and are refered to throughout the rest of this thesis.

1.3.2.2 GnRH regulation of LH

GnRHR activation rapidly causes ERK1/2 phosphorylation through $G\alpha_{q/11}$[476]. In turn, ERK1/2 phosphorylate ETS-like 1 (ELK1), which induces expression of the immediate early gene early-growth response 1 (EGR1)[477,478]. EGR1 then binds the *Lhb* promoter, along with two other transcription factors, SF1 and PITX1[265,479-482] (Fig. 1.7). This triad of transcription factor is critical in gonadotropes for LH production and fertility[263-265,477].

The proximal *Lhb* promoter contains two EGR1, two SF1, and one PITX1 response element, which are required for *Lhb* transcription[251,481,483] (Fig. 1.7). This minimal promoter is also sufficient to induce reporter activity in LβT2 cells in promoter-reporter studies[481,483]. EGR1 is a ubiquitous transcription factor, and *Egr1* global knockout females are infertile due to low *Lhb* mRNA levels and absence of ovulation[477]. Interestingly, *Egr1* global knockout males are not LH-deficient due to compensation by EGR4. *Egr1*/*Egr4* double knockout males produce LH at dramatically reduced levels and are azoospermic[484]. As mentioned in section 1.2.2.1.2, SF1 is critical for normal LH production, but is not essential for GnRH-induced *Lhb* expression *in vivo*[272]. As for PITX1, and its closely related family member PITX2, both are expressed in gonadotropes and can activate the *Lhb* promoter[247,250,485]. Mutation of the PITX binding site in the *Lhb* promoter dramatically decreases reporter activity in response to GnRH in LβT2 cells[483]. Knocking out *Pitx1*

or *Pitx2* in mice leads to decreased *Lhb* expression, though this is coupled with severe hypopituitarism[245,247,250]. As such it is unclear whether PITX1/2 are required for active *Lhb* transcription, play a more general role in pituitary and gonadotrope development, or a combination of both. On the other hand, gonadotrope-specific *Pitx2* knockout mice show no impairment in LH production, suggesting redundancy and/or compensation by PITX1[473]. In sum, these data indicate that *Lhb* expression is regulated by EGR1, SF1, and PITX1.

As discussed in section 1.2.2.2.1, LH is stored in intracellular vesicles close to the plasma membrane[307,311]. Upon calcium signaling generated from $G\alpha_{q/11}$ activation, the contents of these vesicles are released extracellularly[486,487]. Additionally, calcium mediates other functions in the regulation of *Lhb* expression through calcium/calmodulin-dependent kinases (CamK)[488-490]. Pulses of a calcium channel agonist are sufficient to induce *Lhb* in rat pituitary cultures[491]. Moreover, CamK inhibitors block GnRH-induced ERK1/2 phosphorylation, indicating that MAPK activation also requires CamKs[492] (Fig. 1.7). These data indicate that calcium is required for LH release in addition to *Lhb* expression through CamKs.

Overall, *Lhb* induction by GnRH requires activation of the $G\alpha_{q/11}$ pathway, leading to calcium mobilization, ERK1/2 phosphorylation, *Egr1* transcription, and the cooperative activity of EGR1, SF1, and PITX1/2 to induce *Lhb* expression (Fig. 1.7).

1.3.2.3 GnRH regulation of FSH

The absolute necessity for GnRH signaling in FSH production is made clear from the phenotypes of GnRH-deficient (*hpg*) mice[166], *Gnrhr* knockout mice[493], and humans with *GNRH1*/*GNRHR* mutations[19,20]. Serum FSH levels are low to undetectable in all cases. However, the mechanisms through which GnRH stimulates *Fshb* expression and/or FSH secretion remain poorly characterized, especially *in vivo*. More specifically, no clear DNA sequences in the *Fshb* promoter have been directly implicated in GnRH signaling, at least not consistently across different species or between *in vitro* and *in vivo* studies.

Two AP-1 sites in the ovine *Fshb* promoter are essential for GnRH-induction of this promoter *in vitro*[494,495]. GnRH induces the expression of AP-1 family members, such as c-JUN, JUNB, c-FOS, and FOSB[496,497] through p38[498,499], another MAP2K target (Fig. 1.7)[500,501]. These

proteins act as homo- or hetero-dimers at AP-1 sites to activate transcription[502-504]. To test the requirements for these sites *in vivo*, transgenic mice were developed that expressed a luciferase reporter under the control of the ovine *Fshb* promoter[505]. Mutations of the AP-1 sites had no effect on reporter activity *in vivo*, casting doubt about their physiological relevance[505]. It is also important to note that these specific sites are not perfectly conserved in the murine, rat, or human *Fshb*/*FSHB* promoters.

The human *FSHB* promoter contains only one of the two ovine AP-1 sites, and its mutation inhibits GnRH-induced *FSHB* promoter activity in LβT2 cells[497]. Similar inhibition can be seen in the presence of a dominant-negative Fos protein[496]. As for the murine *Fshb* promoter, it contains neither ovine AP-1 site, but has a more proximal AP-1 half-site that is conserved in the human *FSHB* promoter. Mutation of this site impairs GnRH-induced murine *Fshb* and human *FSHB* activation in promoter-reporter assays[496,497]. These data suggest that AP-1 proteins may play a role in *Fshb*/*FSHB* expression following GnRH signaling, but their relevant cis-elements are unclear, particularly *in vivo*.

A known repressor of AP-1 is c-JUN dimerization protein 2 (JDP2), through its heterodimerization with c-JUN[506]. JDP2 and c-JUN form an inhibitory complex on the *Fshb* promoter in LβT2 cells under basal conditions[507]. When the GnRHR is activated, c-FOS can displace JDP2 and form an active AP-1 complex with c-JUN, and induce *Fshb* transcription[507]. Consistent with this idea, *Jdp2*-null female mice display higher circulating FSH levels and increased *Fshb* pituitary expression relative to wild-type females[507]. These data suggest a role for AP-1 proteins in FSH production *in vivo*, at least in mice. However, mice that lack c-Jun specifically in gonadotropes did not display impairments in FSH production[508]. Rather, these animals exhibited lower expression levels of *Gnrhr* along with reduced LH and sex-steroid production[508]. It is unclear if the reduction in LH levels was caused by the loss of c-Jun activity on the *Lhb* promoter or by the decrease in *Gnrhr* expression. Moreover, it is also possible that the lack of effects on FSH production were masked by compensation by other AP-1 proteins[508]. In sum, AP-1 proteins may play a role in *Fshb* expression (Fig. 1.7), though where they bind on the promoter and which proteins they interact with will likely differ across species.

Another pathway through which GnRH regulates *Fshb* expression may involve GnRHR coupling to a different G-protein. As described in 1.3.2.1, GnRHR couples to $G\alpha_{q/11}$ to regulate *Lhb*. It can also couple to $G\alpha_s$, stimulating the adenylyl cyclase-cAMP-PKA pathway. PKA then phosphorylates CREB, which recruits CREB binding protein (CBP) to the rat *Fshb* promoter in LβT2 cells (in the context of a promoter-reporter assay)[496,497,509]. Mechanistically, CBP acts as a histone acetyltransferase (HAT), which adds acetyl groups to lysines on histone N-terminal tails, relaxing the chromatin structure, and allowing transcription factors to access DNA[510]. Interestingly, mutation of the CREB/CBP binding site on the rat *Fshb* promoter abolished preferential *Fshb* promoter activation at low GnRH pulse frequencies[511]. This suggests a role for the $G\alpha_s$-PKA-CREB-CBP pathway in regulating *Fshb* expression, at least in rats, as the CREB/CBP DNA binding site is unique to this species.

Another possible mechanism through which GnRH regulates *Fshb* might be through CamKs. As described in section 1.3.2.1, GnRHR stimulation leads to the activation of CamKs. One of these proteins, CamKI, phosphorylates histone deacetylases (HDACs), leading to their nuclear export in LβT2 cells[512]. HDACs have been described as repressors of transcription, as they counteract HAT activity and cause chromatin compaction[83]. GnRH-induced nuclear export of HDACs could lead to decompaction of the chromatin, allowing recruitment of the transcriptional machinery, inducing *Fshb* expression[512]. This is also demonstrated by the fact that HDAC inhibitors, such as trichostatin A (TSA), can induce *Fshb* expression in both αT3-1 and LβT2 cells[512-514]. These data provide a model through which GnRH can induce *Fshb* expression in a calcium-CamK-HDAC-dependent manner.

Though several pathways have been hypothesized to mediate GnRH-induced *Fshb* expression, it is clear that *Fshb* regulation by GnRH is less understood relative to that of *Lhb*. Particularly, the model in which GnRH prevents HDAC occupancy on the *Fshb* promoter has several caveats and limitations. First, the GnRH treatment paradigms used *in vitro* were not physiologically relevant[512] and did not yield reproducible results across laboratories. Second, the concentrations of TSA used were extremely high[512-514], and TSA is a known cytotoxic agent[515,516]. Third, most of these experiments were conducted in αT3-1 and LβT2 cells[512,513], as opposed to primary pituitary cultures. Fourth, it is unclear how gonadotropes could differentially produce

FSH or LH in response to varying GnRH pulse frequencies, if both *Lhb* and *Fshb* depend on calcium-CamK signaling. Finally, the role (or lack thereof) of HDACs in activin-induced *Fshb* expression has not been described. Therefore, I challenged this model in Chapter 2 of this thesis, in order to rule in or out a role for HDACs in *Fshb* expression in both LβT2 and primary pituitary cells, either following GnRH or activin stimulation.

1.3.3 Sex steroids and gonadotrope cells

Sex steroids clearly play a role in reproduction, as mice lacking progesterone, androgen, and estrogen receptors are sterile[517-520]. However, due to the requirement of these steroids throughout the HPG axis, it is challenging to dissect cell autonomous effects using global knockout models. Cell- and/or tissue-specific knockout of sex steroid receptors enabled the investigation of their role in most organs of the HPG axis.

1.3.3.1 Regulation by estrogens

A role for estradiol in the hypothalamus in regulating GnRH secretion is clear (see section 1.3.1). However, the role for estrogens in direct regulation of gonadotropes is debated. Mice lacking ERα globally show absent estradiol-mediated positive and negative feedback, leading to absent LH surges and elevated basal LH levels, respectively[520-523]. On the other hand, female mice lacking ERα specifically in neurons do not display LH surges, but have normal basal LH levels, indicating that estradiol-mediated negative feedback was unaffected[523]. Collectively, these data suggest that estradiol may act directly in gonadotropes to negatively regulate LH production and/or secretion.

Gonadotrope cells express ERα[524,525]. In one strain of pituitary-specific ERα knockout mice, the females are infertile, but display normal basal LH levels[526]. Though their preovulatory LH production was not directly assessed, failure to generate LH surges may explain the infertility in these animals[526]. However, in a second strain, deletion of ERα in murine pituitaries caused elevated basal LH levels and *Lhb* expression, impaired LH surges, and led to incomplete infertility, with some females displaying subfertility[524]. Both models suggest that estradiol signaling in gonadotropes is required for the LH surge generation, which correlates with data from primary

pituitary cultures, in which estradiol induces *Lhb* expression across species[527-529]. It remains unclear how estradiol inhibits *Lhb* expression, and why the two pituitary ERα-deficient mouse models exhibit different phenotypes.

With respect to FSH, estradiol either inhibits or does not change *Fshb* expression, depending on the species[505,530]. Importantly, though ERα global knockout mice have increased *Fshb* expression[521,531], pituitary-specific ERα knockouts do not[524,526]. This suggests that the inhibitory effects of estradiol on FSH are not directly at the level of gonadotropes, at least in mice.

1.3.3.2 Regulation by androgens

The role androgen signaling in gonadotropes has been well-investigated. One approach has been to inject androgens in mice treated with a GnRHR antagonist. The latter prevents GnRH signaling in gonadotropes, allowing the evaluation of cell autonomous roles of androgens. In this context, androgens negatively regulate *Lhb* and *Cga* expression levels[490], as seen in rat and monkey primary pituitary cultures[495]. Moreover, mice with androgen receptor (AR, encoded by *Ar*) null mutations and humans with androgen insensitivity syndrome secrete elevated levels of LH[532,533]. These data suggest that androgens negatively regulate *Lhb* expression and/or LH production.

Androgens stimulate *Fshb* expression in rats (co-injected with a GnRHR antagonist)[534], in *hpg* mice[535], and in rat primary pituitary cultures[536], and can induce the murine and ovine *Fshb* promoters in LβT2 cells (in promoter-reporter assays)[537,538]. Androgens can also synergize with TGFβ signaling. For example, co-treatment with androgens and activins leads to increased *Fshb* promoter activity relative to either treatment alone in LβT2 cells[537,539,540]. These data suggest that androgens can directly induce *Fshb* expression.

The role for androgen signaling in regulating gonadotrope function *in vivo* became evident from knockout models. Female mice lacking AR in gonadotropes (and thyrotropes) are subfertile, exhibiting lower FSH levels as well as preovulatory LH surges with lower amplitude relative to controls[541]. Basal LH levels are unimpaired. These data confirm the positive role androgen signaling plays in FSH production in female mice, while challenging its role in negatively regulating

LH at the level of the gonadotrope. Interestingly, male mice lacking *Ar* expression in the entire pituitary gland show no changes in FSH or LH production[542].

In sum, androgen signaling through AR positively regulates FSH production, at least in female rodents. Based on *in vivo* data, the role of AR in LH production is less clear, though the receptor may be involved in generating the pre-ovulatory LH surge[541]. It should be noted however that androgen responsive elements (AREs) in the rodent *Fshb* promoter are not perfectly conserved across species, and androgens do not appear to positively regulate *Fshb* expression in human and non-human primates[543-547]. As such, gonadotropin regulation by androgens remains to be fully characterized in humans.

1.3.3.2 Regulation by progestagens

The role for estrogens and androgens has been well investigated in rodent gonadotropes. Though progesterone signaling through the progesterone receptor (PR, encoded by *Pgr*) is critical in the hypothalamus for the generation of the LH surge[522,548-550], only *in vitro* or indirect *in vivo* evidence suggest progesterone action in gonadotropes.

Pgr is expressed in pituitary gonadotropes[267,268,271,551-553], and PR activity is potently induced by the rise in estradiol prior to ovulation[554,555]. At the time of the pre-ovulatory surge, progesterone appears to enhance gonadotrope sensitivity to GnRH[556,557] by mediating GnRH self-priming. This phenomenon is characterized by GnRH enhancing LH production and secretion through its own action[558-560]. GnRH self-priming can be induced by administering progesterone to estradiol-primed mice[549,561] or estradiol-treated primary pituitary cultures[556,562]; this is abolished in *Pgr* global knockout mice[549] and in primary cultures lacking *Pgr* or in which PR is antagonized[556,557,562]. These data suggest that progesterone signaling through PR is required in gonadotropes to amplify and potentiate the preovulatory LH surge.

Second, progesterone has been suggested to positively regulate FSH production. Several binding elements for PR can be found in the *Fshb* promoter, one of which is essential for rat and murine *Fshb* promoter activity in response to steroids in LβT2 cells (in the context of promoter-reporter assays)[538,563]. Similarly, progesterone can induce *Fshb* mRNA expression in rat primary pituitary cultures[564,565]. *In vivo*, prior to the secondary FSH surge, female rodents show high levels

of progesterone (Fig. 1.3), and PR inhibition blocks the increase in FSH on the morning of estrus in rats[566-569], indicating a positive role for PR in mediating the activin-dependent FSH surge.

Overall, the data suggest a role for progesterone and PR in regulating gonadotropin production *in vivo*. However, significant gaps in knowledge remain. First, the requirement for PR in mediating GnRH self-priming has only been described in primary pituitary cultures[556,557,562], in global *Pgr* knockout animals[549], or in animals injected systemically with progesterone[549,561]. Therefore, a gonadotrope autonomous role for PR in mediating GnRH self-priming *in vivo* has not been established. Similarly, PR has been described to positively regulate *Fshb* expression *in vitro*[538,563-565] or in animals injected with a systemic PR antagonist[566-569] (which also blocked activity of the glucocorticoid receptor[570,571]). Last, animals lacking PR in pituitary glands have not been described. As such, in Chapter 3 of this thesis, I (along with Chirine Toufaily) generated animals in which *Pgr* was specifically ablated in gonadotropes, using *Pgr* floxed mice and GRIC animals (which express Cre recombinase specifically in gonadotropes; see section 1.2.2.1.2). This model enabled us to directly assess the role of PR in gonadotropes at the time of the pre-ovulatory LH surge and the secondary FSH surge.

1.3.4 TGFβ superfamily

In sections 1.3.2 and 1.3.3, I described how GnRH and sex steroids regulate gonadotropin production. A third class of factors, which belong to the TGFβ superfamily, specifically regulates FSH production; namely, pituitary-derived activins stimulate *Fshb* expression, while inhibins from the gonads inhibit *Fshb* expression. A description of this pathway is necessary to potentially unveil novel therapeutic strategies to target FSH production in humans.

TGFβ ligands are secreted cytokines that can be divided into different sub-types: activins and inhibins (see section 1.3.4.2 and Fig. 1.8), BMPs (see section 1.3.4.3), TGFβs, AMH, nodals, growth differentiation factors (GDFs), and glial cell-line derived neurotrophic factors (GDNFs).

1.3.4.1 TGF-β signaling pathway

Most TGFβ ligands, including activins and BMPs, signal through heterotetrameric type I and type II serine/threonine kinase receptor complexes[572]. Activins, TGFβs, AMH, nodals, GDFs, and some BMPs (such as BMP6 and 7) first bind two type II receptors on the cell surface, leading to the recruitment and phosphorylation of type I receptors[573-575] (Fig. 1.9). The type I receptors are then phosphorylated by the type II receptors in a serine-rich region termed the GS box[573,576,577]. Other BMPs (such as BMP2 and 4) first bind type I receptors, recruiting type II receptors[575,578,579].

In both scenarios, intracellular signaling requires the formation of a complex composed of two type II and two type I receptors, bound by ligand[572,573]. Once the type I receptors are phosphorylated and activated, they propagate intracellular signaling cascades via serine/threonine phosphorylation on proteins in the homolog of *Drosophila* mothers against decapentaplegic (SMAD) family[572,573,580]. Upon phosphorylation, SMADs dissociate from the receptors, complex with SMAD4 in the cytosol, and accumulate in the nucleus to act as transcription factors and regulate gene expression of target genes[572,573,580].

1.3.4.1.1 Type II and type I receptors

There are five mammalian type II receptors: activin receptor type IIA (ACVR2A, encoded by *Acvr2a*) and IIB (ACVR2B, encoded by *Acvr2b*), BMP receptor type II (BMPR2, encoded by *Bmpr2*), TGFβ receptor type II (TGFBR2, encoded by *Tgfbr2*), and AMH receptor type II (AMHR2, encoded by *Amhr2*)[572,581]. Several TGFβ ligands are promiscuous in their ability to bind type II receptors[582-584]; for example, activins and BMPs can bind ACVR2A, ACVR2B, and BMPR2[585-587], and nodal and GDFs can bind ACVR2A and ACVR2B[588-593] (some GDFs can bind BMPR2[594-596]). On the other hand, TGFβs and AMH are unique in their ability to selectively bind TGFBR2 and AMHR2, respectively[572,597].

There are seven mammalian type I receptors (also known as activin receptor-like kinases or ALKs): activin receptor-like kinase 1 (ALK1, encoded by *Acvrl1*), ALK2 (encoded by *Acvr1a*), ALK3 (encoded by *Bmpr1a*), ALK4 (encoded by *Acvr1b*), ALK5 (encoded by *Tgfbr1*), ALK6 (encoded by *Bmpr1b*), and ALK7 (encoded by *Acvr1c*)[572,598]. TGFβ ligands are subdivided in two groups,

based on their ability to signal through certain type I receptors. Activins, nodal, TGFβs, and certain GDFs bind and signal through ALK4/5/7, while BMPs, AMH, and other GDFs bind and signal through ALK1/2/3/6[572,580,599].

1.3.4.1.2 Canonical cytoplasmic signaling cascade

Activated type I receptors phosphorylate intracellular SMAD proteins. There are three SMAD sub-families: receptor-regulated SMADs (or R-SMADs), consisting of SMADs 1, 2, 3, 5, and 8 (phosphorylated by type I receptors); the common partner SMAD, SMAD4; and the inhibitory SMADs, SMADs 6 and 7[580].

TGFβ ligands that signal through ALK4/5/7 propagate SMAD2/3 signaling, while those that signal through ALK1/2/3/6 induce SMAD1/5/8 signaling[580]. Specific sequences of amino acids in the type I receptor (the L45 loop) and in the R-SMADs (the L3 loop) dictate which R-SMADs are phosphorylated[600-602]. The type I receptor phosphorylates serine residues at the C-terminus of the R-SMADs, within the so-called MH2 domain[603]. This MH2 domain mediates interactions with other SMAD proteins and co-factors[580]. At the N-terminus, R-SMADs contain a MH1 domain, which is necessary for DNA binding[604,605]. The only exception is SMAD2, which has additional amino acids in the MH1 domain that inhibit DNA binding[604,606]. Interestingly, this dogma was recently challenged[607]. SMAD2Δexon3 is a naturally-occurring splice variant that lacks these additional residues and can bind DNA[606].

I-SMADs are target genes of the TGF-β signaling pathway, and they antagonize R-SMAD signaling through a regulatory feedback loop. First, I-SMADs (namely SMAD7) can associate with SMURF1/2[608,609], a ubiquitin ligase which is constitutively trapped in the nucleus[610]. Upon SMAD7/SMURF1/2 interaction, the complex is exported into the cytoplasm, where SMURF2 can target type I receptors for proteasomal degradation[610-612]. Second, I-SMADs can impair the R-SMAD/SMAD4 complex by: 1) binding R-SMADs, preventing their association with SMAD4[613,614], and 2) binding DNA, blocking the interaction between R-SMAD/SMAD4 and DNA[615].

1.3.4.2 Activins and inhibins

Now that I described the general TGFβ signaling cascade, I will focus on TGFβ ligands that modulate FSH production by gonadotrope cells.

Identified as TGF-β ligands, activins rapidly became of interest in the fields of reproductive endocrinology[616,617], as well as embryology[618,619], stem cells[620-622], erythropoiesis[623], neurology[624], energy metabolism[625,626], bone maintenance[627], wound healing[628], and many other physiological systems[629]. Inhibins also belong to the TGF-β superfamily and act as endogenous activin antagonists. Though inhibins have mostly been studied in the context of the HPG axis, their function has been investigated in bone metabolism[627,630], adrenal steroidogenesis and tumorigenesis[631,632], eye development[633,634], and others[635].

1.3.4.2.1 Discovery and purification

It was hypothesized in the early 30's that a water-soluble, non-steroidal gonadal substance inhibited castration-induced pituitary hypertrophy[636]. It was only 40 years later that this idea was revisited, when testicular fluid injected in rats led to decreased FSH, but not LH, levels[637,638]. This was followed by seminal work showing that steroid-deprived bovine and porcine ovarian follicular fluid could also specifically decrease FSH production, without affecting LH[639-642]. Two forms of inhibins, inhibin A and inhibin B, were subsequently purified from porcine and bovine follicular fluid[643-646]. Both ligands share a common α-subunit (inhibin α, encoded by *Inha*) disulfide-linked to one of two unique β-subunits (inhibin βA and inhibin βB, encoded by *Inhba* and *Inhbb*, respectively)[635]. These are not to be confused with the gonadotropin α- and β-subunits. Inhibin α dimerized to inhibin βA yields inhibin A, while dimerization with inhibin βB yields inhibin B (Fig. 1.8).

During the purification of inhibins, certain fractions of porcine follicular fluid were found to stimulate, rather than inhibit, FSH secretion[647-649]. Purification of these ligands revealed that they were homo- or heterodimers of the inhibin β subunits, which were called activins: activin A (βA-βA) and activin AB (βA-βB; Fig. 1.8). Though the existence of gonadal activin B (βB-βB) had been hypothesized[650,651], activin B was only purified later on, first using recombinant technology in mammalian kidney cells[652,653], and eventually using porcine follicular fluid[654].

1.3.4.2.2 Activin regulation of *Fshb* expression

As discussed above, activins can bind the type II receptors ACVR2A, ACVR2B, and BMPR2. Activins bind type I receptors ALK4 and ALK7 (but not ALK5), and therefore induce the SMAD2/3 pathway.

In LβT2 cells, knockdown of *Acvr2a* and *Bmpr2*, but not *Acvr2b*, impaired basal and activin-induced *Fshb* promoter activation[585]. This indicates that activins signal through ACVR2A and BMPR2. A role for ACVR2A was confirmed *in vivo*, as *Acvr2a*-null male and female mice produced lower levels of FSH[655]. Of note, these animals had residual FSH production, perhaps resulting from compensation by another type II receptor. Based on the fact that gonadotropes do not express *Tgfbr2* or *Amhr2*[267,268,271,656], and that ACVR2B is not required for activin signaling *in vitro*[585], BMPR2 was left as the only type II receptor that might mediate activin signaling in the absence of ACVR2A. However, mice in which *Bmpr2* was deleted specifically from gonadotropes showed no impairments in gonadotropin production *in vivo*[657]. These data suggest a potential role for ACVR2B in gonadotropes. To specifically tackle this possibility, I generated gonadotrope-specific *Acvr2a* and *Acvr2b* (alone and in combination) cKO mice in Chapter 5 of my thesis, using the GRIC mouse model. This enabled me to directly assess the relative roles of ACVR2A and ACVR2B in gonadotropes

In terms of the type I receptors, *Fshb* expression is dependent on SMAD3 *in vitro* and *in vivo*[658,659], indicating that ALK4, ALK5, and/or ALK7 are the relevant receptors. Gonadotropes express *Acvr1c* (ALK7) at low levels[267,268], and primary pituitary cultures from *Acvr1c* knockout animals show no impairments in basal or activin-stimulated FSH production[660]. Activins do not signal through ALK5[579,661], which suggests at first glance that ALK4 is required for *Fshb* expression *in vivo*. Given that ALK4-deficient mice are embryonic lethal, animals lacking ALK4 specifically in gonadotropes need to be characterized to investigate the relevance of this receptor *in vivo*.

Regardless of the ligand/receptor complex identity, the intracellular proteins required for FSH production *in vitro* and *in vivo* have been well-characterized (Fig. 1.9). In the nucleus, SMAD3 and SMAD4 recognize a SMAD binding element (GTCT or AGAC) to regulate gene expression[580,662,663]. GTCT or AGAC is a minimal SMAD binding element, and an 8-bp (base pairs) palindromic sequence (GTCTAGAC) is typically required for strong binding of two SMAD

proteins[662,664]. Such a sequence can be found in the mouse *Fshb* promoter and is required for maximal activin induction of the *Fshb* promoter in reporter assays[659,665-668]. However, mutating this site does not completely block activin induction of transcription[665,666,668], and this sequence is unique to the rodent *Fshb* promoter[669], suggesting that other regulatory sites likely mediate SMAD actions.

Given the low-affinity binding of SMADs for sites, as well as the commonality of SMAD elements, SMADs typically require other proteins to carry out their transcriptional functions[580,664,670]. One such transcription factor is forkhead box L2 (FOXL2), which is expressed in gonadotropes and thyrotropes within the pituitary gland[671]. FOXL2 was first described in porcine *Fshb* expression, as the pig promoter contains a unique FOXL2 binding site, which completely abolishes activin responsiveness when mutated[672,673]. When the same site is introduced into the human or murine *FSHB*/*Fshb* promoters, they become more sensitive to activin signaling[672,673]. Such a FOXL2 binding site is also adjacent to a SMAD binding element in the proximal *Fshb* promoter, both of which are near-perfectly conserved across species. These adjacent sequences are essential for activin-sensitivity and activin-mediated *Fshb* promoter activation *in vitro*[673-675].

Through its MH2 domain, SMAD3 can physically interact with FOXL2[673,676-678], and these proteins form a complex along with SMAD4 on the proximal *Fshb* promoter, with FOXL2 and SMAD4 bound to DNA and SMAD3 acting as a linker between the two proteins[674] (Fig. 1.9). The requirement for these factors has also been confirmed *in vivo*, as: 1) *Foxl2*-null animals express low *Fshb* mRNA levels[679]; 2) gonadotrope-specific *Smad4*[680] and *Foxl2*[681] cKO mice are subfertile and have lower serum FSH levels relative to controls; and 3) *Smad4/Foxl2*[680] and *Smad3/Smad4*[658] double cKO (dcKO) mice are sterile and FSH-deficient.

1.3.4.2.3 Other genes regulated by activins in gonadotropes

While *Fshb* is most potently induced by activins (at least in rodents), other genes in gonadotropes can be induced by activin signaling *in vitro*. *Gnrhr* has long been considered to be positively regulated by activins in gonadotropes *in vitro*[682-685]. However, the sequences identified on the murine *Gnrhr* promoter required for this stimulation[685,686] are not conserved across

species[686,687], and activins negatively regulate the ovine *Gnrhr*[688,689]. Moreover, mice with absent *Smad4*[680], *Foxl2*[681], *Smad4/Foxl2*[680], or *Smad3/Smad4*[658] expression in gonadotropes show no impairments in *Gnrhr* mRNA levels. On the contrary, depending on the model, these mice show either no change[680,681] or increases[658,680] in *Gnrhr* expression. Therefore, the regulation of *Gnrhr* by activin signaling *in vitro* is not recapitulated *in vivo*.

Usually described as selective *Fshb* inducers, activins can promote *Lhb* and/or LH secretion *in vitro*, though this is inconsistent across studies. In LβT2 cells, activins can either stimulate[284,690,691] or cause no change in *Lhb* mRNA levels[692]. Moreover, activin-induced *Lhb* expression seems to be murine-specific, as the murine, but not the human, *Lhb*/*LHB* promoter contains SMAD-binding elements adjacent to an EGR1 site[693]. In the context of the murine promoter, SMAD3 and EGR1 can physically interact and enhance *Lhb* expression[693]. However, in the human *LHB* promoter, given the absence of these SMAD-binding elements, SMAD3 acts as an inhibitor of EGR1 function[693]. Introducing SMAD binding elements near the EGR1 site in the human promoter confers activin responsiveness, while mutating them in the murine promoter leads to inhibition by activins[693]. These data suggest that activins may positively regulate *Lhb* expression, at least in a murine cell model.

The notion that activins play a role in LH production *in vivo* is challenged by the fact that gonadotrope-specific *Smad4*/*Foxl2* and *Smad3*/*Smad4* dcKO females have elevated pituitary *Lhb* mRNA and serum LH levels compared to controls[658,680]. However, it should be noted that in these models, males consistently have decreased *Lhb* expression relative to controls[658,680]. It may be that the increased LH production in *Smad4*/*Foxl2* and *Smad3*/*Smad4* dcKO females stems from a decrease in ovarian estradiol production (due to low FSH levels), causing a loss of estradiol-mediated negative feedback to the hypothalamus and/or pituitary gland, leading to increased LH secretion (Fig. 1.1). As for males, it is unclear why *Lhb* expression is decreased, and may stem from the loss of functional interaction between EGR1 and SMAD3 (discussed above)[693] or between androgens and TGFβ signaling[537,539,540] (as described in section 1.3.3.2).

Still, based on *in vitro* and *in vivo* data, activin signaling in gonadotropes is mainly required for FSH production. This is also demonstrated by the fact that the secondary FSH surge in rodents is not accompanied by a rise in LH production (Fig. 1.3).

1.3.4.2.4 Inhibin regulation of gonadotropin production

Inhibins are produced by the gonads and participate in a negative feedback loop to specifically inhibit FSH production by gonadotrope cells[635]. Importantly, inhibin bioneutralization leads to increased production of FSH[694-697], as does deletion of the inhibin α subunit (*Inha*)[698]. Though the primary production sites of inhibins are the gonads, some cell populations within the rodent pituitary gland express *Inha* and *Inhbb*[267,268,271,521,699,700]. This indicates that potential autocrine/paracrine effects of locally produced inhibin in the pituitary should be investigated in future studies[650,700,701].

Mechanistically, inhibins bind to the activin type II receptors, mainly ACVR2A, without recruiting type I receptors (Fig. 1.9)[702]. This binding is mediated by the inhibin β subunit, which is shared between activins and inhibins (se Fig. 1.8)[703,704]. However, the inhibin α subunit is unable to recruit a second type II receptor. Therefore, inhibins act as endogenous antagonists by binding type II receptors without forming the heterotetrameric type I/type II receptor complex. It is important to note that inhibin binding does not cause intracellular signaling, and it decreases FSH production by competing with activins for the type II receptors[635,705].

Importantly, the affinity of inhibins for ACVR2A is ~10-fold lower than that of activins[706], suggesting a poor inhibitory capacity. Yet, primary pituitary cultures co-treated with equimolar amounts of inhibin and activin show complete inhibition of the activin signaling cascade[707-710]. This suggests the existence of another mechanism through which inhibins can effectively antagonize activins. Indeed, it is now well-established that inhibins require the presence of a co-receptor, namely the TGFβ type III receptor (TGFBR3 or betaglycan, encoded by *Tgfbr3*; see Fig. 1.9)[711,712]. The presence of TGFBR3 on the cell surface greatly enhances the affinity of inhibins for the activin type II receptor, and this ternary complex is resistant to disruption by activins[711].

In accordance with this model, gonadotrope-specific *Tgfbr3* cKO female mice displayed enhanced fertility[710], presumably caused by increased FSH production. However, serum FSH levels in these females were comparable between control and cKO animals at all stages of the estrous cycle[710]. This may have been due to more subtle changes in FSH production that could only be captured by more frequent blood sampling. It may also have been caused by a change in the relative proportion of hypo- and hyperglycosylated FSH (see section 1.2.2.2.2), which could

not be captured by standard FSH measurements. Importantly, in pituitary cultures, the loss of TGFBR3 impaired inhibin A, but not inhibin B, antagonism[710]. These data suggest that inhibin B utilizes another co-receptor to antagonize activin signaling[710,713]. This idea is reinforced by the observation that inhibin B is more potent in repressing FSH production than is inhibin A, but has a lower affinity for TGFBR3 than inhibin A[714].

In summary, inhibins act as endogenous antagonists of activin signaling. Though TGFBR3 promotes inhibin A for binding to ACVR2A, the co-receptor of inhibin B remains to be identified.

1.3.4.3 Bone morphogenetic proteins

Though activin signaling has been extensively studied in gonadotrope cells, other TGFβ ligands, namely BMPs, have also been implicated in FSH regulation[715]. As their name suggests, BMPs were initially characterized in the context of bone and cartilage formation[716]. As described in section 1.3.4.1.1, BMPs signal through BMPR2 and ACVR2A (and ACVR2B, to a lesser extent)[585,586], and ALK1, ALK2, ALK3, and/or ALK6[572,580,581,599]. As such, BMPs activate the SMAD1/5/8, or BMP, pathway[572,580,581,599]. Given the restricted expression of ALK1 (*Acvrl1*) to endothelial cells, this specific receptor will not be discussed[717].

As mentioned in section 1.3.4.2, *Acvr2a*-null animals produced lower FSH levels relative to controls[655]. Given that BMP7 (as well as BMP2 and BMP6) can bind ACVR2A, it may be that the decrease in FSH production in these animals stemmed from impaired BMP signaling. Notably, a BMP7-bioneutralizing antibody inhibited reporter activity in primary pituitary cultures from transgenic mice expressing an ovine *Fshb* promoter-reporter construct[649]. Similarly, this antibody also suppressed endogenous FSH production in murine, rat, and ovine pituitary cultures. These data suggest that BMP7 is produced within the pituitary gland[718,719], and is required for basal FSH production in different species, at least *in vitro*. Moreover BMP2 (which can bind ACVR2A[720]) stimulated the murine, porcine, and ovine *Fshb* promoters (in the context of promoter-reporter assays)[718], as well as endogenous *Fshb* expression in LβT2 cells[718,721,722]. Overall, these data suggest that BMP signaling can stimulate *Fshb* expression, at least *in vitro*.

However, the role for these ligands in FSH regulation has not been investigated as intensively as activins *in vivo*. Fertility and serum FSH levels were normal in *Bmpr1a* cKO mice,

which lack ALK3, the preferred type I receptor for BMP2, in gonadotropes[723]. The absence of a phenotype in these animals may be due to BMP7 signaling through ALK2, but litter sizes were normal in gonadotrope-specific *Bmpr1a* and *Acvr1a* (ALK2) dcKO animals[723]. It should be noted that, although BMP7 can also signal through ALK6, this receptor has low expression levels in the pituitary[267,724], making compensation by ALK6 unlikely.

Overall, these data suggest that BMPs are unlikely to regulate endogenous *Fshb* expression and FSH production *in vivo*, at least in mice.

1.4 Rationale for the thesis

In order to define and establish novel therapeutic strategies to treat infertility, we require a better understanding of the pathways controlling FSH and LH production. While LH is dependent on GnRH signaling, FSH requires both GnRH and TGFβ signaling[63,166,390,407,535,658,680]. Moreover, both gonadotropins can also be modulated by sex steroids. The goal of this thesis is to better understand how each of these pathways induces the expression of gonadotropin subunits, in order to potentially identify novel therapeutic targets that can be stimulated or inhibited in the context of IVF, or other infertility treatments.

My thesis addresses important unanswered questions in the field of gonadotropin regulation: 1) How does GnRH induce *Fshb* expression?, 2) Is progesterone signaling in gonadotropes required for the pre-ovulatory LH surge and/or FSH production?, 3) What type II receptors in the TGFβ family are required for FSH synthesis *in vivo*?, and 4) Is GATA2 required for *Fshb* expression in gonadotropes?

As described in section 1.3.2.2, the model in which GnRH induces *Fshb* by stimulating the nuclear export of HDACs[512] has several caveats and limitations. This model originated from experiments in cell lines, the GnRH treatment paradigms were not physiological, TSA (a HDAC inhibitor) was used at high concentrations (potentially causing cytotoxicity), and the proposed pathway did not align with other established signaling cascades (namely activin-induced *Fshb* expression, and GnRH-induced *Lhb* expression through $G\alpha_{q/11}$). Based on these caveats, I challenged this model in Chapter 2, using a multi-pronged approach in both LβT2 and primary pituitary cells, using a variety of HDAC inhibitors in the presence or absence of GnRH and/or activin[692].

Next, though the function of the estrogen and androgen receptors has been characterized in gonadotropes[524,526,541], a direct involvement for progesterone and its receptor, PR, in gonadotropes has not been investigated *in vivo*. In Chapter 3, I describe the reproductive phenotype of gonadotrope-specific *Pgr* cKO mice, which allowed me to evaluate the cell autonomous role of PR in gonadotropes. Specifically, I tested existing hypothesese that PR mediates GnRH self-priming and/or contributes to FSH production in gonadotrope cells[725].

FSH secretion is also under the control of TGFβ ligands. The necessity for TGFβ signaling in *Fshb* expression *in vitro* and *in vivo* is clear, and the intracellular cascade they induce in gonadotropes is well-described. However, recent evidence casts questions regarding the identity of the type II receptors that mediate for this signaling pathway *in vivo*[585,655,657]. In Chapter 4, I generated gonadotrope-specific *Acvr2a* and *Acvr2b* cKO mice to directly assess the relative roles of these two receptors *in vivo*.

Finally, how gonadotropes become "gonadotropes" remains unclear, as no single transcription factor driving this cell lineage differentiation has been identified. Moreover, activin signaling is ubiquitous throughout physiological systems, and transcription factors such as SMAD3, SMAD4, and FOXL2 are not restricted to gonadotropes. Therefore, it is unclear which transcription factor(s) provide gonadotropes the unique ability to express *Fshb* in response to activins and other TGFβ ligands. In Chapter 5, I interrogated the role of GATA2[276,726] in this system, by generating gonadotrope-specific *Gata2* cKO mice.

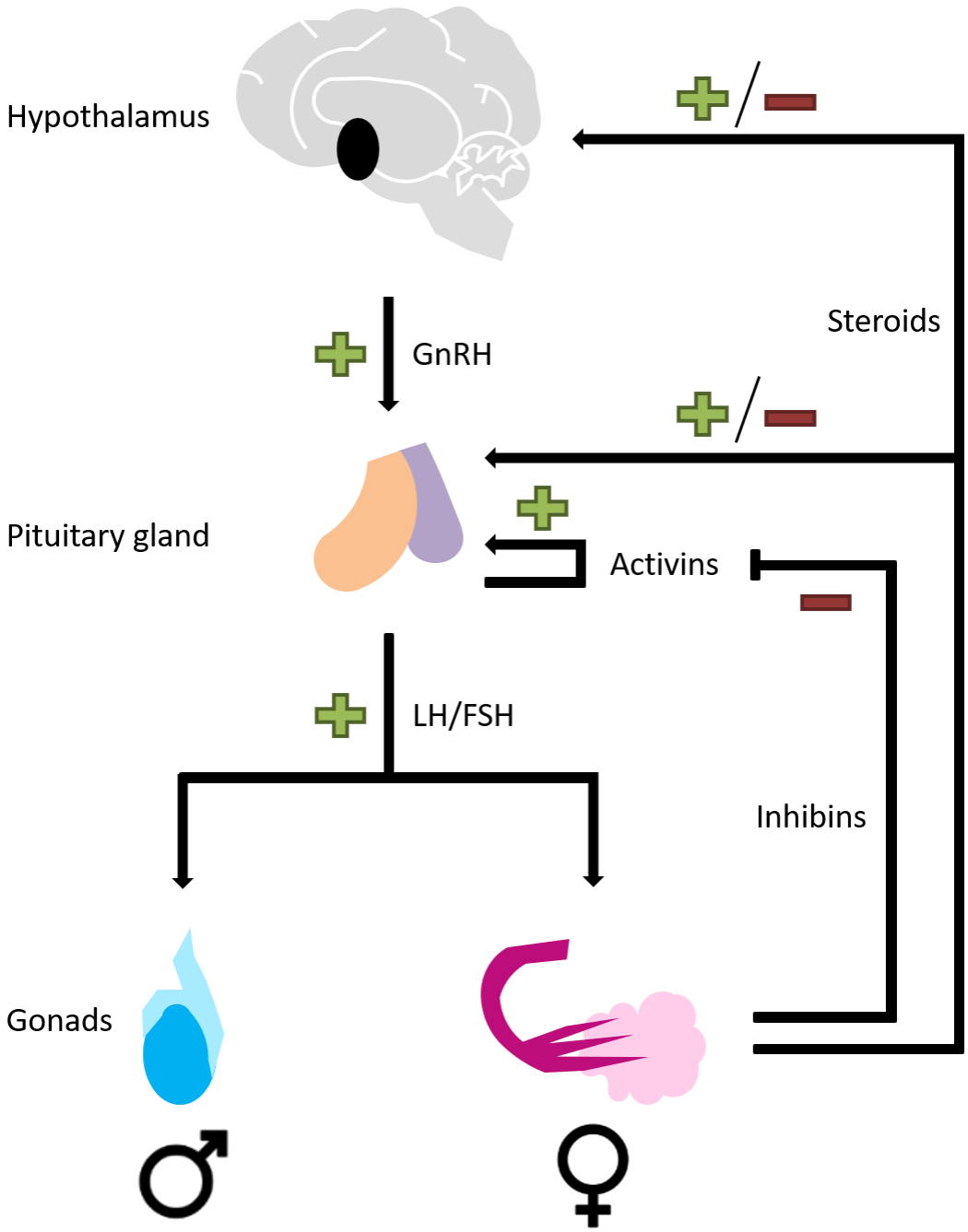
Hypothalamus
Steroids
GnRH
Pituitary gland
Activins
LH/FSH
Inhibins
Gonads

Figure 1.1

Figure 1.1: *Overview of the hypothalamic-pituitary gonadal (HPG) axis.* Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic GnRH neurons and stimulates pituitary gonadotrope cells to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both LH and FSH enter the general circulation to reach the gonads, where they stimulate gametogenesis and steroidogenesis. Sex steroids negatively feedback to the hypothalamus and/or pituitary gland. However, just prior to ovulation, estradiol positively feeds back to the hypothalamus and pituitary gland to generate the LH surge. FSH also stimulates the production of gonadal inhibins, which antagonize the actions of activins in the pituitary gland. Activins are TGFβ ligands that act in an autocrine/paracrine manner to specifically stimulate FSH production. Original artwork by Gabryël Hamel, used and modified with permission.

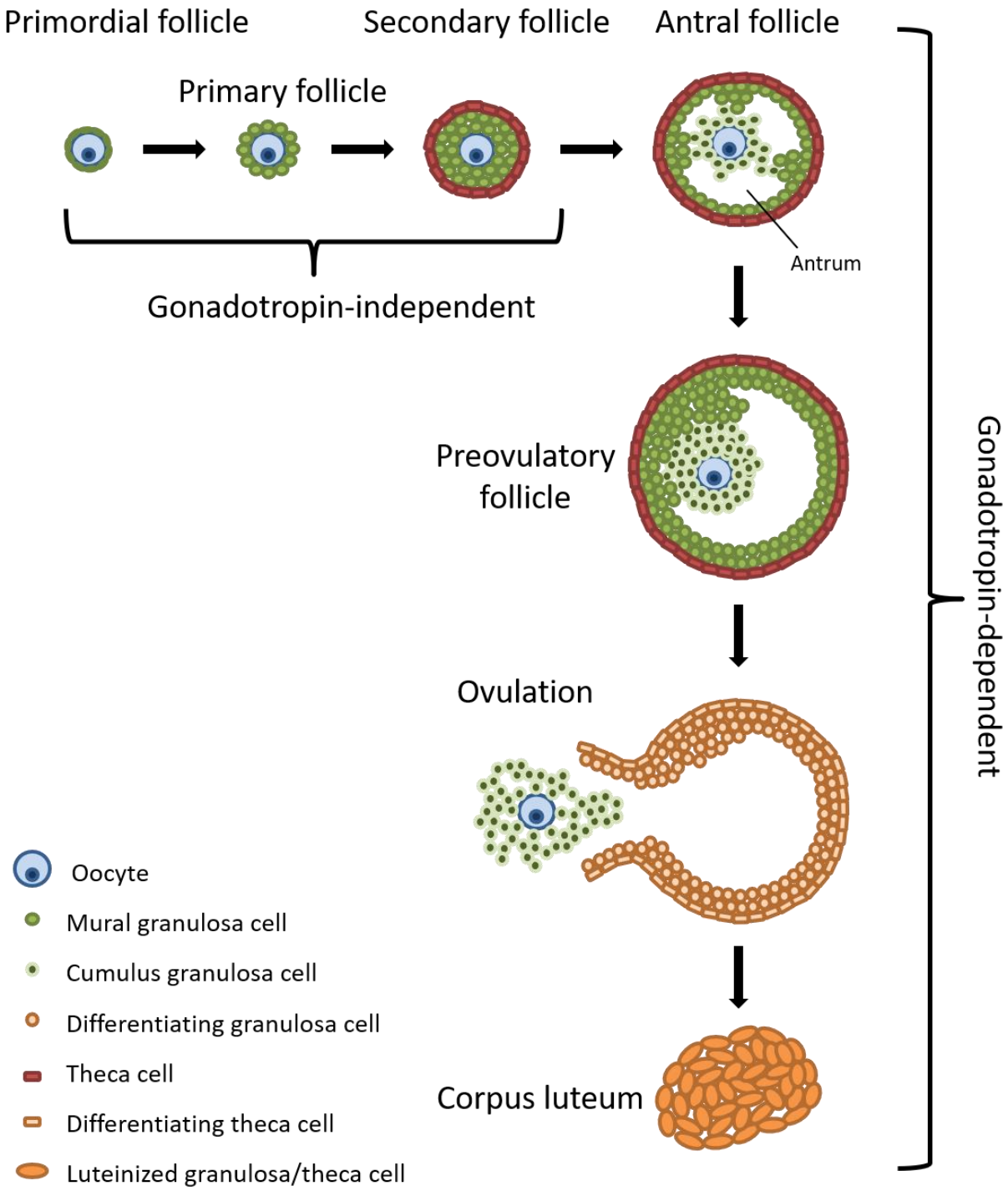
Primordial follicle
Secondary follicle
Antral follicle
Primary follicle
Antrum
Gonadotropin-independent
Preovulatory follicle
Ovulation
Corpus luteum
Gonadotropin-dependent
Oocyte
Mural granulosa cell
Cumulus granulosa cell
Differentiating granulosa cell
Theca cell
Differentiating theca cell
Luteinized granulosa/theca cell

Figure 1.2

Figure 1.2: *Overview of ovarian folliculogenesis*. The follicle is the functional unit of the ovary, consisting of an oocyte (female gamete) surrounded by granulosa cells. Follicles progressively grow from a primordial to a secondary stage, in a gonadotropin-independent manner. At the secondary stage, the follicle recruits theca cells at the outermost layer of somatic cells. Follicular growth becomes gonadotropin-dependent beyond the secondary stage. As granulosa cells proliferate, a fluid-filled antrum separates cumulus cells (surrounding the oocyte) from mural granulosa cells (surrounding the antrum). The LH surge triggers, in part, cumulus cell expansion, luteinization of mural granulosa and theca cells, and follicular rupture, liberating the cumulus-oocyte complex (COC). The remaining mural granulosa and theca cells become the corpus luteum (CL).

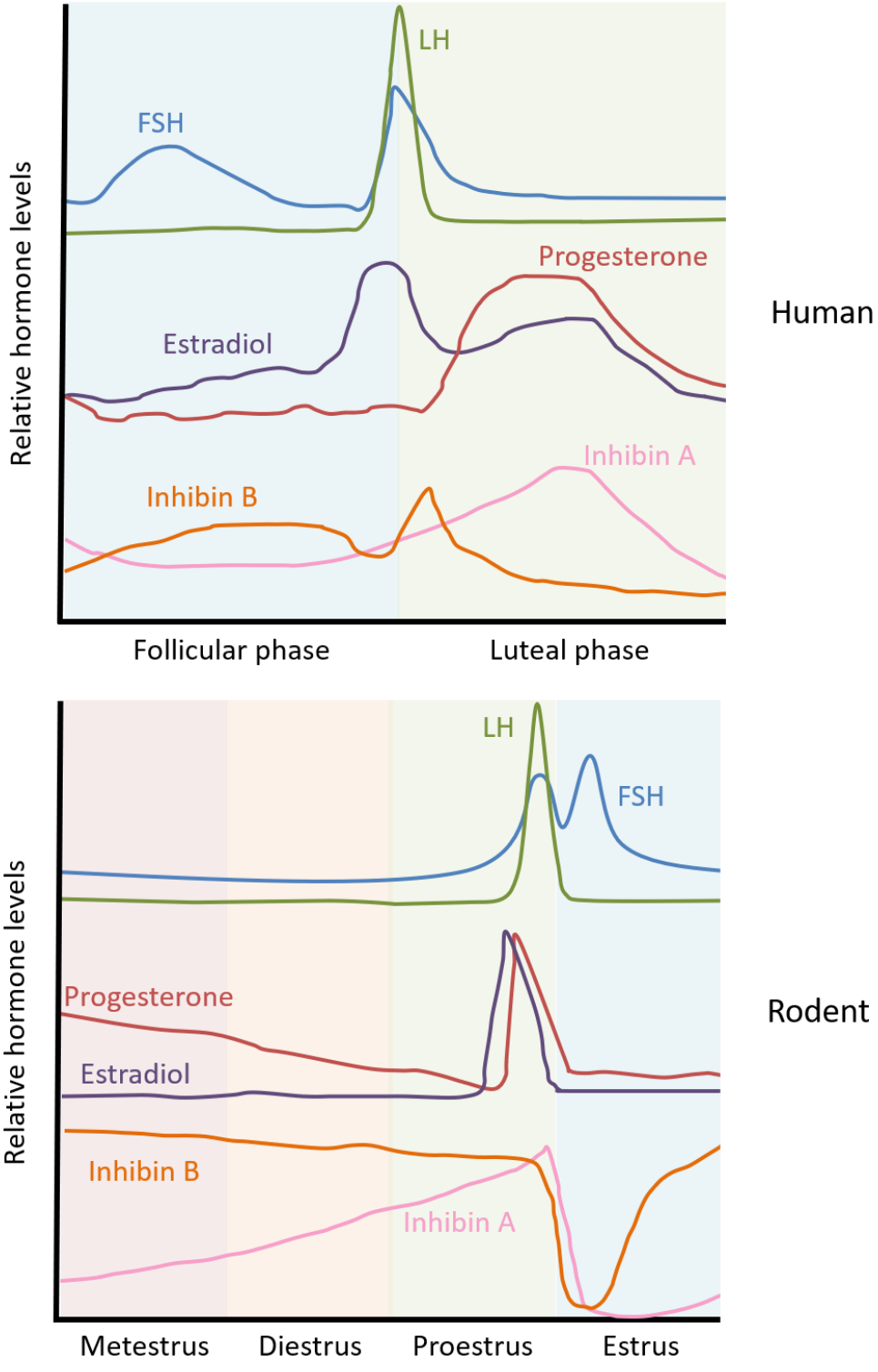
LH
FSH
Progesterone
Estradiol
Inhibin A
Inhibin B
Relative hormone levels
Follicular phase
Luteal phase
Human
LH
FSH
Progesterone
Estradiol
Inhibin B
Inhibin A
Relative hormone levels
Metestrus
Diestrus
Proestrus
Estrus
Rodent

Figure 1.3

Figure 1.3: *Representative profile of hormonal secretion patterns over the course of the human menstrual cycle and rodent estrous cycle*. The top panel represents semi-quantitative hormonal profiles in women throughout the menstrual cycle. The bottom panel depicts qualitative hormonal profiles in female rodents throughout the estrous cycle. Hormones represented are LH (green), FSH (blue), estradiol (purple), progesterone (red), inhibin A (pink), and inhibin B (orange). This figure was inspired by and redrawn from *Bernard D., Li Y., Toufaily C., &* ***Schang G.*** *(2019). Regulation of gonadotropins. In Oxford Research Encyclopedia of Neuroscience. Oxford University Press.*

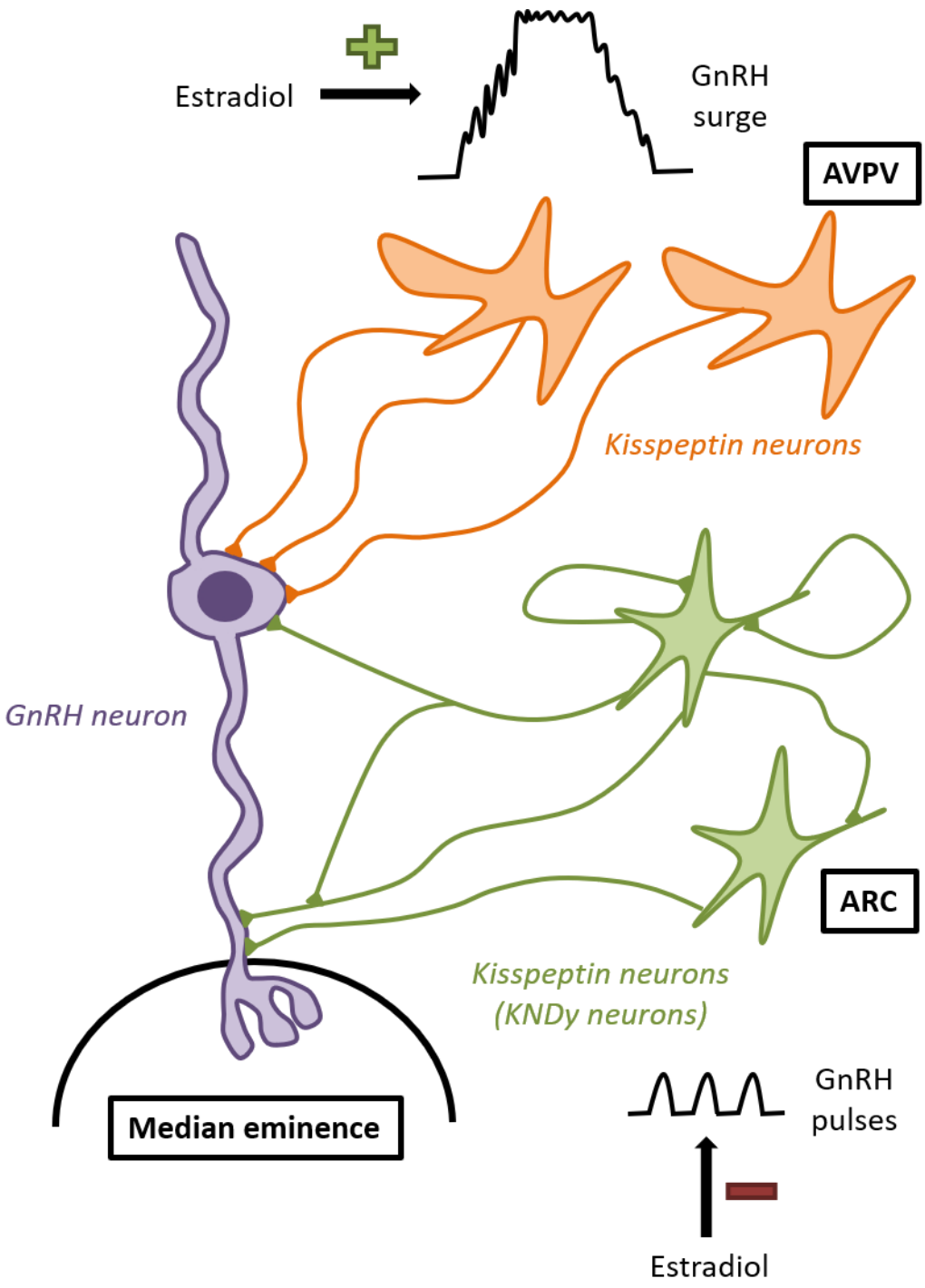
Estradiol
GnRH
surge
AVPV
Kisspeptin neurons
GnRH neuron
ARC
Kisspeptin neurons
(KNDy neurons)
Median eminence
GnRH
pulses
Estradiol

Figure 1.4

Figure 1.4: *Model of negative and positive regulation of kisspeptin neurons by estradiol*. GnRH neurons project their nerve terminals to the median eminence, where they secrete GnRH. Kisspeptin neurons that produce neurokinin B and dynorphin (KNDy neurons) in the arcuate nucleus (ARC) mainly project to the GnRH neurons' nerve terminals. KNDy neurons are inhibited by estradiol, and act as the GnRH pulse generator. Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) project to GnRH cell bodies and are positively regulated by estradiol. Prior to ovulation, when estradiol levels are high, these neurons act as the GnRH surge generator, leading to an LH surge.

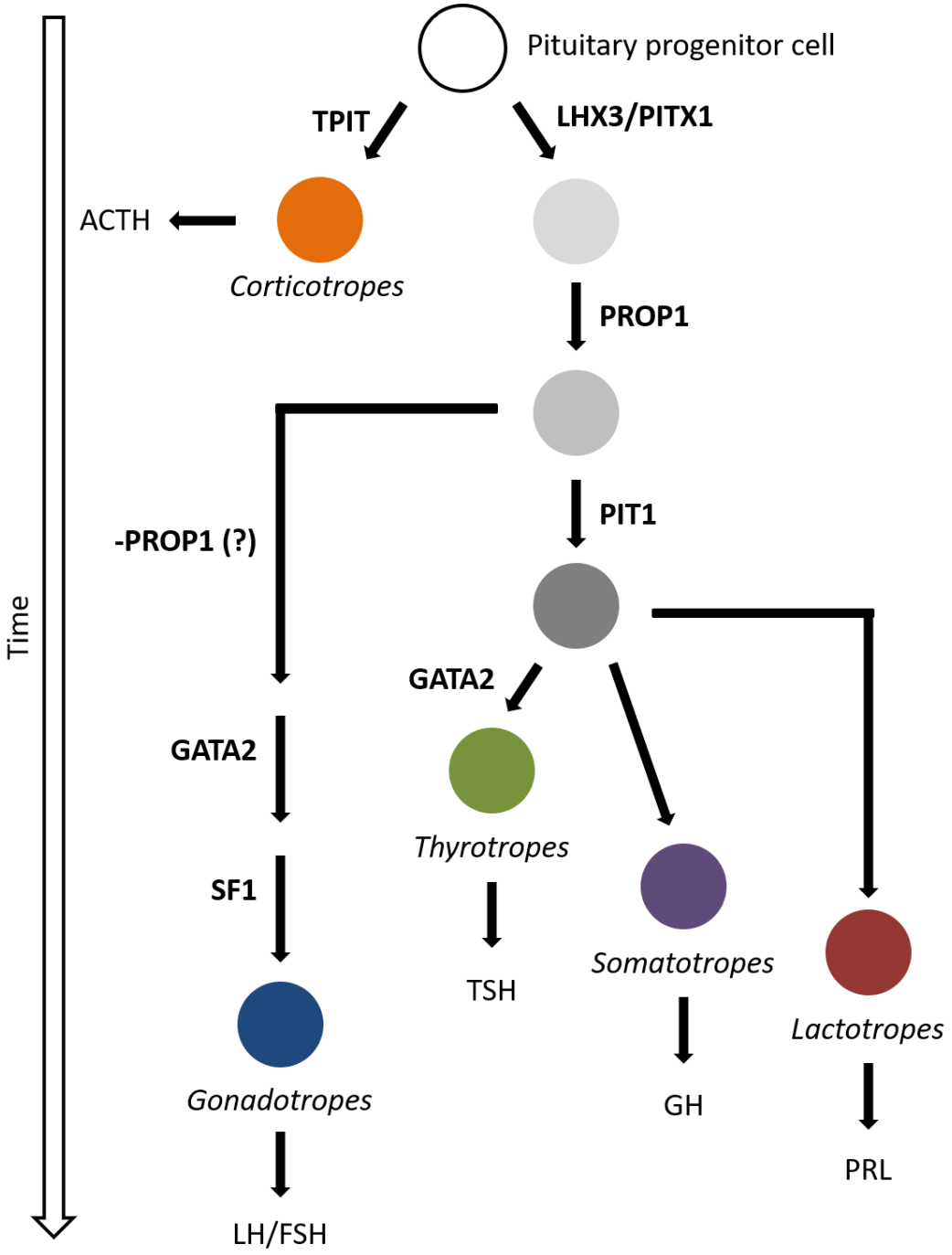
Pituitary progenitor cell
TPIT
LHX3/PITX1
ACTH
Corticotropes
PROP1
PIT1
-PROP1 (?)
GATA2
GATA2
SF1
Thyrotropes
TSH
Somatotropes
GH
Lactotropes
PRL
Gonadotropes
LH/FSH
Time

Figure 1.5

Figure 1.5: *Timeline of pituitary organogenesis and cell lineage commitment*. From a common progenitor, TPIT is responsible for the early differentiation of corticotrope cells that secrete adrenocorticotropic hormone (ACTH). The rest of the anterior pituitary cells express *paired*-like homeodomain transcription factor 1 (PITX1) and LIM homeodomain transcription factor 3 (LHX3), leading to *paired*-like homeobox 1 (PROP1) expression. From there emerges the POU domain, class 1, transcription factor 1 (PIT1) lineage, progressively giving rise to thyrotropes (that also express GATA-binding factor 2 [GATA2]), somatotropes, and lactotropes. Thyrotropes secrete thyroid-stimulating hormone (TSH), somatotropes secrete growth hormone (GH), and lactotropes secrete prolactin (PRL). Gonadotrope cells arise from PROP1+ cells that sequentially lose PROP1 and gain GATA2 and steroidogenic factor 1 (SF1) expression. Gonadotropes, which secrete LH and FSH, are the last cells to terminally differentiate in the anterior pituitary gland.

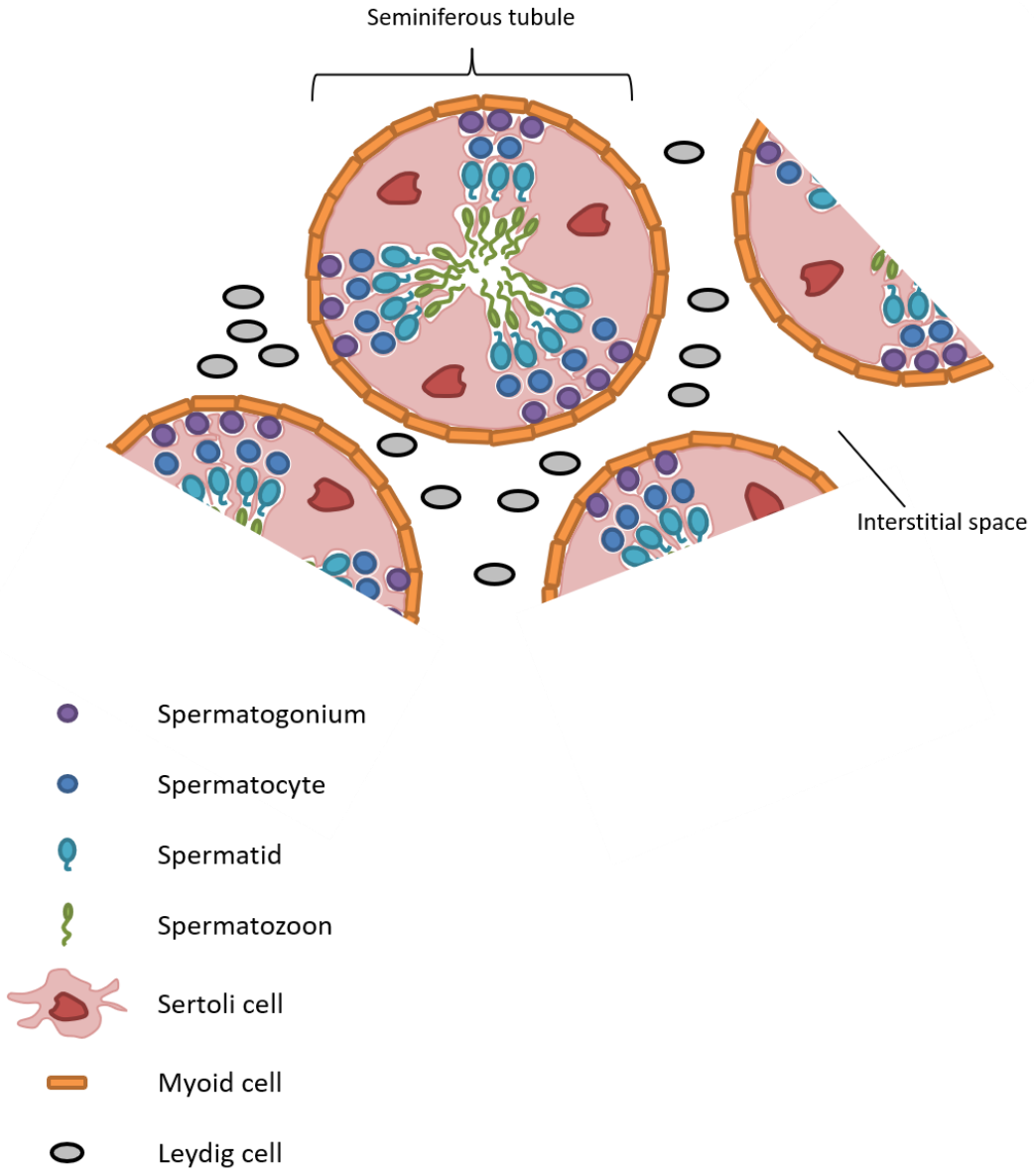
Seminiferous tubule
Interstitial space
Spermatogonium
Spermatocyte
Spermatid
Spermatozoon
Sertoli cell
Myoid cell
Leydig cell

Figure 1.6

Figure 1.6: *Schematic representation of testicular seminiferous tubules and spermatogenesis*. Testes mostly contain seminiferous tubules, which are delimited by peritubular myoid cells. Spermatogenesis occurs within these tubules, where stem cells (spermatogonia) progressively differentiate into spermatocytes, spermatids, and spermatozoa. This process is dependent on Sertoli cells, which act as large nurse cells. Sertoli cells also form a blood-testis barrier, which protects male germ cells (except spermatogonia). Once mature, spermatozoa are released into the lumen of the tubule to migrate out of the testes. Leydig cells are androgen-producing cells and are located in the interstitial space between seminiferous tubules. The androgens produced are necessary for spermatogenesis.

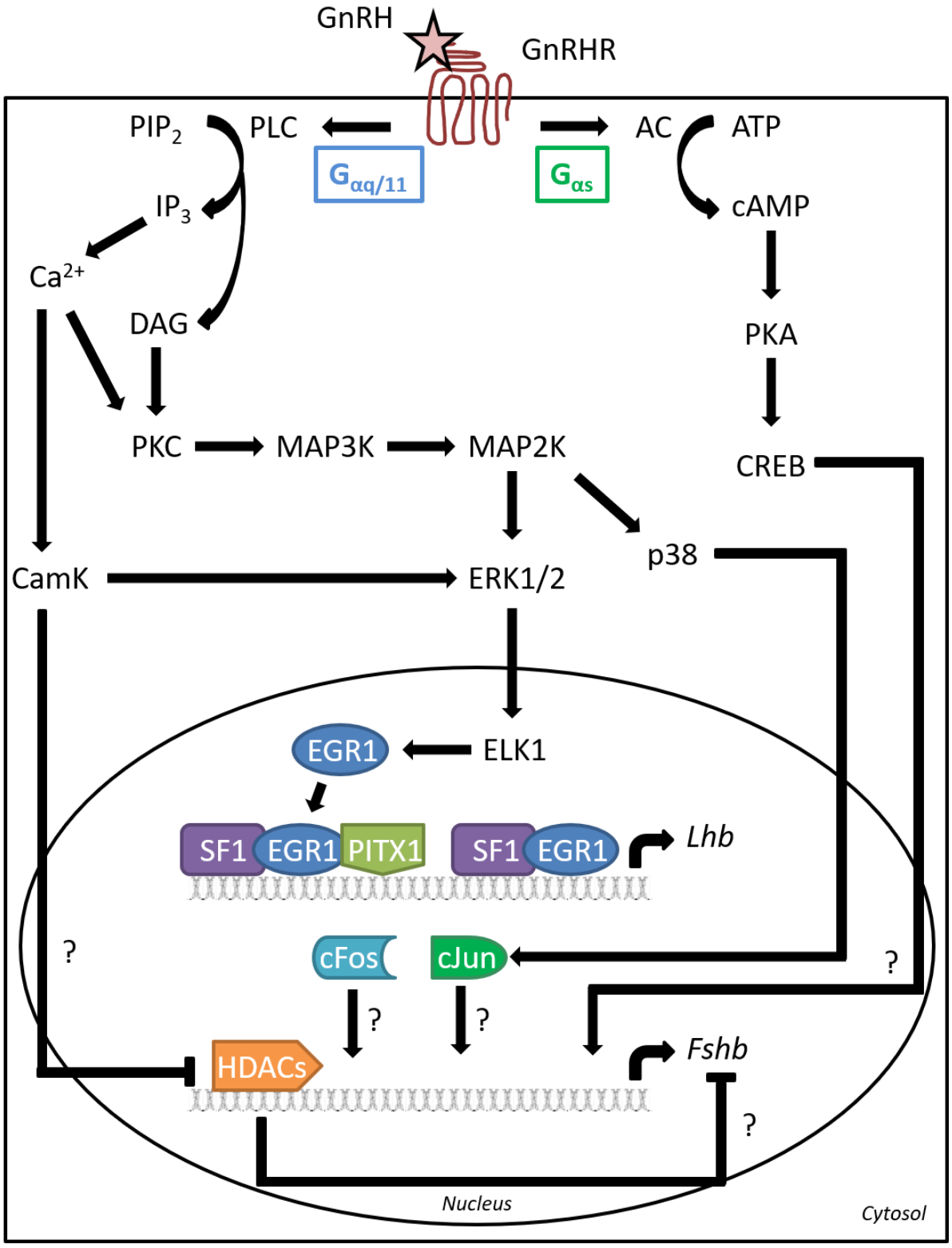
GnRH
GnRHR
PIP2
PLC
Gαq/11
Gαs
AC
ATP
IP3
cAMP
Ca2+
DAG
PKA
PKC
MAP3K
MAP2K
CREB
CamK
ERK1/2
p38
EGR1
ELK1
SF1
EGR1
PITX1
SF1
EGR1
Lhb
?
cFos
cJun
?
?
?
HDACs
Fshb
?
Nucleus
Cytosol

Figure 1.7

Figure 1.7: *Model of GnRH signaling in gonadotrope cells*. Upon binding to the GnRH receptor (GnRHR), two signaling cascades are activated ($G\alpha_{q/11}$ on the left and $G\alpha_s$ on the right). $G\alpha_{q/11}$ activates phospholipase C beta (PLCβ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates calcium mobilization from the endoplasmic reticulum. DAG activates protein kinase C (PKC), which causes the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 through the sequential phosphorylation of mitogen-activated protein kinase (MAPK) kinase kinases (MAP3K) and MAPK kinases (MAP2K). ERK1/2 phosphorylates ETS like-1 (ELK1), which leads to expression of early growth response 1 (EGR1). EGR1 binds the luteinizing hormone beta subunit (*Lhb)* promoter along with SF1 and PITX1 to activate transcription of the gene. p38 may induce activator protein-1 (AP-1) members c-Jun and c-Fos, which may positively regulate follicle-stimulating hormone beta subunit (*Fshb*) expression. The calcium released from the endoplasmic reticulum also activates calmodulin kinases (CamKs), which further promote ERK1/2 activity and phosphorylate histone deacetylases (HDACs) that may repress *Fshb* expression. $G\alpha_s$ activates adenylyl cyclase (AC), which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA), which phosphorylates cAMP response element binding protein (CREB), which may stimulate *Fshb* expression.

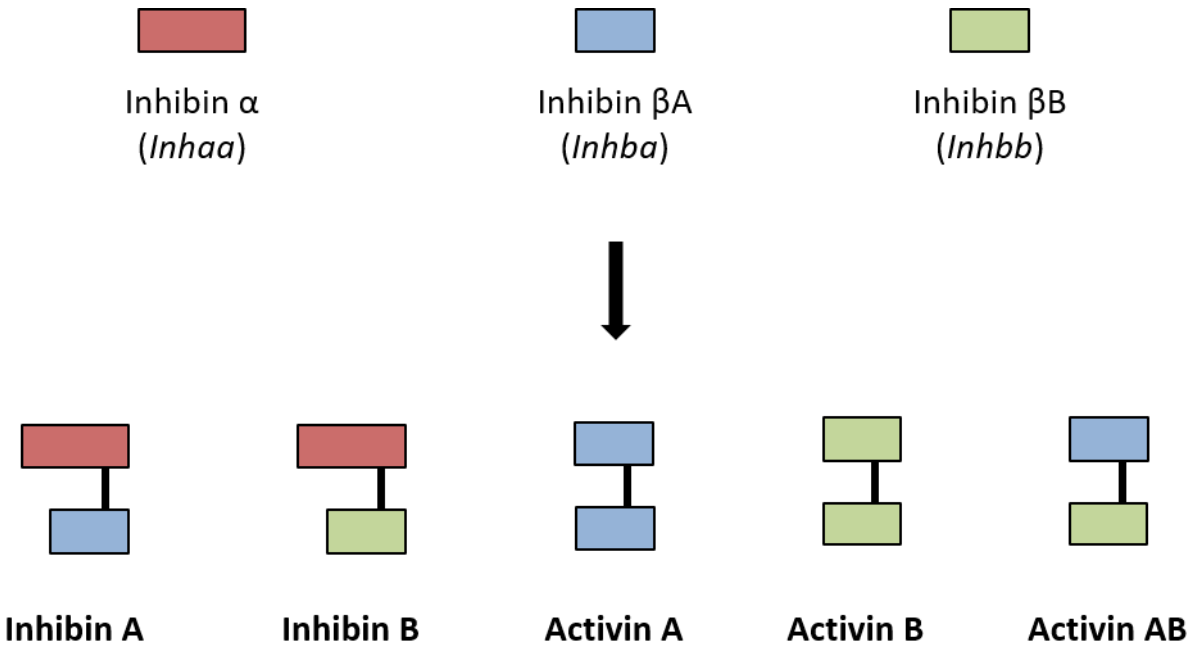
Inhibin α
(*Inhaa*)
Inhibin βA
(*Inhba*)
Inhibin βB
(*Inhbb*)
Inhibin A
Inhibin B
Activin A
Activin B
Activin AB

Figure 1.8

Figure 1.8: *Structure of activins and inhibins*. Inhibins are heterodimers of inhibin α (encoded by *Inha*, shown in red) and inhibin βA (encoded by *Inhba*, shown in blue) or inhibin βB (encoded by *Inhbb*, shown in green). Activins are homo- or heterodimers of inhibin beta subunits. Activin A is βA-βA, activin AB is βA-βB, and activin B is βB-βB. This figure was adapted from *Bernard, D. J., **Schang, G.**, Li, Y., Ongaro, L., & Thompson, T. B. (2018). Activins and Inhibins in Female Reproduction. In M. K. Skinner (Ed.), Encyclopedia of Reproduction. vol. 2, pp. 202–210. Academic Press: Elsevier,* with permission from Elsevier.

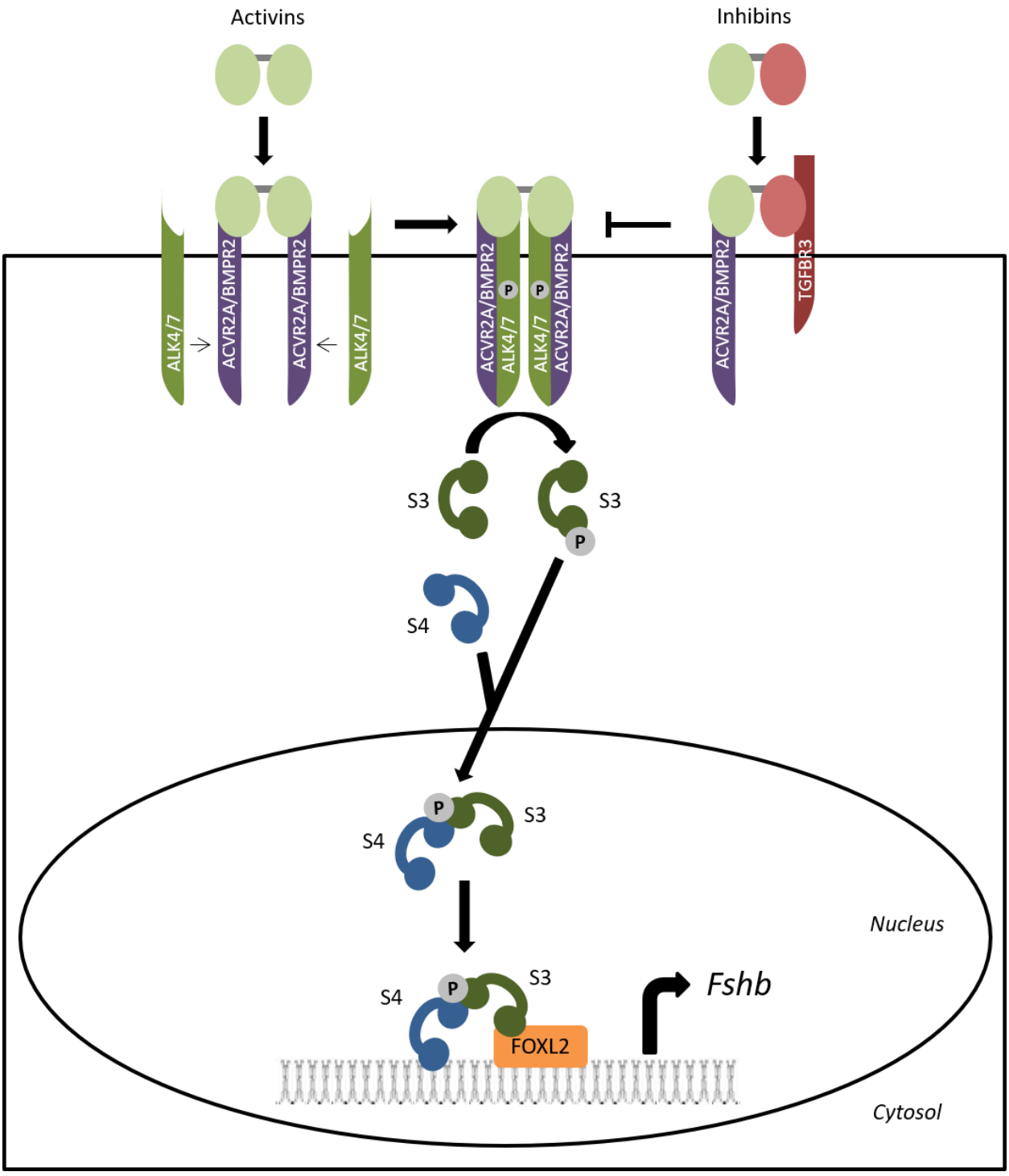
Activins
Inhibins
ALK4/7
ACVR2A/BMPR2
ACVR2A/BMPR2
ALK4/7
ACVR2A/BMPR2
ALK4/7
ALK4/7
ACVR2A/BMPR2
ACVR2A/BMPR2
TGFBR3
P
P
P
S3
S3
S4
P
S3
S4
P
S3
S4
FOXL2
Fshb
Nucleus
Cytosol

Figure 1.9

Figure 1.9: *Model of transforming growth factor beta (TGFβ) signaling in gonadotropes*. Activins bind activin receptor type 2A (ACVR2A) or bone morphogenetic protein receptor type 2 (BMPR2). Upon binding, the complex recruits type I activin receptor-like kinase 4 (ALK4) and/or ALK7, forming a heterotetrameric complex. ALK4/7 are phosphorylated and activated by the type II receptors, and, in turn, phosphorylate intracellular SMAD3. Phospho-SMAD3 complexes with SMAD4, accumulates in the nucleus, and binds the *Fshb* promoter along with forkhead box L2 (FOXL2) to drive transcription. Due to the single β subunit in inhibins (shown in green), they bind one ACVR2A receptor and do not recruit the rest of the heterotetrameric complex. Therefore, inhibins do not induce intracellular signaling. TGFβ receptor type 3 (TGFBR3) enhances inhibin affinity for ACVR2A. This figure was adapted from ***Schang G.****, Ongaro L.**, Ho CC., Zhou X., Bernard DJ. (2019) TGF-β superfamily regulation of follicle-stimulating hormone synthesis by gonadotrope cells: Is there a role for bone morphogenetic proteins? Endocrinology 160(3): 675-683*, with permission from Oxford University Press.

Chapter 2

While the mechanisms underlying GnRH-induced LH production and secretion have been well delineated, those responsible for GnRH stimulation of FSH production remain unresolved. Several mechanisms have been proposed.

Due to the presence of AP-1 binding sites in the human *FSHB* and ovine *Fshb* promoters[495,505], and a half-binding site conserved across species (including rodents)[496], AP-1 transcription factors such as c-Fos, FOSB, c-Jun, and JUNB have been investigated. *In vitro*, these factors appeared to mediate GnRH-induced *Fshb* expression[496], though these observations were not fully corroborated using mouse models[505,507,508]. Another model suggested that GnRH-mediated CamK activation induces phosphorylation of histone deacetylases (HDACs), causing their export from the nucleus[512]. As a consequence, chromatin around the *Fshb* promoter opens, allowing recruitment of transcription factors that activate *Fshb* expression. This was demonstrated by treating αT3-1 and/or LβT2 cells with trichostatin A (TSA)[512-514], an HDAC inhibitor. In both cell lines, TSA led to a robust induction in *Fshb* expression. Though this model provided a mechanism through which GnRH can induce *Fshb* transcription, it had several drawbacks and caveats. First, it was demonstrated solely in cell lines. Second, the GnRH treatment paradigms used were not physiologically relevant (up to 24 h of continuous GnRH). Third, TSA is a potent cytotoxic drug, which may have confounded the results. Therefore, in Chapter 2, I challenge the model of GnRH-induced *Fshb* through HDAC inhibition, not only using primary pituitary cells, but also other HDAC inhibitors that are more specific and less toxic than TSA.

HDAC inhibitors impair *Fshb* subunit expression in murine gonadotrope cells

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Short title: HDAC inhibitors impair Fshb expression *in vitro*

Keywords: FSH, histone deacetylase, activin, GnRH, gonadotropes

ABSTRACT

Fertility is dependent on follicle-stimulating hormone (FSH), a product of gonadotrope cells of the anterior pituitary gland. Hypothalamic gonadotropin-releasing hormone (GnRH) and intra-pituitary activins are regarded as the primary drivers of FSH synthesis and secretion. Both stimulate expression of the FSH beta subunit gene (*Fshb*), although the underlying mechanisms of GnRH action are poorly described relative to those of the activins. There is currently no consensus on how GnRH regulates *Fshb* transcription, as results vary across species and between *in vivo* and *in vitro* approaches. One of the more fully developed models suggests that the murine *Fshb* promoter is tonically repressed by histone deacetylases (HDACs) and that GnRH relieves this repression, at least in immortalized murine gonadotrope-like cells (LβT2 and αT3-1). In contrast, we observed that the class I/II HDAC inhibitor trichostatin A (TSA) robustly inhibited basal, activin A-, and GnRH-induced *Fshb* mRNA expression in LβT2 cells and in primary murine pituitary cultures. Similar results were obtained with the class I specific HDAC inhibitor, entinostat, whereas two class II specific inhibitors, MC1568 and TMP269, had no effects on *Fshb* expression. Collectively, these data suggest that class I HDACs are positive, not negative, regulators of *Fshb* expression *in vitro* and that, contrary to earlier reports, GnRH may not stimulate *Fshb* by inhibiting HDAC-mediated repression of the gene.

INTRODUCTION

Follicle-stimulating hormone (FSH) is an essential regulator of mammalian fertility[53,62]. FSH is composed of two subunits: chorionic gonadotropin alpha (CGA, encoded by the *Cga* gene), which is shared with the other members of the glycoprotein hormone family, and a hormone-specific beta subunit (FSHB, encoded by the *Fshb* gene), which confers receptor binding specificity. According to both human and rodent studies, loss-of-function mutations in *FSHB/Fshb* or in the FSH receptor gene lead to amenorrhea and sterility in females[62,389]. Effects in males are species-specific, as male *Fshb* knockout mice are oligozoospermic but still fertile[62], whereas men with inactivating mutations in the *FSHB* gene are azoospermic[14].

According to current models, *Fshb* expression is stimulated by intra-pituitary activins[687,727] and hypothalamic gonadotropin-releasing hormone (GnRH)[728,729] . Activins belong to the TGFbeta superfamily and specifically regulate FSH production in gonadotropes, without affecting luteinizing hormone (LH)[648,649]. The mechanisms through which activins regulate FSH production have been well-delineated *in vitro* and *in vivo,* at least in the mouse[658,659,673,680,687,730,731]. Activins bind to type II activin receptors on the surface of gonadotrope cells, which leads to the recruitment and phosphorylation of type I activin receptors. Once phosphorylated, these receptors phosphorylate intracellular signaling proteins, SMADs 2 and 3, which then associate with SMAD4 in the cytoplasm, accumulate in the nucleus, and bind the *Fshb* promoter[732,733]. SMADs can also form a complex with the transcription factor forkhead box L2 (FOXL2) to promote *Fshb* transcription[672,674,680].

GnRH is a decapeptide secreted in pulses from the hypothalamus and regulates both FSH and LH production. How GnRH regulates FSH production is presently unresolved. Several transcription factors, including cAMP response element binding protein (CREB), CREB binding protein (CBP), as well as activator protein 1 (AP1) transcription factors have been implicated *in vitro*[496,498,734,735]. *In vivo* studies both support[507,736,737] and refute[505,738] roles for these proteins. One study suggested that histone deacetylases (HDACs) tonically repress the *Fshb* gene and that GnRH relieves this repression by inducing HDAC phosphorylation and nuclear export[512].

There are four classes of HDACs, though only the first two have been implicated in *Fshb* expression. Class I HDACs are mostly nuclear, while class II HDACs can shuttle between the cytoplasmic and nuclear compartments based on their post-translational modifications[739]. HDACs are generally thought to inhibit gene transcription through at least two mechanisms. First, they can deacetylate histone tails, leading to chromatin compaction[740]. Second, HDACs can complex with transcriptional repressors that further prevent binding of transcription initiation factors[741].

In the context of our studies, we attempted to replicate earlier observations that HDACs repress *Fshb* transcription[512]. We were, however, unsuccessful. Rather than stimulating *Fshb* expression, HDAC inhibitors impaired both basal and GnRH-induction of the gene in immortalized gonadotrope-like cells. Given that basal *Fshb* is stimulated by endogenous activin-like signaling[687,742,743], we examined the role of HDACs in activin action. Similar to the effects on GnRH induction, HDAC inhibition impaired activin-induced *Fshb* expression in gonadotrope-like cells as well as in primary murine gonadotrope cells. These results challenge a role for HDAC inhibition in activin- or GnRH-stimulated FSH synthesis.

MATERIALS AND METHODS

Reagents

M199 medium with Hank's salt (M7653), collagenase (C0130), pancreatin (P3292), SB431542 (S4317), GnRH (luteinizing hormone releasing hormone, L8008), and trichostatin A (T8552, CAS 5888-19-16) were obtained from Sigma Aldrich (St. Louis, MO, USA). Entinostat (S1053, CAS 209783-80-2), MC1568 (S1484, CAS 852475-26-4), and TMP299 (S7324, CAS 1314890-29-3) were obtained from SelleckChem (Houston, TX, USA). EvaGreen (ABMMastermix-S) was from Diamed (Mississauga, ON, Canada). RNasin (0000183771), Moloney murine leukemia virus reverse transcriptase (MMLV RT, 0000172807), DNase (0000156360), and random hexamer primers (0000184865) were from Promega Corporation (Madison, WI, USA). TRIzol® reagent (15596026), fetal bovine serum (FBS, 10438026), and horse serum (16050122) were obtained from Life Technologies (Eugene, OR, USA). Deoxynucleotide triphosphates (dNTPs, 800-401-TL),

Hank's Balanced Salt Solution media (HBSS, 311-511-CL), and DMEM (319-005-CL) were from Wisent Inc. (St-Bruno, QC, Canada). Recombinant activin A (338-AC-050) was obtained from R&D Systems (Minneapolis, MN, USA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Total RNA Mini Kit (FA32808-PS) was from Geneaid (New Taipei City, Taiwan). Polyethylenimine (PEI, 23966) was from Polysciences Inc (Warrington, PA).

Cell culture

LβT2 cells[470] were provided by Dr. Pamela Mellon (University of California, San Diego, CA, USA). Cells were maintained in DMEM (4.5 g/L glucose, with L-glutamine and sodium pyruvate) containing 10% (v/v) FBS. Authenticity of the cells was confirmed by activin treatment, which stimulated *Fshb* mRNA expression[732,733]. LβT2 cells are the only immortalized cell line known to produce *Fshb* basally and in response to activins. Cells were seeded in 6-well plates at a density of 2,000,000 cells/well, and in 12-well plates at a density of 1,000,000 cells/well for time-course experiments (between passages 7 and 15). Once they reached 70-80% confluency, cells were serum-starved overnight, and treated in the morning with the indicated compounds in serum-free conditions for 6 h: activin A (1 nmol/L), TSA (45 or 331 nmol/L), entinostat (MS-275, 42.5 µmol/L), MC1568 (2.5 µmol/L) and/or TMP269 (3.9 µmol/L) in serum-free medium. The IC_{50} of the different HDAC inhibitors are: TSA, ~1.8 nmol/L[744]; entinostat, ~1.7 µmol/L[745]; MC1568, ~0.10 µmol/L[746]; and TMP269, ~0.16 µmol/L[747]. The concentrations of 45 nmol/L, 42.5 µmol/L, 2.5 µmol/L, and 3.9 µmol/L for TSA, entinostat, MC1568, and TMP269 are all equal to 25-fold their respective IC_{50}. These concentrations were used to balance between maximal efficacy and off-target effects. The concentration of 331 nmol/L for TSA was sometimes used to allow comparison between our study and previous publications[512,514]. For time-course experiments, cells were starved overnight and RNA was extracted from cells at 2, 6, and 24 h post-treatment (in serum-free conditions). For GnRH induction studies, cells were incubated with GnRH (10 nmol/L) in the presence or absence of TSA (331 nmol/L) for 2 h, followed by a change to medium without GnRH (in the continued presence or absence of TSA) for an additional 2 h.

Pituitaries from 8-week old C57BL/6 male mice were extracted and dispersed as previously described[748]. Between 250,000 and 400,000 cells/well were seeded in 48-well plates.

Cells were cultured for 36 h, after which they were treated with vehicle, TSA (45 or 331 nmol/L), entinostat (42.5 µmol/L), or MC1568 (2.5 µmol/L) in the presence or absence of activin A (1 nmol/L) for 6 h in M199 medium containing 2% (v/v) FBS. Some wells were treated with SB431542 (1 or 10 µmol/L), an activin type I receptor inhibitor[749]. For the entinostat dose-response curve, concentrations of 0, 2, 20, 50, 100, and 200 µmol/L were used (in the presence or absence of activin A). Animal experiments were conducted in accordance with provincial and federal guidelines, and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (Animal Use Protocol #5204).

Protein extraction and western blot

Total protein lysates were extracted as previously described[750]. For cytoplasmic and nuclear protein extraction, cells were washed in cold PBS, resuspended in 0.6 mmol/L EDTA in cold PBS using a cell scraper, and centrifuged at 4°C. The pellet was incubated in cold buffer A (10 mmol/L Tris, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT) containing protease and phosphatase inhibitors for 15 min, following which 10% (v/v) Nonidet P-40 was added. The pellet was vortexed, left on ice for 1 min, and centrifuged at 12,000 x g for 5 s, leaving the cytoplasmic proteins in the supernatant. The remaining pellet was washed three times with additional cold buffer A. After the third wash, cold buffer B (20 mmol/L Tris, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT) containing protease and phosphatase inhibitors was added and the sample was left at 4°C for 1 h on a rotator. This was followed by centrifugation at 4°C at 12,000 x g for 5 min. The supernatant contained the nuclear proteins.

Protein concentration was measured using the Pierce™ BCA protein assay kit (23227, ThermoFisher Scientific, Burlington, ON, Canada), following the manufacturer's instructions. Between 10 and 20 µg of each protein lysate was denatured at 95°C for 10 min prior to being resolved by SDS-PAGE. Proteins were transferred to Protran nitrocellulose membranes (NBA083C001EA; Perkin Elmer, Waltham, MA), blocked in 5% BSA (w/v) in Tris-buffered saline [TBS; 150 mmol/L NaCl, 10 mmol/L Tris (pH 8.0)] containing 0.05% (v/v) Tween 20 (TBST), and incubated in the indicated primary Ab in 5% (w/v) milk in TBST overnight at 4°C with agitation. The rabbit anti-calnexin (1:1000, sc-11397) and mouse anti-acetylated alpha-tubulin (1:5000, sc-

23950) were purchased from Santa Cruz BioTechnology Inc. The mouse anti-nucleoporin (1:3000, 610498) was purchased from BD Transduction Laboratories. The polyclonal rabbit anti-phospho-SMAD2 (1:2000, 3101) was purchased from Cell Signaling. The rabbit polyclonal anti-SMAD2/3 (1:2000, 07-408) and the anti-acetyl-Histone H4 Lys 12 (H4K12; 1:2000, 04-119) were purchased from Millipore. The mouse anti-alpha-tubulin (1:10000, Ab7291) was purchased from Abcam. The next day, the membranes were washed in TBST and subsequently incubated in horseradish peroxidase–conjugated rabbit or mouse secondary Ab (1:5000, goat anti-mouse 170-6516, goat anti-rabbit 170-6515; Bio-Rad Laboratories, Mississauga, ON, Canada) in 5% (w/v) milk in TBST for 1 h at room temperature. Membranes were washed in TBST, enhanced chemiluminescence substrate (ECL; NEL105001 PerkinElmer, Woodbridge, ON, Canada) was applied, and membranes were digitally visualized with an Amersham Imager 600 (G&E Healthcare, Piscataway, NJ). Membranes were stripped in 0.2 M sodium hydroxide solution, washed in TBST, and new primary antibody was applied.

For protein quantification, the Bio-Rad QuantityOne software was used, and phospho-SMAD2 levels were normalized to total SMAD2 levels.

Validation of HDAC inhibitors

LβT2 cells were plated in 6-well plates at a density of 2,000,000 cells/well. The next day, cells were serum-starved overnight, and treated with vehicle, TSA (45 nmol/L), entinostat (42.5 µmol/L), or TMP269 (3.9 µmol/L). Proteins were extracted as described above.

C2C12 cells were provided by Dr. Simon Rousseau (McGill University, Montreal, Canada). Cells were maintained in growth medium (GM; DMEM supplemented with 10% FBS (v/v)). Cells were plated in 24-well plates at a density of 75,000 cells/well. On the next day, cells were treated in GM with vehicle, MC1568 (2.5 µmol/L), or TSA (45 nmol/L) for 24 h. Then, medium was switched to differentiation medium (DM; DMEM supplemented with 2% horse serum (v/v)) containing vehicle, MC1568, or TSA using the same concentrations. An additional control was maintained on GM. After another 24 h of treatment, RNA was harvested, and gene expression studies were performed as described below.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell lines with TRIzol following the manufacturer's guidelines. RNA from primary cells was extracted using the Geneaid Total RNA Mini kit. Between 500 ng (12 well plates) and 1 µg (6 well plates) of RNA were reverse-transcribed for LβT2 cells, 500 ng for C2C12 cells, and 100 ng for primary cells. RNA concentrations were determined by NanoDrop. Reverse transcription was performed as previously described[732], using MMLV reverse transcriptase and random hexamer primers. The obtained cDNA was then used for qPCR analysis on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen™ and primers listed in Table 2.1. mRNA levels of target genes were determined using the $2^{-\Delta\Delta CT}$ method. Ribosomal protein L19 (*Rpl19*) was used for normalization. All primers were validated for efficiency and specificity.

Promoter-reporter assay

For promoter-reporter assays, LβT2 cells were seeded in 48-well plates at a density of 150,000 cells/well. Approximately 24 h after seeding, the cells were transfected with 225 ng/well of CAGA-luciferase reporter plasmid[663] using Lipofectamine 2000 (11668019, ThermoFisher Scientific, Burlington, ON, Canada) overnight. The next day, cells were starved in serum-free medium, following which they wereand treated with vehicle (DMSO), TSA (45 nmol/L), entinostat (42.5 µmol/L), MC1568 (2.5 µmol/L), or TMP269 (3.9 µmol/L), in the presence or absence of activin A (1 nmol/L) for 2, 6, and 24 h.

HEK293 cells were provided by Dr. Terry Hébert (McGill University, Montreal, Canada). Cells were maintained in DMEM supplemented with 5% FBS (v/v), and seeded in 48-well plates at a density of 50,000 cells/well. Approximately 24 h after seeding, cells were transfected with 225 ng/well of -326/+1 porcine *Fshb*-promoter-luciferase reporter plasmid [672] in the presence or absence of 4.15 ng/well of a FOXL2 expression construct[672], using polyethylenimine (PEI) for 2 h in serum-free medium. After this incubation, medium was replaced with fresh culture medium (5% FBS (v/v)). The next day, cells were starved in serum-free medium, following which they wereand treated with vehicle (DMSO) or TSA (45 nmol/L) in the presence or absence of activin A (1 nmol/L) for 6 h.

For all luciferase assays, lysates were prepared and collected as previously described[733]. Luciferase assays were performed on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN). All conditions were performed in duplicate wells in four independent experiments.

Statistical analysis

Data were analyzed by one-way or two-way ANOVA, followed by post-hoc Holm-Sidak multiple comparison test. Statistical analyses were performed using GraphPad Prism 7. Results were considered statistically significant when $p < 0.05$.

RESULTS

HDAC inhibition impairs Fshb expression in LβT2 cells

According to previous reports[512-514], *Fshb* mRNA expression was significantly increased in LβT2 and αT3-1 gonadotrope-like cell lines treated with 20-200 ng/ml (66.1-661 nmol/L) TSA. This was a remarkable result, particularly for αT3-1 cells, which were never before shown to express *Fshb*. However, our efforts to replicate these results using the published assay conditions were unsuccessful, as TSA failed to stimulate *Fshb* mRNA levels in either cell line in our hands (Fig. 2.1 and data not shown). In contrast, GnRH- stimulated *Fshb* expression in LβT2 cells was robustly inhibited by TSA (Fig. 2.1A). GnRH induction of other known targets, *Fos* and *Egr1*, were not impaired (Figs. 2.1B and 2.1C); TSA actually potentiated GnRH-stimulated *Egr1* expression. TSA also suppressed basal *Fshb* mRNA levels (although it was not statistically significant) in these cells (Fig. 2.1A), which are dependent on the actions of an endogenous activin-like ligand[687,742,743]. TSA also significantly decreased *Fshb* mRNA levels following exogenous activin A stimulation (Fig. 2.1D). TSA-mediated inhibition of basal and activin A-stimulated *Fshb* mRNA expression was observed whether cells were treated for 2, 6, or 24 h (Fig. 2.1E). The effects were specific, as basal and activin A-stimulated *Lhb* expression were not significantly affected by TSA at 2 or 6 h (Fig. 2.1F). An impairment was observed in the activin-treated condition at 24 h, which may be a side-effect of prolonged exposure to TSA, which can have cytotoxic effects[751].

A class I, but not class II, HDAC inhibitor recapitulates the effects of TSA

TSA targets a wide range of HDACs. We therefore treated LβT2 cells with class-specific inhibitors to better refine the relevant class(es) of HDACs in gonadotrope-like cells. Entinostat (MS-275), a class I HDAC inhibitor[752], significantly impaired activin A-induced, but not basal, *Fshb* expression (Fig. 2.2A). On the other hand, neither MC1568 (Fig. 2.2A) nor TMP269 (Fig. 2.2B), two class II HDAC inhibitors[746,747], impacted *Fshb* expression.

To confirm the activity of all of the HDAC inhibitors, we examined known markers and targets: acetylated histone 4 lysine 12 (H4K12) for TSA and entinostat[753] and myogenin for MC1568[754]. There are no well-described targets for TMP269, so we assessed its effects on α-tubulin acetylation. Both TSA and entinostat induced acetylation of H4K12 in LβT2 cells (Fig. 2.2C), while TMP269 increased levels of acetylated α-tubulin (Fig. 2.2D). As previously reported, MC1568 (and TSA) blunted the upregulation of myogenin expression (encoded by *Myog*) following differentiation of C2C12 myoblasts (Fig. 2.2E).

HDAC inhibitors impair Fshb expression in primary pituitary cultures

The discrepancy between our results and those of earlier reports[512,514] could have derived from batch to batch differences in LβT2 cells. To address this possibility, we investigated the effects of HDAC inhibitors on *Fshb* expression in murine primary pituitary cell cultures. As we observed in LβT2 cells, basal and activin A-induced *Fshb* mRNA levels were reduced by 95% and 70%, respectively, in the presence of 331 nmol/L TSA compared to control (Fig. 2.3A). In contrast, neither *Lhb* nor *Cga* mRNA levels were affected by TSA (Figs. 2.3B and C). To better compare TSA with the class-specific inhibitors, we also treated primary pituitary cells with 45 nmol/L TSA, instead of 331 nmol/L (see Methods; all inhibitors were used at a concentration equivalent to 25-fold their IC_{50}). The lower concentration of TSA affected the amplitude, but not the directionality nor the significance of its inhibitory effects on *Fshb* (Fig. 2.3A vs Fig. 4A). The class I (entinostat), but not class II HDAC inhibitor (MC1568), suppressed basal and activin A-induced *Fshb* expression in primary cells (Fig. 2.4A). Next, we treated primary cells with 0, 2, 20, 50, 100, and 200 µmol/L of entinostat. Entinostat affected activin A-induced *Fshb* expression at a concentration as low as 2 µmol/L (equivalent to its IC_{50}; Figure 2.4B). Higher concentrations did not affect the amplitude

of this effect. Basal *Fshb* was reduced in a more dose-dependent manner, although the differences were not statistically significant. *Lhb* and *Cga* mRNA levels were not inhibited by TSA, entinostat, or MC1568 (Figs. 2.4C and D). The apparent induction of *Cga* expression by entinostat was not statistically significant (p=0.067).

A consistent effect of TSA and entinostat was to increase the apparent potency of exogenous activin A in both LβT2 and primary cells. That is, the fold-induction by activin A was often increased in the presence of HDAC inhibitors. This effect was principally driven by the decreases in basal *Fshb* expression. It is not clear how to interpret these results, as we have seen similar results with direct antagonists of activin signaling. For example, we treated primary pituitary cells with 1 or 10 µmol/L of SB431542, an activin type I receptor inhibitor (Fig. 2.4E). At 10 µmol/L, both basal and activin A-induced *Fshb* mRNA expression levels were severely impaired. However, at a sub-optimal concentration (1 µmol/L), basal *Fshb* was impaired, while the fold (but not absolute) activin A response was increased from 2.9 to 11.6. At a minimum, these data suggest that the amount of inhibitor required to antagonize endogenous activin-like signaling is less than that needed to inhibit exogenous activin A.

TSA impairs expression of another activin target, Hsd17b1

Results from both immortalized and primary gonadotrope cells indicated that inhibition of class I HDACs impaired activin A-stimulated *Fshb* expression. The results did not indicate, however, whether these effects were gene-specific or reflected a generalized antagonism of activin-like signaling. We therefore examined the effects of TSA on a second activin A-responsive gene in LβT2 cells, 17β-hydroxysteroid dehydrogenase type I (*Hsd17b1*)[755]. Similar to the case with *Fshb*, TSA greatly attenuated basal and activin A-induced *Hsd17b1* expression (Fig. 2.5).

TSA does not impair activin A-induced SMAD signaling

The above results suggested that TSA (and entinostat) somehow antagonized activin action but did not indicate how. A key step in activin signaling is phosphorylation of receptor-regulated SMAD proteins (SMAD2 and SMAD3) by activin type I receptors. Phosphorylated SMADs then partner with SMAD4 and accumulate in the nucleus. To investigate whether or not

TSA impaired this part of the pathway, we first measured levels of phosphorylated SMAD2. However, TSA appeared to enhance, rather than inhibit, activin A induction of SMAD2 phosphorylation in LβT2 cells (Fig. 2.6A). Activin A-stimulated nuclear accumulation of pSMAD2 was also unimpaired by TSA (Fig. 2.6B). These data suggested that TSA did not affect the ability of activin receptors to bind ligand or to activate intracellular signaling.

Activin A-induction of *Fshb* mRNA expression depends on SMAD3 and SMAD4. To determine whether TSA or other HDAC inhibitors impaired SMAD3/4 signaling, we examined their effects on activin A-induction of the SMAD3/4-dependent promoter-reporter CAGA-luc[663] in LβT2 cells. In vehicle-treated conditions (DMSO alone), activin A robustly induced luciferase activity after 2, 6, and 24 h, though statistical significance was only reached at the latter time points (Fig. 2.7). TSA, at 45 nmol/L, did not suppress basal reporter activity, but impaired the activin A response by ~30-50% at 2 h, though not statistically significantly (Fig. 2.7A). This apparent reduction was reversed by 6 h. At 24 h, activin A-stimulated reporter activity was enhanced by TSA by about two-fold. We obtained comparable data when using 331 nmol/L TSA (data not shown). Entinostat showed similar effects to those of TSA: impaired activin stimulation at 2 h, no effect at 6 h, and a robust rebound effect at 24 h, with a ~12-fold increase in activin responsiveness compared to DMSO-treated cells (Fig. 2.7B). The class II-specific inhibitors had no effects at any of the time points investigated (Fig. 2.7C and D). Collectively, these data suggest that any inhibitory effects of HDAC inhibitors on SMAD3/4-signaling are short-lived. If anything, these inhibitors appeared to enhance SMAD3/4 signaling.

TSA does not impair FOXL2-mediated Fshb transcription

Activin-induction of *Fshb* is FOXL2-dependent[672]. We therefore examined whether HDAC inhibition affected FOXL2 activity. We previously reported that a porcine *Fshb*-luciferase reporter was unresponsive to activin A in heterologous cells[672]. However, co-transfection of a FOXL2 expression vector is sufficient to confer activin-sensitivity. We repeated those results here in HEK293 cells (Fig. 2.8). TSA potentiated the effects of FOXL2 in the presence and absence of activin A, suggesting that HDAC inhibition does not impair FOXL2 function.

DISCUSSION

We observed that TSA robustly inhibited basal as well as GnRH- and activin A-stimulated *Fshb* expression. Our results were surprising on at least two counts. First, they contradict those of earlier studies. Second, histone acetylation is usually associated with chromatin de-compaction and promotion of transcription. However, it is now clear that histone deacetylases mediate other functions in addition to histone deacetylation. We explored some of these putative functions in an effort to provide a mechanistic explanation for our results.

We investigated the potential role for non-histone substrate acetylation[756,757]. FOXL2, an essential transcription factor for *Fshb* expression[674,680,681,730], undergoes several post-translational modifications, including acetylation[758]. To our knowledge, the role of FOXL2 acetylation in its transactivation function has not been investigated. However, acetylation of other forkhead proteins can impair their binding to DNA[759]. We therefore asked whether HDAC inhibitors might enhance FOXL2 acetylation and block the protein's binding to the *Fshb* promoter. TSA induced FOXL2 acetylation (data not shown), but only at concentrations that exceeded those needed to inhibit *Fshb* expression. Entinostat did not alter FOXL2 acetylation (data not shown). Finally, TSA did not impair FOXL2-dependent induction of a porcine *Fshb*-luciferase reporter in heterologous cells (Fig. 2.8). We did not investigate FOXL2 recruitment to the *Fshb* promoter in TSA- or entinostat-treated LβT2 cells, as we lack a ChIP-grade antibody for FOXL2. Nonetheless, the available data suggest that the effects of TSA and entinostat are likely to be FOXL2-independent.

A second mechanism by which HDACs are thought to promote transcription is through the control of elongation of paused genes. On the promoters of such genes, negative elongation factor (NELF) interacts with RNA Poll II to prevent the elongation phase from taking place[760]. HDACs inhibit this interaction, releasing the paused state and promoting elongation. As a consequence, HDAC inhibitors can be used to maintain genes in a paused state[760]. It is not yet known whether *Fshb* is a paused gene. To begin to address this question, we inhibited HSP90, a chaperone protein required for the Pol II and NELF interaction[761], using geldanamycin. Assuming that *Fshb* is in a paused state, preventing this interaction should promote elongation and increase

mRNA levels. However, we observed a robust inhibition of *Fshb* expression (data not shown), which, at first blush, might suggest that *Fshb* is not paused. However, SMAD signaling is also dependent on HSP90[762], which confounds a clear interpretation of these data. Thus, whether or not HDAC inhibitors affect *Fshb* expression by impairing transcriptional elongation is unresolved. What is clear, however, is that the HDAC inhibitors did not block SMAD2 phosphorylation (Fig. 2.6) or SMAD3/4-dependent signaling (Fig. 2.7). Therefore, impaired *Fshb* (and *Hsd17b1*) expression appears to be SMAD-independent.

As our data contrast with those of previously published work, we employed several approaches to uncover the potential sources of variation. First, we used different TSA concentrations. However, *Fshb* was inhibited at both 45 and 331 nmol/L, indicating that the directionality of the effect was not concentration-dependent. This is an important consideration as previous studies used between 331 nmol/L and 5 µmol/L[512-514], even though TSA's IC_{50} is in the low nanomolar range (~ 1.8 nmol/L)[744]. Given the high concentrations that we and others used, it is possible that the effects observed stemmed from off-target effects of the drug. Second, we compared different durations of TSA treatment. TSA is a known cell cycle inhibitor[751] and previous studies mostly used 24 h treatments[512-514]. We treated cells for 2, 6, or 24 h, and *Fshb* expression was inhibited at all time points. Third, we compared TSA from two different vendors. Again, both inhibited *Fshb* expression (data not shown). Fourth, we used four different *Fshb* qPCR primer sets, including those used by the groups mentioned above. All revealed a decrease in *Fshb* mRNA levels following TSA treatment (data not shown). Fifth, we compared different HDAC inhibitors, a hydroxamic acid (TSA) and a benzamide (entinostat), and both impaired *Fshb* expression. Moreover, two different class II inhibitors, MC1568 and TMP269 (the activities of which were confirmed), failed to affect *Fshb* expression. This indicates that the inhibitory effects on *Fshb* expression observed in this study are robust and specifically mediated by class I HDACs.

Collectively, our multi-pronged approach indicates that HDACs play a permissive, rather than inhibitory role in *Fshb* expression *in vitro*. Importantly, the results in immortalized cells were corroborated in murine primary pituitary cultures. Thus, these effects were likely not due to batch to batch differences in the cell line and were not specific to immortalized cells. Therefore, our data fail to support a role for HDAC inhibition in GnRH or activin regulation of *Fshb*

expression. In contrast, HDAC inhibitors appear to impair activin stimulation of *Fshb* through a SMAD- and FOXL2-independent, but still uncharacterized mechanism.

DECLARATION OF INTEREST

The authors declare that they have no conflicts of interest.

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AUTHORS CONTRIBUTION

GS and DJB were responsible for the experimental design, data analyses, and manuscript preparation. GS conducted most of the experiments. CT conducted the experiments involving GnRH stimulation. All authors approved the final version of the manuscript.

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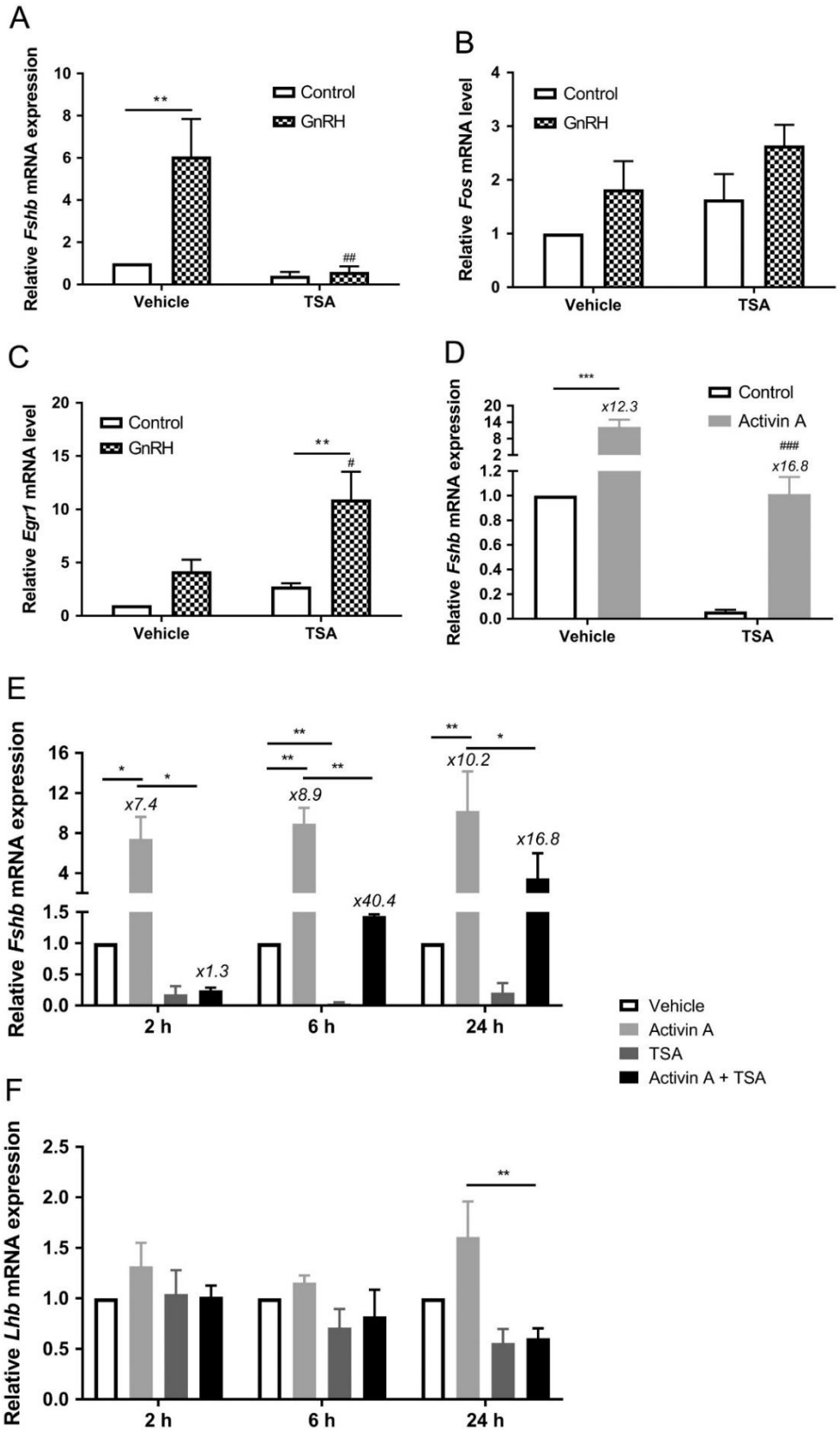
A
Relative Fshb mRNA expression
Control
GnRH
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Vehicle
TSA
B
Relative Fos mRNA level
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Activin A
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2 h
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Relative Lhb mRNA expression
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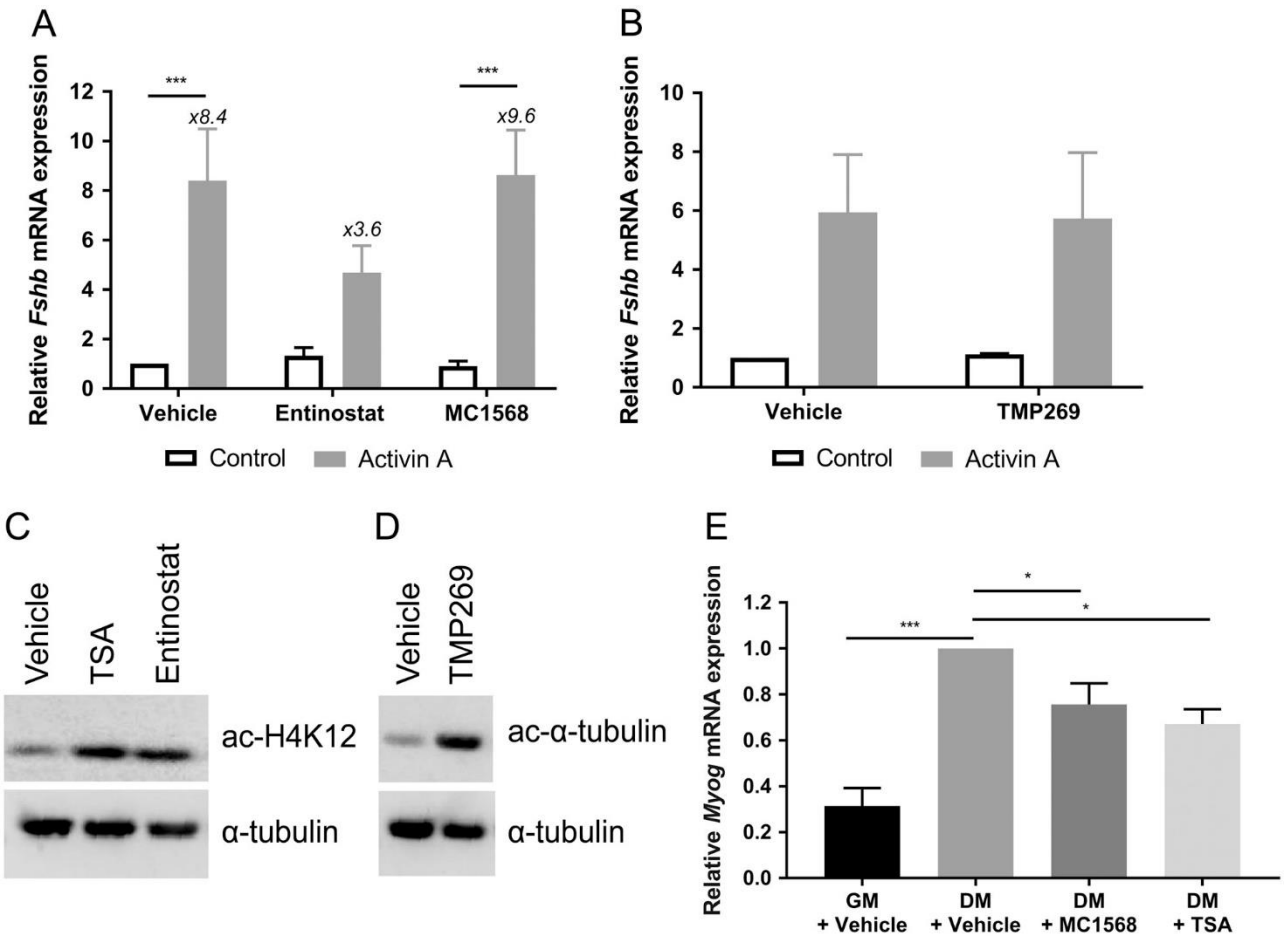
2 h
6 h
24 h

Figure 2.1

Figure 2.1: *HDAC inhibition impairs basal, GnRH-, and activin A-induced Fshb expression*. **A-C)** LβT2 cells were treated for 2 h with GnRH (10 nmol/L) in the presence or absence of TSA (331 nmol/L), followed by incubation in GnRH-free medium for an additional 2 h, in the presence or absence of TSA. N=3 independent experiments. **A)** *Fshb*, **B)** *Fos*, and **C)** *Egr1* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19.* **D)** LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). N=5 independent experiments. *Fshb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19.* **E)** and **F)** Cells were treated as in **D)** and RNA was collected after 2, 6, or 24 h of treatment. N=3 independent experiments. *Fshb* and *Lhb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19.* For each time point, data were normalized to the control condition. In all panels, bars represent mean values (+/- SEM). Activin A's fold induction is indicated above the appropriate bars. Data were analyzed by two-way ANOVAs followed by a post-hoc Holm-Sidak multiple comparison test. *p<0.05, **p<0.01, *** p<0.001 when comparing activin A vs control; ### p<0.001 when comparing TSA vs corresponding vehicle condition.


A
Relative Fshb mRNA expression
***
x8.4
***
x9.6
x3.6
Vehicle
Entinostat
MC1568
Control
Activin A
B
Relative Fshb mRNA expression
Vehicle
TMP269
Control
Activin A
C
Vehicle
TSA
Entinostat
ac-H4K12
α-tubulin
D
Vehicle
TMP269
ac-α-tubulin
α-tubulin
E
Relative Myog mRNA expression
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*
*
GM + Vehicle
DM + Vehicle
DM + MC1568
DM + TSA

Figure 2.2

Figure 2.2: *Class I, but not class II, HDAC inhibition inhibits activin A-induced Fshb mRNA levels*. **A)** LβT2 cells were treated for 6 h with a class I (entinostat) or a class II HDAC inhibitor (MC1568), at a concentration of 42.5 µmol/L and 2.5 µmol/L, respectively (N=4). **B)** LβT2 cells were treated in separate experiments for 6 h with TMP269, a second class II HDAC inhibitor, at a concentration of 3.9 µmol/L (N=2). In **A)** and **B)**, *Fshb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19.* Activin A's fold induction is indicated above the appropriate bars in **A)**. **C)** and **D)** LβT2 cells were treated for 6 h with vehicle (DMSO), TSA (45 nmol/L), entinostat (42.5 µmol/L), or TMP269 (3.9 µmol/L). Total protein lysates were extracted and levels of acetylated H4K12 **(C)**, acetylated α-tubulin **(D)**, and α-tubulin (**C** and **D**) were analyzed by western blot. **E)** C2C12 cells were treated for 24 h with vehicle (DMSO), MC1568 (2.5 µmol/L), or TSA (45 nmol/L) in growth medium (GM). After incubation, cells were grown for another 24 h in GM or differentiation medium (DM) in the presence of vehicle (DMSO), MC1568 (2.5 µmol/L), or TSA (45 nmol/L). *Myog* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*; data were normalized to the vehicle-treated DM condition (N=3). Bars represent mean values (+/- SEM). Data were analyzed by two-way ANOVA (**A** and **B**) or one-way ANOVA **(E)** followed by post-hoc Holm-Sidak multiple comparison test; *$p<0.05$, *** $p<0.001$.

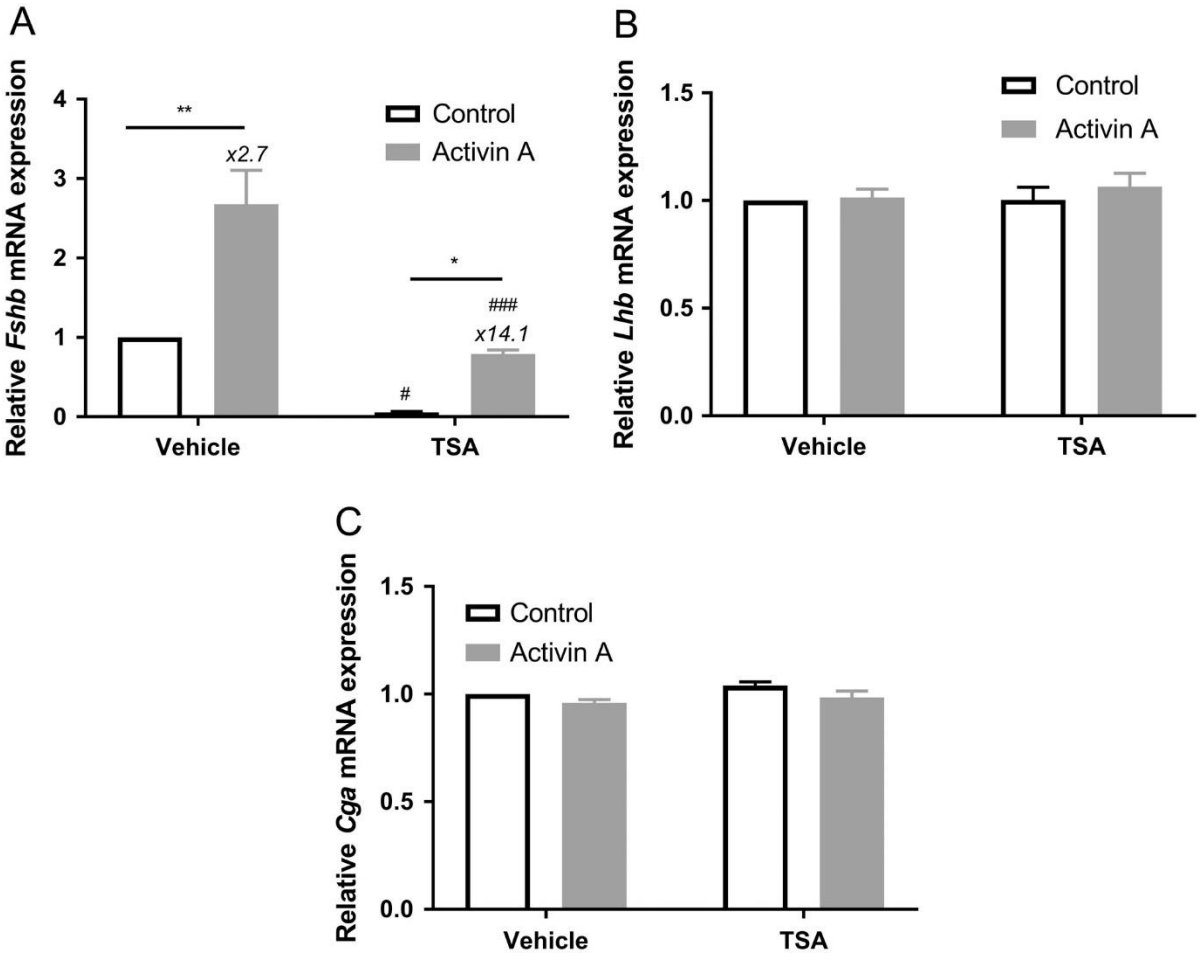
A
Relative Fshb mRNA expression
4
3
2
1
0
**
x2.7
*
###
x14.1
#
Control
Activin A
Vehicle
TSA
B
Relative Lhb mRNA expression
1.5
1.0
0.5
0.0
Control
Activin A
Vehicle
TSA
C
Relative Cga mRNA expression
1.5
1.0
0.5
0.0
Control
Activin A
Vehicle
TSA

Figure 2.3

Figure 2.3: *Fshb expression in primary pituitary cells is reduced by HDAC inhibition*. Pituitaries from 8-week-old male mice were dispersed and cultured. After two days in culture, cells were treated with activin A (1 nmol/L), TSA (331 nmol/L), or both, for 6 h. mRNA levels of gonadotropin subunits (*Fshb* **[A]**, *Lhb* **[B]**, and *Cga* **[C]**) were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (+/- SEM) of N=3 independent experiments. Activin A's fold induction is indicated above the appropriate bars. Two-way ANOVA followed by a post-hoc Holm-Sidak multiple comparison test was used. * $p<0.05$; ** $p<0.01$ when comparing activin A vs control; # $p< 0.05$; ### $p<0.001$ when comparing TSA vs corresponding vehicle condition.

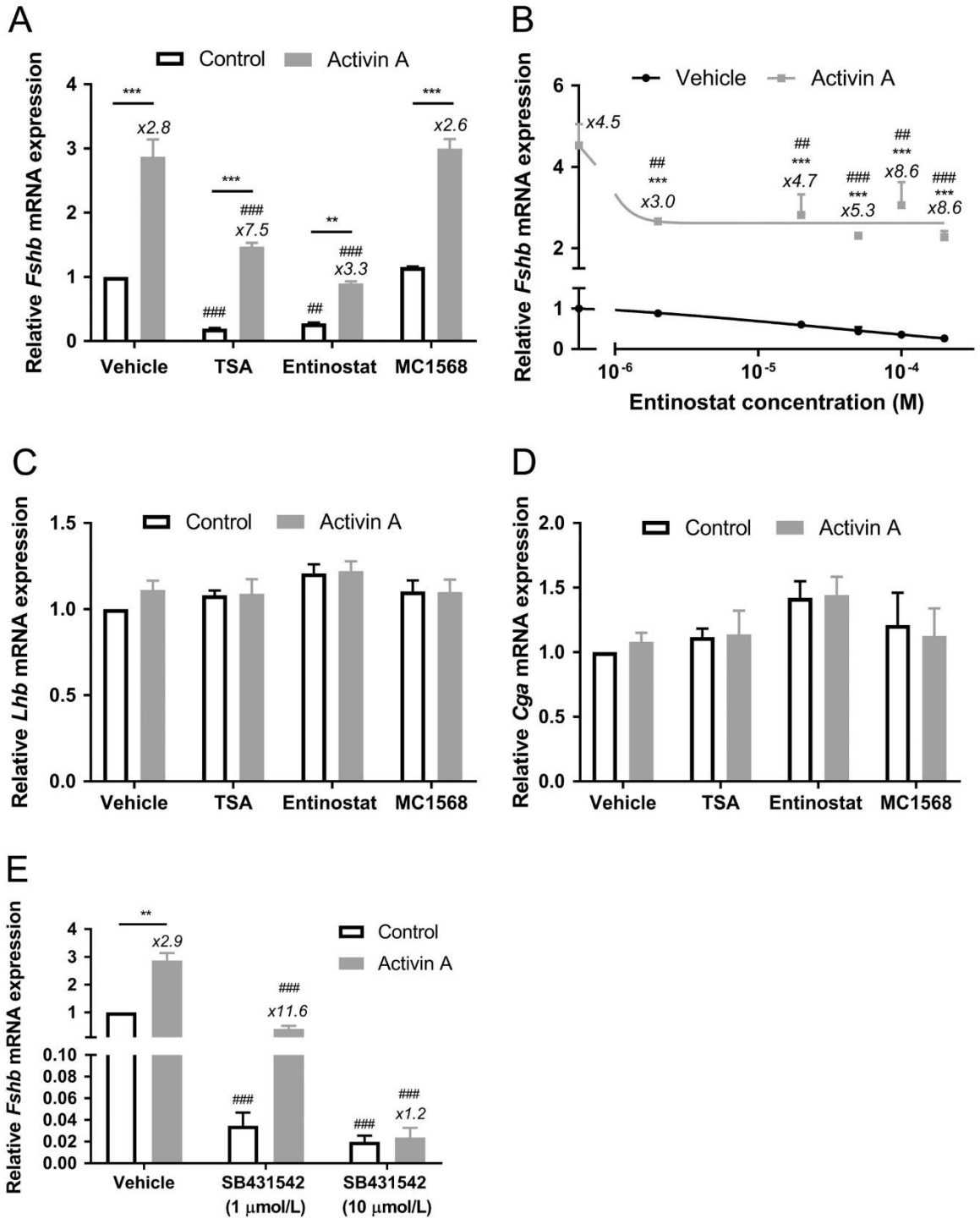
A
Control
Activin A
Relative Fshb mRNA expression
Vehicle
TSA
Entinostat
MC1568
x2.8
x7.5
x3.3
x2.6
B
Vehicle
Activin A
Relative Fshb mRNA expression
Entinostat concentration (M)
x4.5
x3.0
x4.7
x5.3
x8.6
x8.6
C
Control
Activin A
Relative Lhb mRNA expression
Vehicle
TSA
Entinostat
MC1568
D
Control
Activin A
Relative Cga mRNA expression
Vehicle
TSA
Entinostat
MC1568
E
Control
Activin A
Relative Fshb mRNA expression
Vehicle
SB431542 (1 μmol/L)
SB431542 (10 μmol/L)
x2.9
x11.6
x1.2

Figure 2.4

Figure 2.4: *Class I, but not class II, HDAC inhibition suppresses Fshb expression in primary pituitary cultures*. Pituitaries from 8-week-old male mice were dispersed and cultured. After two days in culture, cells were treated with TSA (45 nmol/L in [**A**, **C**, and **D**]), entinostat (42.5 µmol/L in [**A**, **C**, and **D**] or a range of concentrations in [**B**]), MC1568 (2.5 µmol/L in [**A**, **C**, and **D**]), or SB431542 (1 or 10 µmol/L in **[E]**), in the presence or absence of activin A (1 nmol/L), for 6 h. mRNA levels of gonadotropin subunits (*Fshb* [**A**, **B**, and **E**], *Lhb* [**C**], and *Cga* [**D**]) were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (+/- SEM) of N=3 independent experiments, or N=2 independent experiments for the dose-response curve in panel B. Activin A's fold induction is indicated above the appropriate bars or data points. Data were analyzed by two-way ANOVA followed by a post-hoc Holm-Sidak multiple comparison test. *** $p<0.001$ when comparing activin A vs control; ### $p<0.001$ when comparing the inhibitor vs the corresponding vehicle condition.

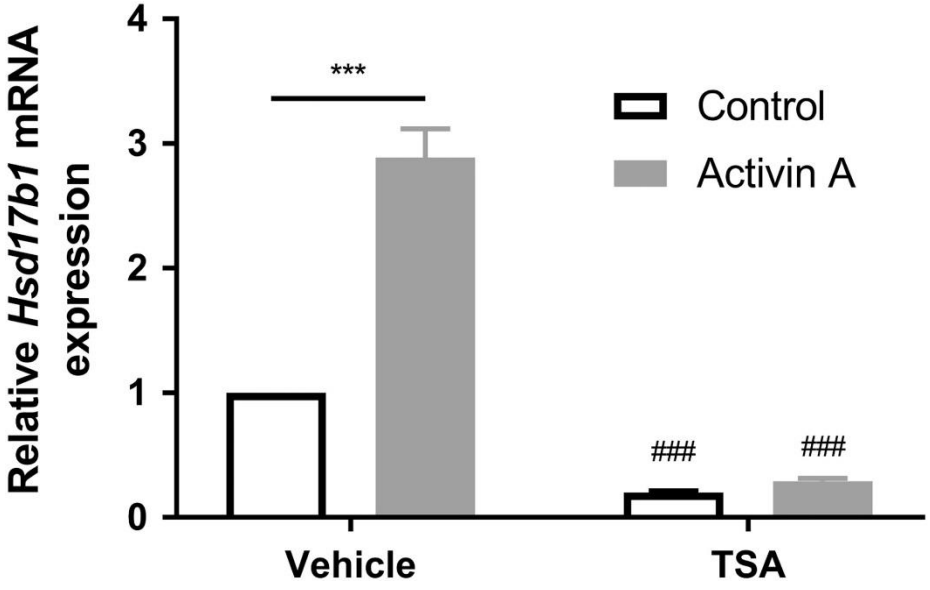
Relative Hsd17b1 mRNA expression
4
3
2
1
0
***
###
###
Control
Activin A
Vehicle
TSA

Figure 2.5

Figure 2.5: *HDAC inhibition impairs Hsd17b1 mRNA expression*. LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). *Hsd17b1* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (+/- SEM) of N=3 independent experiments. Data were analyzed by two-way ANOVA followed by post-hoc Holm-Sidak multiple comparison test. ** $p<0.01$ when comparing activin A vs control; # $p< 0.05$, ### $p<0.001$ when comparing TSA vs corresponding vehicle condition.

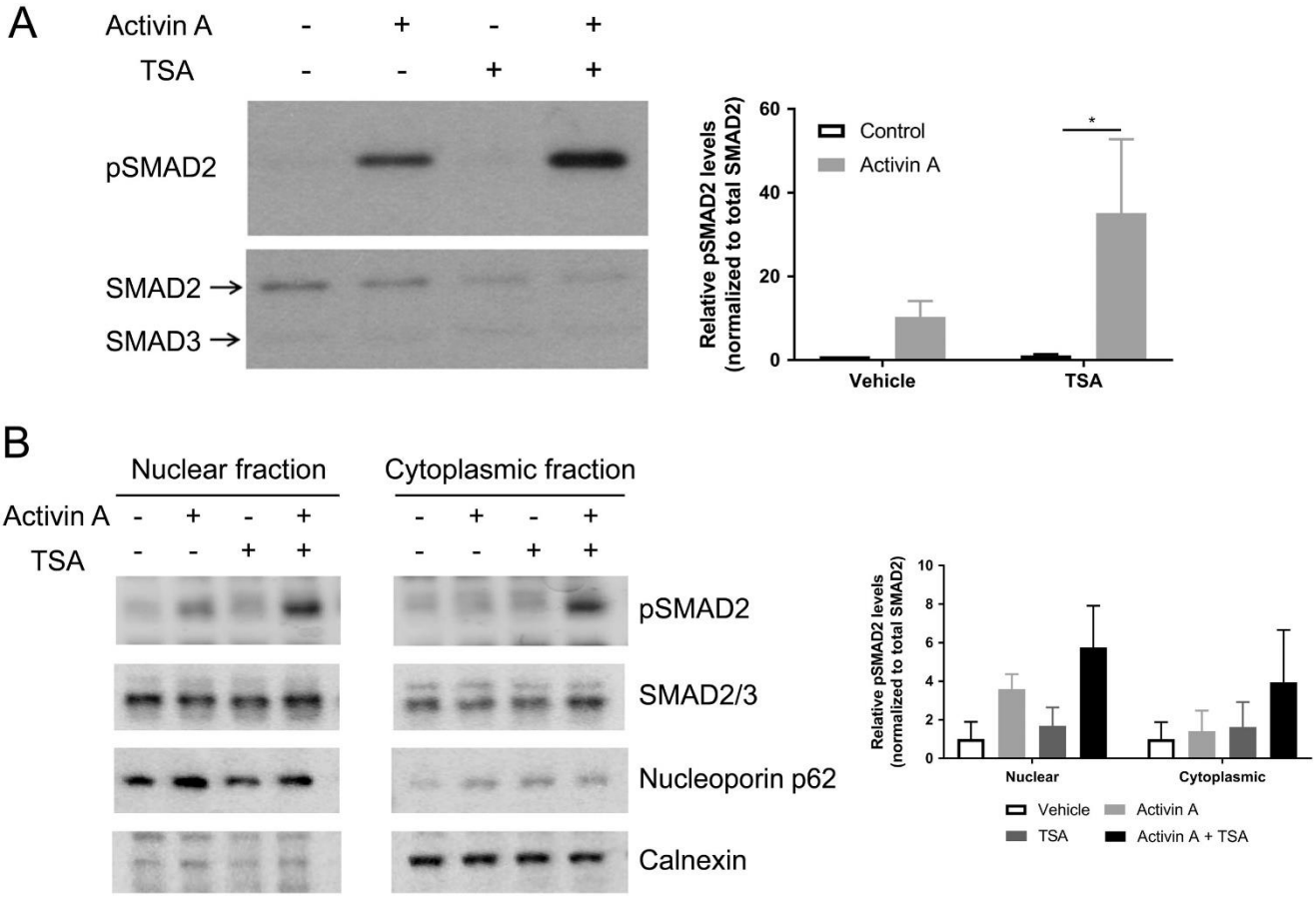
A
Activin A - + - +
TSA - - + +
pSMAD2
SMAD2
SMAD3
Relative pSMAD2 levels (normalized to total SMAD2)
60
40
20
0
Control
Activin A
*
Vehicle
TSA
B
Nuclear fraction
Cytoplasmic fraction
Activin A - + - + - + - +
TSA - - + + - - + +
pSMAD2
SMAD2/3
Nucleoporin p62
Calnexin
Relative pSMAD2 levels (normalized to total SMAD2)
10
8
6
4
2
0
Nuclear
Cytoplasmic
Vehicle
Activin A
TSA
Activin A + TSA

Figure 2.6

Figure 2.6: *HDAC inhibition does not block activin A-induced SMAD2 phosphorylation or nuclear import*. **A)** LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). Total lysates were extracted and levels of phospho-SMAD2 and total SMAD2 were analyzed by western blot. A representative blot is shown. The data at the right represent the mean (+/- SEM) of N=4 independent experiments, and were analyzed by two-way ANOVA, followed by post-hoc Holm-Sidak multiple comparison test. *$p<0.05$ when comparing activin A vs control. **B)** A similar experiment was conducted, but nuclear and cytoplasmic protein fractions were prepared. Levels of pSMAD2, nucleoporin p62 (nuclear marker), and calnexin (cytoplasmic marker) were assessed by western blot. Both cytoplasmic and nuclear fractions were run on the same gel, and exposure times were the same between both compartments (intervening lanes were cropped for purposes of figure preparation). For protein quantification, phospho-SMAD2 levels were normalized to total SMAD2 levels, using the Bio-Rad QuantityOne software.

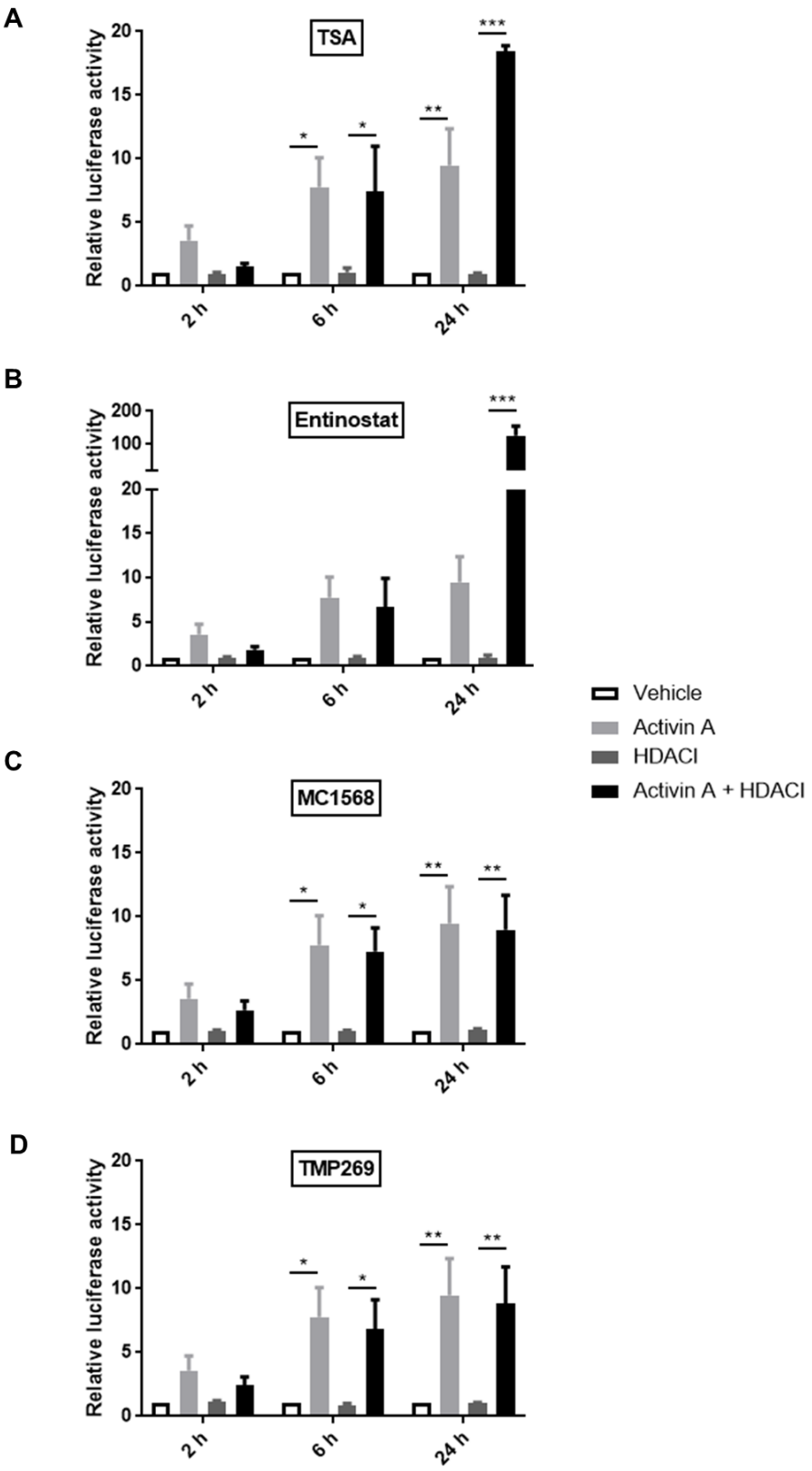
A
TSA
Relative luciferase activity
20
15
10
5
0
*
*
**
***
2 h
6 h
24 h
B
Entinostat
Relative luciferase activity
200
100
20
15
10
5
0
***
2 h
6 h
24 h
C
MC1568
Relative luciferase activity
20
15
10
5
0
*
*
**
**
2 h
6 h
24 h
D
TMP269
Relative luciferase activity
20
15
10
5
0
*
*
**
**
2 h
6 h
24 h
Vehicle
Activin A
HDACI
Activin A + HDACI

Figure 2.7

Figure 2.7: *HDAC inhibition does not significantly affect SMAD3/4 signaling over short periods of time, and promotes it following longer incubation times*. LβT2 cells were transfected with 225 ng of the CAGA-luc reporter plasmid, followed by treatment for 2, 6, or 24 h with **A)** TSA (45 nmol/L), **B)** MS-275 (42.5 µmol/L), **C)** MC1568 (2.5 µmol/L), or **D)** TMP269 (3.9 µmol/L) in the presence or absence of activin A (1 nmol/L). For each time point, values were normalized to the vehicle, control-treated condition. Bars represent mean values (+/- SEM) of N=4 independent experiments. Data were analyzed by two-way ANOVA followed by post-hoc Holm-Sidak multiple comparison test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

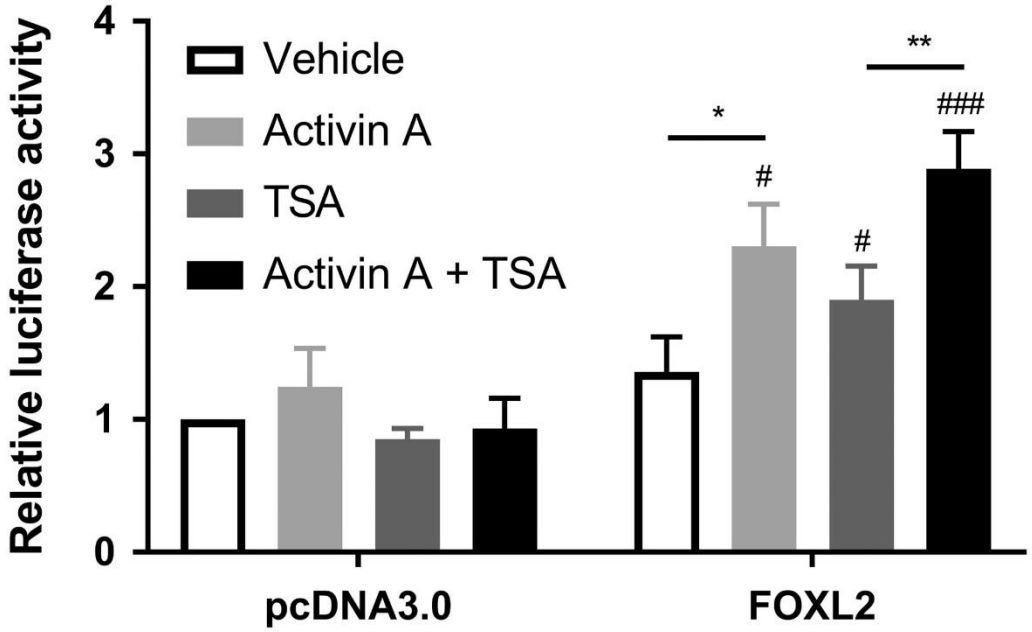
Relative luciferase activity
4
3
2
1
0
Vehicle
Activin A
TSA
Activin A + TSA
*
**
#
#
###
pcDNA3.0
FOXL2

Figure 2.8

Figure 2.8: *HDAC inhibition does not impair FOXL2 regulation of the porcine Fshb promoter*. HEK293 cells were transfected with 225 ng of a porcine *Fshb*-promoter–luciferase reporter plasmid, along with 4.15 ng of an empty vector (pcDNA3.0) or a FOXL2 expression vector. Cells were treated for 6 h with TSA (45 nmol/L) in the presence or absence of activin A (1 nmol/L). Values were normalized to the vehicle, control-treated condition (empty vector). Bars represent mean values (+/- SEM) of N=3 independent experiments. Data were analyzed by two-way ANOVA followed by post-hoc Holm-Sidak multiple comparison test. * $p<0.05$, ** $p<0.01$ when comparing treatments to the vehicle condition; # $p<0.05$, ## $p<0.001$ when comparing pcDNA3.0 and FOXL2 for a given treatment condition.

Gene	Primer sequence
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Lhb	
*F*orward	ACTGTGCCGGCCTGTCAACG
*R*everse	AGCAGCCGGCAGTACTCGGA
Cga	
*F*orward	TCCCTCAAAAAGTCCAQGAGC
*R*everse	GAAGAGAATGAAGAATATGCAG
Hsd17b1	
Forward	GTTATGAGCAAGCCCTGAGC
Reverse	AAGCGGTTCGTGGAGAAGTA
Myog	
Forward	GCACTGGAGTTCGGTCCCA
Reverse	GATGGACGTAAGGGAGTGCAGA
Fos	
Forward	GGAGCTGACAGATACACTCCAA
Reverse	GAGGCCACAGACATCTCCTC
Egr1	
Forward	GAGCGAACAACCCTATGAGC
Reverse	GAGTCGTTTGGCTGGGATAA

Table 2.1: qPCR primers

Chapter 3

In Chapter 2, I challenged the model that GnRH induces *Fshb* by alleviating HDAC-mediated repression of the promoter. I showed that in both LβT2 and primary pituitary cells, HDACs play a permissive, rather than an inhibitory role in *Fshb* expression[692]. This was mainly mediated by class I HDACs. Critically, HDACs were required for basal, GnRH-induced, and activin-induced *Fshb* expression. This is an important point to make, as the previously established model never described how HDACs may or may not influence activin signaling[512]. Indeed, should HDACs maintain the *Fshb* promoter in a closed conformation, it is unclear how activins could induce expression of the subunit. Activins induce phosphorylation of SMAD proteins, promoting their accumulation in the nucleus where they act as transcription factors. Regardless, I demonstrated that, contrary to the previous model, HDACs do not play an inhibitory role in basal, GnRH-, or activin-induced *Fshb*, at least in LβT2 and primary pituitary cells.

Contrary to *Fshb*, the mechanisms underlying GnRH-induced *Lhb* expression are well understood (see section 1.3.2.1). A unique characteristic of GnRH-induced LH production is apparent at the time of the pre-ovulatory LH surge, specifically involving GnRH self-priming, which appears dependent on PR. Global *Pgr* knockout mice fail to mount an LH surge[548,549], as do mice treated with PR inhibitors[763]. Interestingly, PR antagonists also inhibit the activin-dependent secondary FSH surge[566,568,569]. Moreover, in promoter-reporter assays in LβT2 cells, activins synergize with androgens and progestagens in inducing *Fshb* expression[537-539]. These data suggest that 1) PR is necessary for the pre-ovulatory LH surge, and 2) PR mediates the rise in FSH on estrous morning. However, the relevance for PR in gonadotropes has not been demonstrated directly *in vivo*. In Chapter 3, I tackle this unresolved problem by describing the reproductive phenotypes of gonadotrope-specific *Pgr* conditional knockout mice.

Impaired LH surge amplitude in gonadotrope-specific progesterone receptor knockout mice

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Short title**:** Progesterone receptor function in gonadotropes *in vivo*

Keywords: Progesterone receptor, LH, FSH, gonadotropes

ABSTRACT

The progesterone receptor (PR, encoded by *Pgr*) plays essential roles in reproduction. Female mice lacking the PR are infertile, due to the loss of the protein's functions in the brain, ovary, and uterus. PR is also expressed in pituitary gonadotrope cells, but its specific role therein has not been assessed *in vivo*. We therefore generated gonadotrope-specific *Pgr* conditional knockout mice (cKO) using the Cre-LoxP system. Overall, both female and male cKO mice appeared phenotypically normal. cKO females displayed regular estrous cycles (vaginal cytology) and normal fertility (litter size and frequency). Reproductive organ weights were comparable between wild-type and cKO mice of both sexes, as were production and secretion of the gonadotropins, LH and FSH, with one exception. On the afternoon of proestrus, the amplitude of the LH surge was blunted in cKO females relative to controls. Contrary to predictions of earlier models, this did not appear to derive from impaired GnRH self-priming. Collectively, these data indicate that PR function in gonadotropes may be limited to regulation of LH surge amplitude in female mice via a currently unknown mechanism.

INTRODUCTION

The progesterone receptor (PR, product of the *Pgr* gene) plays fundamental and pleiotropic roles in the control of reproduction. This is perhaps most clearly demonstrated in female *Pgr* knockout mice, which are infertile because of impairments in: LH surges from the pituitary, LH-induced meiotic maturation and ovarian follicle rupture, uterine decidualization, and sexual behavior[517,549]. The LH surge is driven by ovarian estrogens, which have positive feedback effects at both the hypothalamic and pituitary levels. In the hypothalamus, estrogens stimulate expression of kisspeptin (*Kiss1*) in neurons of the anteroventral periventricular nucleus (AVPV); kisspeptin, in turn, stimulates GnRH release[196,764]. In the pituitary, high levels of estrogens increase the sensitivity of gonadotrope cells to GnRH, amplifying LH release[765,766]. Estrogens stimulate PR expression in multiple cell types, and PRs in kisspeptin neurons play essential roles in estrogen positive feedback. Indeed, estradiol-induced LH surges, ovulation (as reflected by corpora lutea numbers), and fertility (e.g., litter size) are impaired in female mice with conditional deletion of *Pgr* in kisspeptin neurons[522,550]. Though kisspeptin expression appears to be normal in the AVPV of these animals, the data indicate that LH surges in mice depend, at least in part, on intact PR function in kisspeptin neurons.

These results do not, however, rule out an additional role for the PR in estrogen positive feedback and LH surge generation at the pituitary level. Estrogens induce PR expression in gonadotropes and PR has been suggested to modulate GnRH action (i.e., self-priming) therein [556,767]. GnRH self-priming is a phenomenon in which prior GnRH pulses potentiate the actions of subsequent GnRH pulses on LH release[768,769]. Self-priming, which is more pronounced in rats than mice, is estrogen and protein-synthesis dependent, but does not require increases in GnRH receptor numbers[770,771]. Rather, GnRH actions appear to be enhanced in a cAMP- and PR-dependent manner[556,772]. For example, GnRH self-priming is reduced, though not completely eliminated, in estradiol-treated pituitary cultures of *Pgr* knockout mice[562]. Similarly, GnRH self-priming is blocked in ovariectomized, estradiol-treated *Pgr* knockout mice *in vivo*[549]. Nevertheless, the necessity for PR function in gonadotropes for gonadotropin production, LH

surge dynamics, and fertility have not been assessed. To address these gaps in knowledge, we generated gonadotrope-specific *Pgr* knockout mice.

MATERIALS AND METHODS

Animals

The *Pgr*$^{fx/fx}$ and *Gnrhr*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (GRIC) mice were described previously[283,773]. *Pgr*$^{fx/fx}$ males were crossed with GRIC females to generate *Pgr*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ progeny. *Pgr*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females were then crossed to *Pgr*$^{fx/fx}$ males to generate *Pgr*$^{fx/fx}$;*Gnrhr*$^{+/+}$ controls and *Pgr*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ conditional knockouts (cKOs). In order to purify gonadotropes by fluorescence-activated cell sorting (FACS), we crossed *Pgr*$^{fx/fx}$ animals with *Gt(ROSA26)*$^{ACTB\text{-}tdTomato\text{-}EGFP}$ mice (mTmG/mTmG, stock 007676 from Jackson Laboratories) to generate *Pgr*$^{fx/fx}$;*Rosa26*$^{mTmG/mTmG}$ males, which were then crossed to *Pgr*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females to generate *Pgr*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ males and females. Controls for FACS were generated by crossing *Rosa26*$^{mTmG/mTmG}$ and GRIC mice to generate *Pgr*$^{+/+}$;*Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ progeny. Genotyping and assessment of genomic recombination were conducted as previously described [723] (primers listed in Table 3.1). All animal experiments were performed in accordance with institutional and federal guidelines and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (Protocol 5204).

Fluorescence-activated cell sorting (FACS) of gonadotropes

FACS was performed at the Cell Vision Core Facility for Flow Cytometry and Single Cell Analysis of the Life Science Complex at the Rosalind and Morris Goodman Cancer Research Centre at McGill University. Pituitary cell dispersion and cell sorting were performed as previously described[658,748]. Here, EGFP-positive (gonadotropes) and tdTomato-positive (non-gonadotropes) cells were sorted from control and cKO animals. On average, 1.2 x 10^4 EGFP-positive and 2.5 x 10^5 tdTomato-positive cells were obtained from each group (10 mice per group).

Assessment of female puberty onset, estrous cyclicity, and fertility

Females were monitored daily after weaning (postnatal day 21) to determine the onset of vaginal opening. At 6 weeks of age, estrous cyclicity was assessed by daily vaginal swabs for three weeks. Vaginal cells were smeared on glass slides and stained with 0.1% methylene blue to identify cycle stages[774]. The number of days spent in each stage (proestrus, estrus, or diestrus/metestrus) was then counted and divided by the total number of days to determine the relative proportion of time spent in each stage. At 9 weeks of age, a group of females (n=6 per genotype) was mated with wild-type age-matched C57BL/6 males (Charles River) for 6 months. Breeding cages were monitored daily, and the date of birth and number of pups were recorded.

Reproductive organ collection

Testes, seminal vesicles, ovaries, and uteri were dissected from 10-week-old control and cKO males and females. Females were sacrificed at 7:00 AM on the morning of estrus. All organs were weighed on a precision balance.

Blood collection

Blood was collected from 10-week-old control and cKO males and females (7:00 AM on estrus morning) by cardiac puncture, allowed to clot for 30 min at room temperature, and spun down at 3,000 rpm for 10 min to collect serum. Sera were stored at -20^{o}C until assayed for LH and FSH. To assess LH pulsatility in males, four microliters of blood was collected from the tail tip, every 10 min over four hours, starting two hours before lights off. To assess LH surge amplitude in females, four blood samples (four microliters each) were collected from the tail tip over 10 consecutive days: at 10:00 AM and at 6:00, 7:00 and 8:00 PM (light cycle on/off at 7:00 AM/7:00 PM). For all tail tip blood collections, the animals were acclimatized to the procedure by massaging the tail for two weeks prior to the start of the blood collection. All tail tip blood samples were immediately diluted (1:30) in 1X PBS containing 0.05% of Tween, gently vortexed, and placed on dry ice. Blood dilutions were stored at -80^{o}C until LH ELISAs were performed.

In the LH surge experiment, we compared the maximal LH level obtained from each animal on proestrus afternoon (peak). In females surging more than once over the 10-day

sampling period, an average of the maximal value was calculated and used in the analysis. With the sampling method used, we may have missed the true peak of the LH surge. Nevertheless, it enabled us to observe one or more surges in all animals, which would not have been the case if we relied exclusively on vaginal smears for staging proestrus. Moreover, the same approach was used for all animals, and the pattern of results was comparable between the two genotypes.

Hormone analyses

Serum FSH levels were assessed by a Milliplex kit (Millipore, MPTMAG-49K, custom-made for FSH only) following the manufacturer's instructions (minimal detection limit: 9.5 pg/mL; intra-assay CV < 15%). Serum and whole blood LH levels were measured using an in-house sandwich ELISA as previously described[658,775,776] (detection limit: 0.117 to 30 ng/mL; an intra-assay CV < 10%). As we reported previously (and as seen here), LH levels are higher in serum than whole blood samples[390].

Reverse transcription and quantitative-PCR

Pituitary glands were dissected from control and cKO animals (10-weeks-old; females were euthanized at 7:00 AM on estrous morning), snap-frozen in liquid nitrogen, and stored at -80^{o}C. Pituitaries were homogenized in TRIzol reagent (15596018, ThermoFisher Scientific, Burlington, ON, Canada), and total RNA was extracted following the manufacturer's guidelines. For cells from the FACS experiments, total RNA was extracted using a Total RNA Mini Kit (Geneaid, RB300, New Taipei City, Taiwan). Reverse transcription was performed as previously described[750] using Moloney murine leukemia virus reverse transcriptase (0000172807, Promega, Madison, WI, USA) and random hexamers (0000184865, Promega, Madison, WI, USA). qPCR was run on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen qPCR Mastermix (ABMMmix-S-XL; Diamed, Mississauga, ON, Canada) and the primers listed in Table 3.2. Expression levels of genes of interest were determined using the $2^{-\Delta\Delta Ct}$ method[777] and ribosomal protein L19 (*Rpl19*) for normalization. All primers were validated for efficiency and specificity.

GnRH self-priming

The protocol to examine GnRH self-priming was adapted from Chappell, Schneider, Kim, Xu, Lydon, O'Malley, Levine [549], with some modifications based on Higuchi, Kawakami [768]. Indeed, changes were required because we were unsuccessful in reliably and consistently observing GnRH self-priming in wild-type mice using a protocol described in Chappell et al. (1999)[549]. We developed a reliable and reproducible self-priming method after several rounds of optimization. Females (7- to 9-weeks of age) were ovariectomized in accordance with standard operating procedure 206 of the McGill University and Goodman Cancer Centre Facility Animal Care Committee. Briefly, an incision was made at the midline of the mid-dorsum of the animal. On each side of the animal, a small incision was made in the muscle above the ovary, and the ovary pulled out of the body cavity with forceps. The tissue was then cauterized at the level of the oviduct and the ovary removed. All incisions were closed by sutures. One week following ovariectomy, each female was given a subcutaneous injection of 2 µg estradiol benzoate (EB, E8515, Sigma-Aldrich, Oakville, ON, Canada) dissolved in sesame oil (100 µL of a 0.02 µg/µL solution) between 9:30 and 10:00 AM. At 7:00 AM on the next day, blood from the tail tip was collected as described above. Each female was then given six consecutive subcutaneous injections of 50 ng GnRH (L8008, Sigma-Aldrich, Oakville, ON, Canada) (100 µL of a 0.5 ng/µL solution) at 1 hour intervals. Blood from the tail tip was collected 10 min after each GnRH administration. To minimize stress associated with frequent sampling and injections, mice were handled daily for two weeks prior to the onset of injections. Blood samples were diluted and frozen as described above. Samples were then stored at -80^{o}C until LH ELISAs were performed.

Immunofluorescence

GRIC mice were crossed to eR26-τGFP mice[778] to produce animals in which Cre-expressing cells are tagged with τGFP (GRIC/eR26-τGFP). Eight week-old animals were transcardially perfused with 4% paraformaldehyde (158127 Sigma-Aldrich, Munich, Germany) in 0.1 M PBS (P3813, Sigma-Aldrich Munich, Germany) under ketamine/xylazine (7005294 and 10124950, Serumwerk Bernburg, Bernburg, Germany) anesthesia. Brains were removed, soaked in fixative for 2 hours and 18% sucrose overnight, and then frozen in optimal cutting temperature (OCT)

(14020108926, Leica, Nussloch, Germany). Serial 14-µm coronal cryosections were thaw mounted onto SuperFrost Plus slides (10149870 Thermo Fischer, Waltham, Massachusetts, USA). Brain sections were blocked in 0.1 M PBS, 0.3% Triton X-100, 10% donkey serum (017-000-121, Jackson ImmunoResearch, Cambridgeshire, UK) and 3% BSA (A2153, Sigma-Aldrich, Munich, Germany) and incubated with chicken anti-GFP (1:1000, A10262, Thermo Fischer, Waltham, Massachusetts, USA) and rabbit anti-kisspeptin (1:500, AB9754, Millipore, Burlington, Massachusetts, USA) overnight at 4°C, followed by goat anti-chicken 488 (1:500, A11039, Thermo Fischer, Waltham, Massachusetts, USA) and biotinylated donkey anti-rabbit (1:500, BA-1000, Vector Laboratory, Burlingam, CA) and streptavidin CY5 (1:500, 016-170-084, Jackson ImmunoResearch Inc, Cambridgeshire, UK). For nuclear staining, sections were incubated with 5 µg/ml Hoechst 33258 dye (14530, Sigma-Aldrich, Munich, Germany) in 0.1 M PBS for 5 min and mounted with Fluoromount-G (0100-01, Southern Biotech, Birmingham, Alabama, USA). Sections were analyzed on an Imager.M2 microscope equipped with AxioVision software (Zeiss, Jena, Germany).

Statistical analysis

All data were analyzed on GraphPad Prism 6 using Student *t*-tests. Results were considered statistically significant when $p < 0.05$. For LH pulses, data were deconvoluted using MatLab[779] and number of pulses were compared between genotypes using Student t-test. Comparisons between the two groups of mice challenged with GnRH were done with the GLM procedure for repeated measurements. Calculations were performed with Systat 13 (Systat Software, Inc, San Jose, CA).

RESULTS

Generation of progesterone receptor conditional knockout mice

To address the role of the PR in gonadotropes, we generated gonadotrope-specific *Pgr* knockout (cKO) mice by crossing floxed *Pgr* ($Pgr^{fx/fx}$) and GRIC mice ($Gnrhr^{GRIC/GRIC}$). First, we verified *Pgr* recombination in different tissues from controls and cKOs of both sexes. As expected,

recombination was restricted to the pituitary of both females and males, and to the testes and epididymides of males (Fig. 3.1A). Next, we quantified the efficiency and specificity of *Pgr* recombination by assessing the level of *Pgr* mRNA expression in purified gonadotropes (Cre-recombinase expressing cells, EGFP-positive) compared to other cell populations in the pituitary (Cre-negative, tdTomato-positive). In both male and female cKOs, *Pgr* expression was markedly reduced in gonadotropes (Fig. 3.1B, green bars). *Pgr* expression in non-gonadotropes (lactotropes) was intact (Fig. 3.1B, red bars). Recent single-cell RNA-seq data confirm that *Pgr* expression is enriched in gonadotropes and lactotropes in murine pituitaries[267].

Normal fertility and gonadal development in Pgr cKO mice

cKO females and their control littermates reached puberty at similar ages, as assessed by vaginal opening (Fig. 3.2A). We did not measure the day of first estrus, which is considered by some to be a more robust measure of puberty onset than vaginal opening. However, the other phenotypes in these mice (or lack thereof) suggest that day of first estrus was unlikely to be affected. *Pgr* cKO females had normal estrous cyclicity, as assessed by vaginal cytology (Fig. 3.2B). In breeding trials, frequency of delivery, inter-litter interval, and average litter size were comparable between control and cKO females (Fig. 3.2C-E). Ovarian and uterine masses were also normal in cKO females (Fig. 3.3A-B). In males, testicular and seminal vesicle masses were equivalent between genotypes (Fig. 3.3C-D).

FSH and LH production is intact in Pgr cKO mice

Although fertility and gonadal development were apparently unaffected in the absence of gonadotrope PR function, we measured serum LH and FSH levels in both females and males. Blood samples were collected from control and cKO females on the morning of estrus, just after lights on. Serum FSH (Fig. 3.4A-B) and LH levels (Fig. 3.4C-D) were equivalent between genotypes. Similarly, pituitary expression of the gonadotropin subunit genes (*Fshb, Lhb*, and *Cga*) and the GnRH receptor (*Gnrhr*, Fig. 3.5) did not differ between control and cKO mice, with two exceptions (Fig. 3.5F, H). *Cga* mRNA levels were reduced and *Gnrhr* mRNA levels increased in cKO males relative to controls. The variation in serum FSH and pituitary *Fshb* mRNA levels is likely explained

by the fact that some females were still in the midst of the secondary surge while others were not at the time of sampling.

Pulsatile LH secretion is normal in Pgr cKO males

While there were no apparent effects of PR loss on LH production, we next examined LH pulse frequency. We focused on males, as LH pulsatility varies markedly across the estrous cycle in females[775]. There were no genotype-dependent differences in LH pulse amplitude or frequency (Fig. 3.6 and Table 3.3).

Female Pgr cKO mice have blunted preovulatory LH surges

We assessed the role of PR in gonadotropes on the LH surge. Profiles of the LH surge in controls (left) and cKOs (right) are shown in Fig. 3.7A and B, respectively. The number of surges detected across the 10-day sampling period appeared to be reduced in cKO females, but this was not statistically significant (Fig. 3.7C). Next, we compared the amplitude of the LH surge between controls and cKOs. Consistent with data from the morning of estrus (Fig. 3.4C), there was no genotype difference in LH levels on the morning of proestrus. Although most of the mice surged during the 10-day period (Fig. 3.7A-C), the maximal LH levels measured during the surge were significantly lower in cKO relative to control females (Fig. 3.7D).

Female Pgr cKO mice do not display impaired GnRH self-priming

Finally, based on previous reports[549,556,557,562,767,780], we assessed the effects of gonadotrope-specific PR loss on GnRH self-priming as a potential mechanism underlying reduced LH surge amplitude in these animals. Mean serum LH levels before the first GnRH injection and 10 min after each injection (six injections in total) are shown in Fig. 3.8. The amplitude of GnRH-stimulated LH release increased following successive GnRH injections, indicating that self-priming occurred in both genotypes. Blood LH concentrations increased after GnRH administration (ANOVA: $P<0.0001$), but there was no difference between genotypes ($P=0.90$) and no interaction between time and genotype ($P=0.70$). There was a highly significant difference in LH increase

between the first and subsequent GnRH injections, and the second and the fourth and following administrations (P-values between 0.0001 and 0.012), but thereafter this effect leveled off.

DISCUSSION

We generated conditional *Pgr* knockout mice to assess PR function in pituitary gonadotrope cells. The data suggest that PR's primary role in this cell type is to regulate the amplitude of the LH surge in females. We did not observe any other alterations in reproductive physiology in either sex. As *Pgr* knockdown was highly efficient, the apparently normal gonadotropin synthesis and secretion in males and females (except on proestrus) is unlikely to derive from preservation of some PR function (i.e., incomplete recombination by Cre). Indeed, global *Pgr* knockout mice similarly show normal LH and FSH production under most conditions[548]. Overall, our results confirm and extend some previous observations, while challenging other *in vivo* and *in vitro* findings.

Results from global *Pgr* knockout mice suggest that the PR plays an important role in GnRH self-priming in gonadotropes[549,562]. Therefore, the most parsimonious explanation of the blunted LH surge in *Pgr* cKO mice would be impaired GnRH self-priming. However, we did not observe any such impairment, at least under the conditions used here. We attempted to employ the protocol described in Chappell et al. (1999)[549], but the GnRH dose used previously (~4 ng) was insufficient to stimulate LH release in our hands (data not shown). We developed a new protocol based on a previous report in rats[768]. Here, we modified the estrogen priming (no 17β-estradiol implant added during the ovariectomy, and we increased the amount of EB to 2 μg), GnRH dose (50 ng per injection), and frequency of GnRH treatment (once per hour, for a total of 6 injections) relative to Chappell et al (1999)[549]. We observed enhanced LH release with successive GnRH injections (indicative of self-priming), but there was no difference between genotypes. The data suggest that PR is not required for GnRH self-priming in murine gonadotropes and that PR regulates LH surge amplitude via a distinct mechanism in these cells.

It is also possible that reduced LH surge amplitude might be explained by effects originating outside of the gonadotrope. For example, *Pgr* cKO females could have reduced

circulating estradiol levels relative to control. We did not assess this parameter as, in our experience, measurements of serum estradiol levels in mice are unreliable[658,680,781]. However, as FSH and LH production, and ovary and litter sizes, are normal in *Pgr* cKO mice, there is no reason to suspect impairments in gonadotropin-stimulated estradiol production. Alternatively, as Cre activity has been observed in some neurons of GRIC mice[475,782], it is possible that the reduced LH surge amplitude might derive from loss of PR in the brain in addition to the pituitary. Indeed, PR expression in the AVPV is necessary for LH surge induction[783]. However, there is no Cre activity in either GnRH neurons[475] or kisspeptin neurons in the AVPV or arcuate nucleus (Fig. 3.9) of GRIC mice. In addition, female mice lacking PR in kisspeptin neurons show more dramatic reproductive deficits than what we observe here, including advanced onset of puberty, reduced fertility, and impaired ovulation[550]. We therefore conclude that it is unlikely that the phenotypes described in the *Pgr* cKO mice derive from loss of the PR in GnRH or kisspeptin neurons.

Based on previous reports, we would have expected effects of PR deletion on FSH. Estradiol-treated pre-menopausal women show increased FSH production in response to exogenous progesterone administration[784]. Also, pre-menopausal women co-treated with estradiol benzoate and progesterone produce more FSH in response to exogenous GnRH compared to women treated with estradiol benzoate alone[765]. In rodents, PR and progesterone also appear to stimulate FSH production. For example, progesterone and its analogs (e.g., R5020), both alone or in synergy with activin A, strongly stimulate the activity of murine *Fshb* promoter-reporters in immortalized murine gonadotrope cells, LβT2[538,539]. There was some suggestion that GnRH might stimulate *Fshb* mRNA expression in a PR-dependent fashion in these cells[785]. In rats, the PR antagonists, RU486 and Org31710, attenuate the primary LH and FSH surges on proestrus, while blocking the secondary FSH surge on the morning of estrus[566,568,763,786]. Nevertheless, we did not detect differences in serum FSH or pituitary *Fshb* mRNA levels between genotypes in either sex. One interpretation of these data is that we did not sample females at the appropriate cycle stage to observe effects of the gene deletion on FSH. Though we collected blood and pituitaries on the morning of estrus, it was a few hours after the peak of the secondary surge[710]. Nonetheless, litter size is normal, arguing against an impairment in FSH production at any cycle stage. Although the data suggest that PR is dispensable for FSH production, it is important to

consider that there may be functional redundancy in the system. Specifically, the related androgen and/or glucocorticoid receptors might compensate for the absence of PR in gonadotropes[541,668,787].

In summary, LH surge amplitude is reduced in gonadotrope-specific *Pgr* knockout mice, but gonadotropin production and fertility are otherwise intact in these animals. The data suggest that progesterone negative feedback at the level of the pituitary may be negligible, but that PR contributes to positive feedback effects of estrogens at this level of the HPG axis. Future studies should determine the mechanisms through which PR regulates LH surge amplitude as the receptor does not appear to play a necessary role in GnRH self-priming.

DECLARATION OF INTEREST

The authors declare that they have no conflicts of interest.

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AUTHORS CONTRIBUTION

CT and GS shared equal workloads in terms of experimental design, data collection and analysis, and manuscript preparation, under the supervision of DJB. XZ provided assistance with serial blood collections. PW generated the immunofluorescence images under the supervision of UB. UB provided the GRIC mouse strain. JL provided the floxed *Pgr* mouse strain. FR assisted with statistical analyses. All authors approved the final version of the manuscript before submission.

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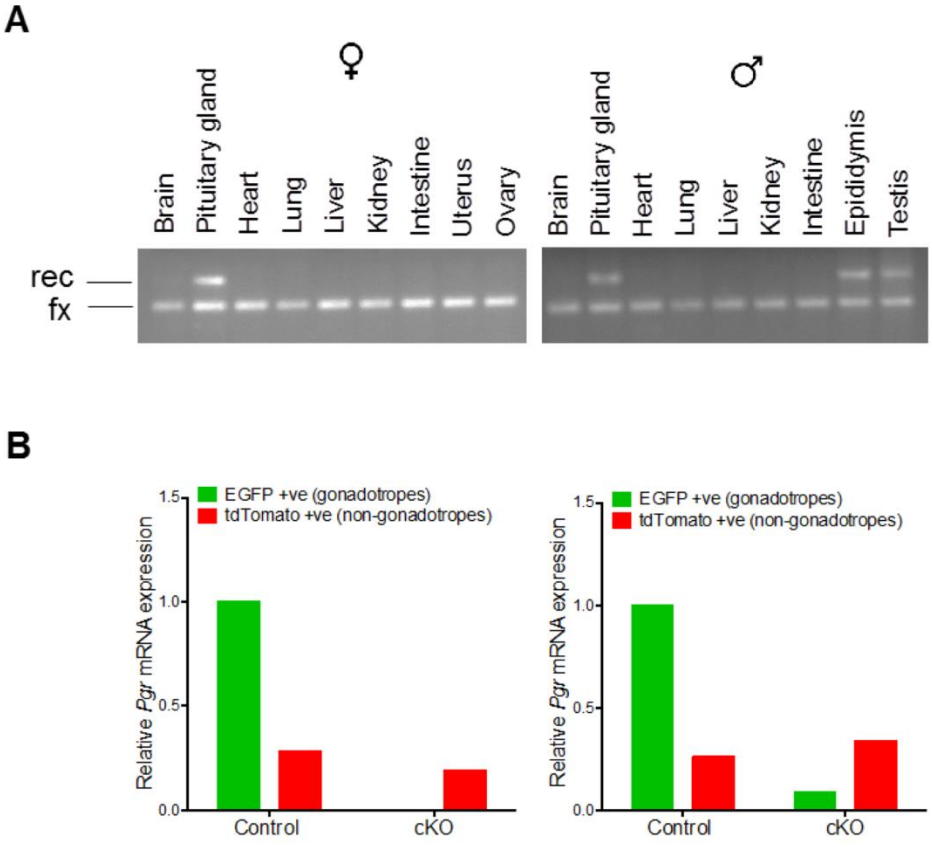
A
♀
Brain
Pituitary gland
Heart
Lung
Liver
Kidney
Intestine
Uterus
Ovary
♂
Brain
Pituitary gland
Heart
Lung
Liver
Kidney
Intestine
Epididymis
Testis
rec
fx
B
EGFP +ve (gonadotropes)
tdTomato +ve (non-gonadotropes)
Relative Pgr mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
EGFP +ve (gonadotropes)
tdTomato +ve (non-gonadotropes)
Relative Pgr mRNA expression
1.5
1.0
0.5
0.0
Control
cKO

Figure 3.1

Figure 3.1: *Pgr recombination efficiency and specificity in gonadotrope cells.* **A)** PCR of genomic DNA from different tissues shows the specificity of recombination in the pituitary glands of female (left) and male cKOs (right), as well as in the epididymis and testis in males (right). rec: recombined; fx: floxed **B)** Quantitative-PCR of cDNA from control (*Pgr*$^{+/+}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) and cKO (*Pgr*$^{fx/fx}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) mice, showing *Pgr* expression in purified gonadotrope (EGFP +ve, green) versus non-gonadotrope (tdTomato +ve, red) cells, in females (left) and males (right).

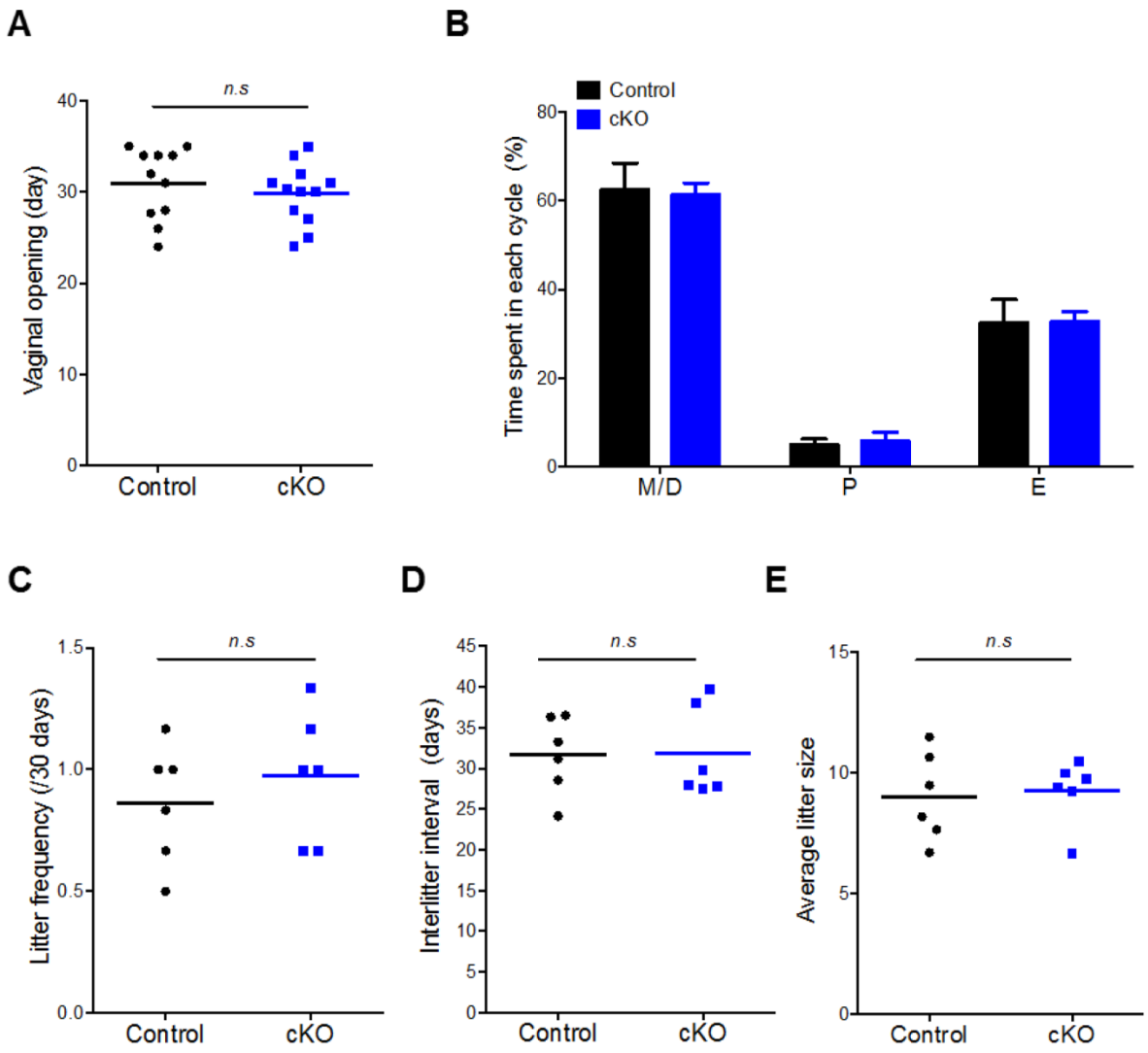
A
n.s
Vaginal opening (day)
40
30
20
10
0
Control
cKO
B
Control
cKO
Time spent in each cycle (%)
80
60
40
20
0
M/D
P
E
C
n.s
Litter frequency (/30 days)
1.5
1.0
0.5
0.0
Control
cKO
D
n.s
Interlitter interval (days)
45
40
35
30
25
20
15
10
5
0
Control
cKO
E
n.s
Average litter size
15
10
5
0
Control
cKO

Figure 3.2

Figure 3.2: *Pgr expression in gonadotropes is not essential for normal reproductive function in female mice.* **A)** Age of vaginal opening (days) in female control (black) and cKO mice (blue). **B)** Percentage of time spent in each stage of the estrous cycle in control and cKO females. **C-E)** Fertility in control and cKO females. **C)** Frequency of delivery per 30 days, **D)** inter-litter interval (days), and **E)** average litter size for each mouse (N=6 per genotype). Two samples Student *t*-test was performed for statistical analysis. n.s: non-significant. M/D: Metestrus/Diestrus, P: Proestrus, and E: Estrus.

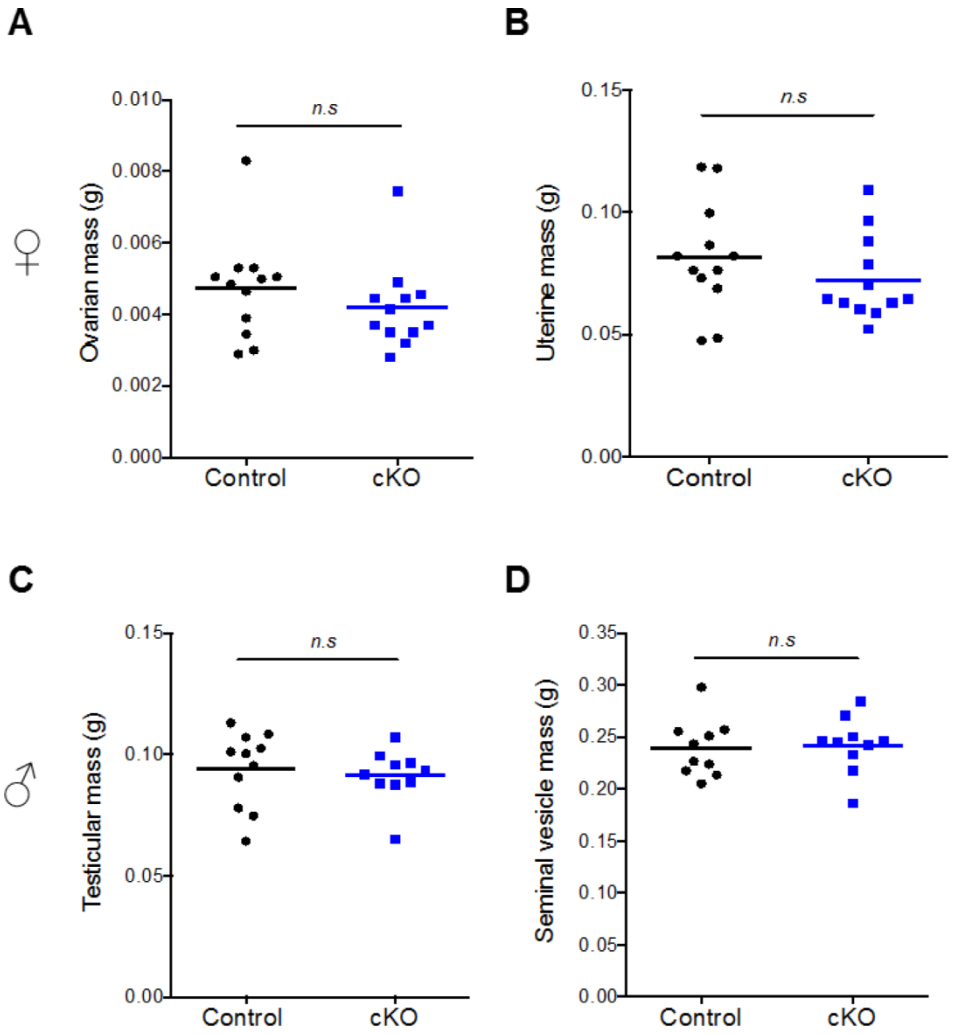
A
B
C
D
♀
♂
Ovarian mass (g)
Uterine mass (g)
Testicular mass (g)
Seminal vesicle mass (g)
n.s
Control
cKO
0.010
0.008
0.006
0.004
0.002
0.000
0.15
0.10
0.05
0.00
0.35
0.30
0.25
0.20

Figure 3.3

Figure 3.3: *Normal reproductive organ weights in female and male Pgr cKO mice.* **A)** Ovarian and **B)** uterine mass in 10-week-old control and cKO females (N=12). **C)** Testicular and **D)** seminal vesicle mass in 10-week-old control and cKO males (N=10). Student *t*-tests were performed for statistical analysis. n.s: non-significant.

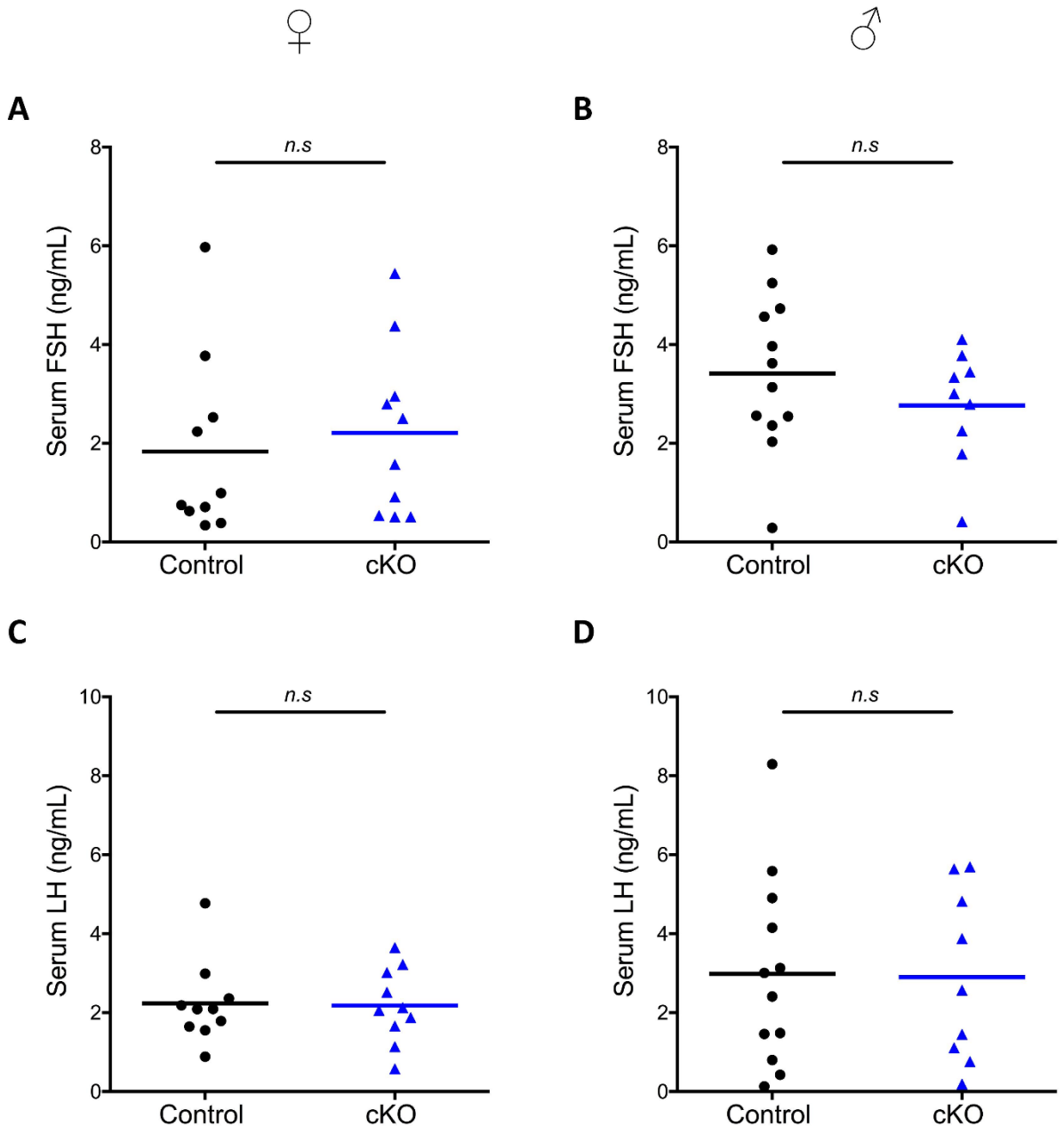

Figure 3.4

Figure 3.4: *Intact serum gonadotropin levels in both female and male Pgr cKO mice.* **A** and **B)** Serum FSH and **C** and **D)** LH levels in 10-week-old control and cKO females (N=10 per genotype, left panels) and males (N=12 controls and N=9 cKO, right panels). Female samples were collected at 7:00 AM on estrous morning. Student *t*-tests were performed for statistical analysis. n.s: non-significant.

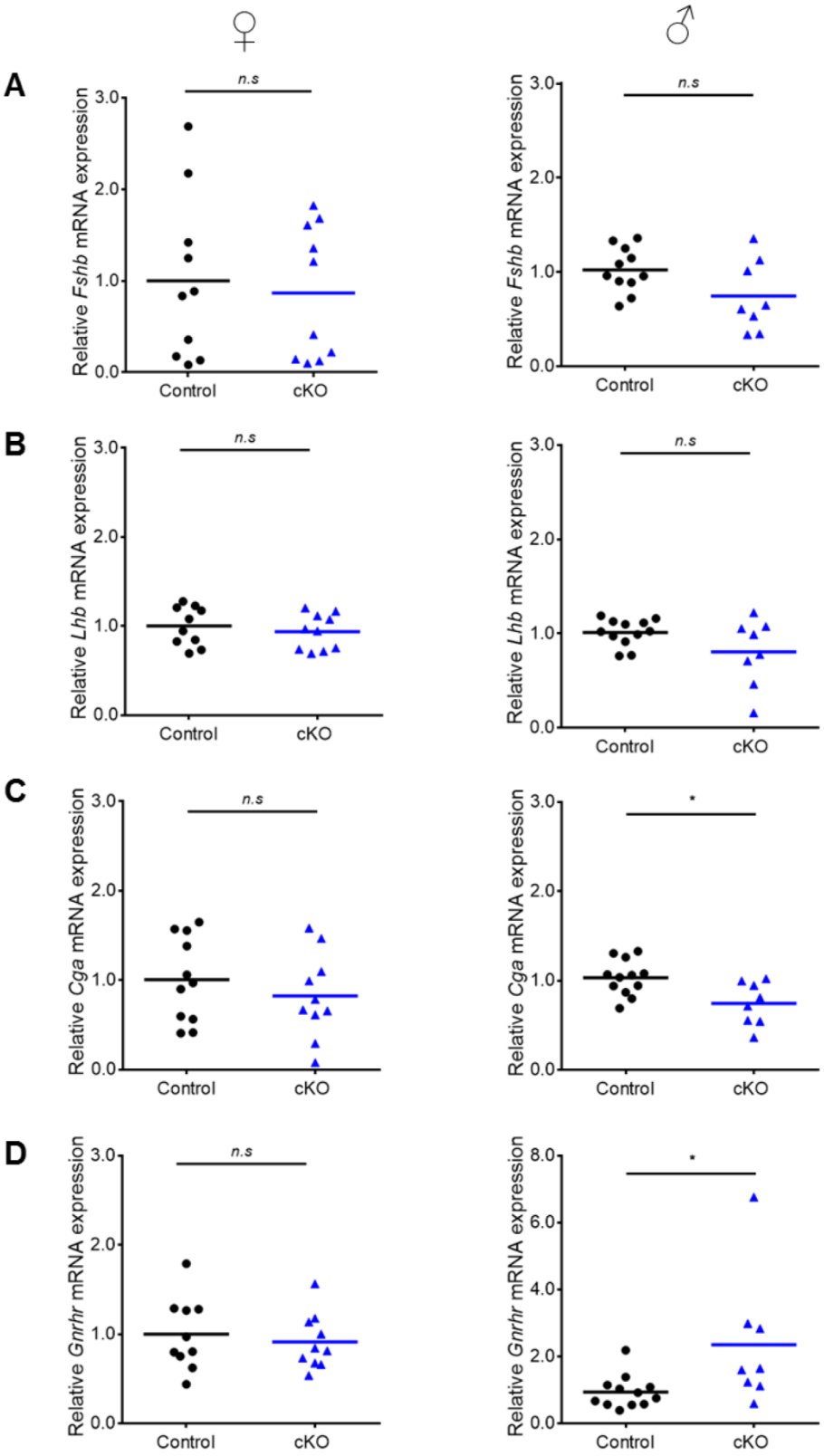
♀
♂
A
B
C
D
Relative Fshb mRNA expression
Relative Lhb mRNA expression
Relative Cga mRNA expression
Relative Gnrhr mRNA expression
n.s
*
Control
cKO
3.0
2.0
1.0
0.0
8.0
6.0
4.0

Figure 3.5

Figure 3.5: *No differences in the expression of pituitary gonadotropin subunits in Pgr cKO mice.* **A** and **B)** FSHβ-subunit (*Fshb)*, **C** and **D)** LH β-subunit (*Lhb)*, **E** and **F)** common α-subunit (*Cga)*, and **G** and **H)** GnRH receptor (*Gnrhr)* mRNA levels in 10-week-old control and cKO females (N=10 per genotype, left panels) and males (N=12 controls and N=8 cKO, right panels). Female samples were collected at 7:00 AM on estrous morning. Student *t*-tests were performed for statistical analysis. *: $p < 0.05$, n.s: non-significant.

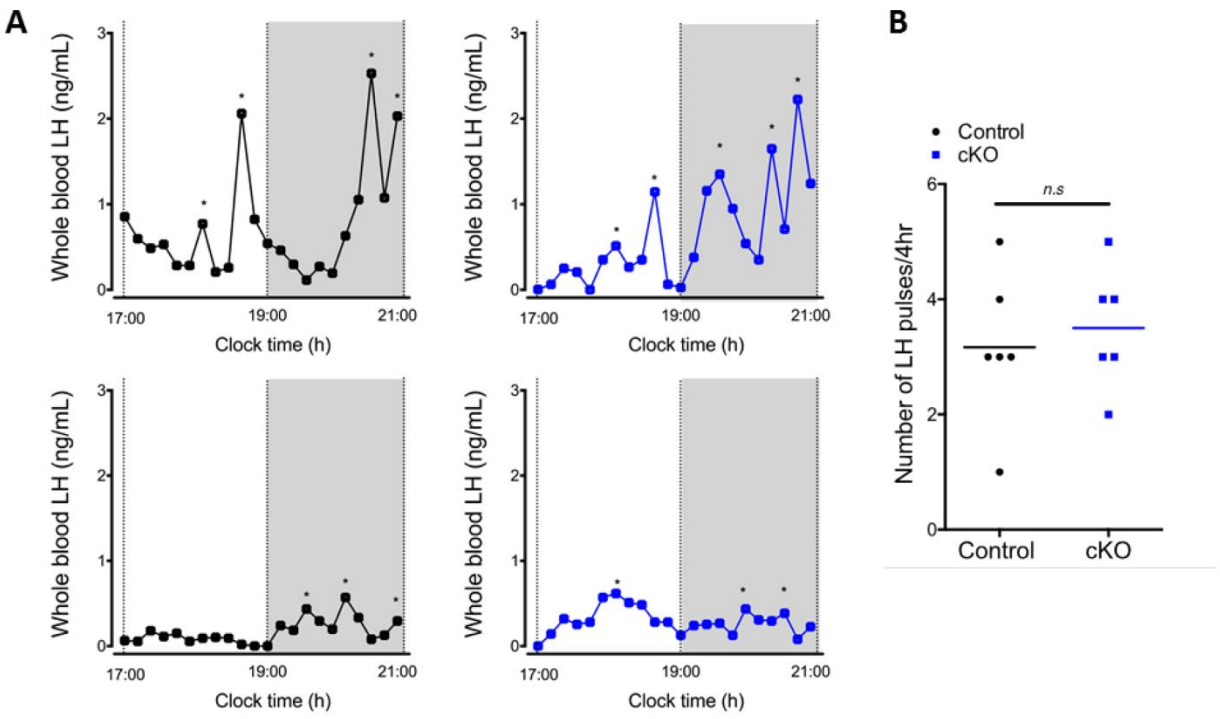
A
Whole blood LH (ng/mL)
Clock time (h)
17:00
19:00
21:00
B
Control
cKO
n.s
Number of LH pulses/4hr
Control
cKO

Figure 3.6

Figure 3.6: *Normal LH pulses in cKO males. Blood samples were collected over 4 hours at 10 min intervals from either control (N=7) and cKO (N=6) males.* **A)** Representative profiles of LH secretion from two control (black graphs, left) and two cKO (blue graphs, right) males. The grey area represents the period of lights-off on a 7AM/7PM (on/off) light/dark cycle. Each Asterisk (*) indicates a pulse. **B)** Quantification of the number of LH pulses in the 4 hr sampling period. Student *t*-tests were performed for statistical analysis. n.s: non-significant.

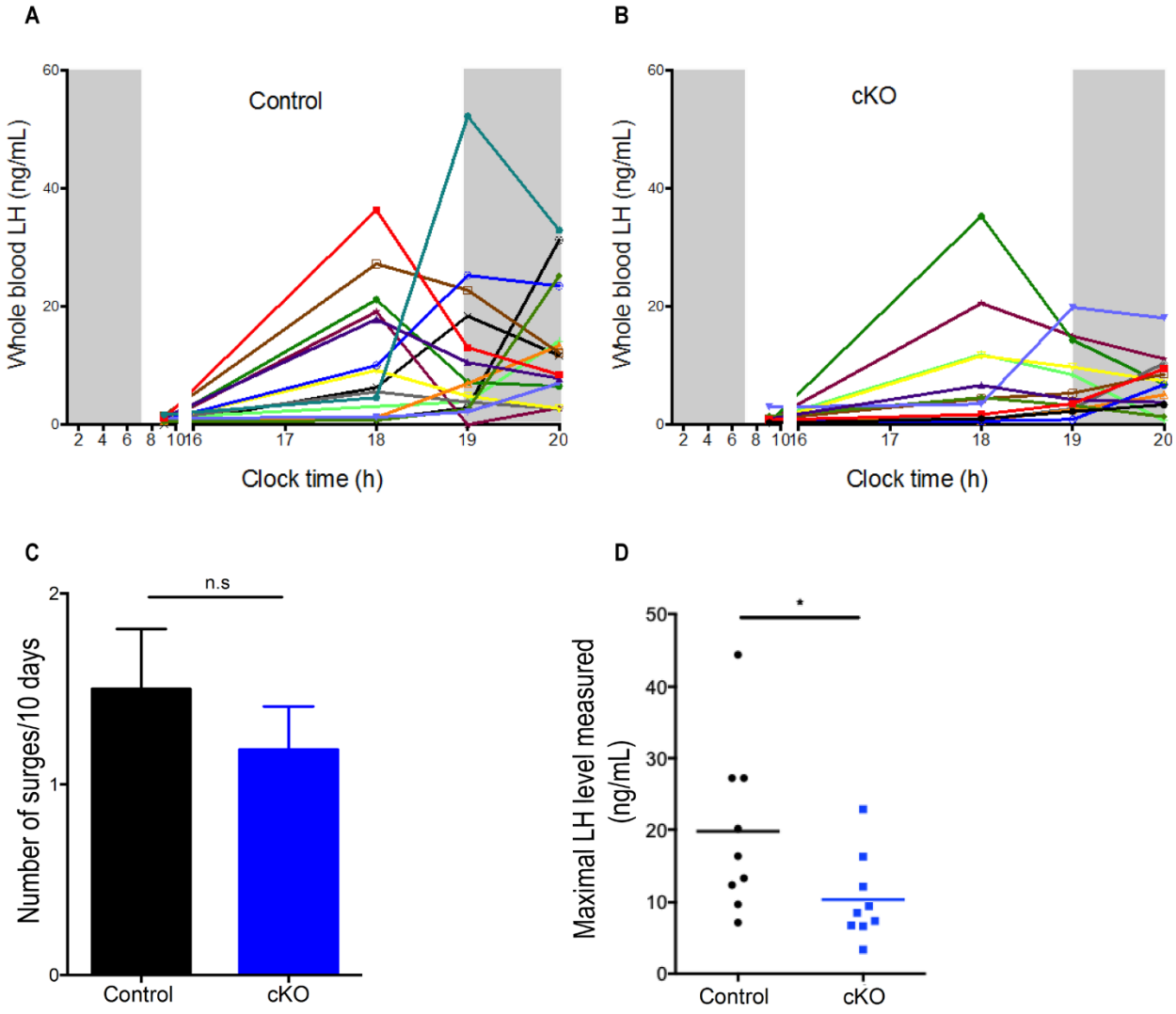
A
Control
Whole blood LH (ng/mL)
Clock time (h)
B
cKO
Whole blood LH (ng/mL)
Clock time (h)
C
n.s
Number of surges/10 days
Control
cKO
D
*
Maximal LH level measured (ng/mL)
Control
cKO

Figure 3.7

Figure 3.7: *The LH surge is blunted in Pgr knockout females.* Blood samples were collected four times daily for 10 consecutive days. Representative profiles of the LH secretion obtained on proestrus from control **(A)** and cKO **(B)** female mice. Different colors indicate different mice. Grey areas represent the dark phase of the light/dark cycle. **C)** Number of surges observed in each mouse during the 10 days of the experiment. **D)** Maximal LH levels measured on proestrus from control (N=9) and cKO (N=9) females. Student *t*-tests were performed for statistical analysis. *: $p < 0.05$. n.s: non-significant. Note: maximal values in panel D are lower than in panels A and B because averages were used in panel D in mice that surged more than once (see Methods).

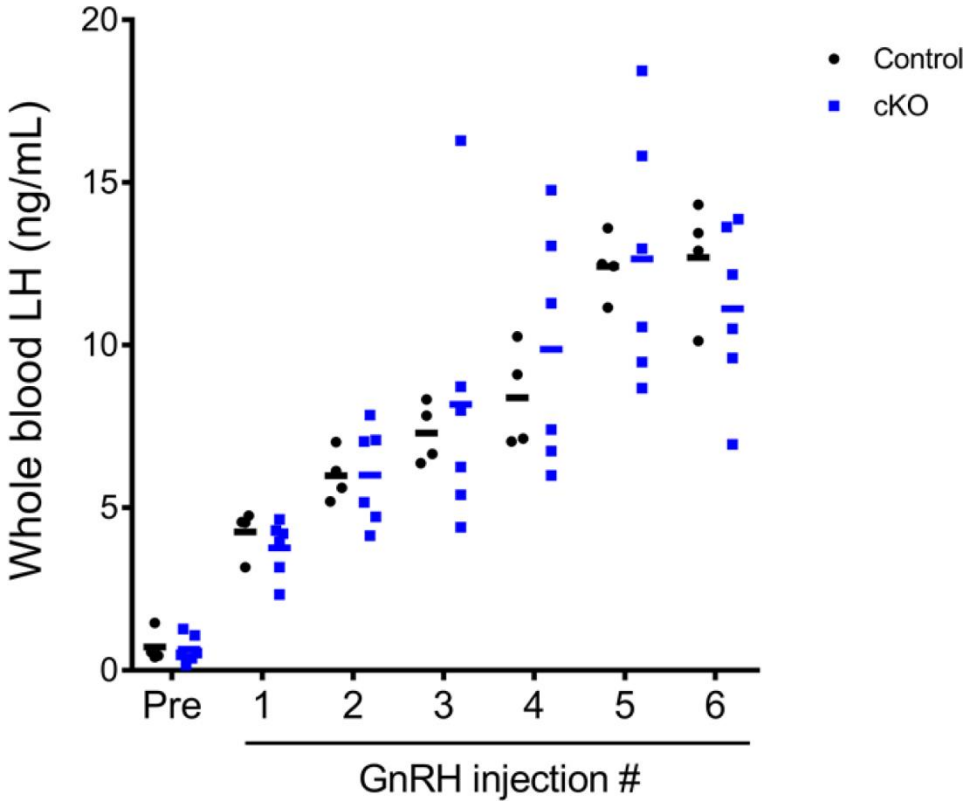
Whole blood LH (ng/mL)
20
15
10
5
0
Pre
1
2
3
4
5
6
GnRH injection #
Control
cKO

Figure 3.8

Figure 3.8: *Control and cKO females show equivalent levels of GnRH self-priming.* Blood samples were collected from ovariectomized, EB-injected females at 7:00 AM. Females were then given six injections of GnRH (50 ng per injection) at 1-hour intervals. Blood samples were collected 10 min after each injection. Mean whole blood LH levels in control (N=4) and cKO (N=6) females are shown. GLM procedure for repeated measurements was used for statistical analysis.

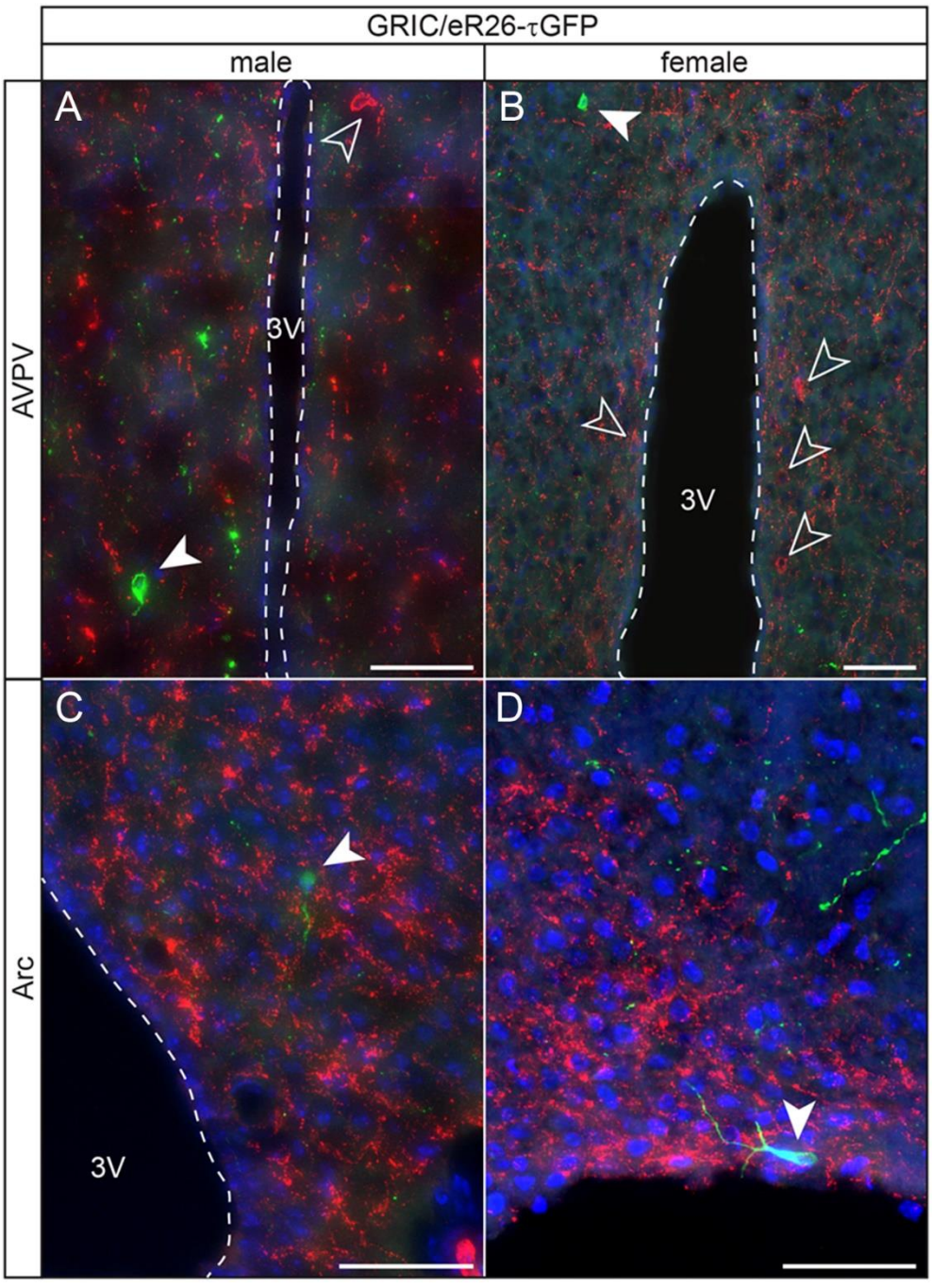

Figure 3.9

Figure 3.9: *Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (Arc) do not exhibit GRIC activity.* **A – D)** Immunofluorescence staining for kisspeptin (red) in brain sections showing the AVPV **(A** and **B)** and the Arc **(C** and **D)** from adult male and female GRIC/eR26-τGFP mice. Note that GnRHR neurons (green, filled arrowheads) do not colocalize with kisspeptin neurons (empty arrowheads). Nuclei were stained with Hoechst 33258 (blue); Scalebars: 50 µm. 3V = third ventricle.

Gene	Primer sequence
Pgr	
Forward	GTATGTTTATGGTCCTAGGAGCTGGG
Reverse (fx)	TGCTAAAGGTCTCCTCATGTAATTGGG
Reverse (rec)	CTGGAAGTAGGATAGAATAATTGGCCTT
Gric	
Forward	GGACATGTTCAGGGATCGCCAGGC
Reverse	GCATAACCAGTGAAACAGCATTGCTG
Rosa26 mTmG	
Forward (WT)	AGGGAGCTGCAGTGGAGTAG
Forward (mut)	TAGAGCTTGCGGAACCCTTC
Reverse	CTTTAAGCCTGCCCAGAAGA

Table 3.1 : Genotyping primers

Gene	Primer sequence
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Lhb	
*F*orward	ACTGTGCCGGCCTGTCAACG
*R*everse	AGCAGCCGGCAGTACTCGGA
Cga	
*F*orward	TCCCTCAAAAAGTCCAQGAGC
*R*everse	GAAGAGAATGAAGAATATGCAG
Gnrhr	
Forward	TTCGCTACCTCCTTTGTCGT
Reverse	CACGGGTTTAGGAAAGCAAA
Pgr	
Forward	GTCACTATGGCGTGCTTACCT
Reverse	TCAGACGACATGCTGGGCA

Table 3.2: qPCR primers

	Control mice	KO mice	P-value	P-value (log-transformed data)
Burst number (#/4h)	3.2±0.5	3.5±0.5	0.64	0.55
Fast half-life (min)	1.0	1.0		
Slow half-life (min)	5.5±1.1	6.5±0.7	0.44	0.36
Mode (min)	10.8±2.0	11.3±2.1	0.84	0.90
Basal secretion (IU/mL)	10.6±2.7	7.7±2.7	0.46	0.54
Pulsatile secretion (IU/mL)	8.8±2.9	10.8±4.1	0.70	0.79
Total secretion (IU/mL)	19.4±3.9	18.5±5.5	0.89	0.69
Mean pulse mass (IU/mL)	2.7±1.0	3.1±1.2	0.78	0.98
Data are mean ± SEM. Statistical comparisons were done with the Student's two-sided t-test for unpaired data.				

Table 3.3:
Deconvolution analysis of circulating LH in six control and six cKO mice sampled at 10-min intervals for 4 h

Chapter 4

In Chapter 3, I demonstrated that PR plays minimal roles in gonadotropes. Serum gonadotropin levels were unchanged in both male and female *Pgr* cKO animals relative to controls[725]. Though female cKOs displayed LH surges with a decreased amplitude, this did not affect their fertility. Finally, cKO females had comparable serum FSH levels and similar litter sizes relative to controls, suggesting that *Pgr* is not required in gonadotropes for the generation of the secondary FSH surge[725].

As explained in Chapter 1, this rise in FSH is activin-dependent, occurs on the morning of estrus, and is required to stimulate the subsequent wave of folliculogenesis[635]. As part of the TGFβ superfamily, activins signal through heterotetrameric type II/type I receptor complexes. The downstream activin signaling cascade in gonadotropes has been well-defined *in vitro* and *in vivo,* and it is clear that SMAD3, SMAD4, and FOXL2 are necessary transcription factors for *Fshb* expression[390,658,674,675,679-681]. However, the exact identity of the receptors initiating this pathway *in vivo* is unknown. Global *Acvr2a* knockout mice have decreased serum FSH levels, but produce residual FSH[655]. Given the absence of *Amhr2* and *Tgfbr2* expression in murine gonadotropes[267,268,656], BMPR2 and ACVR2B are the only type II receptors that could mediate TGFβ signaling in the absence of ACVR2A. Gonadotrope-specific *Bmpr2* cKO mice have normal FSH levels[657], suggesting that ACVR2B may contribute to FSH production. However, activins do not signal through ACVR2B to activate the *Fshb* promoter in a gonadotrope-like cell line[585]. It may be that the residual FSH production in *Acvr2a* global knockouts is the product of GnRH signaling. Though possible, it is important to note that GnRH was unable to compensate for impaired activin signaling in gonadotrope-specific *Smad4*/*Foxl2*[680] and *Smad3*/*Smad4*[658] dcKO mice. Therefore, in Chapter 4, I describe gonadotrope-specific *Acvr2a*, *Acvr2b*, and *Acvr2a*/*Acvr2b* cKO mice to test the hypothesis that ACVR2B plays a necessary role in FSH production *in vivo*.

Murine FSH production depends on the activin type II receptors ACVR2A and ACVR2B

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ABSTRACT

Activins are selective regulators of follicle-stimulating hormone (FSH) production by pituitary gonadotrope cells. In a gonadotrope-like cell line, LβT2, activins stimulate FSH via the activin type IIA receptor (ACVR2A) and/or bone morphogenetic protein type II receptor (BMPR2). Consistent with these observations, FSH is greatly reduced, though still present, in global *Acvr2a* knockout mice. In contrast, FSH production is unaltered in gonadotrope-specific *Bmpr2* knockout mice. In light of these results, we questioned whether an additional type II receptor might mediate the actions of activins or related TGFβ ligands in gonadotropes. We focused on the activin type IIB receptor (ACVR2B), even though it does not mediate activin actions in the gonadotrope-like cell line. Using a Cre-lox strategy, we ablated *Acvr2a* and/or *Acvr2b* in murine gonadotropes. The resulting conditional knockout (cKO) animals were compared to littermate controls. *Acvr2a* cKO (cKO-A) females were subfertile (~70% reduced litter size), cKO-A males were hypogonadal, and both sexes showed marked decreases in serum FSH levels compared to controls. *Acvr2b* cKO (cKO-B) females were subfertile (~20% reduced litter size), cKO-B males had a moderate decrease in testicular weight, but only males showed a significant decrease in serum FSH levels relative to controls. Simultaneous deletion of both *Acvr2a* and *Acvr2b* in gonadotropes led to profound hypogonadism and FSH deficiency in both sexes; females were acyclic and sterile. Collectively, these data demonstrate that ACVR2A and ACVR2B are the critical type II receptors through which activins or related TGFβ ligands induce FSH production in mice *in vivo*.

INTRODUCTION

The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced by gonadotrope cells in the anterior pituitary gland, and are key regulators of gonadal function in males and females [17,53,61,62]. These dimeric hormones are composed of a common alpha subunit (CGA, encoded by *Cga*) non-covalently linked to hormone-specific beta subunits (LHβ and FSHβ, encoded by *Lhb* and *Fshb*). CGA is produced in excess; therefore, expression of the beta subunits is rate-limiting in gonadotropin synthesis [284,285]. Beta subunit expression is regulated by hypothalamic gonadotropin-releasing hormone (GnRH), which binds to its receptor (GnRHR, encoded by *Gnrhr*) on the gonadotrope cell surface [483,728,729]. *Fshb* expression is also strongly regulated by TGFβ superfamily ligands such as the activins. Activins stimulate *Fshb* transcription and their mechanisms of action have been extensively investigated *in vitro* [659,666,731,788,789] and *in vivo* [390,658,680].

Activins are dimeric ligands and bind type II serine-threonine kinase receptors on the cell surface of gonadotropes. Upon binding, the ligand-type II receptor complex recruits type I receptor serine-threonine kinases, which are phosphorylated and activated by the type II receptors. The type I receptors then phosphorylate intracellular SMAD proteins (mainly SMAD3 in this system), which complex with SMAD4 and accumulate in the nucleus. There, they act as transcription factors together with forkhead box L2 (FOXL2) to promote *Fshb* transcription [390,672-674,680,681]. While it is clear that FSH production absolutely depends on this signaling cascade, the specific type II and type I receptors required for *Fshb* expression *in vivo* are not known. Here, we focused on the type II receptors.

Within the TGFβ family, there are five type II receptors: TGFBR2, AMHR2, BMPR2, ACVR2A, and ACVR2B (22). TGFBR2 and AMHR2 are not expressed in murine gonadotropes [267,268,656] and activins preferentially bind ACVR2A, ACVR2B, and BMPR2 [585,790]. Activin induction of *Fshb* promoter activity requires ACVR2A and/or BMPR2, but not ACVR2B, in LβT2 cells (a murine gonadotrope-like cell line) [585]. In contrast, FSH levels are reduced in global *Acvr2a* knockout mice [655], but not in mice lacking BMPR2 specifically in gonadotropes [657]. A role for ACVR2B in FSH production has not been investigated *in vivo,* in part because *Acvr2b* global

knockout mice die shortly after birth [791]. Collectively, these data suggest that ACVR2A is either the only TGFβ type II receptor required for quantitatively normal FSH production *in vivo* or that a second type II receptor, most likely ACVR2B, compensates in its absence. To test this hypothesis, we generated animals in which *Acvr2a*, *Acvr2b*, or both were specifically ablated in murine gonadotropes using the Cre-lox system.

MATERIALS AND METHODS

Animals

The *Acvr2a*$^{fx/fx}$, *Acvr2b*$^{fx/fx}$, and *Gnrhr*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (GRIC) mice were described previously [283,792,793]. *Acvr2a*$^{fx/fx}$ males were crossed with GRIC females to generate *Acvr2a*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ progeny. *Acvr2a*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females were then crossed to *Acvr2a*$^{fx/fx}$ males to generate *Acvr2a*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (control) and *Acvr2a*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (conditional knockout; *Acvr2a* cKO [cKO-A]) animals. The same strategy was used to generate *Acvr2b*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (control) and *Acvr2b*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (*Acvr2b* cKO [cKO-B]) animals. *Acvr2a*$^{fx/fx}$ and *Acvr2b*$^{fx/fx}$ mice were crossed to generate *Acvr2a*$^{fx/+}$;*Acvr2b*$^{fx/+}$ animals, which were then intercrossed to generate *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ mice. *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ males were crossed to GRIC females to generate *Acvr2a*$^{fx/+}$;*Acvr2b*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females. These females were then mated to *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ males to generate *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (control) and *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (double cKO; dcKO) animals. Genotyping and assessment of genomic recombination were conducted as previously described [723] (primers listed in Table 1). Animals were housed *ad libitum* on a 12:12 lights on/lights off cycle (lights on at 7:00, lights off at 19:00). All animal experiments were performed in accordance with institutional and federal guidelines and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (Protocol 5204).

To purify gonadotropes by fluorescence-activated cell sorting (FACS), we crossed *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ animals with *Gt(ROSA26)*$^{ACTB\text{-}tdTomato\text{-}EGFP}$ mice (*Rosa26*$^{mTmG/mTmG}$, stock 007676 from Jackson Laboratories) to eventually generate *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$; *Rosa26*$^{mTmG/mTmG}$ males, which were then crossed to *Acvr2a*$^{fx/+}$;*Acvr2b*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females to

produce *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ males and females. Controls for FACS were generated by crossing *Rosa26*$^{mTmG/mTmG}$ and GRIC mice to produce *Acvr2a*$^{+/+}$;*Acvr2b*$^{+/+}$; *Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ males and females. FACS of gonadotropes was performed at the Flow Cytometry Core at the Montreal Clinical Research Institute (IRCM). Protocols for pituitary cell dispersion and cell sorting were adapted from previous publications [658,748]. Briefly, we dispersed 3-4 pituitary glands per digestion per genotype per sex. Each sample was then sorted individually, and we obtained ~2.0x10^4 EGFP-positive and ~4.4x10^5 tdTomato-positive cells per sample. Male and female cells of a given genotype were then pooled together for RNA analysis.

Blood collection

Blood was collected from 8- to 10-week-old male or 9- to 10-week-old female control and cKO animals by cardiac puncture. Females were euthanized at 0700 h on estrous morning. As dcKO females were acyclic, they were collected alongside controls at 0700 h. Blood was allowed to clot for 30-60 min at room temperature, and was then spun down at 3,000 rpm for 10 min to collect serum. Sera were stored at -80^{o}C until assayed for LH and FSH.

Hormone analyses

Serum FSH was assessed with Milliplex kits (Millipore-Sigma, MPTMAG-49K [custom-made for FSH only], Oakville, Ontario, Canada) following the manufacturer's instructions (lower detection limit (LOD): 23.7 pg/mL; dynamic range: 61.0 pg/mL to 250,000 pg/mL; limit of quantification (LOQ): 61.0 pg/mL; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described [658,775,776] (LOD: 0.117 ng/mL; dynamic range: 0.117 ng/mL to 30 ng/mL; LOQ: 0.516 ng/mL ; intra-assay CV < 10%). Serum FSH and LH values that fell below each assay's LOQ were set to the LOQ for purposes of statistical analyses and plotting of the data.

The LOQ for each assay was defined as the lowest concentration of analyte that could be reliably quantified. As described elsewhere [794], the LOQ was calculated by adding two times the standard deviation of blank matrix absorbance values to the mean absorbance value of the blanks (based on six independent experiments). In other words, LOQ = A + 2σ, where A is the mean

absorbance value of the blank matrix (i.e., no analyte present), and σ is the standard deviation of absorbance values using blank matrix. The obtained absorbance value (from A + 2σ) was then interpolated on a standard curve.

Organ collection

Pituitary glands, testes, and seminal vesicles were dissected from 8- to 10-week-old control and cKO males. Control and cKO females were sacrificed at 9- to 10-weeks of age at 0700 h on the morning of estrus (confirmed by assessing vaginal cytology), at the approximate time of the secondary FSH surge, to collect pituitary glands and ovaries. Acyclic dcKO females were collected along with control females at 0700 h. Pituitary glands were snap frozen in liquid nitrogen and stored at -80^{o}C. All other organs were weighed on an analytical scale.

One ovary per female was fixed in 10% formalin (HT501128, Millipore-Sigma, Oakville, Ontario, Canada) overnight at room temperature, and then stored in 70% ethanol. Fixed ovaries were sent for embedding, sectioning, and hematoxylin/eosin staining at the McGill Centre for Bone and Periodontal Research. Follicle counting (done by H.S. and blinded to genotype) was done on fully cut-through ovaries using an inverted microscope. For each female, the other ovary was snap frozen in liquid nitrogen and stored at -80^{o}C.

One testis per male was immersed in Bouin's fixative solution (1120-16, Ricca Chemical Company, Pocomoke City, MD, USA) overnight at room temperature, followed by an overnight incubation in 100% ethanol. Finally, the testis was left in 70% ethanol and processed like the ovaries at the McGill Centre for Bone and Periodontal Research. For each male, the second testis was homogenized in a solution containing 10% DMSO and 0.9% NaCl. Sperm were then counted in a 0.1% trypan blue solution on a hemocytometer. The data represent the average of two counts performed by two independent observers (G.S. and L.O.; both were blinded to genotype).

Assessment of female puberty onset, estrous cyclicity, and fertility

Vaginal opening was monitored daily following weaning (postnatal day 21). At 6 weeks of age, females were swabbed daily for three weeks to assess estrous cyclicity. Vaginal cytology was assessed using 0.1% methylene blue, following previously established guidelines [774].

At 9 weeks of age, females were mated with wild-type, age-matched C57BL/6 males (Charles River, Senneville, Quebec, Canada) for 3-4 months. Breeding cages were monitored daily to record the number of pups produced. Pups were euthanized two weeks after birth.

Primary pituitary cultures

Pituitaries from 8- to 10-week-old male and female mice (*Acvr2a*$^{fx/fx}$, *Acvr2b*$^{fx/fx}$, or *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$) were extracted and dispersed as previously described [657,692,710]. On day 1, cells pooled from both sexes were seeded in 48-well plates (250,000-400,000 cells/well). At the time of plating, cells were treated with adenoviruses expressing GFP (Ad-GFP) or Cre-IRES-GFP (Ad-Cre) (Baylor College of Medicine Vector Development Laboratory, Houston, Texas, USA) at a multiplicity of infection of 600 in medium 199 (M199; 31100-035, Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; 10438026, ThermoFisher Scientific, Burlington, Ontario, Canada). Approximately 24 h later, on day 2, the medium was changed to fresh culture medium (10% FBS) without viruses. On day 4, RNA was harvested as described below.

Reverse transcription and quantitative-PCR

Pituitaries and ovaries were homogenized in TRIzol reagent (15596018, ThermoFisher Scientific, Burlington, Ontario, Canada), and total RNA was extracted following the manufacturer's guidelines. For FACS samples and primary pituitary cultures, total RNA and DNA were extracted using Total RNA Mini Kits (Geneaid, RB300, New Taipei City, Taiwan).

Reverse transcription was performed as previously described [777] using Moloney murine leukemia virus reverse transcriptase (0000172807, Promega, Madison, WI, USA) and random hexamers (0000184865, Promega, Madison, WI, USA). qPCR runs were conducted on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen qPCR Mastermix (ABMMmix-S-XL; Diamed, Mississauga, Ontario, Canada) and the primers listed in Table 2. Expression levels of genes of interest were determined using the $2^{-\Delta\Delta Ct}$ method (41) and ribosomal protein L19 (*Rpl19*) for normalization (unless otherwise specified). All primers were validated for efficiency and specificity (primers listed in Table 2).

Superovulation

Superovulation was performed in juvenile females (23-30 days of age). Control and dcKO animals were i.p. injected with 5 IU equine chorionic gonadotropin (HOR-272-a, East Brunswick, NJ, USA) between 1600 h and 1700 h. Two days later, females were i.p. injected with 5 IU of human chorionic gonadotropin (C1063, Millipore-Sigma, Oakville, Ontario, Canada) between 1700 h and 1800 h. On the next morning, between 0700 h and 0730 h, mice were euthanized, and cumulus-oocyte complexes (COCs) were harvested from the ampullae of the oviduct. COCs were enzymatically digested with 0.5 mg/ml hyaluronidase (H3884, Millipore-Sigma, Oakville, Ontario, Canada) for 20 min at 37 °C. The number of oocytes was counted (by G.S., who was blinded to genotype) using an inverted microscope.

Gonadectomy

Males and females were gonadectomized at 7-8 weeks of age in compliance with standard operating procedures 206 and 207 of the McGill University and Goodman Cancer Centre Facility Animal Care Committee. Briefly, for males, an incision was made at the midline of the scrotum at the level of the skin, and then the tunica. Each testis was pulled out with tweezers, and the tissue was cauterized at the level of the fat pad such that entire testes and epididymides could be removed. The wound was then closed with sutures and veterinary glue. For females, an incision was made at the midline of the mid-dorsum of the animal. A small incision was made in the muscle above the ovary on each side, and the ovary pulled out with forceps by the surrounding fat pad. The oviduct was then cauterized so that the entire ovary could be removed. All incisions were closed by sutures. In the case of sham-operated animals, all the procedures were the same, except that there was no cauterization. Animals were left to recover for two weeks following surgery, at which point they were euthanized to collect serum and pituitary glands. All analyses from gonadectomized animals were conducted as described above, except that control females were collected at random estrous cycle stages.

Statistical analysis

All data were analyzed on GraphPad Prism 8 using Student *t*-tests, unless otherwise specified. In situations where two-way ANOVA was used, analyses were corrected for multiple comparisons using the Holm-Sidak method (all *p*-values were adjusted for multiple comparisons). Results were considered statistically significant when $p<0.05$.

RESULTS

Generation of Acvr2a and Acvr2b conditional knockout mice

To investigate the relative roles of ACVR2A and ACVR2B in gonadotropes, we generated gonadotrope-specific *Acvr2a* and *Acvr2b* conditional knockout (cKO-A and cKO-B) mice. First, we examined the specificity of *Acvr2a* and *Acvr2b* gene recombination in the conditional KO animals. To that end, we collected several tissues from cKO-A or cKO-B males and females and extracted genomic DNA. We confirmed by PCR that recombination occurred specifically in the pituitary glands of males and females, and in testes and epididymides of males (Figs. S1A-B [795]). The Cre-driver line used here (GRIC) is active in the male germline in addition to gonadotropes [283,475,658,725]. Therefore, the recombination in testes and epididymides was expected. It is for this reason that the GRIC allele is always introduced via the female parent.

We then generated animals in which both *Acvr2a* and *Acvr2b* were ablated in gonadotropes (double conditional knockouts, dcKOs). We detected little to no *Acvr2a* and *Acvr2b* mRNA in purified gonadotropes of dcKOs relative to control mice (~93-94% reduction) (GFP+ cells in Figs. S1C-D [795]). In contrast, expression of both receptors was intact in non-gonadotropes from dcKO mice (Tomato+ cells in Figs. S1C-D [795]). *Bmpr2* mRNA expression was intact in both cell populations in dcKOs (Fig. S1E [795]). Finally, we confirmed by PCR that Cre-mediated recombination of the floxed alleles occurred exclusively in the purified gonadotrope population (Fig. S1F [795]).

FSH production is impaired in male and female Acvr2a cKO mice

Serum FSH levels were significantly reduced in male and female *Acvr2a* cKO (cKO-A) animals compared to controls (Figs. 1A and 1C). The average serum FSH levels were 19.6 ng/mL (+/- 4.7 ng/mL) and 0.52 ng/mL (+/- 0.15 ng/mL) in control and cKO-A males, respectively. In females, they were 2.78 ng/mL (+/- 2.97 ng/mL) in control and 0.65 ng/mL (+/- 0.48 ng/mL) in cKO-A mice. Serum LH levels were unaffected in cKO-A males (Fig. 1B) but elevated in cKO-A females (Fig. 1D). Pituitary analyses revealed decreased *Fshb*, *Lhb*, and *Cga* mRNA expression in cKO-A relative to control males (Fig. 1E). *Gnrhr* mRNA levels were normal in cKO-A males. *Fshb* expression was decreased in cKO-A females, whereas their *Lhb* and *Gnrhr* levels were significantly elevated (Fig. 1F). *Cga* expression was unaltered in female cKO-A animals relative to controls. The variability in serum FSH and pituitary *Fshb* levels in control females (for this and the following models) likely stemmed from the fact that some females had completed the secondary FSH surge by 0700 h on the morning of estrus while others had not.

In primary pituitary cultures from *Acvr2a*$^{fx/fx}$ animals, *Fshb* mRNA expression levels were reduced by ~60% in cells transduced with a Cre-expressing adenovirus (Ad-Cre), relative to cells transduced with a GFP-expressing adenovirus (Ad-GFP; Fig. S2A [795]). *Acvr2a* mRNA levels were reduced by ~85% in Ad-Cre relative to Ad-GFP cells (Fig. S2A [795]).

Acvr2a cKO mice are hypogonadal

cKO-A males exhibited decreased testicular weights compared to controls (Fig. 2A), while their seminal vesicles weights were unaffected (Fig. S3A [795]). The hypogonadism was associated with a decrease in testicular sperm counts (Fig. 2B), with no structural abnormalities in the testes (Fig. S3B [795]).

Female cKO-A mice did not display impairments in puberty onset (measured by vaginal opening, Fig. S3C [795]) or estrous cyclicity (Fig. S3D [795]). When paired to wild-type males, however, cKO-A females had significantly smaller litters compared to controls (Fig. 2C), despite producing litters at normal intervals (data not shown). cKO-A females had smaller ovaries than controls (Fig. 2D-E), which was associated with a reduced number of antral follicles (Fig. 2F) and corpora lutea (Fig. 2G).

Male, but not female, Acvr2b cKO mice show a mild impairment in FSH production

Acvr2b cKO (cKO-B) males showed a measurable impairment in FSH production relative to controls (Fig. 3A; from 15.2 ng/mL (+/- 5.06 ng/mL) in controls to 7.52 ng/mL (+/- 1.77 ng/mL) in cKO-Bs), with serum LH unaffected (Fig. 3B). In contrast, FSH and LH levels did not differ between cKO-B and control females (Figs. 3C-D). Mean serum FSH levels were 2.51 ng/mL (+/- 2.18 ng/mL) and 2.40 ng/mL (+/- 1.88 ng/mL) in control and cKO-B females, respectively. In the pituitary glands, there were no significant changes in gene expression in *Fshb*, *Lhb*, or *Cga* in males (Fig. 3E) or females (Fig. 3F). However, *Gnrhr* mRNA levels were statistically significantly increased in both male and female cKO-B animals relative to controls (Figs. 3E-F).

In primary pituitary cultures from *Acvr2b*$^{fx/fx}$ animals, *Fshb* mRNA expression levels were not statistically significantly different between Ad-GFP and Ad-Cre-transduced cells (Fig. S2B [795]). However, *Acvr2b* mRNA levels were reduced by ~85% in Ad-Cre transduced cells, relative to controls (Fig. S2B [795]).

Acvr2b cKOs show milder reproductive phenotypes than Acvr2a cKOs

Gonadotrope-specific *Acvr2b* deletion in males led to a decrease in testicular weight (Fig. 4A) with no change in seminal vesicle weight (Fig. S4A [795]). However, there were no detectable changes in testicular sperm production or testis morphology between genotypes (Fig. 4B and S4B [795]).

cKO-B females had normal puberty onset (Fig. S4C [795]) and estrous cyclicity (Fig. S4D [795]) relative to controls; however, they produced modestly smaller litters relative to controls when paired to wild-type males (Fig. 4C). While cKO-B females had no measurable changes in ovarian weights (Fig. 4D) or ovarian morphology (Fig. 4E), their ovaries contained fewer antral follicles (Fig. 4F) and corpora lutea than controls (Fig. 4G).

Pituitary FSH production is abolished in dcKO mice

Serum FSH was below detection levels in *Acvr2a* and *Acvr2b* dcKO males (Fig. 5A), whereas their serum LH levels were normal (Fig. 5B). dcKO females similarly had low to undetectable serum FSH (Fig. 5C), but also exhibited elevated serum LH levels (Fig. 5D) compared

to controls. FSH, but not LH, protein levels were also profoundly decreased in pituitaries of dcKO males and females, as assessed by immunofluorescence (Figs. S5A-B [795-800]). dcKO males showed decreased pituitary *Fshb*, *Lhb*, and *Cga* mRNA levels (Fig. 5E), while expression of *Fshb* was decreased and *Lhb* increased in dcKO females (Fig. 5F). *Gnrhr* expression was normal in dcKO males (Fig. 5E), whereas *Gnrhr* was elevated and *Cga* was normal in dcKO females (Fig. 5F).

In primary pituitary cultures from *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ animals, *Fshb* mRNA expression levels were reduced by ~70% in cells transduced with Ad-Cre, relative to cells transduced with ad-GFP (Fig. S2C [795]). In Ad-Cre transduced cells, *Acvr2a* and *Acvr2b* mRNA levels were reduced by ~90% and 80%, respectively (Fig. S2C [795]).

Acvr2a and Acvr2b dcKO mice are hypogonadal and females are sterile

Consistent with the hormone data, testicular (Figs. 6A and S6A [795]) but not seminal vesicle weights (Figs. S6A-B [795]) were significantly reduced in dcKO males relative to controls. Testicular sperm production in these animals was also impaired (Fig. 6B) without morphological changes in testicular structure (Fig. S6C [795]).

dcKO females were sterile, hypogonadal, and had thread-like uteri (Figs. 6C, 6D, and S6D [795]). There was no delay in vaginal opening in dcKO females (Fig. S6E [795]), but they did not exhibit estrous cycles (Fig. S6F [795]). Consistent with their sterility, dcKO females had no late antral follicles or corpora lutea in their ovaries (Fig. 6E). Expression of *Cyp19a1* (encoding aromatase), an FSH target gene, was significantly decreased in ovaries of dcKOs relative to controls (Fig. S6G [795]). To examine ovarian integrity in these animals, we superovulated control and dcKO females. Both genotypes were able to ovulate in response to exogenous gonadotropins, although dcKO females ovulated fewer oocytes on average compared to controls (Fig. S6H [795]).

To assess potential *Acvr2a* and *Acvr2b* gene dosage effects, we measured gonadal weights in males and females with varying degrees of allelic recombination of *Acvr2a* and/or *Acvr2b* (Fig. S7A-B [795]). Of note, the loss of a single *Acvr2a* allele did not decrease testicular weights relative to controls (Fig. S7A [795]; see *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{+/+}$;*Gnrhr*$^{+/+}$ compared to *Acvr2a*$^{fx/+}$;*Acvr2b*$^{+/+}$;*Gnrhr*$^{GRIC/+}$); however, in the context of ACVR2B-deficient gonadotropes, the loss of a single *Acvr2a* allele led to a decrease in gonadal weights (see

Acvr2a$^{fx/fx}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{+/+}$ compared to *Acvr2a*$^{fx/+}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$). Finally, though *Acvr2a*$^{fx/+}$;*Acvr2b*$^{+/+}$;*Gnrhr*$^{GRIC/+}$ females were not investigated, *Acvr2a*$^{fx/+}$;*Acvr2b*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ females did not appear to have a significant decrease in ovarian weights (Fig. S7B [795]).

Sex differences in LH levels in dcKO mice are dependent on gonadal and extra-gonadal factors

LH levels were elevated in dcKO females (Fig. 5D), but not males (Fig. 5B). This could have stemmed from the loss of estrogen negative feedback in females (Fig. S6G [795]) or might reflect a sex difference in LH regulation via ACVR2A and ACVR2B at the pituitary level. To begin to address these possibilities, we compared sham-operated or gonadectomized control and dcKO mice. In males, castration (GDX) led to a modest increase in serum FSH levels in control animals, whereas FSH remained undetectable in both sham-operated and castrated dcKOs (Fig. 7A). Both genotypes displayed increases in serum LH following castration, though levels were lower in dcKO males relative to controls (Fig. 7B). These results were paralleled by the pattern of pituitary mRNA expression. *Fshb* in control, but not dcKO males, modestly increased post-castration (Fig. 7C). *Lhb* and *Cga* expression increased in males of both genotypes post-castration (Figs. 7D-E), though levels were lower in dcKOs relative to controls. *Gnrhr* mRNA levels were not significantly different between groups (Fig. 7F).

In females, serum FSH increased markedly post-ovariectomy (OVX) in control mice, but remained undetectable in intact and OVX dcKOs (Fig. 8A). In sham-operated females (collected at random estrous cycle stages, as opposed to morning of estrus in Fig. 5), serum LH levels were increased in dcKO relative to control mice (Fig. 8B). Post-OVX, LH levels increased in control but not dcKO females (Fig. 8B). Pituitary gene expression matched the serum hormone levels, with *Fshb* and *Lhb* increasing only in control females following OVX (Fig. 8C-D). In dcKO females, *Fshb* and *Lhb* expression remained low and high, respectively (Fig. 8C-D). Importantly, there were no differences in *Lhb* mRNA levels between OVX controls and dcKOs (Fig. 8D). *Cga* expression patterns closely matched those of *Lhb* (Fig. 8E), while *Gnrhr* expression was not significantly different between groups (Fig. 8F).

DISCUSSION

We generated conditional *Acvr2a* and *Acvr2b* knockout mice to investigate the relative role of these TGFβ family type II receptors in gonadotrope function *in vivo*. Our data suggest that FSH production is dependent on ACVR2A and, to a lesser extent, ACVR2B. Given the high recombination efficiency in the context of the double conditional (dcKO) mice, it is unlikely that the residual reproductive function and FSH production observed in the single *Acvr2a* or *Acvr2b* cKO animals stemmed from preservation of *Acvr2a* or *Acvr2b* expression.

ACVR2A and ACVR2B are required for FSH production in murine gonadotropes

ACVR2A had already been suggested to be a necessary type II receptor for FSH production *in vivo*, based on the phenotype of *Acvr2a* global knockout mice [655]. By specifically ablating *Acvr2a* in gonadotropes, we both confirmed and extended these earlier observations. One important difference between the two studies is the extent to which FSH was reduced. While we observed a reduction of ~98% and ~80% in serum FSH levels in *Acvr2a* cKO males and females, respectively, *Acvr2a* global KO animals (of both sexes) showed a ~60% reduction in FSH [655]. Inter-study variation may stem from differences in the genetic backgrounds of the mice and/or from the hormone assays used. Indeed, there were reportedly no sex differences in serum FSH levels in either global *Acvr2a* KOs or their wild-type counterparts [655], even though male mice are well known to have higher FSH levels than females [389,390,801], as we show here. Moreover, the loss of ACVR2A outside of gonadotropes in the global KOs, such as in the gonads, may have contributed to the milder decrease in serum FSH levels observed in these animals relative to *Acvr2a* cKO mice.

Despite differences between the two studies, FSH was still detected in circulation in both the global and gonadotrope-specific *Acvr2a* knockout models, raising the possibility that another type II receptor may compensate for the absence of ACVR2A. Given that *Amhr2* and *Tgfbr2* are not expressed in gonadotropes [267,268,656], and that gonadotrope-specific *Bmpr2* cKO animals produce FSH at normal levels [657], ACVR2B was the only other candidate type II receptor remaining that might mediate TGFβ signaling in gonadotropes.

Gonadotrope-specific *Acvr2b* cKO mice showed milder forms of the phenotypes observed in *Acvr2a* cKO mice. This was most notable in males, which had detectable decreases in testicular weights and serum FSH levels. *Acvr2b* cKO females produced smaller litters and had impaired folliculogenesis relative to controls, though their FSH production was apparently normal. It is possible that we missed alterations in serum FSH and *Fshb* mRNA expression in these mice due to intrinsic biological variability. Regardless, ACVR2B's role in gonadotropes of both males and females was made evident by the complete loss of FSH production in dcKO animals, and the sterility observed in dcKO females. The data conclusively demonstrate that FSH production in mice depends on ACVR2A and ACVR2B in gonadotropes.

Sex differences

Interestingly, ablation of *Acvr2a* alone or in combination with *Acvr2b* produced sex-specific effects. First, *Acvr2a* cKO and dcKO males, but not females, had decreased pituitary *Lhb* and *Cga* mRNA levels. We attempted to elucidate the basis for these sex differences. Castrated dcKO males continued to express reduced levels of *Lhb* and *Cga* relative to controls, indicating that androgens or other testicular factors likely do not explain the sex differences in *Lhb* and *Cga* expression. Moreover, there did not appear to be a 'protective' factor from the ovary, as *Lhb* and *Cga* mRNA levels were not reduced in ovariectomized dcKO relative to control females. It may be that TGFβ signaling regulates *Lhb* and *Cga* expression [284,543,691,802] *in vivo* either alone or in combination with GnRH [693,803,804]. However, it remains unclear why this signaling and regulation might be male-specific.

Second, we observed that *Acvr2a* cKO and dcKO females, but not males, had elevated *Lhb* mRNA expression and serum LH levels relative to controls. We previously reported similar observations in other FSH-deficient models and argued that this may reflect endocrine effects [658,680]. Although we did not measure estradiol levels here because of concerns about the reliability of estradiol assays in mice [658,680,781], *Cyp19a1* mRNA levels were decreased in *Acvr2a*/*Acvr2b* dcKO ovaries relative to controls. As a consequence, estradiol production was likely impaired in these mice. This is supported by their hypoplastic uteri, a biological indicator of hypoestrogenemia. The loss of estradiol negative feedback in *Acvr2a*/*Acvr2b* dcKO females could

therefore explain their increased LH. This idea is substantiated by the equivalency of LH levels in ovariectomized dcKO and control females. In males, testosterone is the principal testicular steroid that negatively regulates GnRH and LH secretion. Testosterone production is FSH independent, helping to explain why elevated LH levels were not observed in dcKO males. Indeed, their LH levels were reduced, likely due to reductions in *Lhb* and *Cga* expression.

Are activins the TGFβ ligands driving FSH synthesis in vivo?

Our observations raise questions regarding the necessity for activins in FSH production *in vivo*. In the gonadotrope-like cell line, LβT2, activins act via ACVR2A and BMPR2, but not ACVR2B, to regulate *Fshb* transcription [585]. However, mice lacking *Bmpr2* expression in gonadotropes produce FSH normally [657] and the data reported here clearly indicate that the TGFβ ligand regulating FSH *in vivo* acts via ACVR2A and ACVR2B. Therefore, activins either use different receptors in the cell line than in *bona fide* gonadotropes or a non-activin TGFβ ligand may be the principal driver of FSH production *in vivo*. Elevated, rather than reduced, FSH levels in activin B-deficient mice [633], as well as other unpublished observations from our lab, are consistent with the latter possibility.

Study limitations

It is important to note some potential limitations of the study. First, Cre activity in the GRIC mice starts at embryonic day 12.75 [283]. Therefore, it is possible that the phenotypes observed in the different conditional knockout strains stemmed from defects in gonadotrope development. However, we also observed robust decreases in *Fshb* expression following *Acvr2a*, but not *Acvr2b*, knockdown in pituitary cultures from adult mice (Fig. S2 [795]). The loss of both *Acvr2a* and *Acvr2b* led to an even more pronounced decrease in *Fshb* mRNA levels. These data indicate that: 1) the endogenous pituitary TGFβ ligand that regulates *Fshb* synthesis use(s) ACVR2A and ACVR2B, with preference for ACVR2A, and 2) the effects observed in single cKO and dcKO animals *in vivo* are likely not developmental in nature. Indeed, intact LH production in these mice suggests that gonadotrope development was likely unimpaired.

Second, we assessed the extent of recombination in the double knockout model (Fig. S1 [795]), but not in either of the individual receptor knockout lines. While there is no reason to suspect that recombination efficiency would be lower in the latter lines (especially as there are fewer floxed alleles), it would have been valuable to assess the expression of one receptor subtype in the absence of the other. For example, it is possible that *Acvr2a* may have been upregulated in *Acvr2b* cKO gonadotropes, which could have contributed to the relatively modest FSH phenotype in these mice. Finally, deleting the type II receptors would also be predicted to affect inhibin as well as activin action [705,805,806]. However, impairments in inhibin activity should manifest as increased FSH production [696,710]. In all three models presented here, FSH production was either modestly or profoundly reduced. Therefore, the reported phenotypes are not likely explained by alterations in inhibin action or any such changes are, at least, superseded by the impairments in activin-like signaling.

In summary, TGFβ signaling via ACVR2A and ACVR2B in gonadotropes is required for FSH production in mice. Whether the same receptors mediate activin or activin-like regulation of FSH synthesis in other mammals, including humans, is not yet clear. However, it should be noted that an ACVR2A-Fc fusion protein dose-dependently inhibits FSH levels in post-menopausal women [807]. Similarly, an ACVR2A neutralizing antibody suppresses FSH levels in the same clinical population [808]. Therefore, common TGFβ ligands and type II receptors may regulate FSH production in mice and humans.

DECLARATION OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTION

G.S. and D.J.B. were responsible for the experimental design, data analyses, and manuscript preparation. G.S. conducted most of the experiments. L.O. and X.Z. helped with tissue collection, mouse colony management, and analyses. H.S. was responsible for the follicle counts. Y.W. conducted the FSH assays. E.B. performed the immunofluorescence. U.B. and S.J.L. generated and provided the mouse strains. All authors approved the final version of the manuscript.

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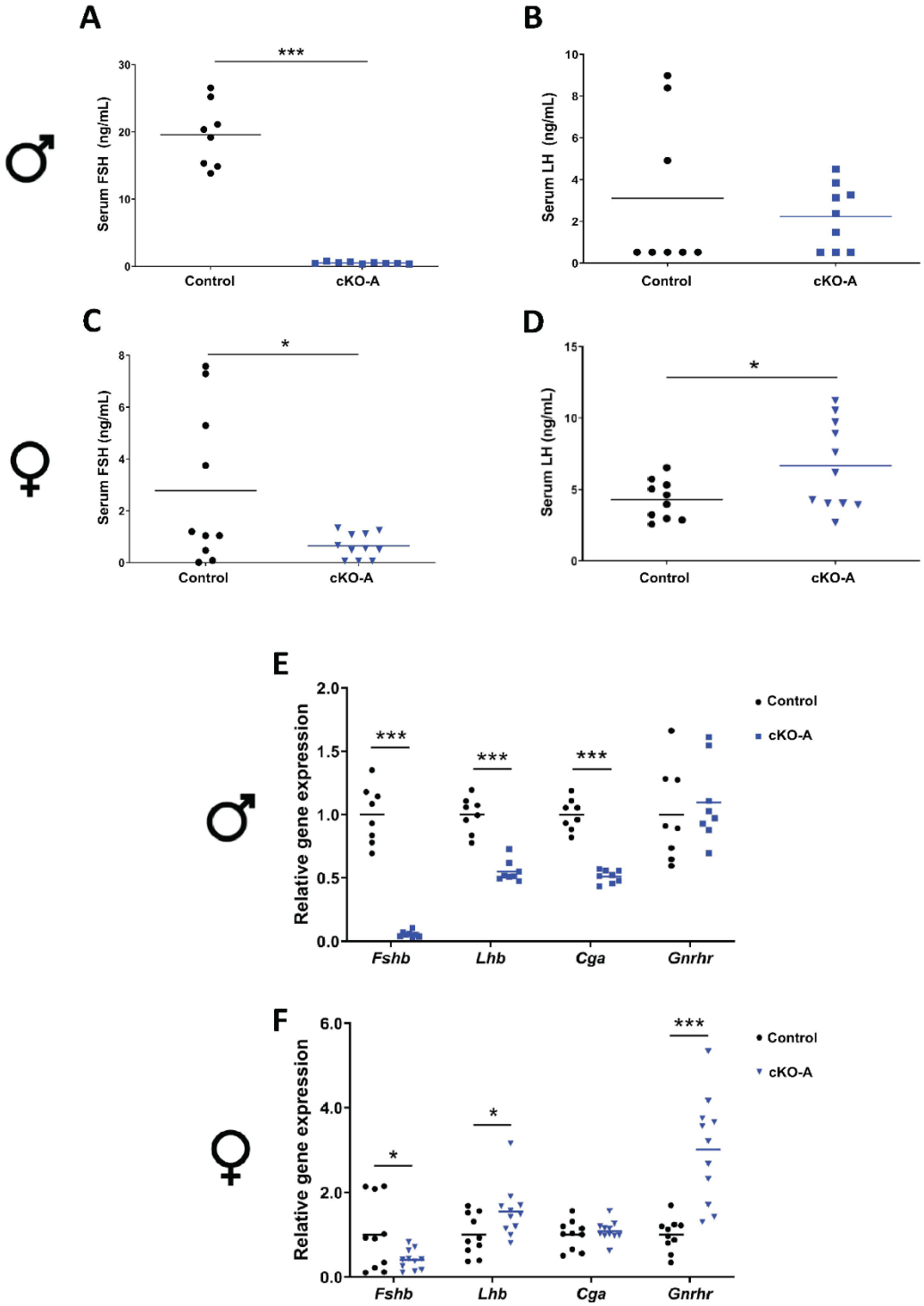
A
B
C
D
E
F
♂
♀
Serum FSH (ng/mL)
Serum LH (ng/mL)
Control
cKO-A
***
*
Relative gene expression
Fshb
Lhb
Cga
Gnrhr

Figure 4.1

Figure 4.1: *Acvr2a expression in gonadotropes is required for quantitatively normal FSH production and Fshb expression in both male and female mice.* Serum FSH levels in control and *Acvr2a* cKO (cKO-A) **(A)** males and **(C)** females. Serum LH levels in control and cKO-A **(B)** males and **(D)** females. Pituitary *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels assessed by RT-qPCR in **(E)** male and **(F)** female control and cKO-A mice. *Rpl19* was used as the housekeeping gene. *t*-tests were used for statistical analyses, **p* <0.05, ****p*<0.001.

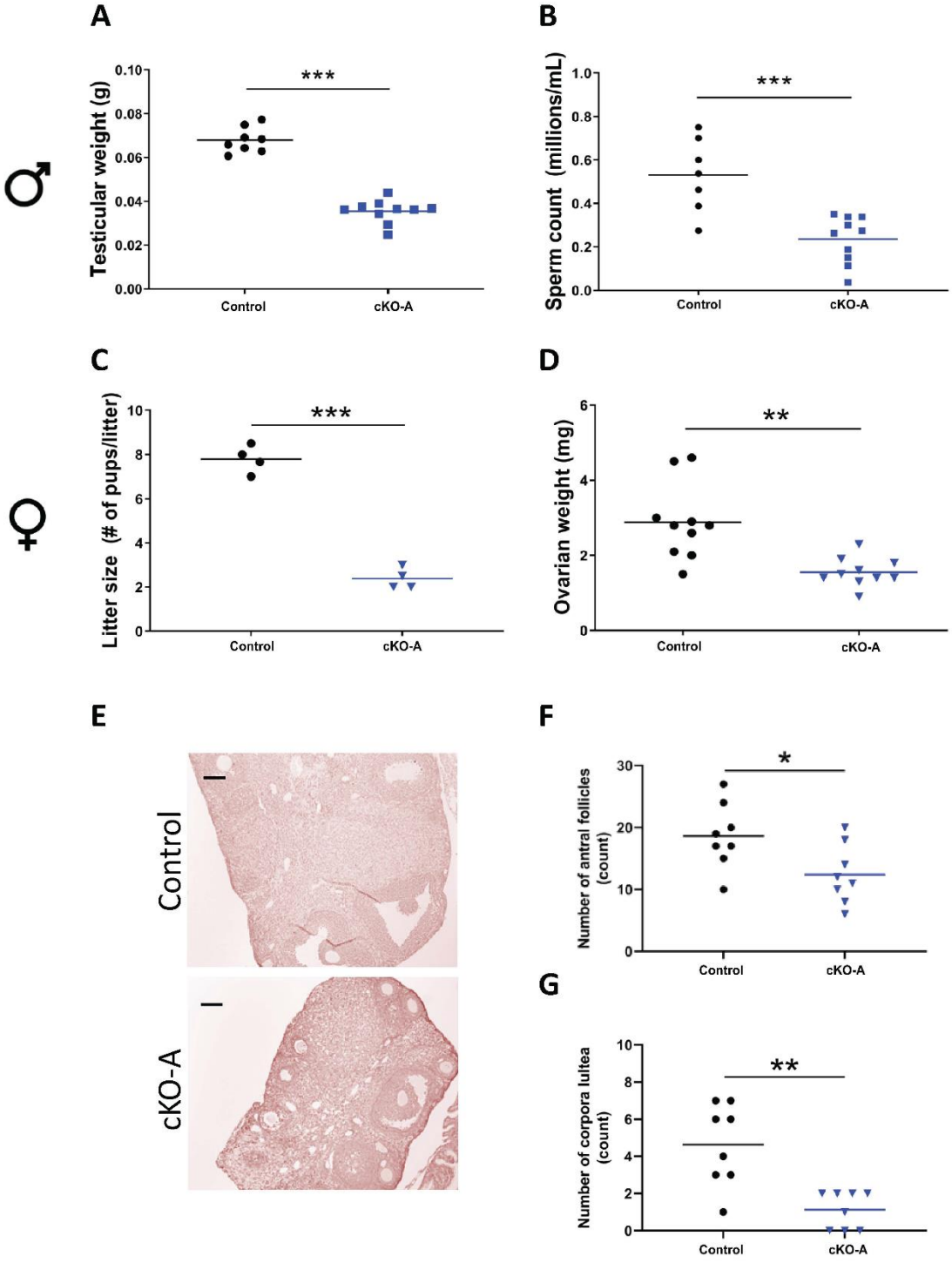
♂
♀
A
Testicular weight (g)
0.10
0.08
0.06
0.04
0.02
0.00
***
Control
cKO-A
B
Sperm count (millions/mL)
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
cKO-A
C
Litter size (# of pups/litter)
10
8
6
4
2
0
***
Control
cKO-A
D
Ovarian weight (mg)
6
4
2
0
**
Control
cKO-A
E
Control
cKO-A
F
Number of antral follicles (count)
30
20
10
0
*
Control
cKO-A
G
Number of corpora lutea (count)
10
8
6
4
2
0
**
Control
cKO-A

Figure 4.2

Figure 4.2: *Acvr2a cKO animals are hypogonadal.* **(A)** Testicular weights and **(B)** testicular sperm counts in 8-10-week-old control and cKO-A males. **(C)** Average litter sizes from control and cKO-A females paired with wild-type males for 3-4 months. Ovarian **(D)** weights, **(E)** histology, **(F)** antral follicle counts, and **(G)** corpora lutea numbers in 9-10-week-old control and cKO-A females. Scale bar: 100 µm. *t*-tests were used for statistical analyses, **p*<0.05, ***p*<0.01, ****p*<0.001.

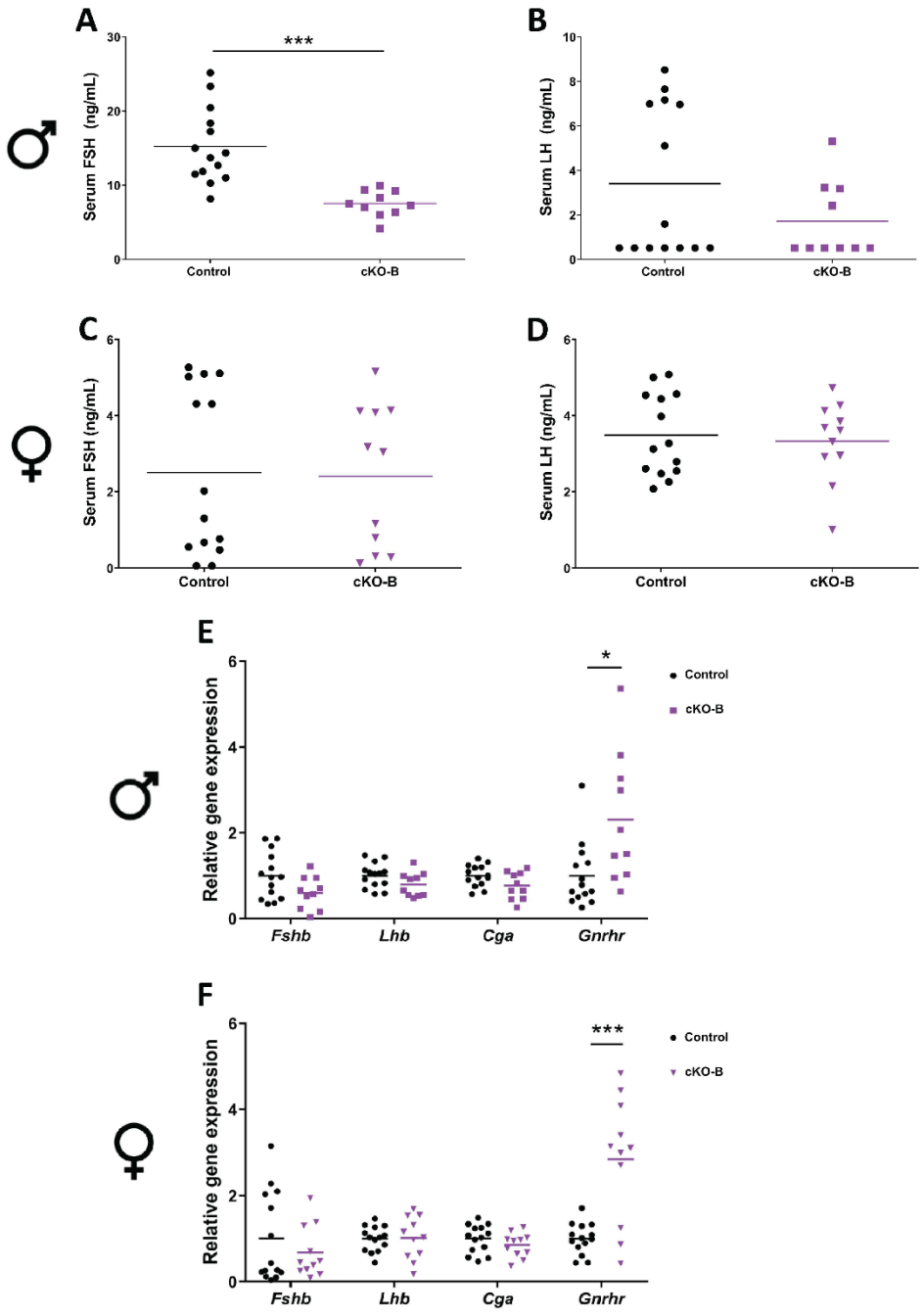
A
B
C
D
E
F
♂
♀
Serum FSH (ng/mL)
Serum LH (ng/mL)
Control
cKO-B
***
*
Relative gene expression
Fshb
Lhb
Cga
Gnrhr

Figure 4.3

Figure 4.3: *Acvr2b expression in gonadotropes is required for quantitatively normal FSH production in male, but not female, mice.* Serum FSH levels in control and *Acvr2b* cKO (cKO-B) **(A)** males and **(C)** females. Serum LH levels in control and cKO-B **(B)** males and **(D)** females. Pituitary *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels assessed by RT-qPCR in **(E)** male and **(F)** female control and cKO-B mice. *Rpl19* was used as the housekeeping gene. *t*-tests were used for statistical analyses, **p* <0.05, ****p*<0.001.

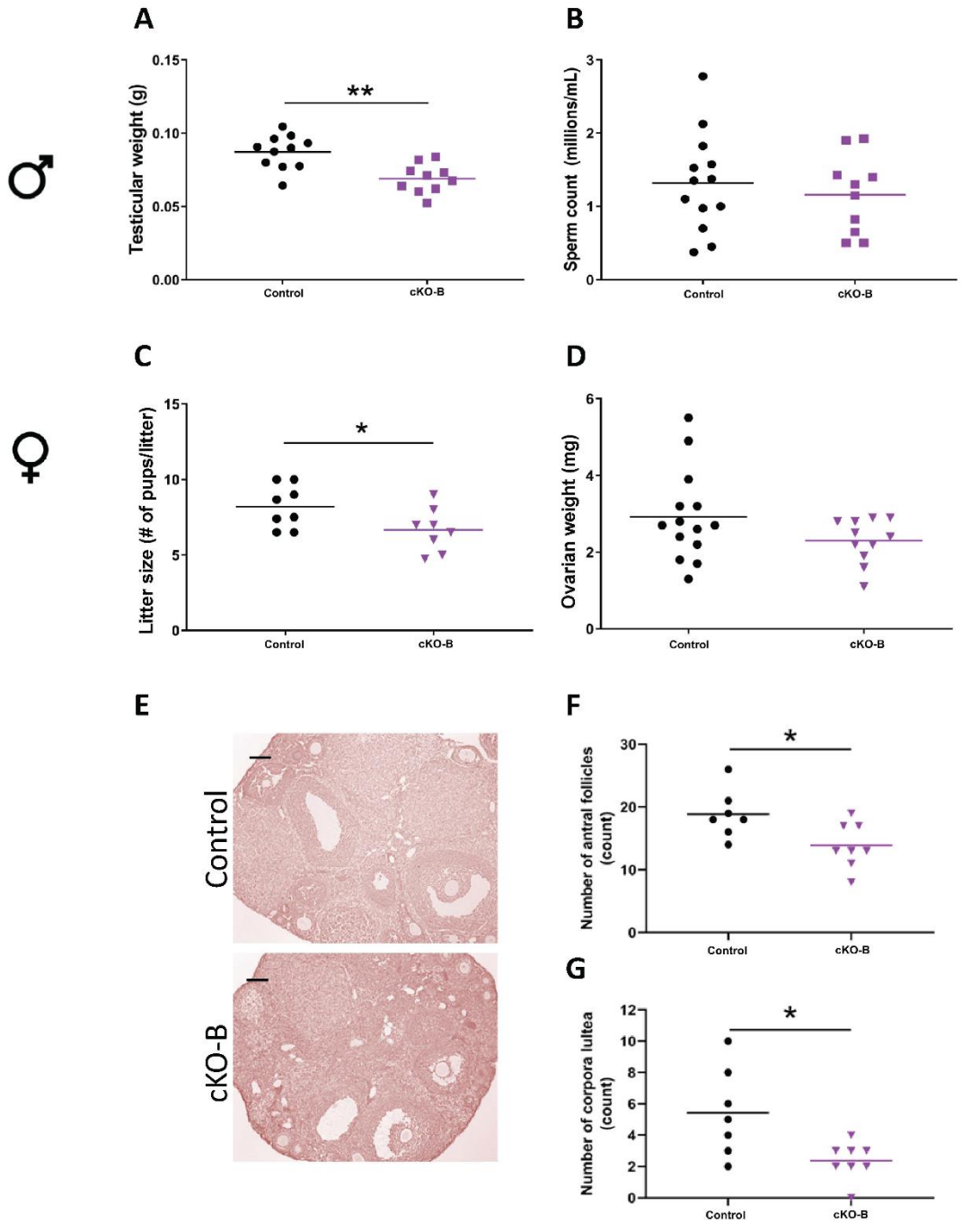
♂
♀
A
Testicular weight (g)
0.15
0.10
0.05
0.00
**
Control
cKO-B
B
Sperm count (millions/mL)
3
2
1
0
Control
cKO-B
C
Litter size (# of pups/litter)
15
10
5
0
*
Control
cKO-B
D
Ovarian weight (mg)
6
4
2
0
Control
cKO-B
E
Control
cKO-B
F
Number of antral follicles (count)
30
20
10
0
*
Control
cKO-B
G
Number of corpora luitea (count)
12
10
8
6
4
2
0
*
Control
cKO-B

Figure 4.4

Figure 4.4: *Acvr2b cKO males are hypogonadal and females display impaired ovarian function.* **(A)** Testicular weights and **(B)** testicular sperm counts in 8-10-week-old control and cKO-B males. **(C)** Average litter sizes from control and cKO-B females paired with wild-type males for 3-4 months. Ovarian **(D)** weights, **(E)** histology, **(F)** antral follicle counts, and **(G)** corpora lutea numbers in 9-10-week-old control and cKO-B females. Scale bar: 100 µm. *t*-tests were used for statistical analyses, **p*<0.05, ***p*<0.01.

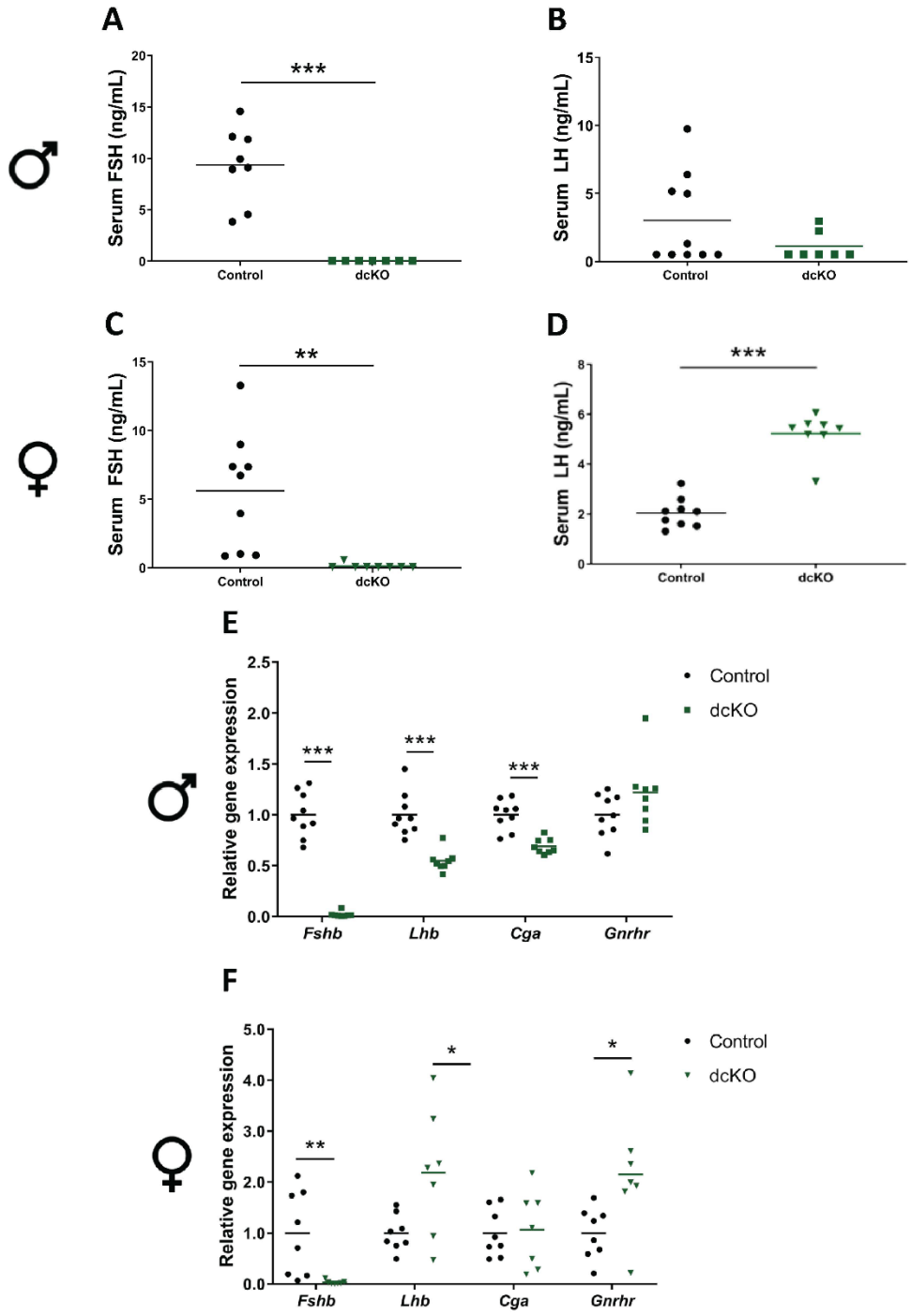
A
B
C
D
E
F
♂
♀
Serum FSH (ng/mL)
Serum LH (ng/mL)
Control
dcKO
***
**
Relative gene expression
Fshb
Lhb
Cga
Gnrhr
*

Figure 4.5

Figure 4.5: *Acvr2a/Acvr2b dcKO males and females are FSH-deficient.* **(A and C)** Serum FSH levels in control and dcKO **(A)** males and **(C)** females. **(B and D)** Serum LH levels in control and dcKO **(B)** males and **(D)** females. **(E and F)** *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels assessed by RT-qPCR in **(E)** male and **(F)** female pituitaries from control and dcKO mice. *Rpl19* was used as a housekeeping gene. *t*-tests were used for statistical analysis, **p* <0.05, **p<0.01, ****p*<0.001.

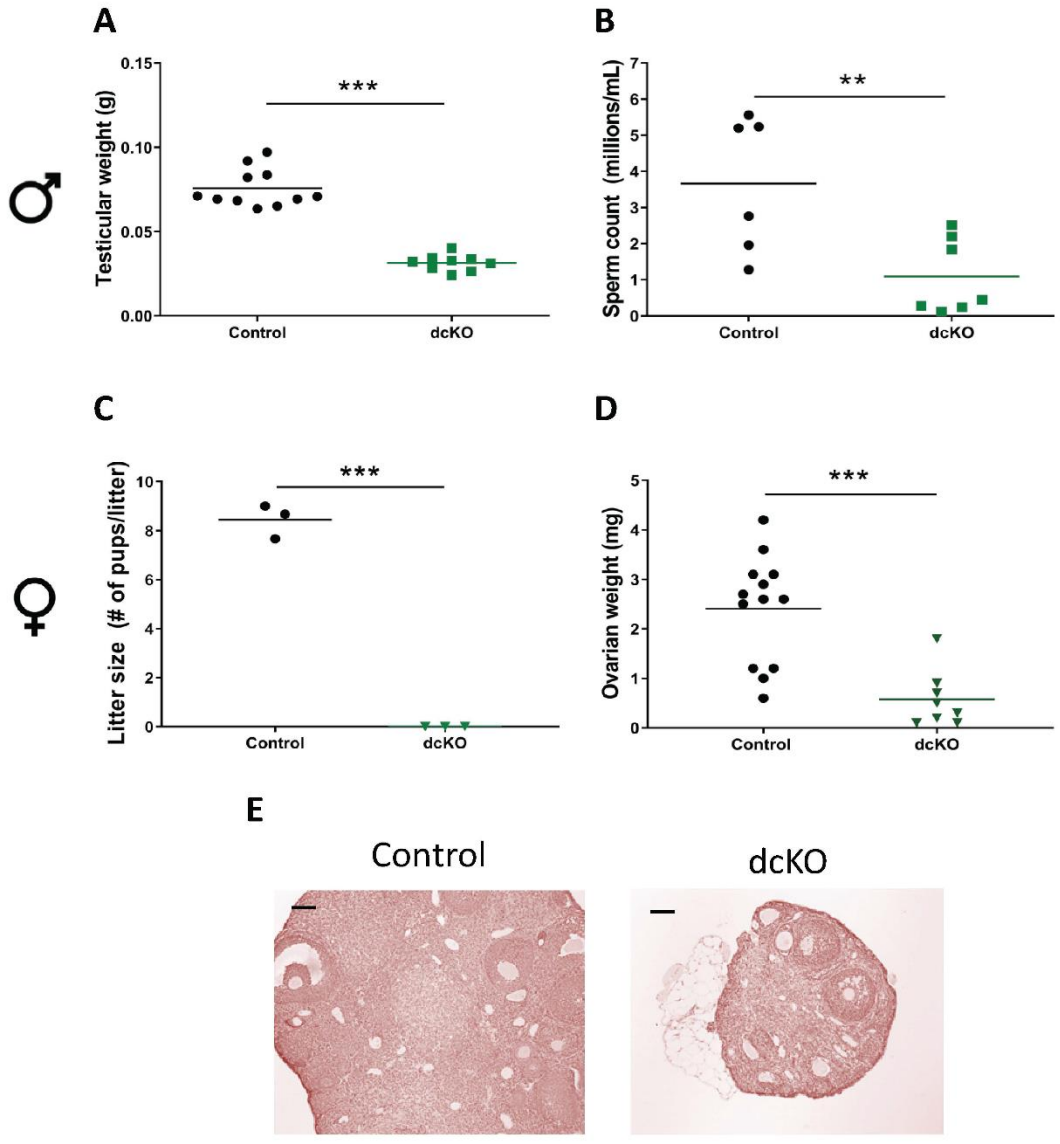
A
B
♂
Testicular weight (g)
0.15
0.10
0.05
0.00
***
Control
dcKO
Sperm count (millions/mL)
7
6
5
4
3
2
1
0
**
Control
dcKO
C
D
♀
Litter size (# of pups/litter)
10
8
6
4
2
0
***
Control
dcKO
Ovarian weight (mg)
5
4
3
2
1
0
***
Control
dcKO
E
Control
dcKO

Figure 4.6

Figure 4.6: *dcKO animals show profound hypogonadism.* **(A)** Testicular weights and **(B)** testicular sperm count in 8-10 week-old control and dcKO males. **(C)** Average litter sizes from control and dcKO females paired with WT males for 3 months. **(D)** Ovarian weights and **(E)** ovarian histology in 9-10 week-old control and dcKO females. Scale bar: 100 µm. *t*-tests were used for statistical analysis, **p*<0.05, ***p*<0.01.

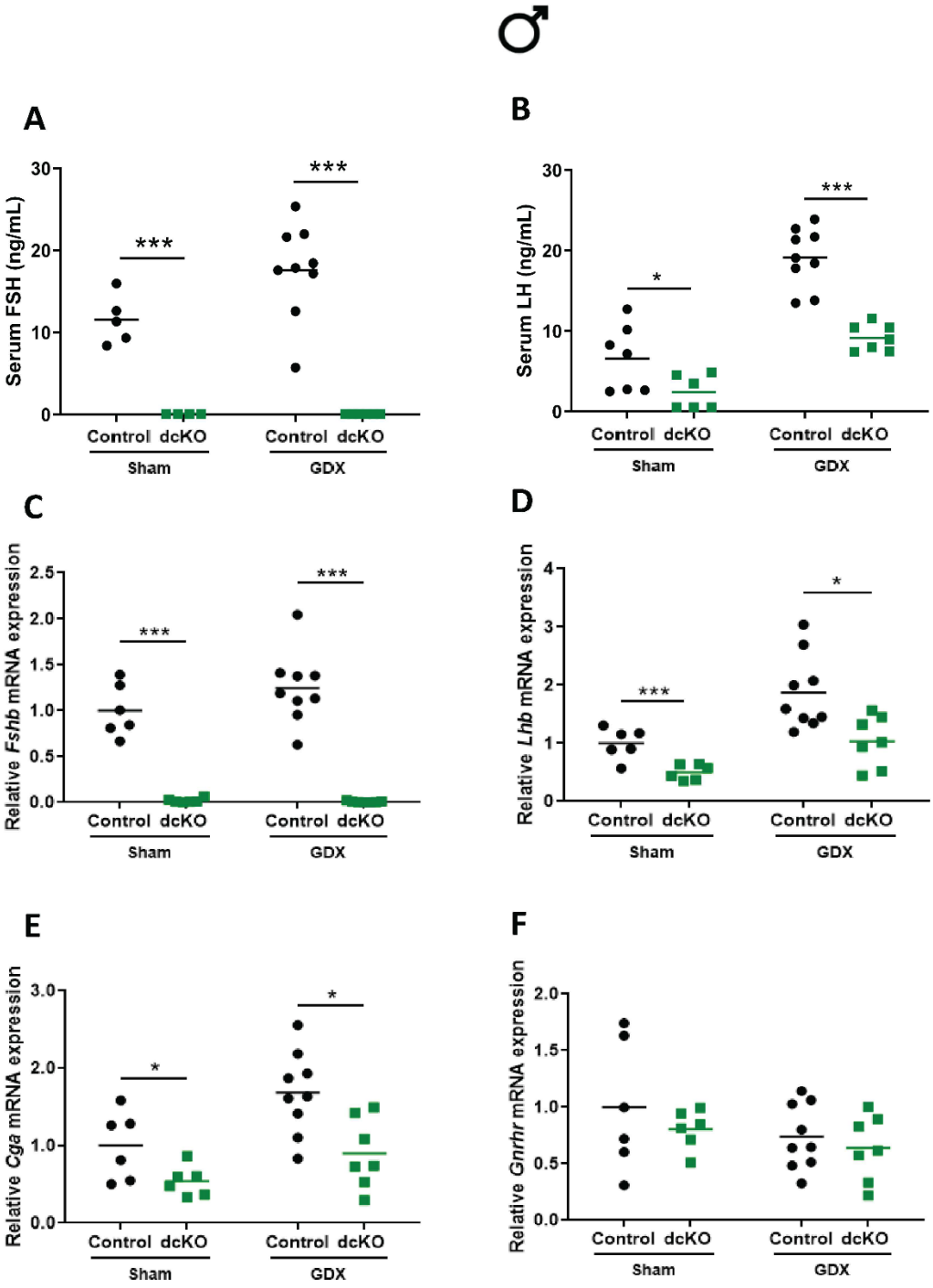
♂
A
Serum FSH (ng/mL)
30
20
10
0
***
***
Control dcKO
Sham
Control dcKO
GDX
B
Serum LH (ng/mL)
30
20
10
0
*
***
Control dcKO
Sham
Control dcKO
GDX
C
Relative Fshb mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Control dcKO
Sham
Control dcKO
GDX
D
Relative Lhb mRNA expression
4
3
2
1
0
***
*
Control dcKO
Sham
Control dcKO
GDX
E
Relative Cga mRNA expression
3.0
2.0
1.0
0.0
*
*
Control dcKO
Sham
Control dcKO
GDX
F
Relative Gnrhr mRNA expression
2.0
1.5
1.0
0.5
0.0
Control dcKO
Sham
Control dcKO
GDX

Figure 4.7

Figure 4.7: *Gonadectomized dcKO males do not produce FSH, but display impaired LH production and Lhb and Cga expression levels.* **(A)** Serum FSH and **(B)** LH in sham-operated (Sham) or castrated (GDX) control and dcKO males. **(C-F)** *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels assessed by RT-qPCR in male pituitaries from Sham or GDX control and dcKO mice. *Rpl19* was used for normalization in all RT-qPCR experiments. Two-way ANOVA, followed by Holm-Sidak multiple comparison post-hoc tests were used for statistical analysis, **p* <0.05, , ****p*<0.001.

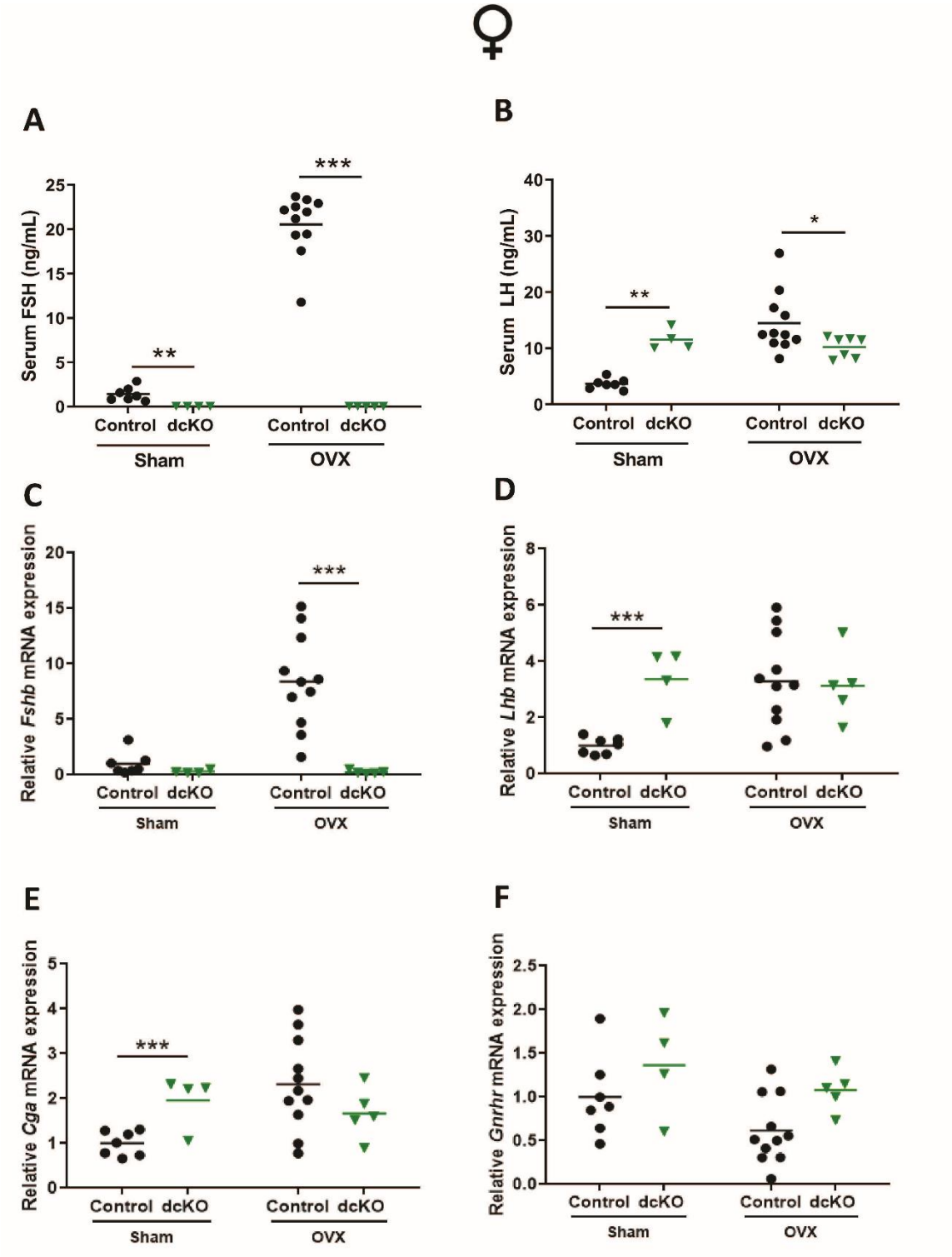
♀
A
Serum FSH (ng/mL)
25
20
15
10
5
0
**
***
Control dcKO
Sham
Control dcKO
OVX
B
Serum LH (ng/mL)
40
30
20
10
0
**
*
Control dcKO
Sham
Control dcKO
OVX
C
Relative Fshb mRNA expression
20
15
10
5
0
***
Control dcKO
Sham
Control dcKO
OVX
D
Relative Lhb mRNA expression
8
6
4
2
0
***
Control dcKO
Sham
Control dcKO
OVX
E
Relative Cga mRNA expression
5
4
3
2
1
0
***
Control dcKO
Sham
Control dcKO
OVX
F
Relative Gnrhr mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Control dcKO
Sham
Control dcKO
OVX

Figure 4.8

Figure 4.8: *Gonadectomized dcKO females do not produce FSH, but secrete LH comparably to controls.* **(A)** Serum FSH and **(B)** LH in sham-operated (Sham) or ovariectomizd (OVX) control and dcKO females. **(C-F)** *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels assessed by RT-qPCR in female pituitaries from Sham or OVX control and dcKO mice. *Rpl19* was used for normalization in all RT-qPCR experiments. Two-way ANOVA, followed by Holm-Sidak multiple comparison post-hoc tests were used for statistical analysis, **p* <0.05, ****p*<0.001.

Gene	Primer sequence
Acvr2a	
Forward (fx)	CCATTATGTAGAGTGCTGTCATTAGTTCAGTGCC
Forward (rec)	CCACTGATACCATTGTCACATGTTATCCTAATGCTAG
Reverse	CTAAGAGACCCAGAAGGCCCAAGGTATTC
Acvr2b	
Forward (fx)	CACTCCACTGTGTCCAGGGGCTG
Forward (rec)	CAGGTGGGTTATTGGAGTAGGCTGGG
Reverse	GATCTCTGGGGTAGCTGACAACAGCG
Gric	
Forward	GGACATGTTCAGGGATCGCCAGGC
Reverse	GCATAACCAGTGAAACAGCATTGCTG
Rosa26 mTmG	
Forward (WT)	AGGGAGCTGCAGTGGAGTAG
Forward (mut)	TAGAGCTTGCGGAACCCTTC
Reverse	CTTTAAGCCTGCCCAGAAGA

Table 4.1: Genotyping primers

Gene	Primer sequence
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Lhb	
Forward	ACTGTGCCGGCCTGTCAACG
Reverse	AGCAGCCGGCAGTACTCGGA
Cga	
Forward	TCCCTCAAAAAGTCCAQGAGC
Reverse	GAAGAGAATGAAGAATATGCAG
Gnrhr	
Forward	TTCGCTACCTCCTTTGTCGT
Reverse	CACGGGTTTAGGAAAGCAAA
Acvr2a	
Forward	AAGATGGCCTACCCTCCTGT
Reverse	ACCAAATCTTCCCCTTGCTT
Acvr2b	
Forward	GGCTGCGTTTGGAAAGCTC
Reverse	GCAACAAGTTTTCGTGCTTCA
Bmpr2	
Forward	GAATGTTGACAGGAGACCGGA
Reverse	TTATCCAGGTCAAGGGAGGGC
Actb	
Forward	TGGCGCTTTTGACTCAGGAT
Reverse	GGGATGTTTGCTCCAACCAA

Table 4.2: qPCR primers

SUPPLEMENTARY MATERIALS AND METHODS

Immunofluorescence

Pituitaries from 8 to 10-week-old animals were fixed in 4% paraformaldehyde (diluted in PBS) for 1.5 h at room temperature, rinsed in PBS, and left in 30% sucrose at 4°C overnight, as described previously (16). The next day, pituitaries were frozen in optimal cutting temperature (OCT; CA95057-838, VWR) compound on dry ice. Pituitaries were sectioned on a cryostat (at a thickness of 5 µm). Sections were blocked with 10% donkey serum (diluted in PBS with 0.2% Triton X-100, or PBST) for 1 h at room temperature. Slides were incubated with a purified unlabeled goat anti-mouse IgG (0.1 mg/mL; 115-007-003, Jackson ImmunoResearch; RRID: AB_2338476) diluted in PBST (10% donkey serum) for 30 min at room temperature. Slides were washed three times (5 min/wash) with PBST. Sections were then incubated with antibodies detecting LHβ (mouse anti-bovine LHβ, 1:500; 518B7, University of California, Davis; RRID: AB_2665514) and FSHβ (rabbit anti-rat FSHβ, 1:500; AFP-7798_1289P, National Institute of Diabetes and Digestive and Kidney Diseases) diluted in PBST (10% donkey serum) overnight at 4°C. The next day, slides were washed and incubated with secondary donkey anti-mouse (Alexa Fluor 488; A21202, Life Technologies; RRID: AB_141607) and donkey anti-rabbit (Alexa Fluor 594; A21207, Life Technologies; RRID: AB_141637) at a dilution of 1:1000 in PBST (10% donkey serum) for 1 h at room temperature. Sections were washed and mounted with ProLong Gold antifade (P10144, ThermoFisher). Images were acquired using a Zeiss Axio Imager M2 microscope.

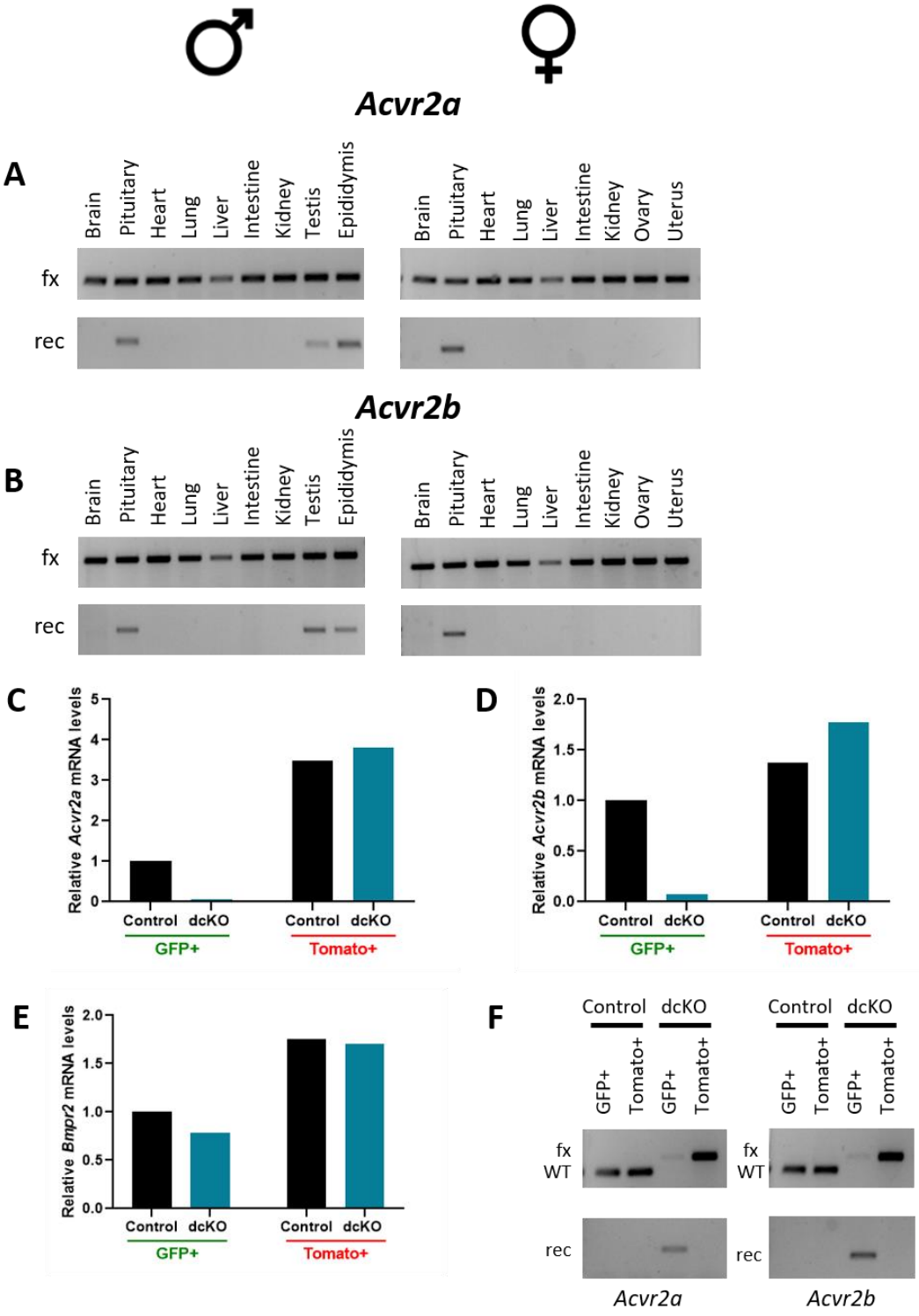
♂
♀
Acvr2a
A
Brain
Pituitary
Heart
Lung
Liver
Intestine
Kidney
Testis
Epididymis
Ovary
Uterus
fx
rec
Acvr2b
B
C
Relative Acvr2a mRNA levels
Control
dcKO
GFP+
Tomato+
D
Relative Acvr2b mRNA levels
E
Relative Bmpr2 mRNA levels
F
WT
Acvr2a
Acvr2b

Figure S4.1

Figure S4.1: *Floxed Acvr2a and Acvr2b alleles were recombined efficiently and specifically in gonadotrope cells.* **(A and B)** PCR of genomic DNA from different tissues showing recombination of **(A)** *Acvr2a* and **(B)** *Acvr2b* in males (left) and females (right); rec: recombined, fx: floxed. **(C-E)** *Acvr2a*, *Acvr2b*, and *Bmpr2* mRNA levels assessed by RT-qPCR in purified gonadotrope (GFP-positive) versus non-gonadotrope (tdTomato-positive) cells from control (*Acvr2a*$^{+/+}$;*Acvr2b*$^{+/+}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) and dcKO (*Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$;*Rosa26*$^{mTmG/+}$; *Gnrhr*$^{GRIC/+}$) mice. *Actb* was used for normalization. **(F)** PCR of genomic DNA from purified gonadotropes and non-gonadotropes showing recombination of *Acvr2a* (left) and *Acvr2b* (right) in control and dcKO cells; WT: wild-type, rec: recombined, fx: floxed.

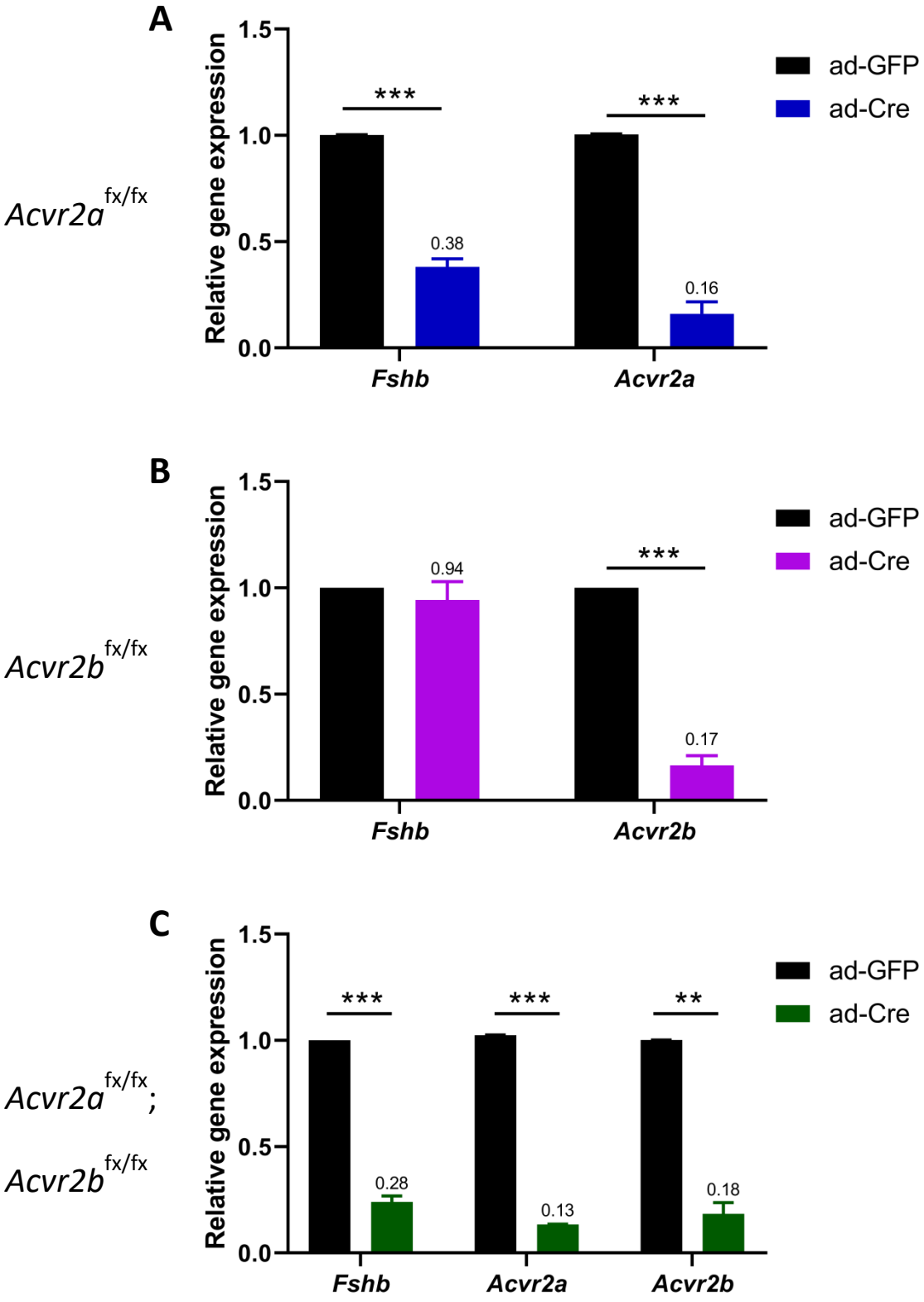
A
Acvr2a fx/fx
Relative gene expression
1.5
1.0
0.5
0.0
***
***
0.38
0.16
Fshb
Acvr2a
ad-GFP
ad-Cre
B
Acvr2b fx/fx
Relative gene expression
1.5
1.0
0.5
0.0
0.94
***
0.17
Fshb
Acvr2b
ad-GFP
ad-Cre
C
Acvr2a fx/fx;
Acvr2b fx/fx
Relative gene expression
1.5
1.0
0.5
0.0
***
***
**
0.28
0.13
0.18
Fshb
Acvr2a
Acvr2b
ad-GFP
ad-Cre

Figure S4.2

Figure S4.2: *Fshb expression depends on a ligand that signals via ACVR2A in cultured pituitaries.* Pituitary cells were isolated from adult **(A)** *Acvr2a*$^{fx/fx}$, **(B)** *Acvr2b*$^{fx/fx}$, and **(C)** *Acvr2a*$^{fx/fx}$;*Acvr2b*$^{fx/fx}$ animals (mixed sexes). N.B. These mice have the floxed, but not Cre (GRIC) allele. At the time of seeding, cells were transduced with a GFP-expressing adenovirus (Ad-GFP; control) or a Cre-expressing adenovirus (Ad-Cre). *Fshb*, *Acvr2a*, and/or *Acvr2b* mRNA expression levels were determined by RT-qPCR. *Rpl19* was used for normalization. Data represent the mean (+/- SEM) of 2-3 independent experiments. *t*-tests were used for statistical analyses. ** $p < 0.01$, *** $p < 0.001$.

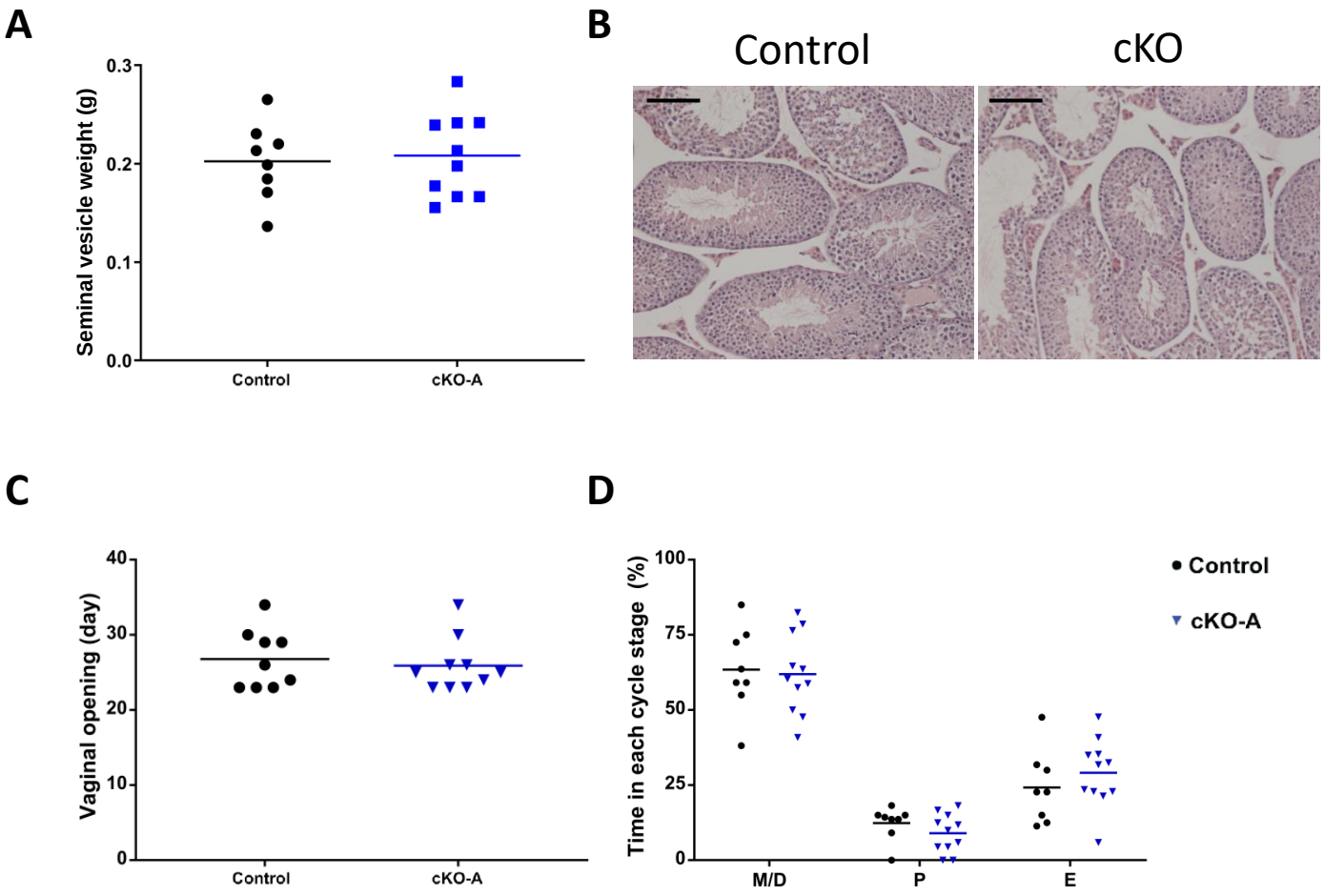
A
Seminal vesicle weight (g)
0.3
0.2
0.1
0.0
Control
cKO-A
B
Control
cKO
C
Vaginal opening (day)
40
30
20
10
0
Control
cKO-A
D
Time in each cycle stage (%)
100
75
50
25
0
M/D
P
E
Control
cKO-A

Figure S4.3

Figure S4.3: *Many reproductive parameters are normal in Acvr2a cKO animals.* **(A)** Seminal vesicle weight and **(B)** testicular histology in 8-10 week-old control and cKO-A males. Scale bar: 100 µm. **(C)** Age of vaginal opening (days) and **(D)** estrous cyclicity profiles (% of time spent in each cycle stage) in control and cKO-A females. M: metestrus, D: diestrus, P: proestrus, E: estrus.

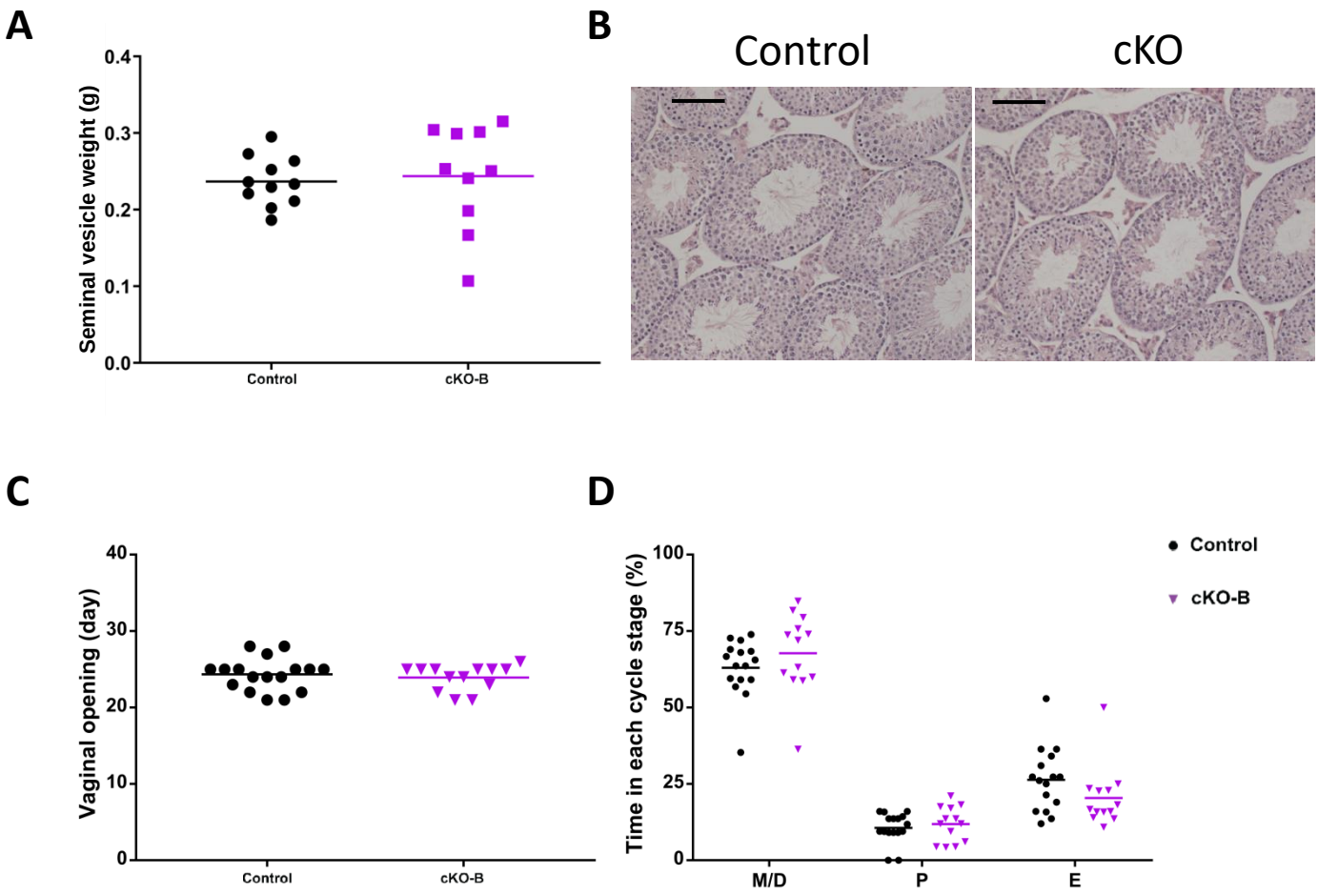
A
Seminal vesicle weight (g)
0.4
0.3
0.2
0.1
0.0
Control
cKO-B
B
Control
cKO
C
Vaginal opening (day)
40
30
20
10
0
Control
cKO-B
D
Time in each cycle stage (%)
100
75
50
25
0
M/D
P
E
Control
cKO-B

Figure S4.4

Figure S4.4: *Many reproductive parameters are normal in Acvr2b cKO animals.* **(A)** Seminal vesicle weight and **(B)** testicular histology in 8-10 week-old control and cKO-B males. Scale bar: 100 µm. **(C)** Age of vaginal opening (days) and **(D)** estrous cyclicity profiles (% of time spent in each cycle stage) in control and cKO-B females. M: metestrus, D: diestrus, P: proestrus, E: estrus.

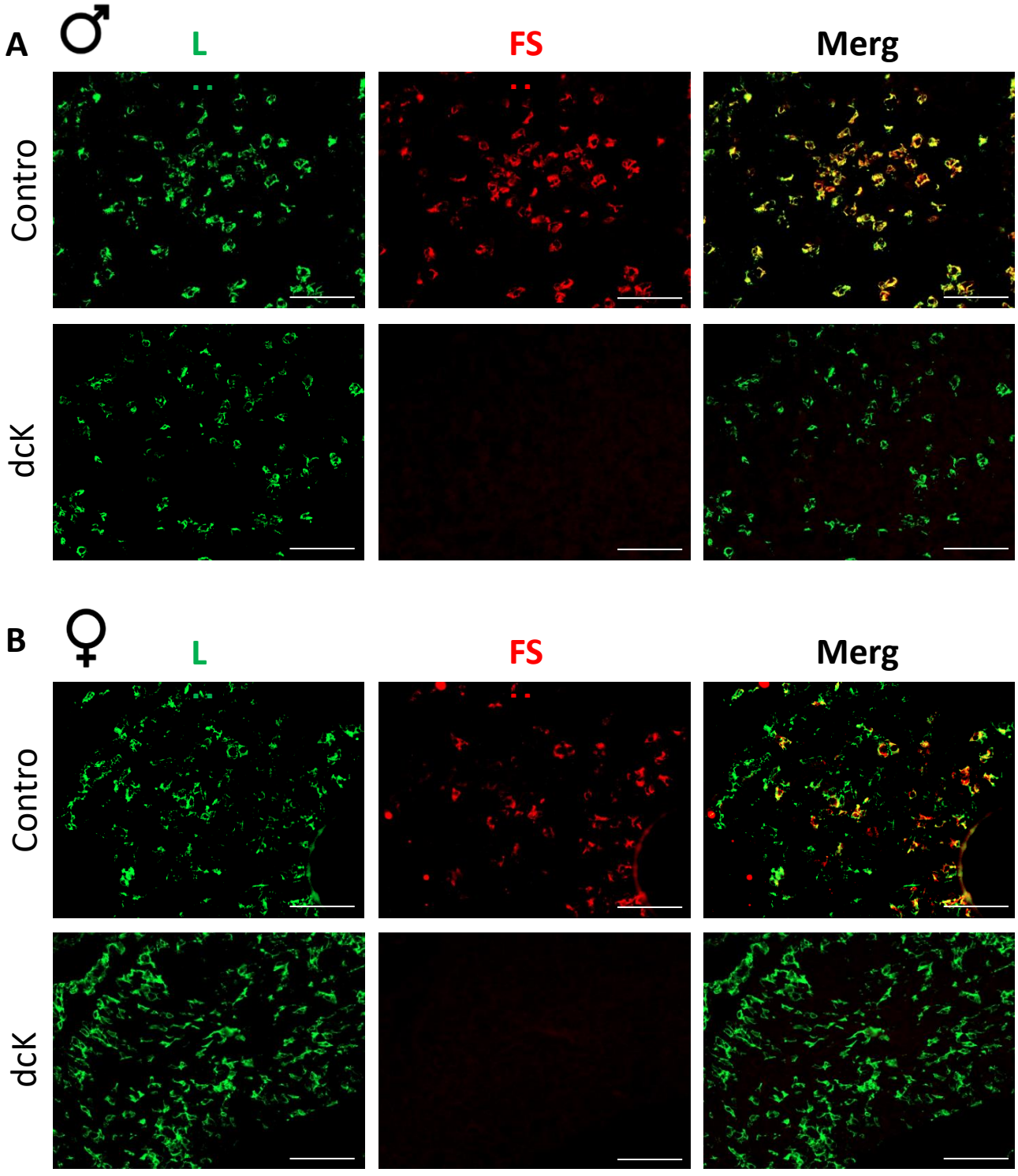

Figure S4.5

Figure S4.5: *FSH protein levels are reduced in dcKO pituitary glands.* Immunofluorescence staining for LHβ (green) and FSHβ (red) in pituitary sections of an adult control and dcKO **(A)** male and **(B)** female. Scale bar: 100 μm.

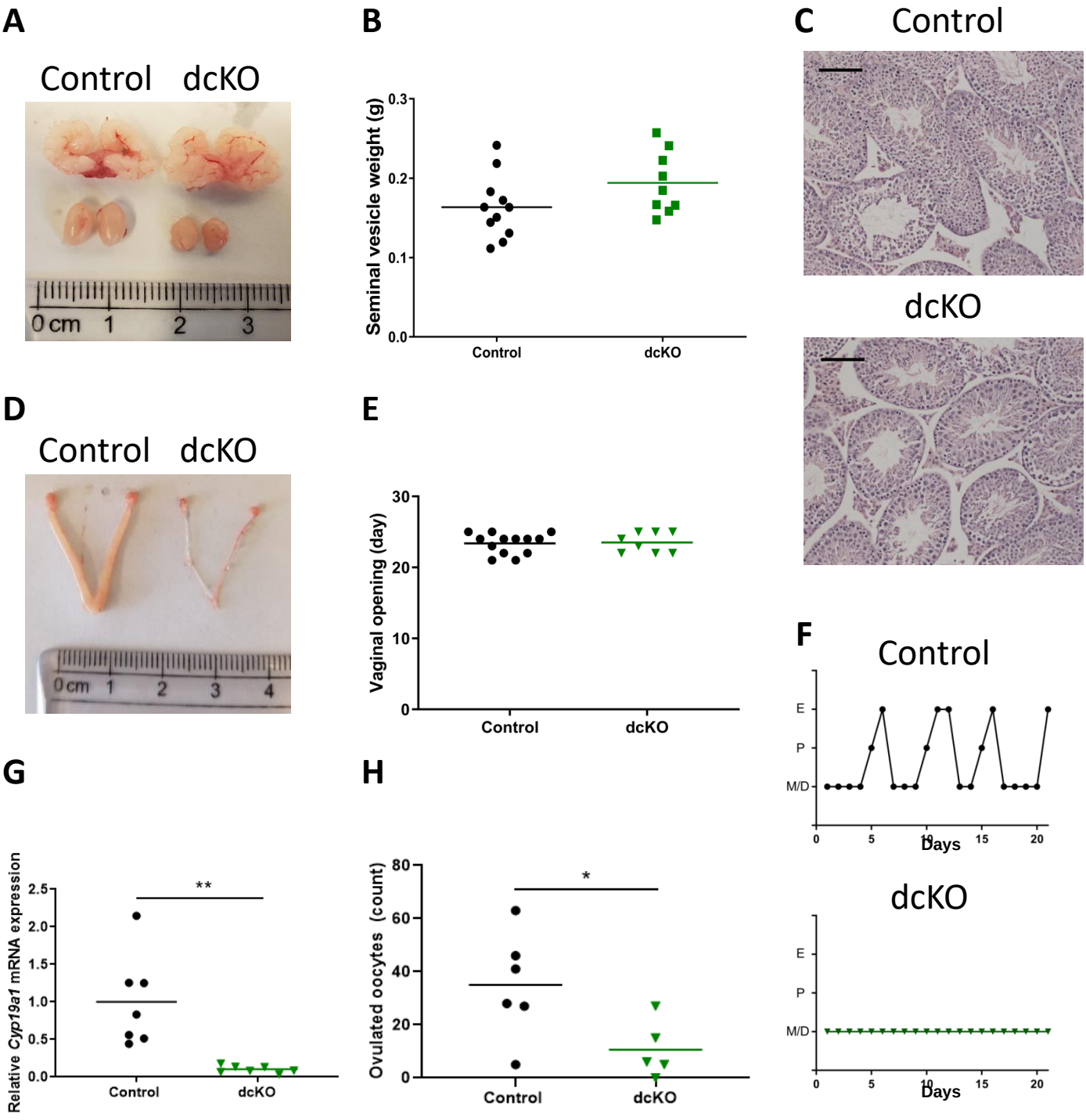
A
Control dcKO
0 cm 1 2 3
B
Seminal vesicle weight (g)
0.3
0.2
0.1
0.0
Control
dcKO
C
Control
dcKO
D
Control dcKO
0 cm 1 2 3 4
E
Vaginal opening (day)
30
20
10
0
Control
dcKO
F
Control
E
P
M/D
0 5 10 15 20
Days
dcKO
E
P
M/D
0 5 10 15 20
Days
G
Relative Cyp19a1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
dcKO
H
Ovulated oocytes (count)
80
60
40
20
0
*
Control
dcKO

Figure S4.6

Figure S4.6: *dcKO mice are hypogonadal.* **(A)** Representative picture of reproductive organs of a control and dcKO male. **(B)** Seminal vesicle weights and **(C)** testicular histology in 8-10-week-old control and dcKO males. Scale bar: 100 µm. **(D)** Representative picture of reproductive organs of a control and dcKO female. **(E)** Age of vaginal opening (days) and **(F)** estrous cyclicity profiles in control and dcKO females (two representative profiles are shown). M: metestrus, D: diestrus, P: proestrus, E: estrus. **(G)** Ovarian *Cyp19a1* mRNA levels in 9-10-week-old control and dcKO females as assessed by RT-qPCR. *Rpl19* was used as a housekeeping gene. **(H)** Number of cumulus-oocyte complexes (COCs) in juvenile control and dcKO females following superovulation with eCG/hCG. *t*-tests were used for statistical analyses, **p* <0.05, ***p*<0.01.

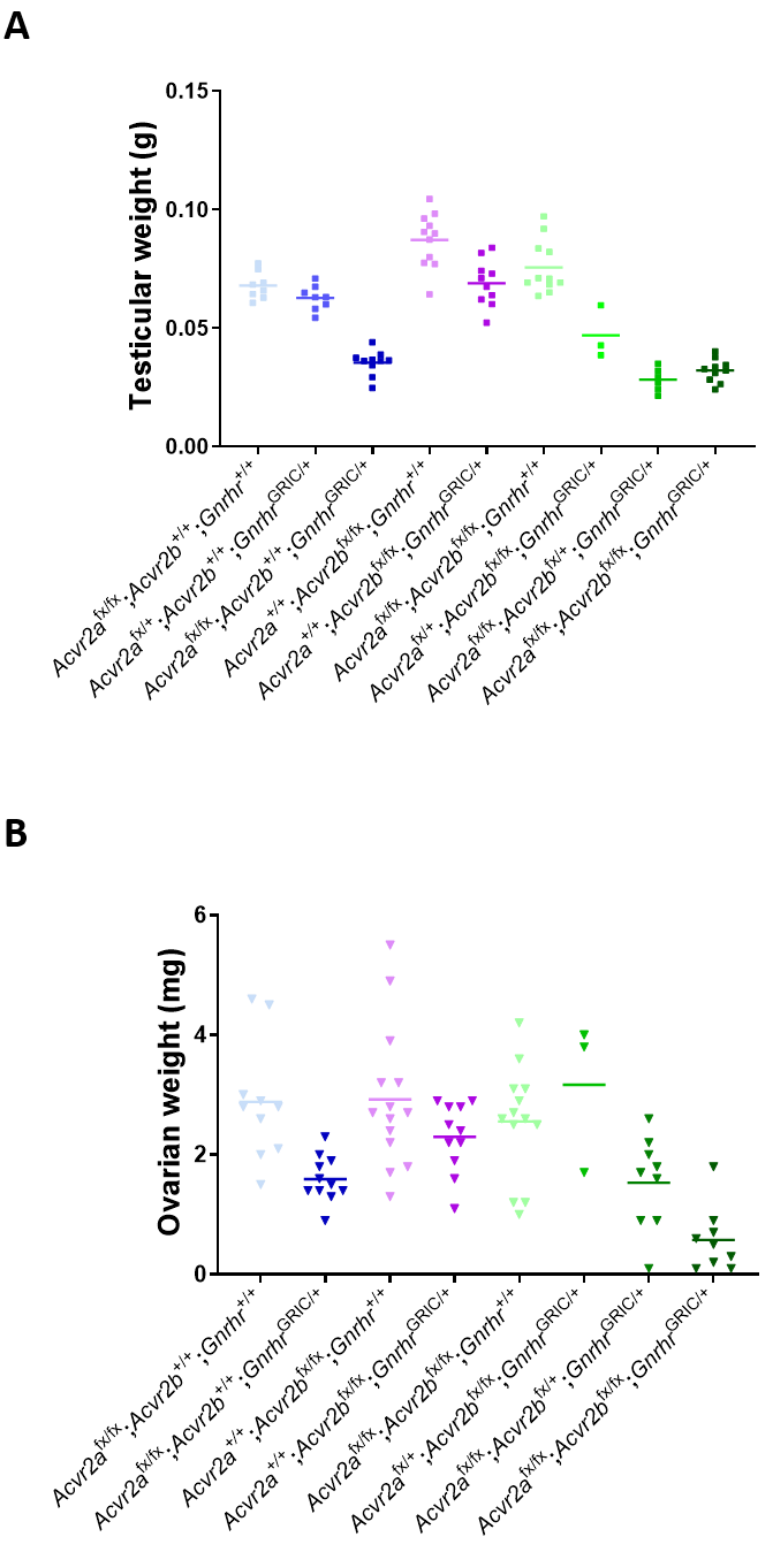
A
Testicular weight (g)
0.15
0.10
0.05
0.00
Acvr2a fx/fx ;Acvr2b +/+ ;Gnrhr +/+
Acvr2a fx/+ ;Acvr2b +/+ ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b +/+ ;Gnrhr GRIC/+
Acvr2a +/+ ;Acvr2b fx/fx ;Gnrhr +/+
Acvr2a +/+ ;Acvr2b fx/fx ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/fx ;Gnrhr +/+
Acvr2a fx/+ ;Acvr2b fx/fx ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/+ ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/fx ;Gnrhr GRIC/+
B
Ovarian weight (mg)
6
4
2
0
Acvr2a fx/fx ;Acvr2b +/+ ;Gnrhr +/+
Acvr2a fx/fx ;Acvr2b +/+ ;Gnrhr GRIC/+
Acvr2a +/+ ;Acvr2b fx/fx ;Gnrhr +/+
Acvr2a +/+ ;Acvr2b fx/fx ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/fx ;Gnrhr +/+
Acvr2a fx/+ ;Acvr2b fx/fx ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/+ ;Gnrhr GRIC/+
Acvr2a fx/fx ;Acvr2b fx/fx ;Gnrhr GRIC/+

Figure S4.7

Figure S4.7: *Acvr2a and/or Acvr2b gene dosage reductions progressively decrease gonadal weights.* **(A)** Testes were collected at 8-10-weeks of age. **(B)** Ovaries were collected at 9-10-weeks of age.

Chapter 5

In Chapter 4, I demonstrated that ACVR2A and ACVR2B are the relevant type II receptors involved in FSH production in mice. The data indicate that ACVR2A is the most critical receptor, with ACVR2B playing a more minor, yet necessary, role. Given that activins do not signal through ACVR2B to activate the *Fshb* promoter[585], these data (along with other observations) cast doubt on the relevance of activins as the TGFβ ligands inducing FSH production *in vivo*. I will further discuss this issue in Chapter 6.

Downstream of the type II receptors, we know that SMAD3, SMAD4, and FOXL2 are the necessary transcription factors to induce *Fshb* expression *in vivo*[390,658,674,675,679-681]. However, other cell types, such as ovarian granulosa cells, express *Smad3*, *Smad4*, and *Foxl2* and are responsive to TGFβ signaling. However, gonadotropes are unique in their ability to express *Fshb*. Therefore, gonadotrope cells must possess some other transcription factor(s) that enable(s) them to produce this gonadotropin subunit. It is well-established that gonadotropes are unique in expressing the EGR1-PITX1-SF1[267,268,479,481] transcription factor triad, which confers them the ability to express *Lhb*[809]. Several transcription factors could fit this description for *Fshb* expression. Though *Nr5a1*-null animals show dramatically reduced *Fshb* pituitary expression[265,266,272], circulating FSH levels are still detectable. Also, SF1 alone can activate the *Lhb*, but not *Fshb*, promoter in heterologous cell lines[479-481,810-812 251,813]. On the other hand, PITX1/2 can stimulate the *Fshb* promoter in heterologous cells[251,482,667,814,815]. However, because of the pan-pituitary function of these two transcription factors[247,250], assessing their direct role in FSH production *in vivo* through knockout strategies is challenging. As described in Chapter 1, GATA2 has restricted expression in the pituitary gland[258,267,268], and male *Gata2* pitKO mice have decreased serum FSH levels[276]. However, gene deletion in these animals was not gonadotrope-specific in the pituitary[280], may have occured outside the gland[280,282], and female *Gata2* pitKO were not investigated. In Chapter 5, I describe gonadotrope-specific *Gata2* cKO animals to directly assess the function of this transcription factor in FSH production.

GATA2 regulation of FSH production in male mice may depend on the BMP antagonist gremlin 1

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Short title: GATA2 regulates gonadotropin production in a sex-specific manner

Keywords**:** GATA2, LH, FSH, gonadotropes

ABSTRACT

Mammalian reproduction is dependent on follicle-stimulating hormone (FSH) and luteinizing hormone secreted by pituitary gonadotrope cells. These dimeric glycoproteins share a common α-subunit linked to hormone-specific β-subunits. Activins are TGFβ family ligands that selectively regulate FSHβ subunit (*Fshb*) expression via the transcription factors FOXL2, SMAD3, and SMAD4. Another transcription factor, GATA2, was previously implicated in FSH production in male mice, although its mechanisms of action and role in females were not determined. To address these gaps in knowledge, we generated and analyzed gonadotrope-specific GATA2 knockout mice using the Cre-lox system. While conditional knockouts (cKO) males exhibited ~50% reductions in serum FSH and pituitary *Fshb* levels relative to controls, there were no effects on FSH production in cKO females. This male-specific phenotype was reminiscent of that observed in mutant mice expressing a stabilized β-catenin in their gonadotropes. In that case, FSH deficiency derived from increased expression or activity of the activin antagonists follistatin and inhibin. These mechanisms did not explain FSH deficiency in *Gata2* cKO males. RNA-seq analysis of purified gonadotropes from cKO mice revealed a profound decrease in expression of gremlin 1 (*Grem1*), a bone morphogenetic protein (BMP) antagonist. *Grem1* is expressed in gonadotropes, but not other cell lineages, in the male mouse pituitary. Both *Gata2* and *Grem1* mRNA levels are significantly lower in pituitaries of wild-type females relative to males, but increase following ovariectomy. Remarkably, ovariectomy-induced increases in FSH are blunted in *Gata2* cKO females. Collectively, the data suggest that GATA2 promotes *Grem1* expression in gonadotropes and that the gremlin 1 protein potentiates FSH production. The mechanisms of gremlin 1 action in this context are not yet clear, but may involve potentiation of activin-like and/or attenuation of BMP signaling in gonadotropes.

INTRODUCTION

Gonadal function is tightly regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH), dimeric glycoproteins produced and secreted by gonadotrope cells of the anterior pituitary gland[61,62]. LH and FSH share a common α subunit (CGA, encoded by *Cga*) that is noncovalently linked to hormone-specific β subunits (LHβ and FSHβ, encoded by *Lhb* and *Fshb*, respectively). The transcriptional regulation of *Lhb*, particularly in response to gonadotropin-releasing hormone (GnRH), is well-characterized[265,466,477-481,810,816,817]. GnRH, a decapeptide secreted by the hypothalamus, signals through the GnRH receptor (GnRHR) on pituitary gonadotrope cells to induce expression of early growth response 1 (*Egr1*), an immediate early gene[478,481,817]. EGR1 then binds the proximal *Lhb* promoter, along with two other transcription factors, steroidogenic factor 1 (SF1) and *paired*-like homeodomain 1 (PITX1), to induce transcription[479,481,810]. This combination of transcription factors (EGR1, SF1, and PITX1) is unique to gonadotropes, which may help explain the unique ability of this cell type to express the *Lhb* gene.

An analogous transcription factor combination has not yet been described for *Fshb*. In an effort to understand what enables gonadotropes to uniquely produce the FSHβ subunit, we focused our attention on activin signaling. Activins are ligands of the TGF-β superfamily that selectively stimulate *Fshb* expression[390,647-649,658,659,674,680,687]. Activins bind and signal via complexes of activin type II and type I receptors. These receptors are transmembrane serine/threonine kinases. Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor. The type I receptor then phosphorylates the intracellular signaling protein SMAD3. Phosphorylated SMAD3 partners with SMAD4 in the cytoplasm, and the complex accumulates in the nucleus. There, SMAD3/SMAD4 act as transcription factors, in concert with forkhead box L2 (FOXL2), on the proximal *Fshb* promoter[390,672-674,680,681].

At first glance, the SMAD3/SMAD4/FOXL2 transcription factor triad appears to provide an appealing explanation for gonadotrope-specific FSHβ production[390,658,680]. However, this combination of transcription factors is present in other cell types and tissues that do not express *Fshb*, most notably ovarian granulosa cells[818-820]. As such, there must be some yet unidentified

transcription factor(s) that provide(s) gonadotropes the unique ability to express *Fshb*. Several proteins stand out as candidates based on decreases in *Fshb* expression that are observed when their encoding genes are knocked out in mice: GATA2[258,276], SF1[263-265], PITX1/PITX2[247,482,814,815,821], or LHX3[249,813]. Here, we focused our attention on GATA2's role in *Fshb* expression based on several independent observations.

First, using cDNA microarrays, we observed that LβT2 cells (a mature murine gonadotrope-like cell line) co-express *Gata2* and *Fshb*, while αT3-1 cells (an immature murine gonadotrope-like cell line) do not (unpublished data). Second, transgenic animals that overexpress GATA2 in pituitary lineages that normally do not express the protein show an expansion of the gonadotrope cell population[258]. Conversely, transgenic mice that express a dominant-negative form of GATA2 have reduced gonadotrope cell numbers[258]. Third, RNA-sequencing (RNA-seq) data show an enrichment of *Gata2* expression relative to other GATA factors in gonadotropes[719] and in gonadotropes relative to other pituitary cell lineages[267,268,271]. Finally, conditional deletion of GATA2 in gonadotropes and thyrotropes (another pituitary cell lineage) leads to decreased FSH production in male mice ("*Gata2* pitKO" males)[276]. Collectively, these data suggest that GATA2 plays a role in FSH production and gonadotrope function.

We selectively ablated GATA2 in gonadotrope cells to interrogate the protein's cell autonomous role in FSH synthesis. The data suggest an unanticipated role for the bone morphogenetic protein (BMP) antagonist, gremlin 1, in sex-specific regulation of FSH.

MATERIALS AND METHODS

Animals

Gata2$^{fx/fx}$ and *Gnrhr*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (GRIC) mice were described previously[276,283]. *Gata2*$^{fx/fx}$ males (MMRC, stock 030290-MU, RRID: MMRRC_030290-MU) were crossed with GRIC females to generate *Gata2*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ progeny. *Gata2*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females were then crossed to *Gata2*$^{fx/fx}$ males to generate *Gata2*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (control) and *Gata2*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (conditional knockout or cKO) animals. Eventually, given that cKO females had normal fertility, *Gata2*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ females were mated to *Gata2*$^{fx/fx}$ males so that all progeny could be used

for experiments (50% control, 50% experimental). Genotyping and assessment of genomic recombination were conducted as previously described [723] (primers listed in Table 1). Animals were housed *ad libitum* on a 12L:12D cycle (lights on at 7:00, lights off at 19:00). All animal experiments were performed in accordance with institutional and federal guidelines, and were approved by the downtown Facility Animal Care Committee of McGill University (Protocol 5204).

To purify gonadotropes by fluorescence-activated cell sorting (FACS), we crossed *Gata2*$^{fx/fx}$ animals with *Gt(ROSA26)*$^{ACTB\text{-}tdTomato\text{-}EGFP}$ mice (mTmG/mTmG, stock 007676 from Jackson Laboratories) to generate *Gata2*$^{fx/fx}$;*Rosa26*$^{mTmG/mTmG}$ males, which were then crossed to *Gata2*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ females to generate *Gata2*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ males and females. Controls for FACS were generated by crossing *Rosa26*$^{mTmG/mTmG}$ and GRIC mice to produce *Gata2*$^{+/+}$;*Gnrhr*$^{GRIC/+}$;*Rosa26*$^{mTmG/+}$ progeny.

Fluorescence-activated cell sorting

FACS was performed at the Flow Cytometry Core at the Montreal Clinical Research Institute (IRCM). Protocols for pituitary cell dispersion and cell sorting were adapted from previous publications[658,748]. Briefly, we dispersed 2-3 pituitary glands per digestion tube per genotype and per sex, each in technical duplicates. Each sample was then sorted individually, after which the purified cells for a given genotype and sex were pooled. On average, 3.6 x 10^4 EGFP-positive (gonadotropes) and 1.1 x 10^6 tdTomato-positive cells (non-gonadotropes) were obtained from each pool (4 to 6 mice per pool). This was repeated twice per sex.

Blood collection

Blood was collected from 8- to 10-week-old males or 9- to 10-week-old females (at 0700 h on estrus morning) by cardiac puncture. Blood was allowed to clot for 30 min to 1 h at room temperature, and was then spun down at 3,000 rpm for 10 min to collect serum. Sera were stored at -80^{o}C until assayed for LH and FSH.

Hormone analyses

Serum FSH levels were assessed using Milliplex kits (Millipore, MPTMAG-49K, custom-made for FSH only) following the manufacturer's instructions (minimal detection limit: 9.5 pg/mL; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described[658,775,776] (detection limit: 0.117 to 30 ng/mL; an intra-assay CV < 10%).

Reverse transcription and quantitative PCR

Pituitary glands were dissected from control and cKO animals (same age as described above), snap-frozen in liquid nitrogen, and stored at -80^{o}C. Pituitaries were homogenized in TRIzol reagent (15596018, ThermoFisher Scientific, Burlington, ON, Canada), and total RNA was extracted following the manufacturer's guidelines. For FACS samples, total RNA was extracted using Total RNA Mini Kits (Geneaid, RB300, New Taipei City, Taiwan).

Reverse transcription was performed as previously described[692] using Moloney murine leukemia virus reverse transcriptase (0000172807, Promega, Madison, WI, USA) and random hexamers (0000184865, Promega, Madison, WI, USA). qPCR runs were conducted on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen qPCR Mastermix (ABMMmix-S-XL; Diamed, Mississauga, ON, Canada) and the primers listed in Table 2. Expression levels of genes of interest were determined using the $2^{-\Delta\Delta Ct}$ method[777] and ribosomal protein L19 (*Rpl19*) for normalization. All primers were validated for efficiency and specificity.

Reproductive organ collection

Testes and seminal vesicles were dissected from 8- to 10-week-old control and cKO males. Control and cKO females were sacrificed at 9- to 10-weeks of age at 0700 h on the morning of estrus (unless otherwise specified) to collect ovaries. All organs were weighed on a precision balance.

Assessment of female fertility

At 9 weeks of age, females (n=5 per genotype) were mated with wild-type, age-matched C57BL/6 males (Charles River) for 3-4 months. Breeding cages were monitored daily to record the numbers of pups produced.

Gonadectomy

Males and females were gonadectomized at between 7- and 8-weeks of age in compliance with the standard operating procedures (SOP) at McGill University (SOPs 206 and 207). Briefly, for males, an incision was made at the midline of the scrotum at the level of the skin, and then the tunica. Each testis was pushed out and cauterized. The wound was then closed with sutures and veterinary glue. For females, an incision was made at the midline of the mid-dorsum of the animal. A small incision was made in the muscle above each ovary, the ovary was then pulled out and cauterized. All incisions were closed by sutures. In the case of sham-operated animals, all the procedures were the same, except that the gonads were not cauterized. Unless specified otherwise, gonadectomized animals were left to recover for two weeks, at which point they were euthanized to collect the pituitary gland and serum (from cardiac puncture blood). For these experiments, sham-operated females were euthanized on metestrus/diestrus.

Estradiol implants

To treat castrated males with estradiol, we followed a previously described protocol[822]. For these experiments, males were castrated at 10- to 12-weeks of age as described above. Two weeks later, males received an implant containing vehicle (sesame oil) or 20 µg 17β-estradiol in sesame oil (E2758, Millipore-Sigma, Oakville, Ontario, CA). Implants were prepared by cutting SILATIC tubing (inner diameter of 0.062 in/1.57 mm and outer diameter of 0.125 in/3.18 mm; Dow Corning, Midland, MI, USA) in 15 mm segments. One end was sealed with SILASTIC adhesive (Dow Corning, Midland, MI, USA) and left to dry for 24 h. On the next day, 20 µL of sesame oil alone (vehicle) or containing 1 µg/µL of 17β-estradiol was pipetted into the implant. The other end was then sealed with SILASTIC adhesive for 24 h. The next day, the implants were left overnight in saline solution at 37^{o}C and then implanted in the animals on the next morning. A

small incision was made on the back of the animal, below the neck, and the implant was placed subcutaneously. The incision was sealed with sutures. The animals were left to recover for 3 weeks. The animals were then euthanized to collect the pituitary gland and serum (from cardiac puncture blood). Submandibular blood was also collected prior to castration and prior to adding the implant. All pituitary and serum analyses from gonadectomized animals were conducted as described above.

RNA-seq

FACS was performed to isolate gonadotropes from two control and two cKO male pools, as described above. On average, each pool contained ~12,000 cells, each coming from 2-3 male mice. RNA extraction was performed according to manufacturers' instructions using the NucleoSpin RNA XS kit (cat # 740902, Macherey-Nagel). Briefly, FACS-isolated gonadotrope cells were lysed in 100 µl of lysis buffer (RA1 reagent + TCEP), DNA was digested with rDNAse for 15 min at room temperature, and the final RNA eluted in 10 µl of RNAse-free water. RNA concentration was measured by Nanodrop-One (Thermo Scientific), and the RNA quality was assessed on a Bioanalyzer (cat# 5067-1513, Eukaryote Total RNA Pico Bioanalyzer kit, Agilent). RNA-seq libraries were prepared with the extracted RNA using the Universal plus mRNA-seq library preparation kit (NuGEN). Library quality control and quantification were assessed by fluorometry (cat # Q32854, Qubit dsDNA High sensitivity Assay Kit), and on a Bioanalyzer (cat # 5067-4626, High-Sensitivity DNA Bioanalyzer kit, Agilent). The four libraries were pooled together at equal concentrations, the pooled sample was first assessed by mi-seq (Illumina), and then sequenced at the New York Genome Center on Novaseq 6000 (Illumina) with a depth of ~30 million paired-end reads per sample.

Analysis of RNA-seq data

Raw data fastq files were aligned to the GENCODE mm10 genome using STAR[823]. Gene expression was quantified with featureCounts[824]. The raw data fastq files and the final gene expression matrix were deposited to GEO. Differential expression analysis was performed using Bioconductor[825] package limman[826] under R version 3.6.1 (RCoreTeam, http://www.R-

project.org/). After filtering for low expression, gene expression was analyzed using a voom method[827] and compared between control and cKO mice. Benjamini-Hochberg correction[828] for multiple comparisons testing was applied. The differentially expressed genes were determined with: i) the false discovery rate of ≤ 0.05; ii) the log2 fold change ≥ 0.5 in either direction; and iii) the mean gene expression in log2 of cpm (counts per million) ≥ 4.0 in at least one of the two groups.

Validation of RNA-seq results was conducted by RT-qPCR on new FACS samples. FACS was performed as described above, and ~70,000 gonadotropes were obtained from 12-16 control and cKO animals. Such a high yield was necessary to detect some rare transcripts by RT-qPCR.

Statistical analysis

Data were analyzed on GraphPad Prism 8 using Student *t*-tests, unless otherwise specified. Experiments involving multiple sampling of the same animals were analyzed by two-way ANOVA, followed by *post-hoc* Holm–Sidak multiple comparison tests. Results were considered statistically significant when $p < 0.05$.

RESULTS

Generation of Gata2 conditional knockout mice

To investigate the role of GATA2 in gonadotropes, we generated gonadotrope-specific *Gata2* conditional knockouts (cKO) by crossing floxed *Gata2* (*Gata2*$^{fx/fx}$) and GRIC mice (*Gnrhr*$^{GRIC/GRIC}$). First, we confirmed the specificity of *Gata2* deletion in the cKO animals; gene recombination occurred solely in the pituitary glands of males and females, and in testes and epididymides of males (Fig. S5.1A). This is consistent with previous studies showing exclusive Cre activity from the GRIC allele in the pituitary and male germline[283,475]. Next, we aimed to quantify *Gata2* deletion in gonadotropes. In both sexes, *Gata2* was markedly reduced in the gonadotrope-enriched population (GFP+ cells in Figs. S5.1B and S5.1C). *Gata2* expression in the other cell lineages (i.e., thyrotropes[258,726]) was unaffected (Tomato+ cells in Figs. S5.1B and S5.1C).

Gata2 cKO males, but not females, produce FSH at reduced levels

Male cKO animals had reduced serum FSH levels relative to controls (Fig. 1A), while serum LH was not affected (Fig. 5.1B). In contrast, female cKO animals showed normal FSH (Fig. 5.1C) and LH (Fig. 5.1D) levels relative to controls. Consistent with these observations, pituitary *Fshb* mRNA expression was significantly reduced in cKO males (Fig. 5.1E) but not females (Fig. 5.1F) relative to controls. *Lhb* was unaffected in both sexes, while *Cga* expression showed a male-specific impairment (Figs. 5.1E and F). *Gnrhr* mRNA levels were elevated in cKO animals of both sexes, although this did not reach statistical significance in males (p=0.07).

Reproductive organs are normal in both male and female Gata2 cKO animals

Male cKO animals showed no changes in testicular (Fig. 5.2A) or seminal vesicle (Fig. 5.2B), weights compared to littermate controls. Testicular sperm production (Fig. S2A) and testicular architecture (Fig. S5.2B) were comparable between control and cKO males. Female cKO animals showed normal: 1) puberty onset, as assessed by vaginal opening (Fig. S5.2C), 2) estrous cyclicity (Fig. S5.2D), 3) fertility (Fig. 5.2C), and 4) ovarian weights (Fig. 5.2D). Finally, ovaries from both control and cKO females contained follicles at all stages of development (Fig. S5.2E).

Given normal testicular weights in the face of reduced FSH production, we assessed serum FSH levels during postnatal development in males. FSH was comparable between the two genotypes at 3-weeks of age (Fig. S5.3). However, control males showed increases in serum FSH levels from 4- to 7-weeks of age, while cKO males did not (Fig. S5.3).

Effects of Gata2 deletion are not caused by increased inhibin sensitivity or follistatin expression in male pituitaries

This male-specific FSH phenotype closely paralleled that of an earlier model in which a gain-of-function (stabilizing) mutation was introduced into the β-catenin gene (*Ctnnb1*) in murine gonadotropes[829]. There, the male-specific FSH deficiency derived from increased pituitary sensitivity to inhibins and increased pituitary expression of the activin antagonist follistatin (*Fst*)[829].

To assess whether *Gata2* cKO males were more sensitive to inhibins, we injected mice with anti-inhibin serum (AIS)[710,830,831]. In our experience, control adult mice have limited inhibin tone[710]. As a result, AIS does not affect their FSH levels. In contrast, FSH levels increase in response to AIS in wild-type females (Fig. S5.4A). Neither control nor cKO males injected with AIS showed significantly elevated serum FSH levels after 6 or 11 h (Fig. S5.4B), suggesting that inhibin sensitivity remains low in cKOs.

With respect to follistatin expression, we could not detect the *Fst* transcript in pituitaries from either genotype (data not shown). These results are consistent with recent single-cell RNA-sequencing data[267,268,271]. Follistatin-like 3 (encoded by *Fstl3*) has overlapping functions with follistatin[832,833], and has high expression levels in gonadotropes[267,268,271]. While we could detect the transcript, control and cKO males showed no differences in pituitary *Fstl3* expression (Fig. S5.5).

Gonadectomy reveals a pleiotropic role for GATA2 in male and female gonadotropes

To begin to understand why male but not female cKOs exhibited FSH-deficiency, we gonadectomized control and cKO males to investigate the potential influence of a testicular factor. Following castration, both control and cKO mice showed increased serum FSH levels; however, cKO animals continued to produce less FSH than controls (Fig. 5.3A). Serum LH levels also increased post-castration in both genotypes, but the response was blunted in cKOs relative to controls (Fig. 5.3B). Pituitary *Fshb* (Fig. 5.3C), *Lhb* (Fig. 5.3D), *Cga* (Fig. 5.3E) mRNA levels increased following gonadectomy in both genotypes, but to a lesser extent in cKOs. There were non-significant trends for *Gnrhr* levels to be: 1) higher in cKOs relative to controls and 2) to be lower following castration (Fig. 5.3F)

We conducted similar experiments in females to assess whether ovarian factors may mask the effects of GATA2 loss in gonadotropes. Females of both genotypes showed increases in serum FSH (Fig. 5.4A) and LH (Fig. 5.4B) levels following ovariectomy. However, there was a non-significant trend (p=0.08) for the FSH response to be blunted in cKO females relative to controls. LH levels were higher in cKOs compared to controls post-ovariectomy. As in males, *Fshb* (Fig. 5.4C), *Lhb* (Fig. 5.4D), and *Cga* (Fig. 5.4E) mRNA levels were lower in ovariectomized cKO females

relative to controls. *Gnrhr* expression was increased in sham-operated and ovariectomized cKO females relative to controls (Fig. 5.4F).

The effects of GATA2 loss are not reversed by estradiol administration

Given that ovariectomized cKO females showed similar gene expression impairments as castrated cKO males, we assessed whether estradiol may mask the effects of GATA2 loss. We treated control males with a 17β-estradiol implant that reduced serum LH to pre-castration levels (Fig. S5.6A). This treatment also reduced pituitary *Lhb*, but not *Fshb*, *Cga*, or *Gnrhr* mRNA expression (Fig. S5.6B). The serum FSH impairment persisted in cKO males relative to controls before castration [Pre-GDX], after castration [Post-GDX], and after estradiol administration [Post-implant] (Fig S5.6C). Because of the manner in which blood samples were collected, we could not measure LH levels in the same experiment. Following estradiol treatment, pituitary *Fshb*, *Lhb*, and *Cga* mRNA levels were significantly lower in cKOs compared to controls. In contrast, *Gnrhr* expression was elevated in cKOs (Fig. S5.6D).

GATA3 does not compensate for the loss of GATA2 in gonadotropes

As a testicular factor or estradiol did not explain the sex difference, we next asked whether there may be distinct compensatory mechanisms in the two sexes. Previously, GATA3 has been shown to compensate for the absence of GATA2 in some contexts[276,834]. However, pituitary *Gata3* mRNA levels did not differ between controls and cKOs in either sex (Figs. S5.7A-B). Although *Gata3* is not detectable in murine gonadotropes[267,268] (data not shown), we nevertheless generated *Gata2*/*Gata3* double cKO (dcKO) mice to investigate a potential compensatory role for GATA3 in gonadotropes. dcKO males' phenotypes were similar to those of *Gata2* cKO males, displaying normal testicular weight (Fig. S5.7C), decreased serum FSH (Fig. S5.7D), and normal LH levels (Fig. S5.7E). Pituitary *Fshb* and *Cga* mRNA levels were reduced, as in *Gata2* cKOs (Fig. S5.7F). The only distinction we observed between the two models was a significant increase in *Gnrhr* levels in dcKOs relative to controls. The basis for this difference is unclear as *Gata3* is not expressed in gonadotropes and *Gata3* mRNA levels are unaffected in the

whole pituitary of dcKO mice. Though we did not systematically characterize dcKO females, their fertility was unaffected (data not shown).

GATA2 is not required for normal gonadotrope development during embryogenesis, but is required for normal Fshb expression in adult animals

Next, we assessed whether the decrease in FSH production in males was caused by embryonic defects of pituitary development. Indeed, Cre activity in the GRIC mice is first observed at embryonic day 12.75[475]. *Gata2* is first expressed approximately two days earlier at embryonic day 10.5[258]. We did not detect differences in gonadotrope cell numbers (Fig. S5.8A) or in *Nr5a1* expression (a gonadotrope-specific marker in the pituitary) between control and cKO males (Fig. S5.8B).

A postnatal role for GATA2 in gonadotrope function was suggested by the emergence of FSH-deficiency in cKO males after 3 weeks of age (Fig. S5.3). However, to rule in or out developmental effects, we used a tamoxifen-inducible gonadotrope-specific Cre-driver[390] to ablate *Gata2* in adult male mice. As in *Gata2* cKOs, inducible knockout mice exhibited significant reductions in pituitary *Gata2*, *Fshb*, and *Cga*, and increases in *Gnrhr*, mRNA levels (Fig. S5.9A). *Lhb* expression was unaffected. Unexpectedly, serum FSH levels were not significantly reduced in inducible knockouts, though there was a trend in this direction (Fig. S5.9B).

Grem1 is profoundly downregulated in gonadotropes of Gata2 cKO males

After ruling out several mechanisms to explain the sex-specific FSH impairment in cKO males, we performed bulk RNA-seq on purified gonadotropes of these and control mice to identify novel candidates. We detected the upregulation 114 and the downregulation of 199 genes in male cKO gonadotropes relative to controls (Figs. 5.5A and B). *Inhbb*, which encodes the activin B subunits, was among the downregulated genes. However, *Inhbb* knockout mice have elevated FSH levels[633], likely ruling out activin B-deficiency as an explanation. The most downregulated gene in cKO gonadotropes was *Grem1*, which encodes the bone morphogenetic protein (BMP) antagonist gremlin 1. Given that BMPs are TGF-β superfamily ligands previously

implicated in FSH regulation[715,718,721,835,836], and that *Grem1* is expressed in male, but not female, pituitaries[719], we investigated potential links between GATA2, gremlin-1, and FSH production.

Pituitary Grem1 expression is male-specific, but increases post-ovariectomy in females

Consistent with a previous report[719], we observed that *Grem1* was expressed at significantly higher levels in gonad-intact wild-type males than females ('Sham' in Fig. 5.6A). Castration did not alter pituitary *Grem1* mRNA levels in wild-type males, but ovariectomy increased *Grem1* expression 7-fold in wild-type females (GDX in Fig. 5.6A). These results correlated with patterns of *Gata2* expression. That is, gonad-intact males had ~2.5-fold higher *Gata2* mRNA levels relative to gonad-intact females (Fig. 5.6B, Sham); castration did not change *Gata2* expression in males, while ovariectomy increased *Gata2* levels by ~75% in females, nearly reaching male levels (p=0.057; Fig. 5.6B, GDX).

Pituitary Grem1 expression decreases in the absence of GATA2

We next examined the effects of gonadectomy on pituitary *Grem1* expression in *Gata2* cKO mice. In males, *Grem1* expression was significantly reduced both in sham-operated and castrated males (Fig. 5.6C), consistent with the RNA-seq results. *Gata2* ablation in adult male gonadotropes also led to decreased *Grem1* mRNA levels (Fig. S5.9A). Castration did not affect *Gata2* expression in pituitaries of either control or cKO males (Fig. 5.6D). In contrast, ovariectomy increased both *Grem1* (Fig. 5.6E) and *Gata2* (Fig. 5.6F) expression in pituitaries of control females, whereas only *Gata2* levels increased in cKO females. Residual *Gata2* mRNA in cKO mice reflects expression in thyrotropes (Figs. S5.1A and B). Therefore, the increase post-ovariectomy likely reflects gene expression changes in this cell lineage.

Pituitary Gata2 and Grem1 expression are regulated by estradiol

In order to explain the sex-differences in both *Gata2* and *Grem1* expression, we analyzed mRNA levels of both genes in castrated control males supplemented with estradiol, relative to vehicle-treated castrated males from Fig. S5.6B. In those animals, both *Gata2* and *Grem1* levels

decreased (Figs. 5.7A and B), but not to the levels observed in castrated cKO males supplemented with estradiol from Fig. S5.6D.

Expression of BMP target genes are increased in gonadotropes of Gata2 cKO males

To assess whether the decrease in *Grem1* expression had a functional impact, we purified gonadotropes from control and cKO males and examined mRNA levels of BMP-target genes. As expected, *Gata2* and *Grem1* were undetectable in cKO relative to control gonadotropes (GFP+ in Figs. 5.8A and B). We also confirmed the selective expression of *Grem1* in gonadotropes (GFP+ cells) relative to non-gonadotropes (Tomato+ cells)[267,268]. Inhibitor of DNA binding (Id) proteins, such as Id1, Id2, and Id3, are well-described BMP-target genes[748,837-839]. *Id2* and *Id3* (but not *Id1*) showed higher expression levels in male cKO relative to control gonadotropes (Figs. 5.8C-E), and *Id2* was significantly upregulated in the RNA-seq data (~2-fold; Figs. 5.5A and B). Increases in *Id2* and *Id3* expression levels were also observed in non-gonadotropes (Tomato+). *Nr5a1*, a gonadotrope marker, was only detected in GFP+ cells (Fig. 5.8F), demonstrating the purity of the cell preparations. Though *Nr5a1* levels were slightly reduced in cKO gonadotropes (Fig. 5.8F), this may reflect biological variability rather than a consequence of GATA2 loss, as we did not observe this decrease consistently (see Fig. S5.8B).

DISCUSSION

We generated *Gata2* cKO mice to investigate GATA2 function in gonadotrope cells. Our results confirm and greatly extend earlier observations. As previously reported in *Gata2* pitKO mice[276], FSH levels were significantly reduced in cKO relative to control males. Though it may have been surprising that testicular weights were not affected in *Gata2* cKO males, the same observation was made in *Gata2* pitKO males[276]. This was consistent with the fact that serum FSH levels were normal in 3-week-old *Gata2* cKO males. Indeed, testicular development mostly occurs before that age[840,841], and decreases in FSH production beyond this window have no effect on testicular weight[390].

On the other hand, gonad-intact cKO females did not show impaired FSH production. These results were unexpected, as the phenotypes observed in *Gata2* cKO males resembled those from previous TGFβ signaling-deficient models, in which both males and females showed impaired FSH production[658] (see Chapter 4). We were able to rule out changes in inhibin sensitivity, increases in *Fst* or *Fstl3* expression, compensation by GATA3, and developmental effects as the causes of this discrepancy between males and females. *Grem1* appeared as an ideal candidate to explain the male-specific FSH production deficiency in gonad-intact *Gata2* cKO animals for at least three reasons: 1) *Grem1* showed the most robust decrease in gene expression in *Gata2* cKO gonadotropes relative to controls, 2) *Grem1* is specifically expressed in male, but not female, gonadotropes[719], and 3) gremlin-1 regulates some TGFβ ligands (namely BMP2, 4, 6, and 7)[842]. Moreover, *Gata2* and *Grem1* expression patterns correlated in both sexes (sham-operated and gonadectomized).

With the assumption that the decrease in *Grem1* expression levels in *Gata2* cKO gonadotropes led to decreased gremlin-1 production, we analyzed expression levels of BMP-target genes[748,838,839]. We observed increased *Id2* and *Id3* mRNA levels in purified gonadotropes from *Gata2* cKO males (relative to controls), which suggests that the loss of *Grem1* expression (and potentially gremlin-1 production) provided a permissive environment for BMPs to signal in gonadotropes. This increased BMP signaling could decrease *Fshb* expression through, at least, two non-mutually exclusive pathways.

First, it may be that BMP signaling directly inhibits *Fshb* expression and FSH production. Indeed, BMP4 and BMP6 dose-dependently inhibit *Fshb* expression and FSH production in ewe pituitary cultures[843]. However, in murine LβT2 cells, BMP2/4 increase *Fshb* expression in synergy with activins, while BMP6/7 modestly activate the *Fshb* promoter[718]. Also, BMP6/7 potently activate the ovine *Fshb* promoter in primary pituitary cultures (from mice expressing an o*Fshb*-luciferase transgene) and LβT2 cells[836]. To investigate the function of BMPs in gonadotropes *in vivo*, gonadotrope-specific BMP receptor knockout mice were characterized[657,723]. The principal type I receptors through which BMP2/4 and BMP6/7 signal are activin receptor-like kinase 3 (ALK3, encoded by *Bmpr1a*) and ALK2 (encoded by *Acvr1a*), respectively[844,845]; BMP2/4 and BMP6/7 preferentially bind BMPR2 and ACVR2A type II receptors, respectively[587,720,844,845].

Gonadotrope-specific *Bmpr1a*, double *Bmpr1a*/*Acvr1a*, and *Bmpr2* cKO animals had comparable *Fshb* expression and/or fertility relative to controls[657,723], which argues against a role for BMP signaling in FSH production *in vivo*.

Second, it is possible that endogenous BMPs compete with activins for receptor binding. BMP2 and BMP7 are both bioneutralized by gremlin-1 and both can bind ACVR2A[587,720,844], the most critical type II receptor for FSH production in mice *in vivo* (see Chapter 4). Given that BMP signaling does not induce *Fshb* expression *in vivo*[657,658,715,723], BMP2 and/or 7 binding to ACVR2A could inhibit activin-induced *Fshb* expression. Under normal conditions, it is possible that gremlin-1 bioneutralizes BMP2 and/or 7, providing a permissive environment for activins to bind ACVR2A and induce FSH production (Fig. 5.9). Therefore, the loss of *Grem1* expression may increase the competition between BMPs and activins to bind ACVR2A, thereby inhibiting *Fshb* expression (Fig. 5.9).

However, the effects of *Grem1* on FSH production are likely male-specific in gonad-intact mice. It remains unclear why male and female mice displayed such differences in *Gata2* and *Grem1* pituitary mRNA levels. Although we showed that estradiol supplementation in castrated *Gata2* cKO males did not rescue the FSH deficiency phenotype, it significantly decreased both *Gata2* and *Grem1* mRNA levels in control males. These data suggest that estradiol likely contributes to the lower *Gata2* and *Grem1* expression levels observed in female pituitaries, but that other factors are also involved in repressing both genes.

Also, we observed that loss of GATA2 impaired other gonadotropin subunit genes. Namely, *Lhb* expression was impaired in *Gata2* cKO animals post-gonadectomy (in males and females). GATA2 has been shown to induce the rat *Lhb* promoter *in vitro*[846] in synergy with SF1, a transcription factor implicated in LH production[265,479-481,483,810]. These data indicate that GATA2 can promote *Lhb* expression *in vitro*. Moreover, *Gata2* knockdown in LβT2 cells decreased endogenous *Lhb* expression[846], suggesting that GATA2 is necessary for quantitatively normal *Lhb* expression in this cell line. However, this effect was modest and, if consistent *in vivo*, this may be why *Lhb* was only detectably decreased following gonadectomy, which dramatically increased *Lhb* expression in both sexes.

Finally, *Cga* expression was also significantly decreased in gonad-intact cKO males and in gonadectomized cKO males and females relative to controls. The *Cga* promoter can be induced *in vitro* by GATA2, which is dependent on a specific GATA sequence [847]. The decrease in *Cga* mRNA levels in sham-operated cKO males, but not females, might reflect a difference in sensitivity to the loss of GATA2, as this transcription factor is more highly expressed in males relative to females. *Gata2* expression increased in ovariectomized control females, which may explain why *Cga* was only detectably decreased in ovariectomized cKO females, relative to controls.

In sum, GATA2 plays pleiotropic roles in gonadotropes, but is mostly required for quantitatively normal FSH production in gonad-intact male, but not female, mice. Our data suggest that GATA2 induces *Grem1* expression, though it is unclear if GATA2 directly or indirectly regulates the *Grem1* promoter. It may be that male mice produce higher levels of FSH relative to females because of elevated *Grem1* expression levels, and that gremlin-1 may contribute to the dramatic rise in FSH production in ovariectomized females. Finally, the potential for gremlin-1 to become a novel injectable factor to directly modulate FSH secretion should be investigated in future studies.

DECLARATION OF INTEREST

The authors declare that they have no conflicts of interest.

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AUTHOR CONTRIBUTIONS

G.S and D.J.B. were responsible for the experimental design, data analyses, and manuscript preparation. G.S. conducted most of the experiments. L.O., E.B., and X.Z. helped with sample collection and analyses. Y.W. conducted the FSH assays. U.B. provided the GRIC strain. F.R.Z. extracted RNA from sorted gonadotropes for the RNA-seq and performed the quality controls, N.S., M.A.A, and V.N prepared the libraries for RNA-seq and performed the quality controls, and Y.G. analyzed the data, all under the supervision of S.S.. All authors approved the final version of the manuscript.

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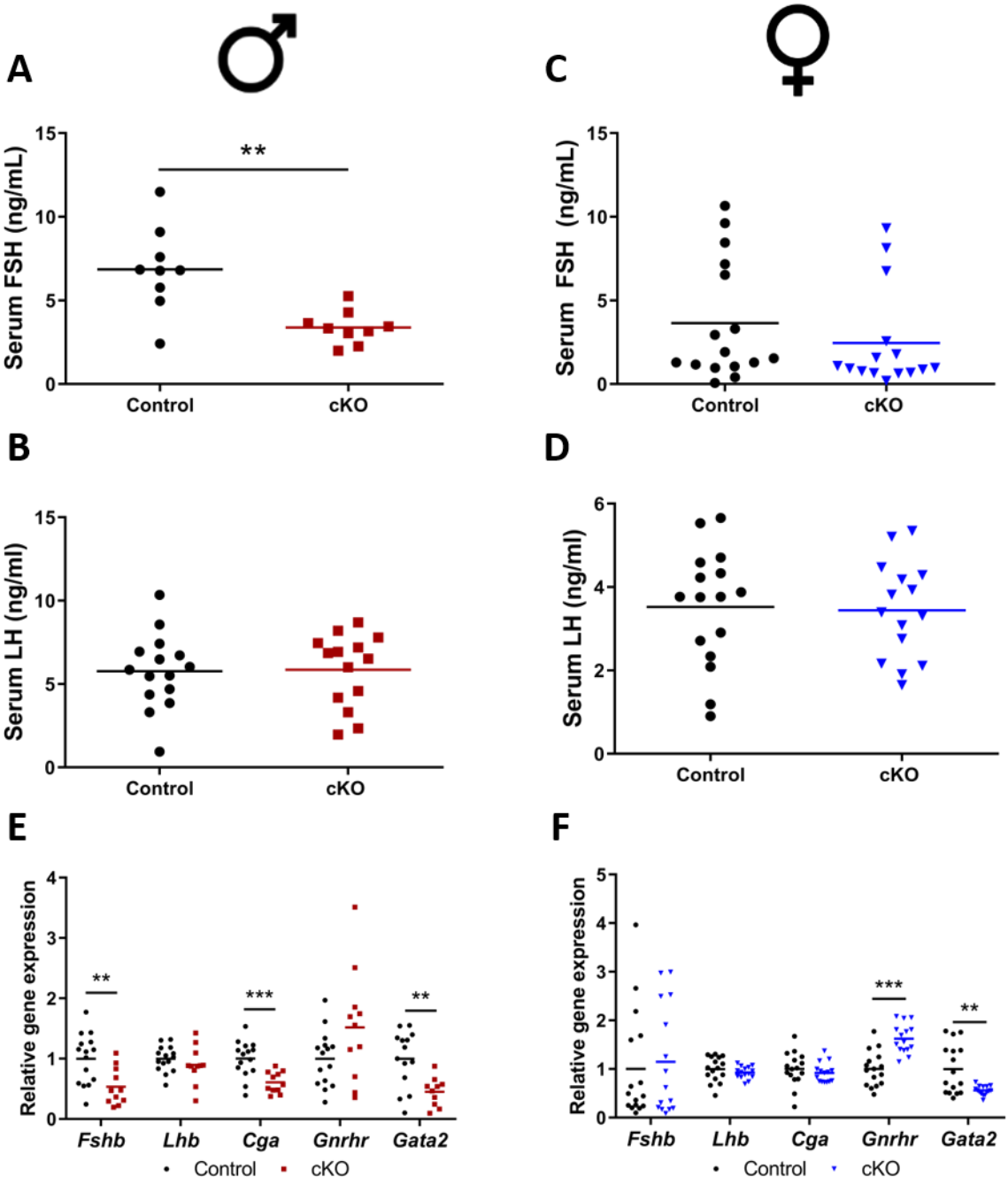
A
♂
Serum FSH (ng/mL)
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C
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Serum FSH (ng/mL)
Control
cKO
B
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Cga
Gnrhr
Gata2
Control
cKO
F
Relative gene expression
***
**
Fshb
Lhb
Cga
Gnrhr
Gata2
Control
cKO

Figure 5.1

Figure 5.1: *Gata2 expression in gonadotropes is required for quantitatively normal FSH production and Fshb expression in male, but not female, mice.* **(A and C)** Serum FSH levels in control and cKO **(A)** males and **(C)** females. **(B and D)** Serum LH levels in *Gata2* control and cKO **(B)** males and **(D)** females. **(E and F)** Pituitary *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels in **(E)** male and **(F)** female control and cKO mice were measured by RT-qPCR. Levels were normalized to the housekeeping gene *Rpl19*. *t*-tests were used for statistical analysis, **p* <0.05, ***p*<0.01, ****p*<0.001.

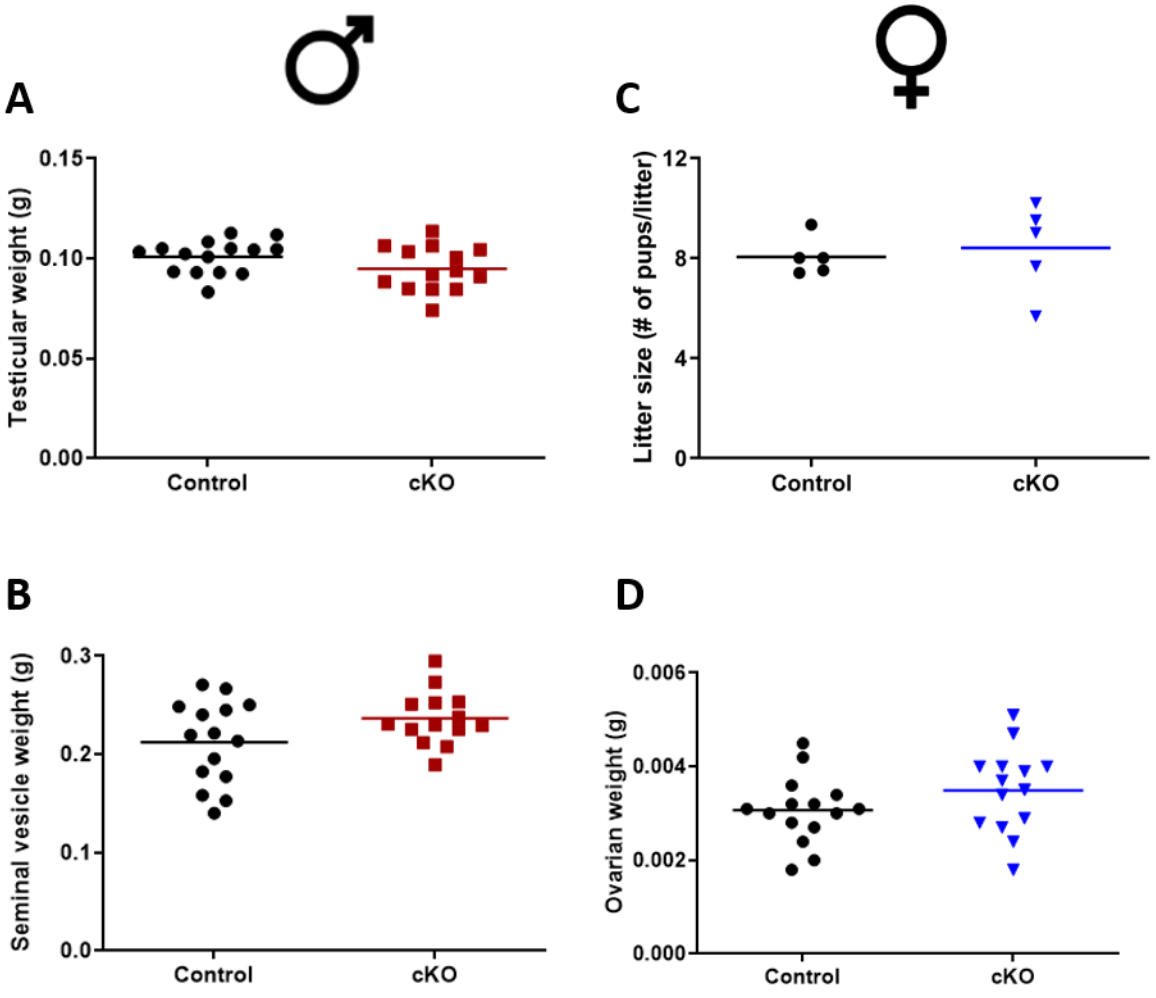

A
♂
Testicular weight (g)
0.15
0.10
0.05
0.00
Control
cKO
C
♀
Litter size (# of pups/litter)
12
8
4
0
Control
cKO
B
Seminal vesicle weight (g)
0.3
0.2
0.1
0.0
Control
cKO
D
Ovarian weight (g)
0.006
0.004
0.002
0.000
Control
cKO

Figure 5.2

Figure 5.2: *Gonads are normal in Gata2 cKO males and females.* **(A)** Testicular weights and **(B)** seminal vesicle weights in control and cKO males (8- to 10-weeks of age). **(C)** Average litter sizes produced by control and cKO females (breeding trials). **(D)** Ovarian weights in control and cKO females (9- to 10-weeks of age, collected at 0700 h on estrous morning). *t*-tests were used for statistical analysis.

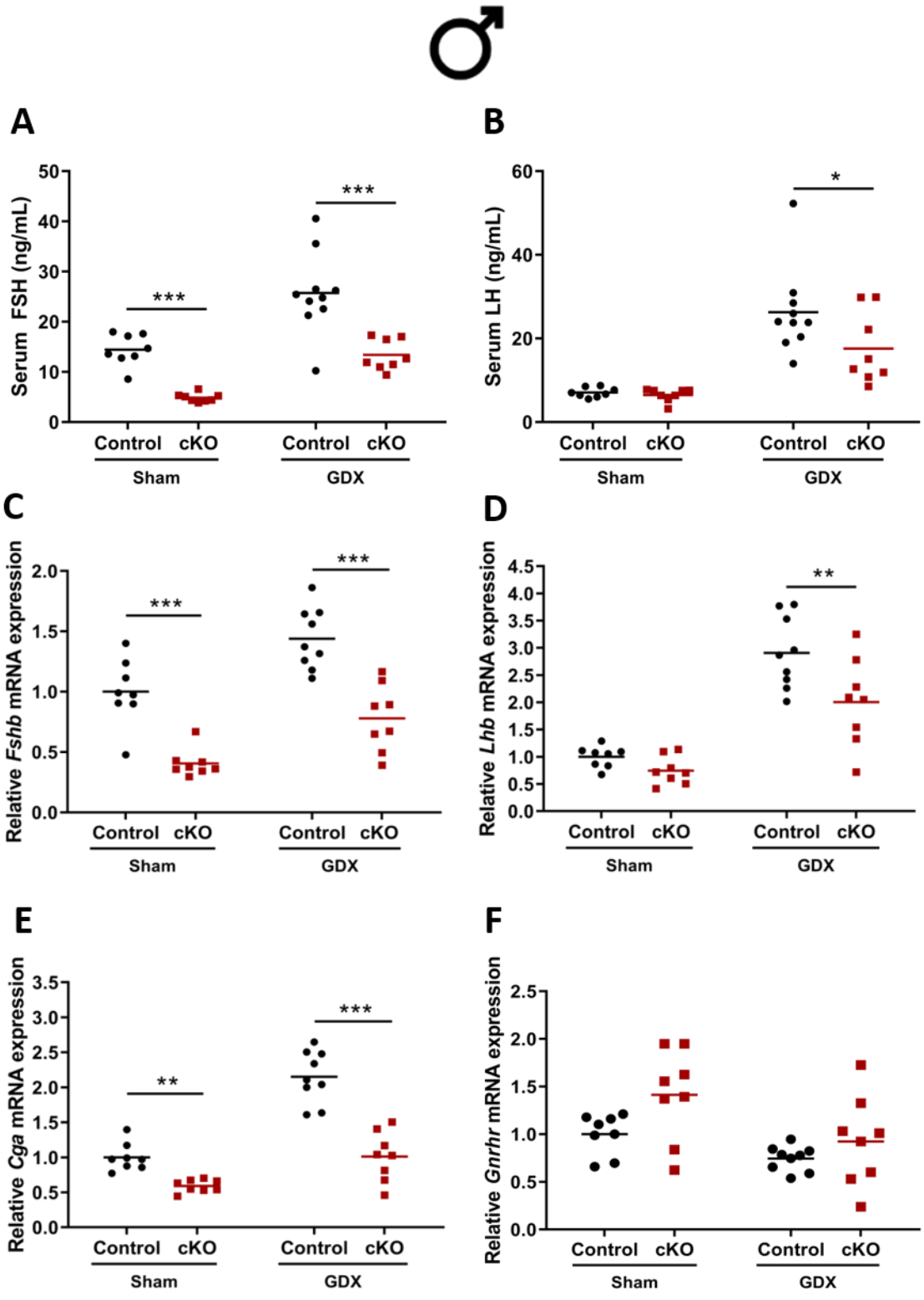

♂
A
Serum FSH (ng/mL)
50
40
30
20
10
0
***
***
Control
cKO
Control
cKO
Sham
GDX
B
Serum LH (ng/mL)
60
40
20
0
*
Control
cKO
Control
cKO
Sham
GDX
C
Relative Fshb mRNA expression
2.0
1.5
1.0
0.5
0.0
***
***
Control
cKO
Control
cKO
Sham
GDX
D
Relative Lhb mRNA expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
cKO
Control
cKO
Sham
GDX
E
Relative Cga mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
***
Control
cKO
Control
cKO
Sham
GDX
F
Relative Gnrhr mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
Sham
GDX

Figure 5.3

Figure 5.3: *cKO males continue to produce lower levels of FSH relative to controls following gonadectomy.* **(A)** Serum FSH and **(B)** LH in sham-operated (Sham) and castrated (GDX) control and *Gata2* cKO males. **(E-F)** Pituitary gene expression profiles in Sham and GDX control and cKO animals (**[C]** *Fshb,* **[D]** *Lhb,* **[E]** *Cga,* and **[F]** *Gnrhr*). mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. Results were analyzed by two-way ANOVA, followed by *post hoc* Holm–Sidak multiple comparison, **p* <0.05, **p<0.01, ****p*<0.001.

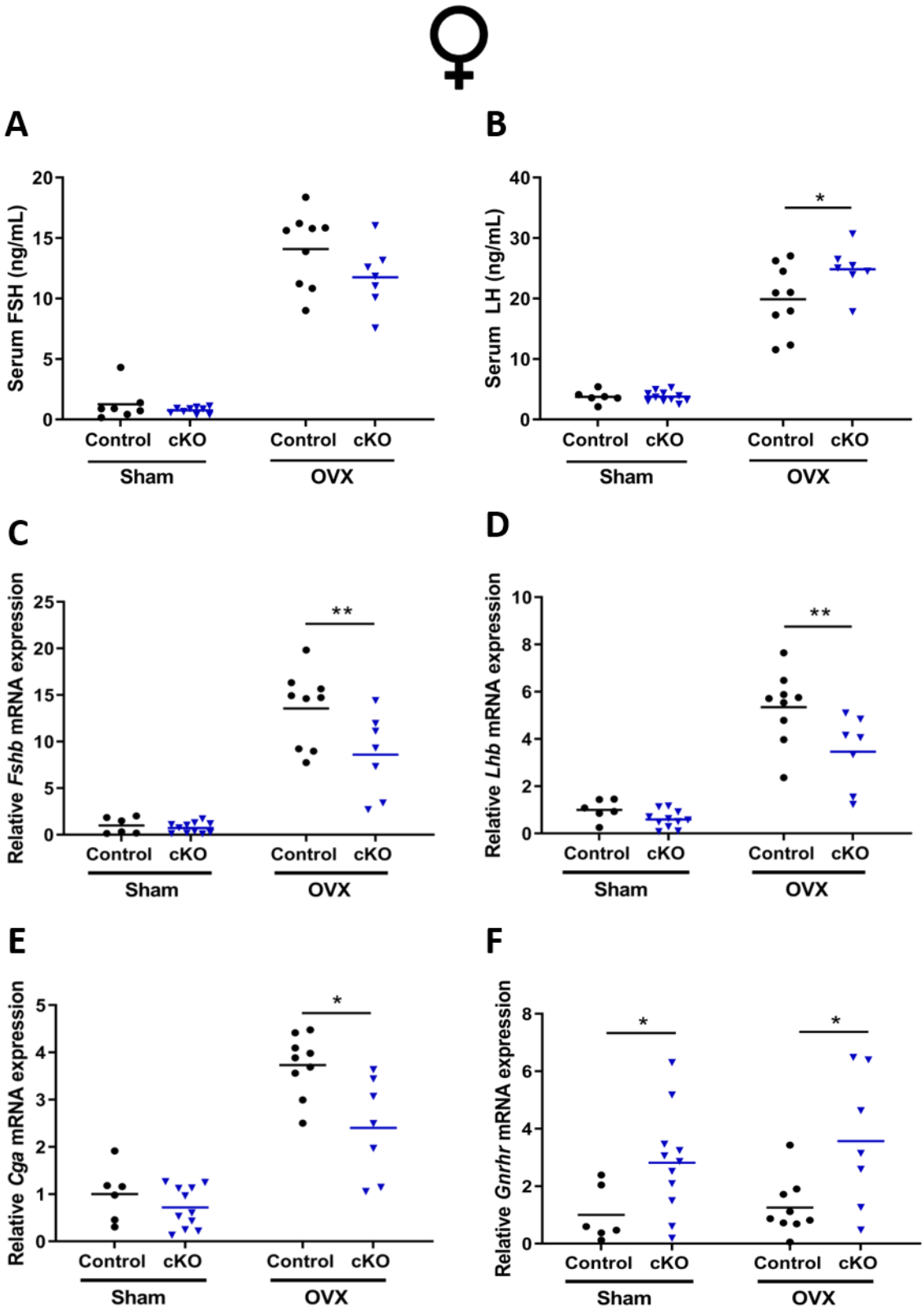

♀
A
Serum FSH (ng/mL)
20
15
10
5
0
Control
cKO
Control
cKO
Sham
OVX
B
Serum LH (ng/mL)
40
30
20
10
0
*
Control
cKO
Control
cKO
Sham
OVX
C
Relative Fshb mRNA expression
25
20
15
10
5
0
**
Control
cKO
Control
cKO
Sham
OVX
D
Relative Lhb mRNA expression
10
8
6
4
2
0
**
Control
cKO
Control
cKO
Sham
OVX
E
Relative Cga mRNA expression
5
4
3
2
1
0
*
Control
cKO
Control
cKO
Sham
OVX
F
Relative Gnrhr mRNA expression
8
6
4
2
0
*
*
Control
cKO
Control
cKO
Sham
OVX

Figure 5.4

Figure 5.4: *Ovariectomized Gata2 cKO females show impairments in gonadotropin subunit expression.* **(A)** Serum FSH and **(B)** LH in sham-operated (Sham) and ovariectomized (OVX) control and cKO females. Sham females of both genotypes were euthanized during metestrus/diestrus. **(C-F)** Pituitary gene expression profiles in Sham and OVX control and cKO females (**[C]** *Fshb,* **[D]** *Lhb,* **[E]** *Cga,* and **[F]** *Gnrhr*). mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. Results were analyzed by two-way ANOVA, followed by *post hoc* Holm–Sidak multiple comparison, **p* <0.05, **p<0.01, ****p*<0.001.

♂

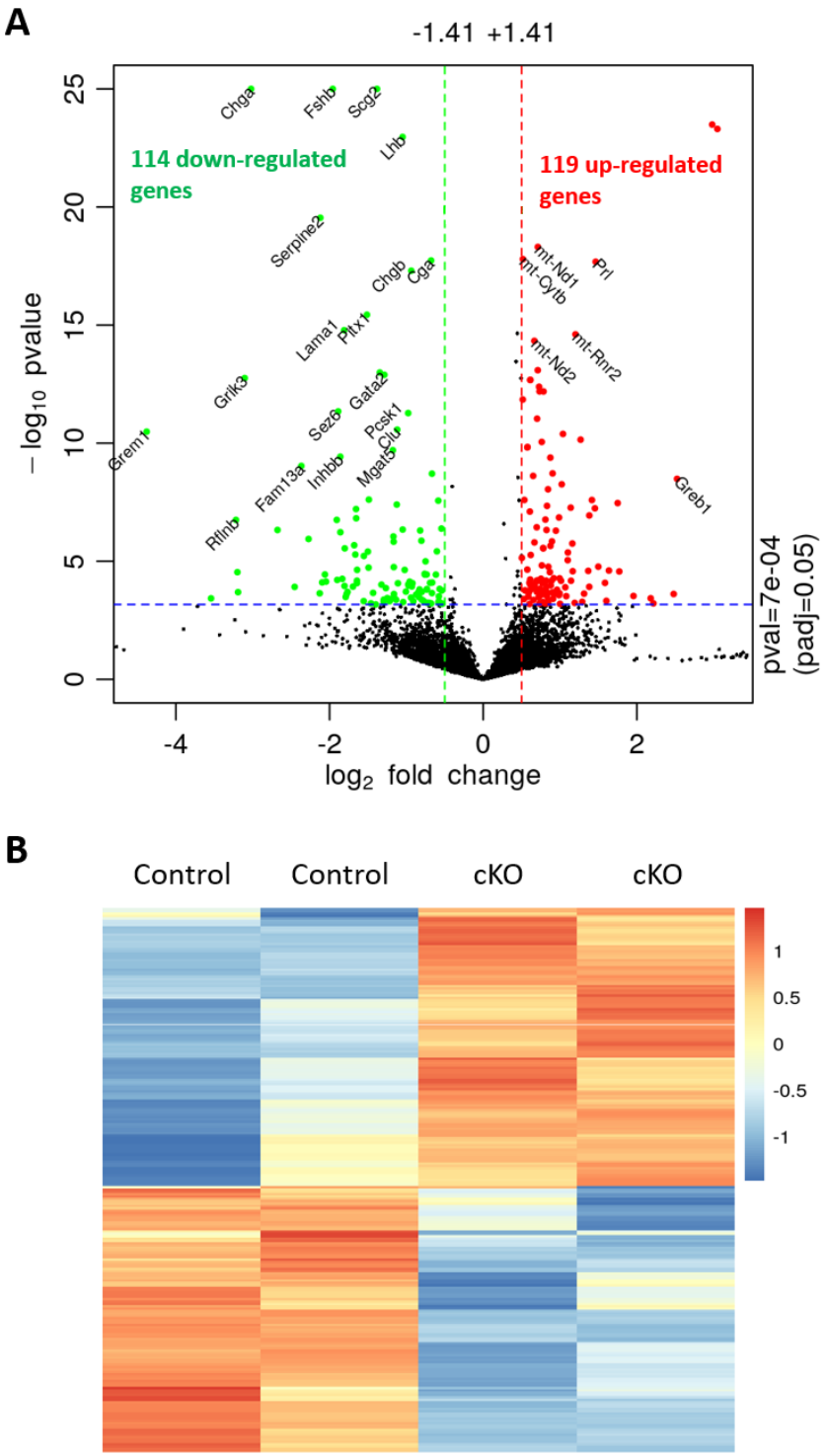

A
-1.41 +1.41
114 down-regulated genes
119 up-regulated genes
Chga
Fshb
Scg2
Lhb
Serpine2
Chgb
Cga
Lama1
Ptx1
Grik3
Sez6
Gata2
Pcsk1
Clu
Mgat5
Inhbb
Fam13a
Rlnh
Grem1
mt-Nd1
mt-Cytb
Prl
mt-Nd2
mt-Rnr2
Greb1
−log10 pvalue
log2 fold change
pval=7e-04
(padj=0.05)
B
Control
Control
cKO
cKO
1
0.5
0
-0.5
-1

Figure 5.5

Figure 5.5: *RNA-seq data analysis of control and Gata2 cKO male pituitaries*. **(A)** Volcano plot with the log2 fold changes in gene expression in gonadotropes isolated from control and *Gata2* cKO males on the x-axis and the statistical significance (−log10 *p*-value) on the y-axis. Differentially expressed genes were selected based on fold change ≥ 0.5 [i.e., log2 fold change ≥ 0.5 in either direction; the mean gene expression (normalized log2 counts per million) of one of the two groups ≥ 4.0; and FDR ≤ 0.05]. The gene symbols of a subset of differentially expressed genes are displayed. **(B)** Heatmap of RNA-seq expression for the 233 genes selected from Panel A that were differentially regulated between gonadotropes isolated from control (2 columns on the left) and *Gata2* cKO (2 columns on the right) mice. Gene expression is shown in normalized log2 counts per million.

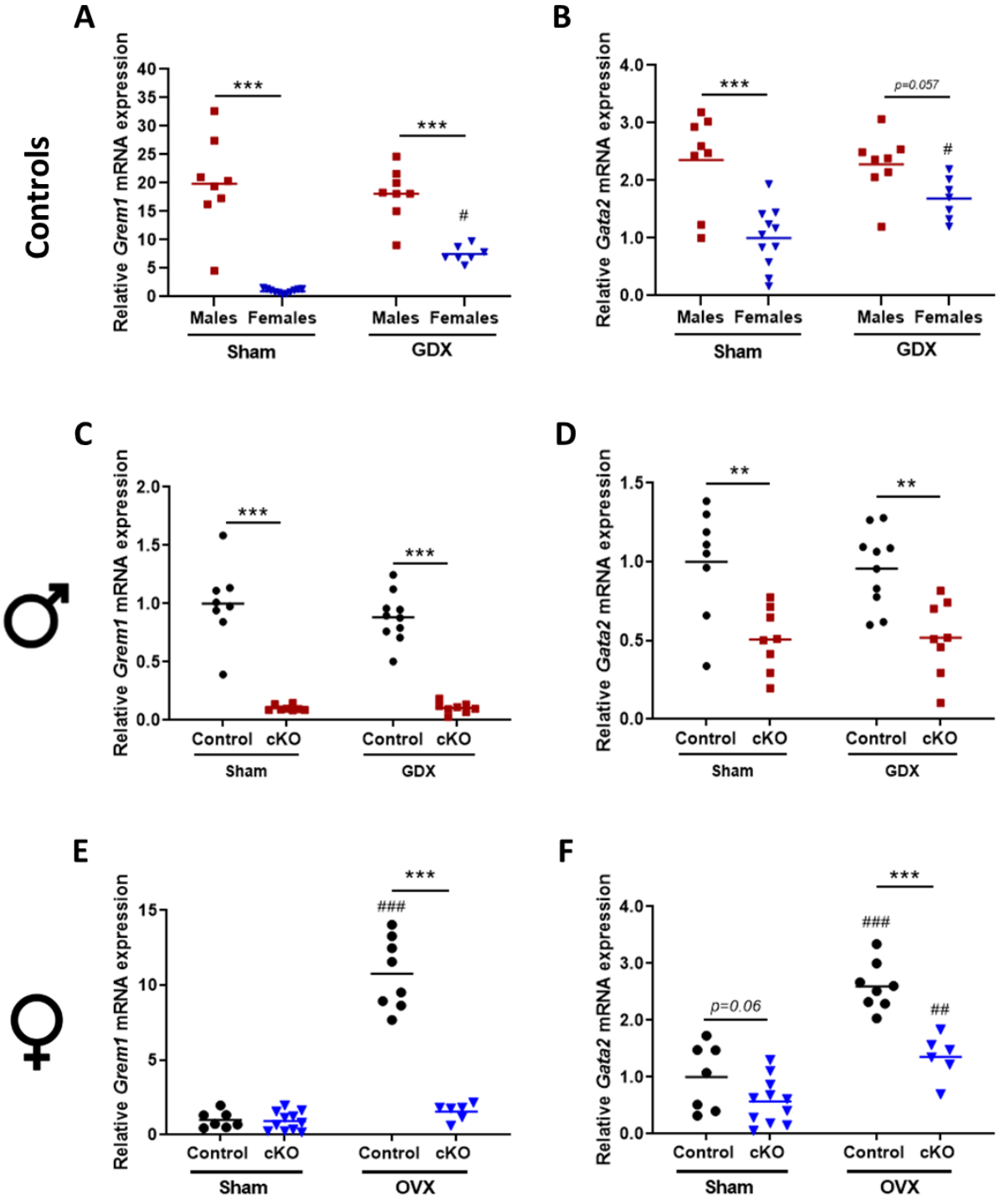

Controls
A
Relative Grem1 mRNA expression
***
***
#
Males Females
Males Females
Sham
GDX
B
Relative Gata2 mRNA expression
***
p=0.057
#
Males Females
Males Females
Sham
GDX
♂
C
Relative Grem1 mRNA expression
***
***
Control cKO
Control cKO
Sham
GDX
D
Relative Gata2 mRNA expression
**
**
Control cKO
Control cKO
Sham
GDX
♀
E
Relative Grem1 mRNA expression
***
###
Control cKO
Control cKO
Sham
OVX
F
Relative Gata2 mRNA expression
***
###
p=0.06
##
Control cKO
Control cKO
Sham
OVX

Figure 5.6

Figure 5.6: *Grem1 and Gata2 expression are correlated in the pituitary gland.* **(A-B)** Pituitary gene expression profiles in sham-operated (Sham) and gonadectomized (GDX) control males and females (**[A]** *Grem1* and **[B]** *Gata2*). In **(A-B)**, expression levels were normalized to those in Sham females. **(C-D)** Pituitary gene expression profiles in Sham and GDX control and cKO males (**[C]** *Grem1* and **[D]** *Gata2*). **(E-F)** Pituitary gene expression profiles in Sham and ovariectomized (OVX) control and cKO females (**[E]** *Grem1* and **[F]** *Gata2*). mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. Although samples may have been used in multiple figures (Figs. 5.3, 5.4, and 5.6), RNA was always processed anew. Results were analyzed by two-way ANOVA, followed by *post hoc* Holm–Sidak multiple comparison, **p* <0.05, **p<0.01, ****p*<0.001 (comparison between sexes **[A-B]** or genotypes **[C-F]**); #*p* <0.05, ##p<0.01, ###*p*<0.001 (comparison between surgeries within sex **[A-B]** or genotype **[C-F]**).

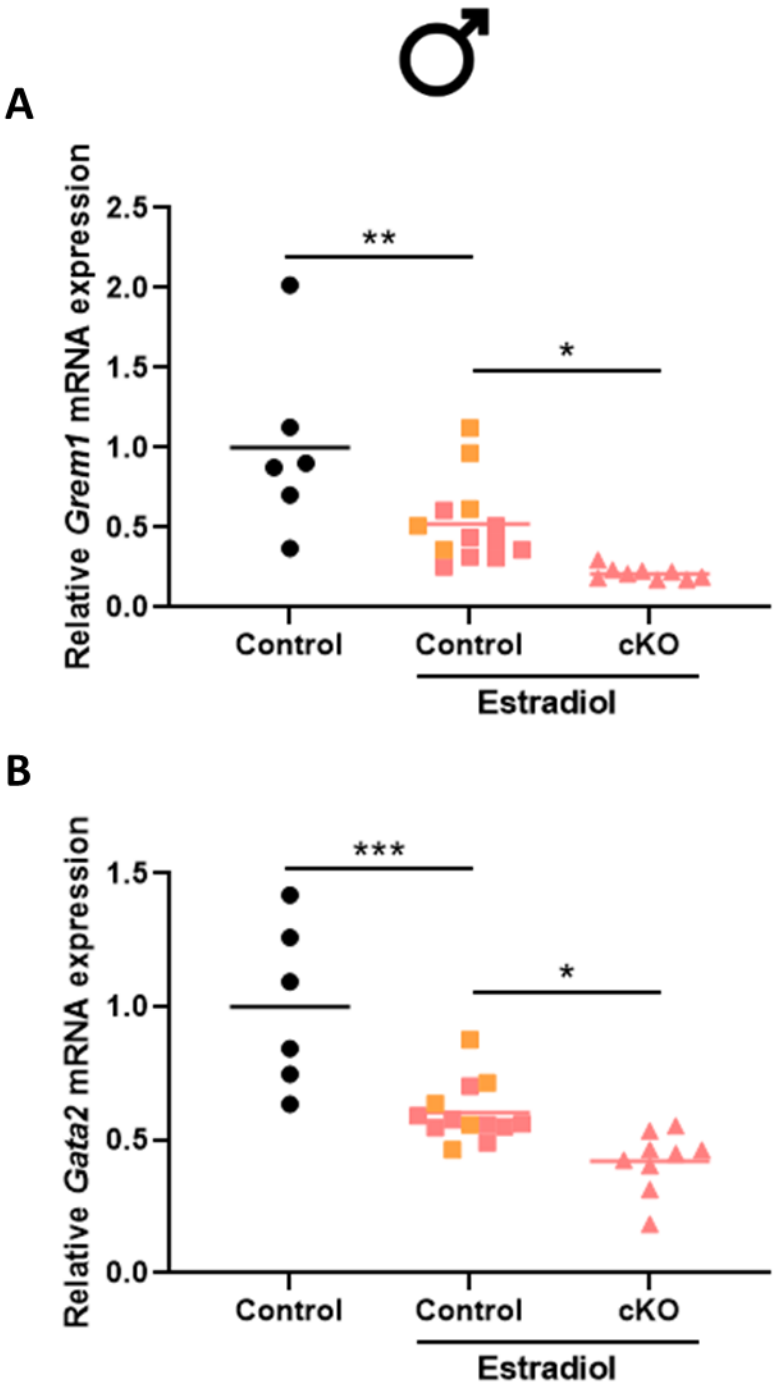

♂
A
Relative Grem1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Control
Control
cKO
Estradiol
B
Relative Gata2 mRNA expression
1.5
1.0
0.5
0.0
***
*
Control
Control
cKO
Estradiol

Figure 5.7

Figure 5.7: *Estradiol inhibits Grem1 and Gata2 expression in control males.* **(A-B)** Pituitary gene expression profiles in castrated controls treated with oil vehicle (Controls) or with estradiol (Controls – Estradiol), and castrated cKOs treated with estradiol (cKO – Estradiol); **(A)** *Grem1* and **(B)** *Gata2* mRNA levels are shown. These results were obtained by combining animals from graphs S5.6.B (shown in black and orange here) and S5.6D (shown in pink here). All RNA samples were processed anew. Results were analyzed by one-way ANOVA, followed by *post hoc* Holm–Sidak multiple comparison, **p* <0.05, **p<0.01, ****p*<0.001.

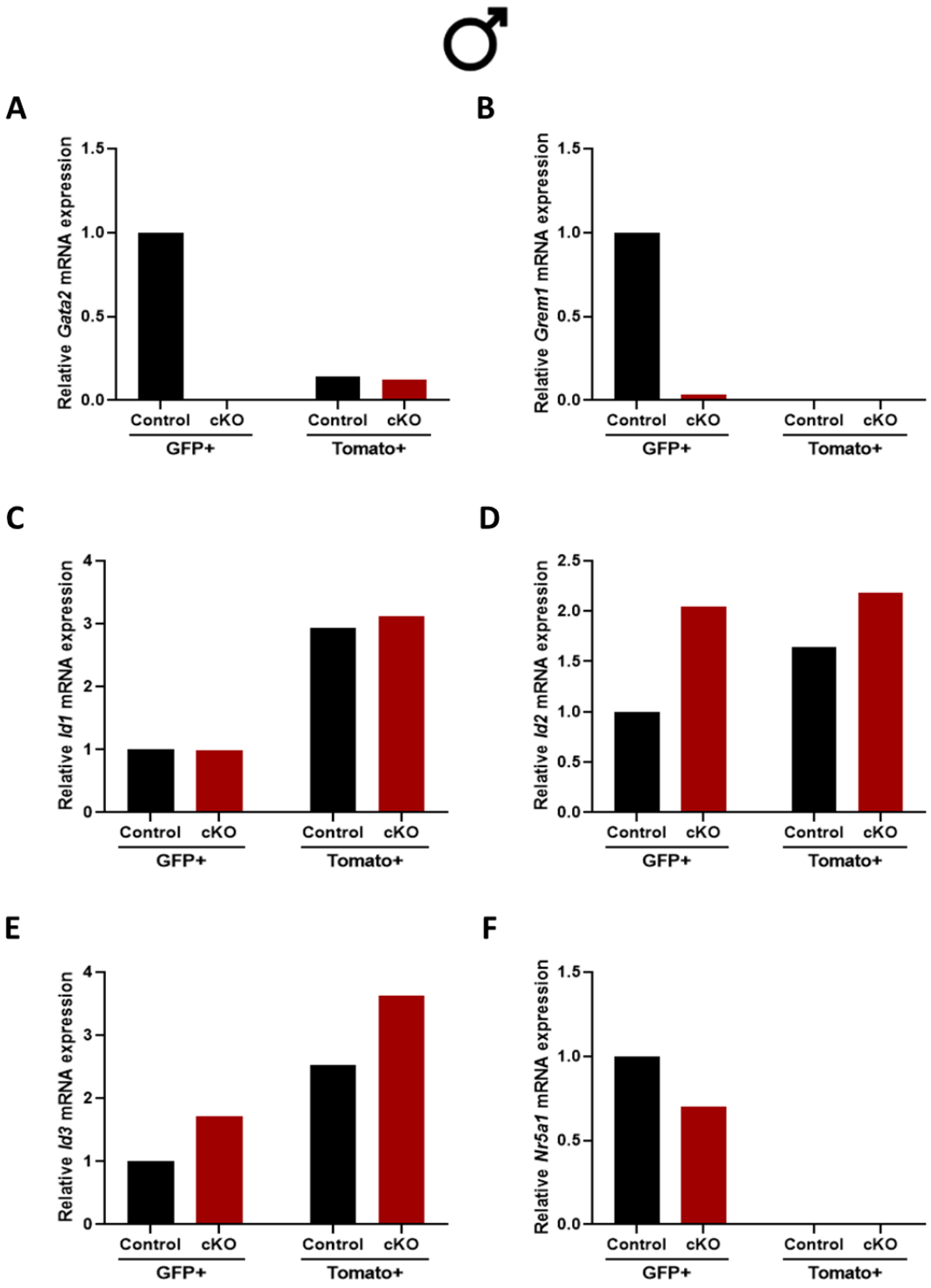

♂
A
Relative Gata2 mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+
B
Relative Grem1 mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+
C
Relative Id1 mRNA expression
4
3
2
1
0
Control
cKO
Control
cKO
GFP+
Tomato+
D
Relative Id2 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+
E
Relative Id3 mRNA expression
4
3
2
1
0
Control
cKO
Control
cKO
GFP+
Tomato+
F
Relative Nr5a1 mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+

Figure 5.8

Figure 5.8: *Gonadotropes from cKO males exhibit increases in expression of BMP target genes.* **(A-F)** qPCR of cDNA from control (*Gata2*$^{+/+}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) and cKO (*Gata2*$^{fx/fx}$; *Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) males, showing **(A)** *Gata2*, **(B)** *Grem1*, **(C)** *Id1*, **(D)** *Id2*, **(E)** *Id3*, and **(F)** *Nr5a1* mRNA expression in purified gonadotrope (GFP+) versus non-gonadotrope (Tomato+). mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. The results come from a single experiment.

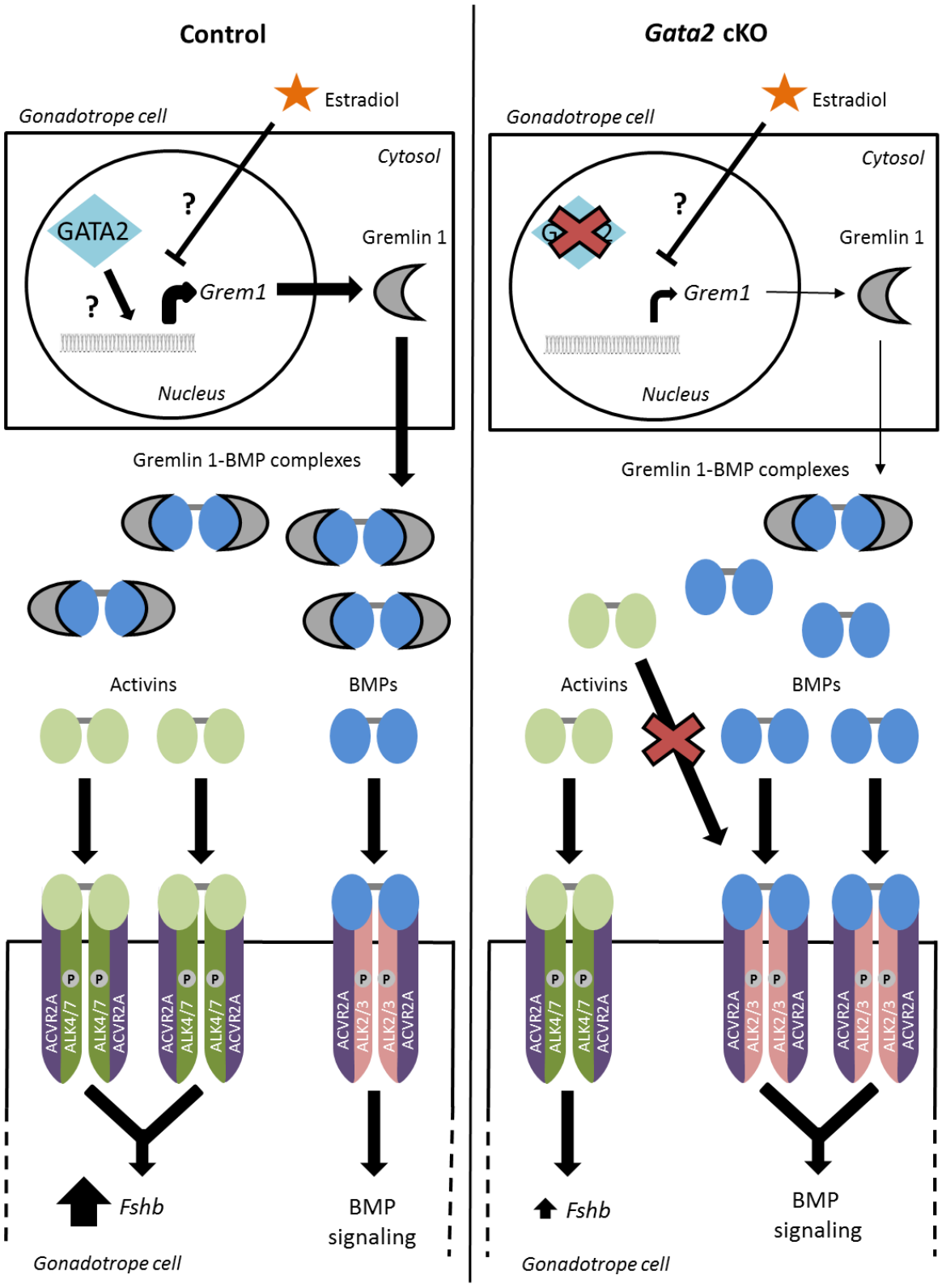

Control
Gata2 cKO
Estradiol
Gonadotrope cell
Cytosol
GATA2
?
?
Grem1
Gremlin 1
Nucleus
Gremlin 1-BMP complexes
Activins
BMPs
ACVR2A
ALK4/7
ALK2/3
P
Fshb
BMP signaling
Gonadotrope cell

Figure 5.9

Figure 5.9: *Proposed model of BMP regulation by gremlin-1 in the presence or absence of GATA2 in gonadotrope cells*. The normal condition is depicted on the left. GATA2 induces (directly or indirectly) *Grem1* expression. Gremlin-1 is secreted and bioneutralizes BMPs in the extracellular environment, providing a permissive environment for activins (or activin-like ligands) to bind ACVR2A without competition and induce *Fshb* expression. The condition in which *Gata2* is ablated is depicted on the right. The absence of GATA2 decreases *Grem1* expression levels. As a consequence, BMPs are less bioneutralized, enabling them to compete with activins (or activin-like ligands) for binding to ACVR2A. Therefore, activin signaling and *Fshb* expression are inhibited. In females, estradiol and perhaps other ovarian factors decrease *Grem1* expression levels (either directly or indirectly through decreased *Gata2* expression). GATA2: GATA-binding factor; ACVR2A: activin receptor type II A; ALK2/3 and ALK4/7: activin receptor-like kinases 2/3 and 4/7.

Gene	Primer sequence
Gata2	
Forward (fx/rec)	GCCTGCGTCCTCCAACACCTCTAA
Reverse (fx/rec)	TCCGTGGGACCTGTTTCCTTAC
Gata3	
Forward	GTCAGGGCACTAAGGGTTGTT
Reverse (fx)	TGGTAGAGTCCGCAGGCATTG
Reverse (rec)	TATCAGCGGTTCATCTACAGC
Gric	
Forward	GGACATGTTCAGGGATCGCCAGGC
Reverse	GCATAACCAGTGAAACAGCATTGCTG
iGric	
Forward	TCAATACCGGAGATCATGCAAG
Reverse	GGTAGGATCATACTCGGAATAG
Rosa26 mTmG	
Forward (WT)	AGGGAGCTGCAGTGGAGTAG
Forward (mut)	TAGAGCTTGCGGAACCCTTC
Reverse	CTTTAAGCCTGCCCAGAAGA

Table 5.1 : Genotyping primers

Gene	Primer sequence
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Fshb	
Forward	GTGCGGGCTACTGCTACACT
Reverse	CAGGCAATCTTACGGTCTCG
Lhb	
*F*orward	ACTGTGCCGGCCTGTCAACG
*R*everse	AGCAGCCGGCAGTACTCGGA
Cga	
*F*orward	TCCCTCAAAAAGTCCAQGAGC
*R*everse	GAAGAGAATGAAGAATATGCAG
Gnrhr	
Forward	TTCGCTACCTCCTTTGTCGT
Reverse	CACGGGTTTAGGAAAGCAAA
Gata2	
Forward	ACTACCTGTGCAATGCCTGT
Reverse	CGCCATAAGGTGGTGGTTGT
Gata3	
Forward	AGGATCCCCTACCGGGTTC
Reverse	GTTCACACACTCCCTGCCTTC
Fstl3	
Forward	CCTGGTCTGGACAGTGGGAT
Reverse	ACACGAGTCTTCAGCACCAGA
Nr5a1	
Forward	AGGAGTTCGTCTGTCTCAAGTTCCT
Reverse	ACAAGGTGTAATCCAACAGGGCAG
Grem1	
Forward	AACAGCCGCACTATCATCAACC
Reverse	GTGAACTTCTTGGGCTTGCAGAA
Id1	
Forward	GGTACTTGGTCTGTCGGAGC
Reverse	GCAGGTCCCTGATGTAGTCG
Id2	
Forward	CTCCAAGCTCAAGGAACTGG
Reverse	ATTCAGATGCCTGCAAGGAC
Id3	
*F*orward	TTAGCCAGGTGGAAATCCTG
*R*everse	TCAGTGGCAAAAGCTCCTCT

Table 5.2: qPCR primers

SUPPLEMENTARY MATERIALS AND METHODS

Histology

One ovary per female was fixed in formalin (HT501128, Millipore-Sigma, Oakville, Ontario, Canada) overnight, and then stored in 70% ethanol. One testis per male was fixed in Bouin's fixative solution (1120-16, Ricca Chemical Company, Pocomoke City, MD, USA) overnight, followed by an overnight incubation in 100% ethanol. Finally, each testis was left in 70% ethanol. Fixed gonads were sent for paraffin-embedding, sectioning, and hematoxylin/eosin staining at the McGill Centre for Bone and Periodontal Research.

The other testis from the males was homogenized in a solution containing 10% DMSO and 0.9% NaCl. Sperm were then counted in a 0.1% trypan blue solution on a hemocytometer. The data represent the average of two counts performed by two independent observers (G.S. and E.B.).

Assessment of female puberty onset and estrous cyclicity

Vaginal opening was monitored daily following weaning (postnatal day 21). At 6 weeks of age, females were swabbed daily for three weeks to assess estrous cyclicity. Vaginal cytology was performed using 0.1% methylene blue, following previously established guidelines[774].

Serum FSH assessment in developing males

We collected submandibular blood from control and cKO males at 3-, 4-, 5-, 6-, and 7-weeks of age (repeated sampling in the same individuals). Blood was processed and sera analyzed as described in the main text.

Anti-inhibin serum injections

Anti-inhibin serum (AIS) was purchased from Central Research (Tokyo, Japan; Lot AIG890208L3)[710,830,831]. Lyophilized AIS was dissolved in sterile H_2O and stored at −80°C. AIS was injected intraperitoneally (100 mg in 0.1 mL) in wild-type females, and control and cKO males.

Blood was collected from the tail vein before injection, and 6 and 11 hours after injection. Blood was processed and serum FSH analyzed as described above.

Generation of Gata2 and Gata3 double cKO mice

The *Gata3*$^{fx/fx}$ mice[848] were kindly provided by Dr. Maxime Bouchard (Biochemistry Department, McGill University) (Jackson Laboratory, stock 028103). *Gata3*$^{fx/fx}$ mice were crossed with *Gata2*$^{fx/fx}$;*Rosa26*$^{mTmG/mTmG}$ animals to generate *Gata2*$^{fx/+}$;*Gata3*$^{fx/+}$;*Rosa26*$^{mTmG/+}$ mice. These were then interbred to generate *Gata2*$^{fx/fx}$;*Gata3*$^{fx/fx}$;*Rosa26*$^{mTmG/mTmG}$ males, which were bred to *Gata2*$^{fx/fx}$;*Gata3*$^{+/+}$;*Gnrhr*$^{GRIC/+}$ females to generate *Gata2*$^{fx/fx}$;*Gata3*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$; *Rosa26*$^{mTmG/+}$ females. These females were then bred to *Gata2*$^{fx/fx}$;*Gata3*$^{fx/fx}$;*Rosa26*$^{mTmG/mTmG}$ males to generate *Gata2*$^{fx/fx}$;*Gata3*$^{fx/fx}$;*Gnrhr*$^{+/+}$; *Rosa26*$^{mTmG/mTmG}$ (control) and *Gata2*$^{fx/fx}$;*Gata3*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$; *Rosa26*$^{mTmG/mTmG}$ (dcKO) males and females. Eventually, given the normal fertility *Gata2*$^{fx/fx}$;*Gata3*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$; *Rosa26*$^{mTmG/mTmG}$ females, they were used to generate control and dcKO animals. The same animals were used to sort gonadotropes by FACS.

Gonadotrope cell count

Seven-week-old animals were either sham-operated or gonadectomized as described in the main manuscript. Two weeks following surgery, pituitaries were dissociated (as described in the main manuscript), and seeded on coverslips previously coated with poly-L-ornithine (P3655 Millipore-Sigma, Oakville, Ontario, CA). The next day, cells were fixed with 4% paraformaldehyde (P6148, Millipore-Sigma, Oakville, Ontario, CA), blocked and permeabilized (0.2% Triton X-100, 10% donkey serum in PBS), followed by incubation with a monoclonal antibody against bovine LHβ (518B7, provided by Dr. A.F. Parlow and the National Hormone and Peptide program)[849] overnight in 5% donkey serum (0.04% Triton X-100 in PBS). The next day, cells were washed three times with PBS (0.2% Triton X-100), incubated with a donkey anti-mouse Alexa 488 secondary antibody (A-21202, Invitrogen) for 1 hour. Cells were washed three times and mounted with ProLong Gold Antifade Mountant with DAPI (P36931, Invitrogen). Ten fields were then captured per coverslip with a fluorescence microscope, following which LH-positive cells were counted manually. DAPI-positive nuclei were counted manually.

Generation of inducible cKO animals

The *Gnrhr*$^{tm1.1(cre/ERT2)Djb}$ (*Gnrhr*^{iGRIC} or iGRIC) mice were described previously[390]. *Gata2*$^{fx/fx}$ mice were crossed with iGRIC/iGRIC animals to generate *Gata2*$^{fx/+}$;*Gnrhr*$^{iGRIC/+}$ progeny. *Gata2*$^{fx/+}$;*Gnrhr*$^{iGRIC/+}$ females were then crossed to *Gata2*$^{fx/fx}$ males to generate *Gata2*$^{fx/fx}$;*Gnrhr*$^{iGRIC/+}$ females, which were then crossed to *Gata2*$^{fx/fx}$ males to generate *Gata2*$^{fx/fx}$;*Gnrhr*$^{+/+}$ and *Gata2*$^{fx/fx}$;*Gnrhr*$^{iGRIC/+}$ males.

At 8- to 10-weeks of age, submandibular blood was collected from males of both genotypes. They were then fed three consecutive cycles of tamoxifen diet (130856, Envigo), with each cycle consisting of 5 days on the diet (*ad libitum*), followed by a 2-day break. Each animal was fed the diet for a total of 15 days. Following the last exposure to tamoxifen, animals were left to recover for 4 weeks. At that point, *Gata2*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (controls) and *Gata2*$^{fx/fx}$;*Gnrhr*$^{iGRIC/+}$ (inducible cKO, or icKO) animals were sacrificed, and pituitary glands and blood were collected. Samples were analyzed as described in the main manuscript.

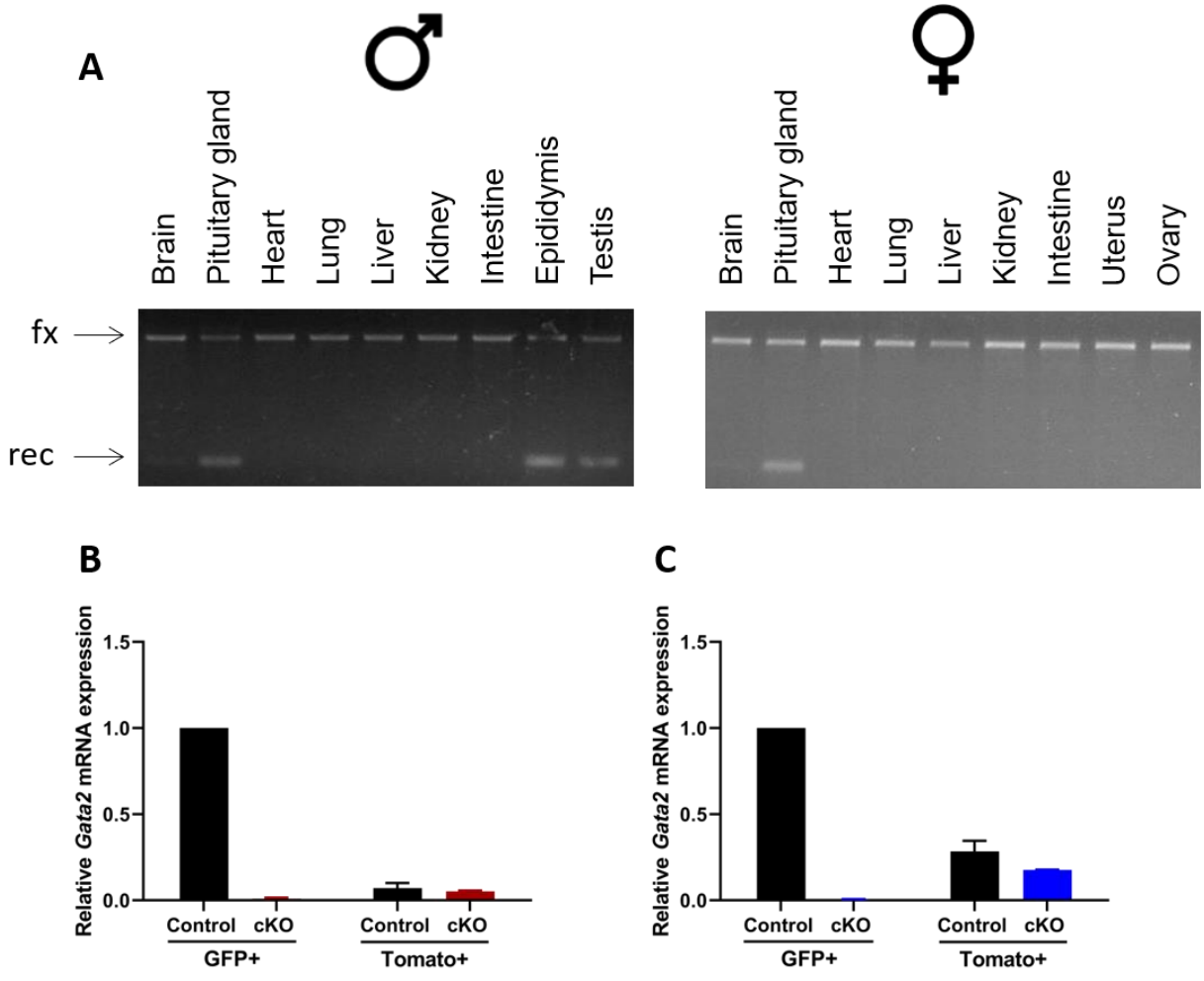

A
♂
Brain
Pituitary gland
Heart
Lung
Liver
Kidney
Intestine
Epididymis
Testis
fx
rec
♀
Brain
Pituitary gland
Heart
Lung
Liver
Kidney
Intestine
Uterus
Ovary
B
Relative Gata2 mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+
C
Relative Gata2 mRNA expression
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
GFP+
Tomato+

Figure S5.1

Figure S5.1: *The floxed Gata2 allele was recombined efficiently and specifically in gonadotrope cells.* **(A)** PCR of genomic DNA from different tissues shows the specificity of recombination for *Gata2* in the pituitary glands of male (left) and female cKOs (right), as well as in the epididymis and testis in males (left); rec: recombined, fx: floxed. **(B-C)** qPCR of cDNA from control (*Gata2*$^{+/+}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) and cKO (*Gata2*$^{fx/fx}$;*Rosa26*$^{mTmG/+}$;*Gnrhr*$^{GRIC/+}$) mice, showing *Gata2* mRNA expression in purified gonadotrope (GFP+) versus non-gonadotrope (Tomato+) in **(B)** males and **(C)** females. *Gata2* mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*.

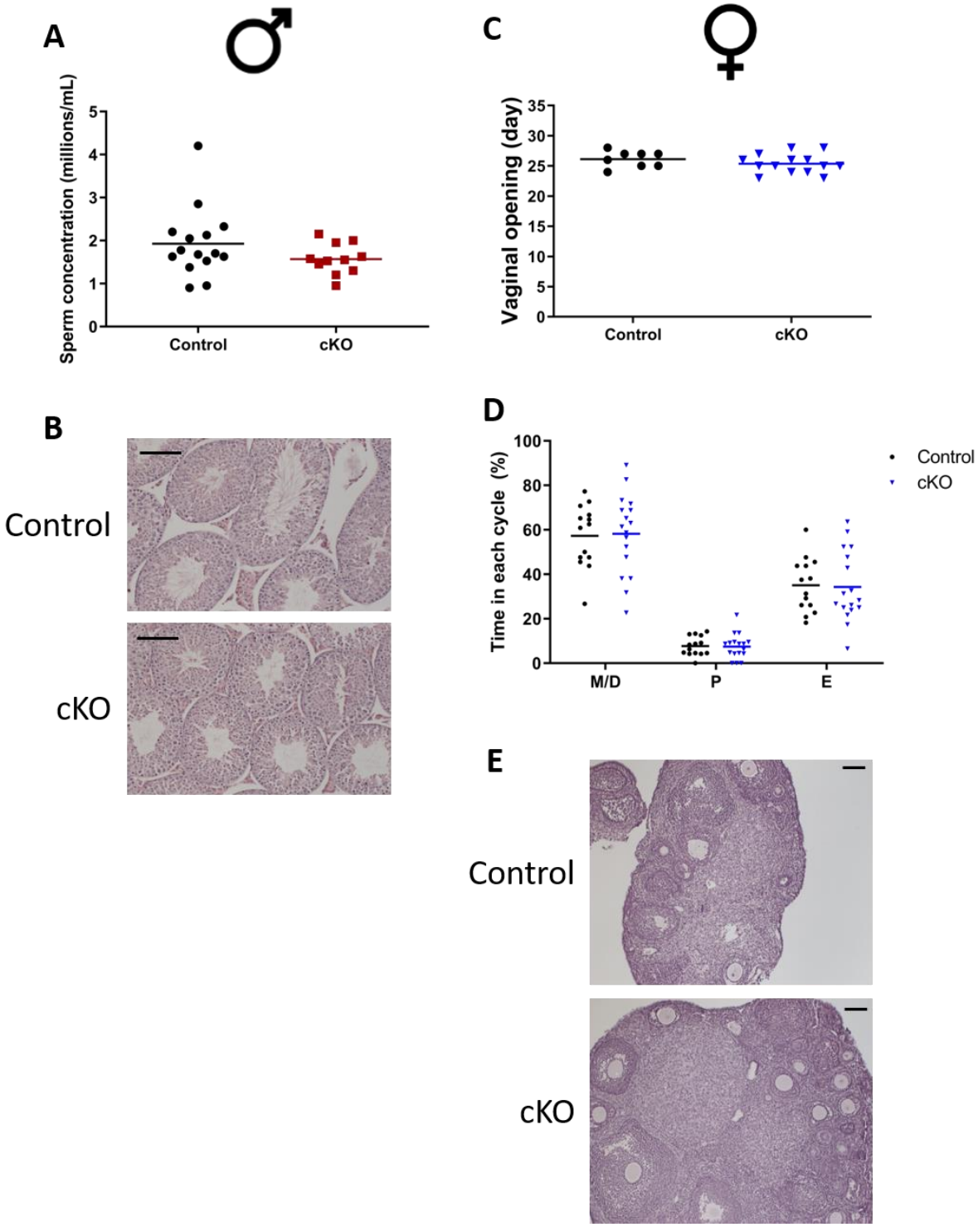

A
♂
Sperm concentration (millions/mL)
0
1
2
3
4
5
Control
cKO
C
♀
Vaginal opening (day)
0
5
10
15
20
25
30
35
Control
cKO
B
Control
cKO
D
Time in each cycle (%)
0
20
40
60
80
100
M/D
P
E
Control
cKO
E
Control
cKO

Figure S5.2

Figure S5.2: *Reproductive profiles and organs in males and females.* Male reproductive organs were collected between 8- and 10-weeks of age. **(A)** Seminal vesicle weights and **(B)** representative testicular sections from control and cKO males. Scale bar: 100 µm. **(C)** Age of vaginal opening (days) in control and cKO females. **(D)** Estrous cyclicity profiles in control and cKO females. M: metestrus, D: diestrus, P: proestrus, E: estrus. **(E)** Representative ovarian sections from control and *Gata2* cKO females. Scale bar: 100 µm.

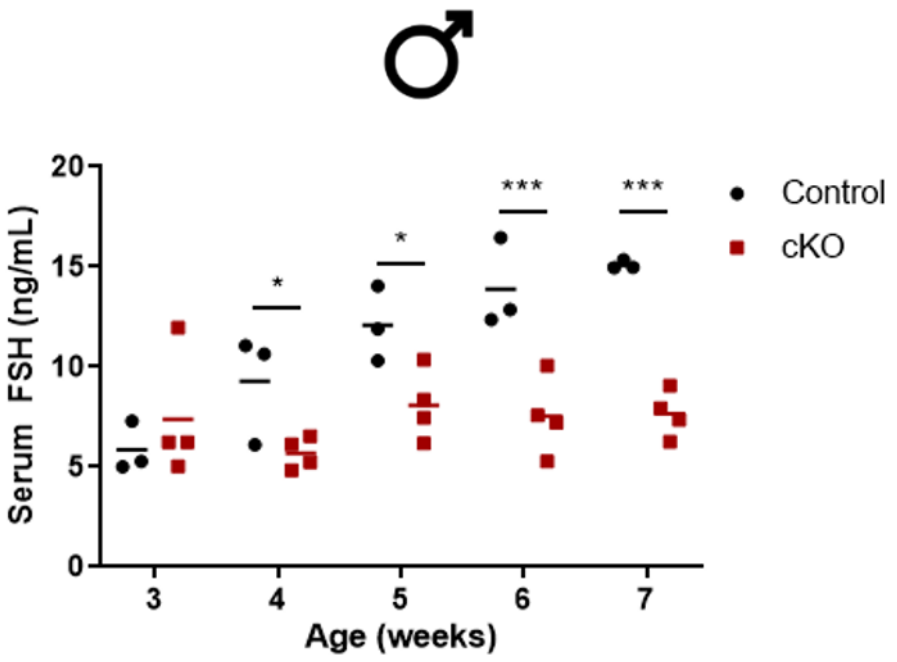
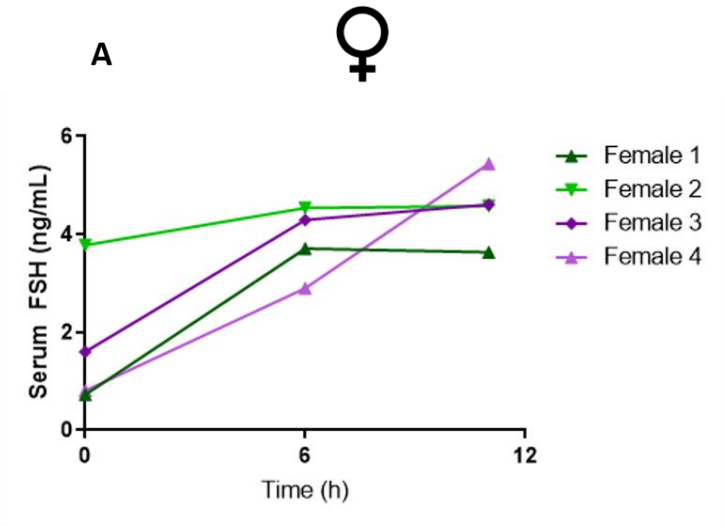


Figure S5.3

Figure S5.3: *FSH deficiency emerges postnatally in male Gata2 cKO mice.* Serum FSH levels in 3-, 4-, 5-, 6-, and 7-week-old control and cKO males. Data were analyzed by two-way ANOVA (repeated measurements) followed by *post-hoc* Holm-Sidak multiple comparison test, *p <0.05, ***p<0.001.


A
♀
Serum FSH (ng/mL)
0
2
4
6
0
6
12
Time (h)
Female 1
Female 2
Female 3
Female 4

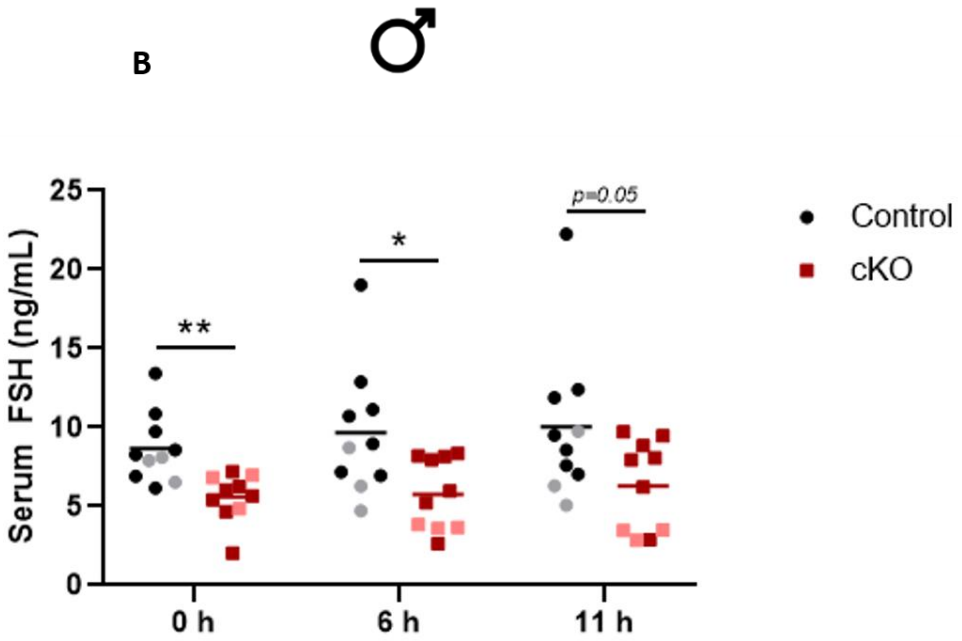

B
♂
Serum FSH (ng/mL)
0
5
10
15
20
25
**
*
p=0.05
0 h
6 h
11 h
Control
cKO

Figure S5.4

Figure S5.4: *Gata2 control and cKO males are insensitive to endogenous inhibins.* Serum FSH levels were analyzed before injection, and 6 and 11 hours after injection of anti-inhibin serum. **(A-B)** Graphs represent data from **(A)** wild-type females and **(B)** control and cKO males. Injections were done immediately after blood collection at the 0 h time point. The results displayed come from two cohorts of injections. Cohort 1: green females and pale males; cohort 2: purple females and dark males. Data were analyzed by two-way ANOVA followed by *post-hoc* Holm-Sidak multiple comparison test, **p* <0.05, ***p*<0.01.

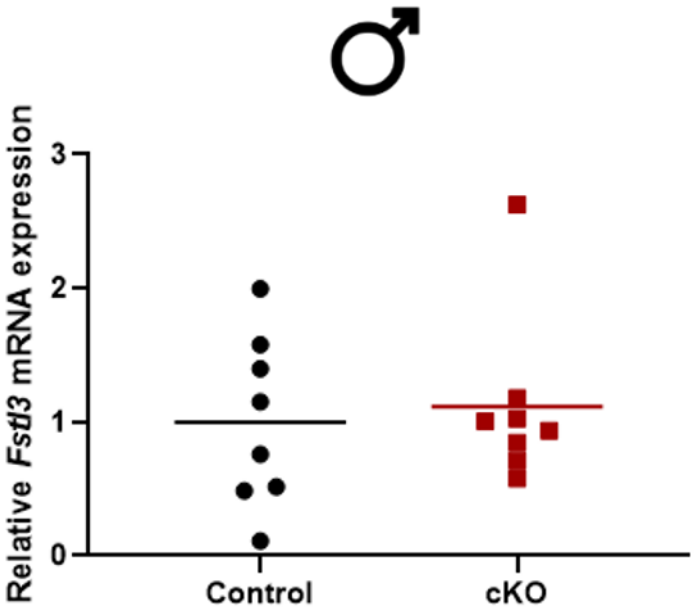


Figure S5.5

Figure S5.5: *Follistatin-like 3 (Fstl3) expression is normal in Gata2 cKO males relative to controls.* Pituitary gene expression of *Fstl3* in control and *Gata2* cKO males. *Fstl3* mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. *t*-test was used for statistical analysis.

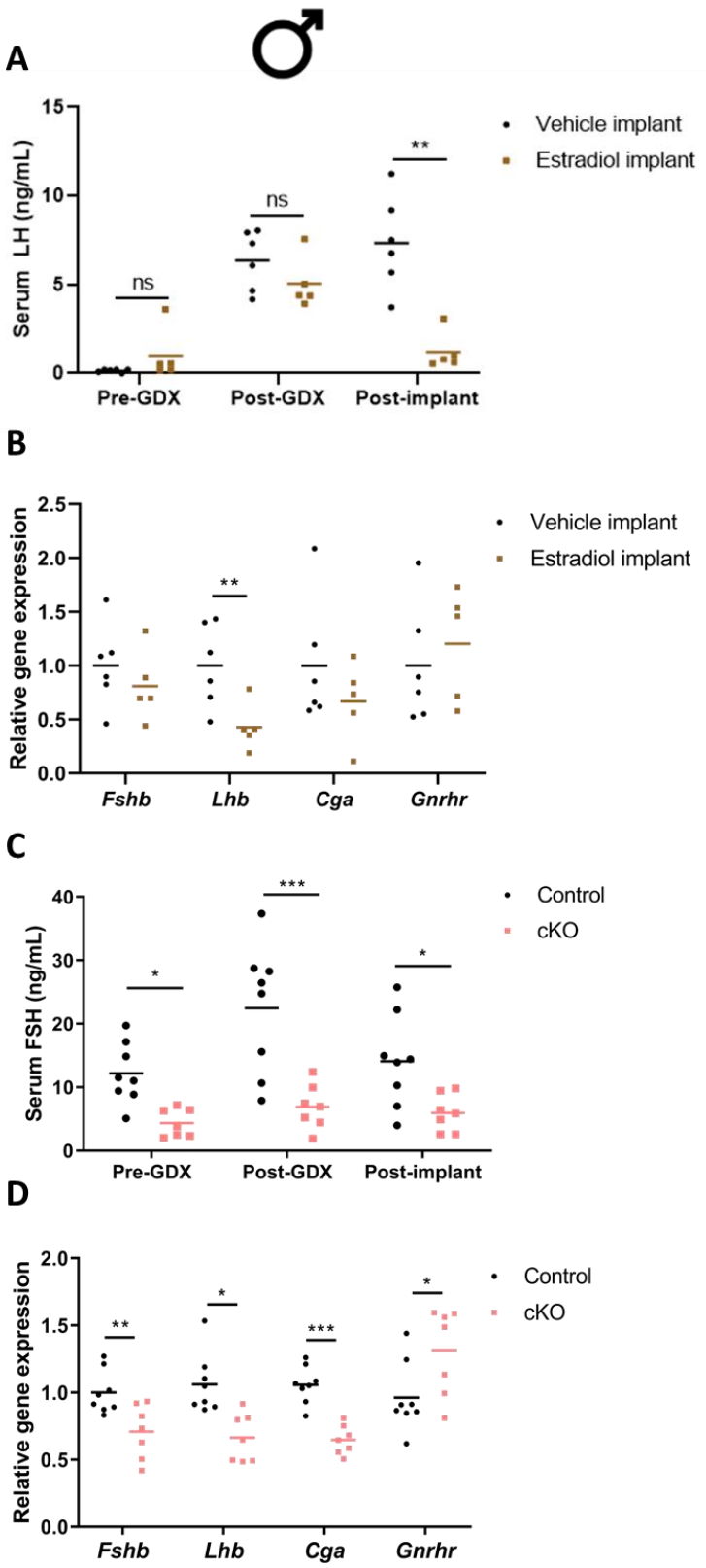

A
♂
Serum LH (ng/mL)
15
10
5
0
ns
ns
**
Pre-GDX
Post-GDX
Post-implant
Vehicle implant
Estradiol implant
B
Relative gene expression
2.5
2.0
1.5
1.0
0.5
0.0
**
Fshb
Lhb
Cga
Gnrhr
Vehicle implant
Estradiol implant
C
Serum FSH (ng/mL)
40
30
20
10
0
*
***
*
Pre-GDX
Post-GDX
Post-implant
Control
cKO
D
Relative gene expression
2.0
1.5
1.0
0.5
0.0
**
*
***
*
Fshb
Lhb
Cga
Gnrhr
Control
cKO

Figure S5.6

Figure S5.6: *The phenotype in Gata2 cKO males is not rescued by exogenous estradiol administration.* **(A)** Serum LH levels in control males before castration (Pre-GDX), two weeks after castration (Post-GDX), and three weeks after treatment with a vehicle or 17β-estradiol implant (Post-implant). **(B)** Pituitary gene expression profiles in control males that received a vehicle or 17β-estradiol implant. **(C)** Serum FSH levels in estradiol-treated control and *Gata2* cKO males Pre-GDX, Post-GDX, and Post-implant. **(D)** Pituitary gene expression profiles in control and cKO animals. In **(B)** and **(D)**, *Fshb*, *Lhb*, *Cga*, and *Gnrhr* mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. Data were analyzed by two-way ANOVA (repeated measurements) followed by *post-hoc* Holm-Sidak multiple comparison test, **p* <0.05, **p<0.01, ****p*<0.001.

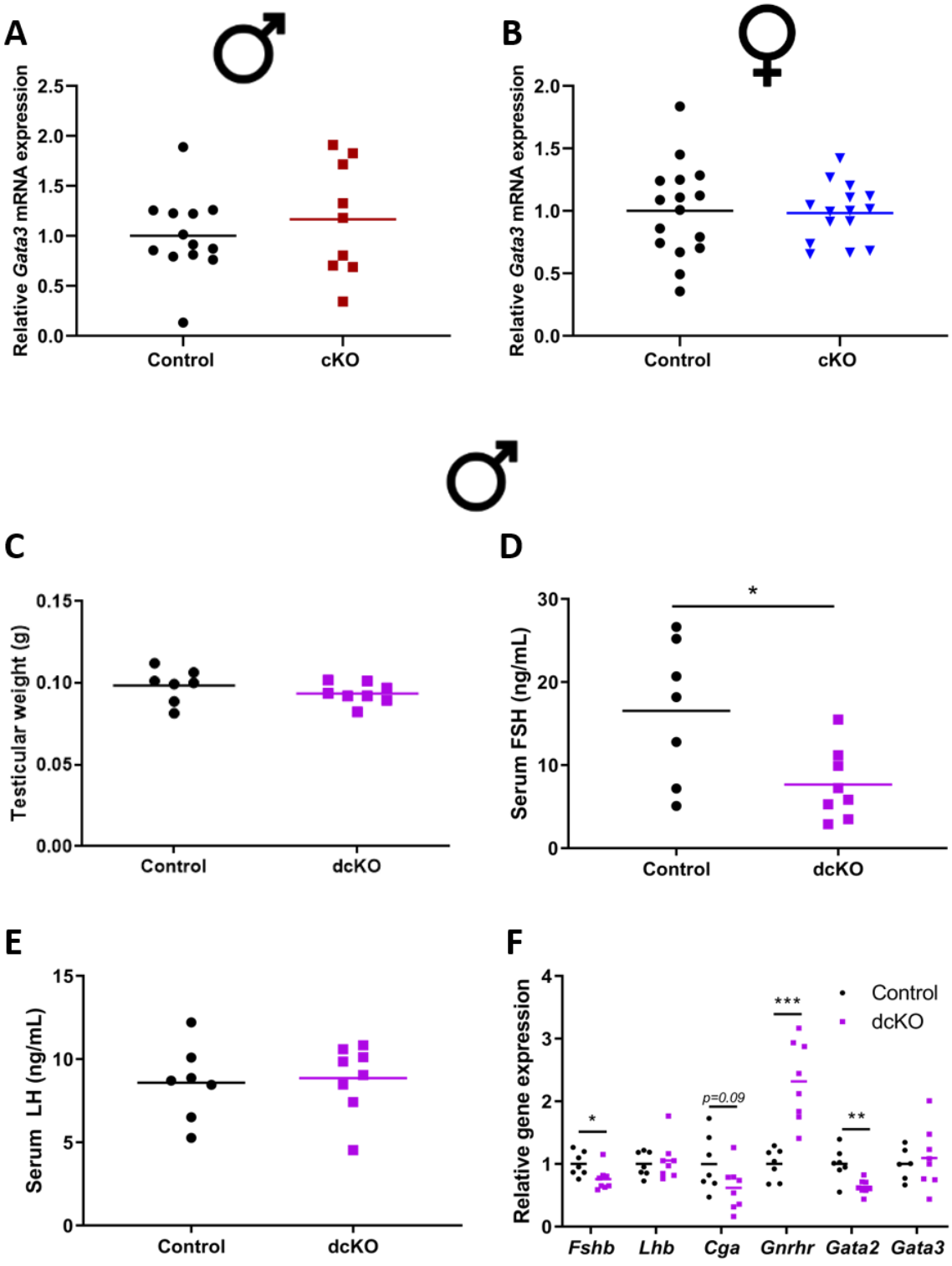

A
♂
Relative Gata3 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
cKO
B
♀
Relative Gata3 mRNA expression
2.0
1.5
1.0
0.5
0.0
Control
cKO
♂
C
Testicular weight (g)
0.15
0.10
0.05
0.00
Control
dcKO
D
Serum FSH (ng/mL)
30
20
10
0
*
Control
dcKO
E
Serum LH (ng/mL)
15
10
5
0
Control
dcKO
F
Relative gene expression
4
3
2
1
0
Control
dcKO
*
p=0.09
***
**
Fshb
Lhb
Cga
Gnrhr
Gata2
Gata3

Figure S5.7

Figure S5.7: *GATA3 is dispensable in murine gonadotropes and does not compensate for the absence of GATA2.* **(A-B)** Pituitary gene expression of *Gata3* in control and *Gata2* cKO **(A)** males and **(B)** females. **(C)** Testicular weights in control and *Gata2/Gata3* double conditional knockout (dcKO) males. **(D)** Serum FSH and **(E)** LH levels in control and dcKO males. **(F)** Pituitary gene expression profiles in control and dcKO males; *Fshb*, *Lhb*, *Cga*, *Gnrhr*, *Gata2*, and *Gata3* mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. *t*-tests were used for statistical analysis, **p* <0.05, **p<0.01, ****p*<0.001.

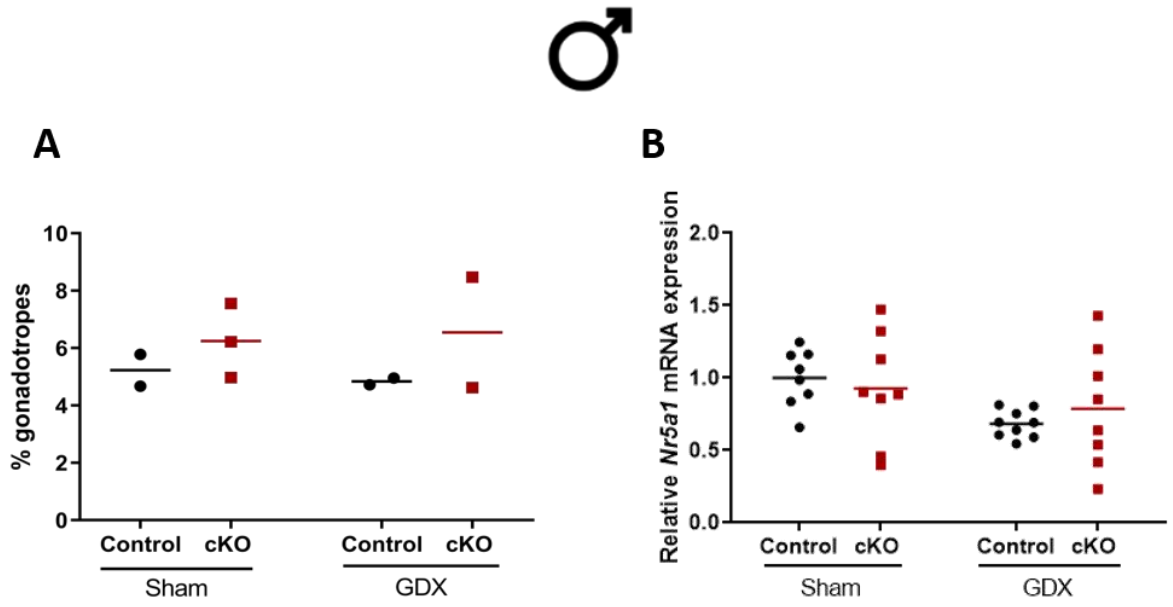

♂
A
% gonadotropes
10
8
6
4
2
0
Control
cKO
Control
cKO
Sham
GDX
B
Relative Nr5a1 mRNA expression
2.0
1.5
1.0
0.5
0.0
Control
cKO
Control
cKO
Sham
GDX

Figure S5.8

Figure S5.8: *Loss of GATA2 during embryonic development does not affect gonadotrope cell numbers.* **(A)** LH-positive cells were counted in pituitary cultures from sham-operated (Sham) and gonadectomized (GDX) control and cKO males. **(B)** Pituitary gene expression of *Nr5a1* in Sham and GDX males, as measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Data were analyzed by two-way ANOVA followed by *post-hoc* Holm-Sidak multiple comparison test.

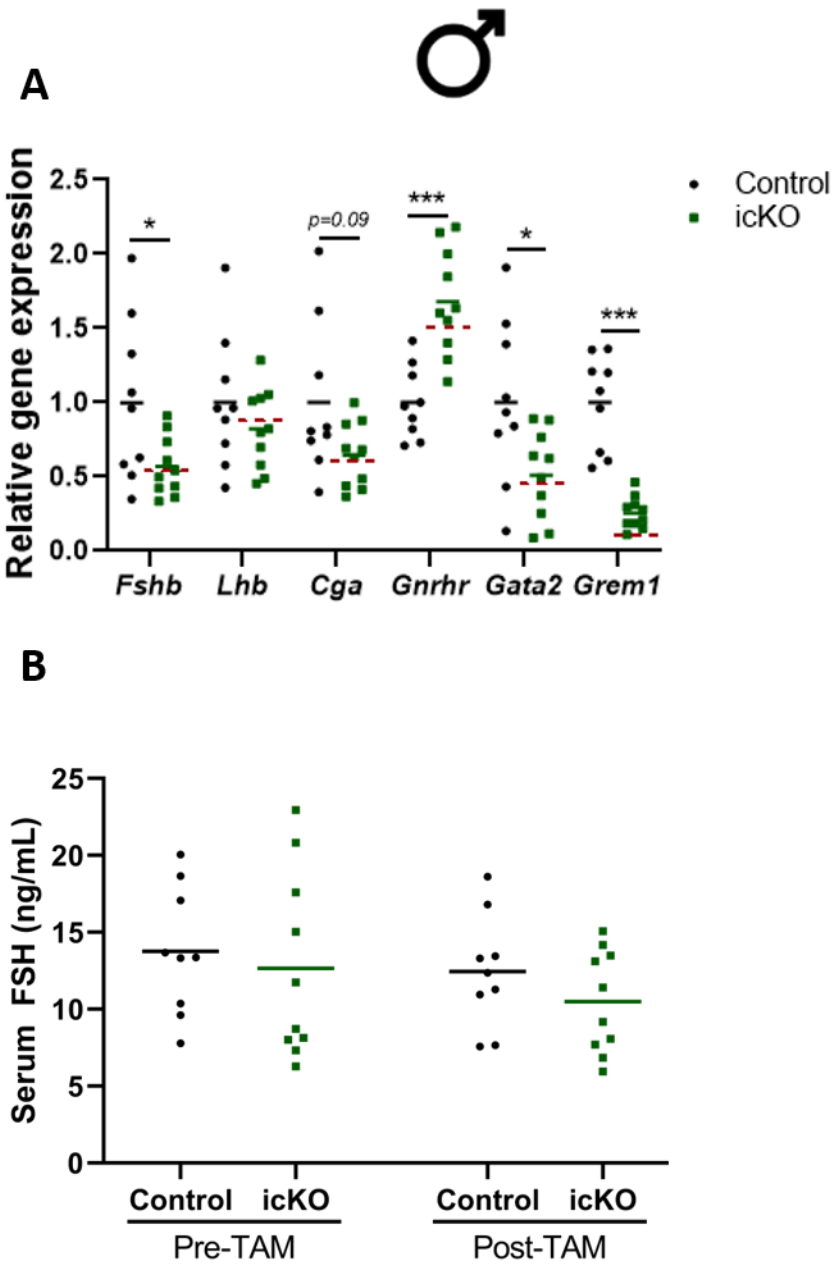

♂
A
Relative gene expression
2.5
2.0
1.5
1.0
0.5
0.0
*
p=0.09
***
*
***
Control
icKO
Fshb
Lhb
Cga
Gnrhr
Gata2
Grem1
B
Serum FSH (ng/mL)
25
20
15
10
5
0
Control
icKO
Control
icKO
Pre-TAM
Post-TAM

Figure S5.9

Figure S5.9: *Loss of Gata2 expression in adult gonadotropes decreases Fshb expression.* **(A)** Pituitary gene expression profiles in control and inducible conditional knockout (icKO) males after being fed a tamoxifen diet. *Fshb*, *Lhb*, *Cga*, *Gnrhr*, *Gata2*, and *Grem1* mRNA levels were measured by RT-qPCR, and normalized to the housekeeping gene *Rpl19*. Dashed red lines represent mRNA levels in *Gata2*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ males (Figs. 5.1E and 5.6C). *t*-tests were used for statistical analysis. **p*<0.05, ****p*<0.001. **(B)** Serum FSH levels in controls and icKOs before (Pre-TAM) and after (Post-TAM) exposure to tamoxifen. Data were analyzed by two-way ANOVA (repeated measurements) followed by *post-hoc* Holm-Sidak multiple comparison test.

Chapter 6: General discussion

The work presented in this thesis provides novel insights into pituitary mechanisms that regulate gonadotropin synthesis and fertility. Below, I will first summarize the main findings presented in my thesis. Then, I will discuss their impact, as well as unresolved and outstanding questions for future research.

In Chapter 2, I challenged the model that GnRH induces *Fshb* expression by inhibiting HDAC activity[512]. My results showed that HDACs play permissive, rather than inhibitory roles, in basal *Fshb* expression *in vitro*[692]. Also, inhibiting HDAC activity blocked both activin- and GnRH-induced *Fshb* expression in a gonadotrope-like cell line (LβT2 cells) and in murine primary pituitary cells. These data call into question the hypothesis that GnRH induces *Fshb* by promoting the nuclear export of HDACs. Though my data were consistent in LβT2 and primary cells, the relevance of these findings remains to be determined *in vivo*.

In Chapter 3, with my co-first author Chirine Toufaily, I investigated the *in vivo* role of the progesterone receptor (PR) in: 1) the secondary, activin-dependent FSH surge, and 2) GnRH self-priming and the preovulatory LH surge. PR function has been well-characterized in the brain[522,550]; more specifically, kisspeptin neuron-specific *Pgr* knockout animals failed to induce LH surges in response to estradiol, showed decreased kisspeptin neuron activation in the AVPV, and were subfertile[522,550]. While the functions of other steroid receptors have previously been assessed in gonadotropes[11-14], I was the first to assess PR function in these cells *in vivo*. I showed that *Pgr* cKO animals had normal gonadotropin production (with the exception of LH surge amplitude), and that females had normal fertility[725]. These data indicate that PR is not required for quantitatively normal FSH production, at least at the time point assessed. Though female fertility was unaffected, the amplitude of the LH surge in these animals was significantly blunted. Contrary to the predictions of earlier studies, this did not appear to derive from impaired GnRH self-priming. As such, these data confirm and challenge previous studies in knockout mouse models and cultured cells.

Although these observations call into question the concept that PR regulates FSH production, the results of Chapter 4 confirm the necessity for TGFβ family signaling in FSH

synthesis[568,640,709]. Previously, the requirement for TGFβ signaling in *Fshb* expression *in vivo* was demonstrated in gonadotrope-specific *Smad3*/*Smad4*[658] and *Smad4*/*Foxl2*[390,680] dcKO males and females. However, prior to my work, the receptors through which TGFβ ligands (canonically thought to be activins, but see more below) bind and signal *in vivo* to induce *Fshb* expression were unidentified. In Chapter 4, I demonstrated that the type II receptors ACVR2A and ACVR2B are required for FSH production. However, activins act via the type II receptors ACVR2A and BMPR2, but not ACVR2B[585], in the LβT2 cell line. These and other data in the field (and unpublished work from our laboratory) challenge the dogma that activins, namely activin B, are the TGFβ ligands that induce FSH production *in vivo*. I will discuss this in greater detail below.

Finally, in Chapter 5, I explored how GATA2 regulates *Fshb* expression. GATA2-deficient males, but not females, displayed impaired FSH production, which correlated with the loss of *Grem1* expression. On the other hand, *Gata2*, *Grem1*, and *Fshb* expression levels increased in control females following ovariectomy; these changes in gene expression were either blunted (*Gata2* and *Fshb*) or blocked (*Grem1*) in ovariectomized *Gata2* cKO females. These data suggest a potential regulatory mechanism in which gremlin-1, induced by GATA2, promotes *Fshb* expression and FSH production. Given that gremlin-1 bioneutralizes certain BMPs (BMP2, 4, 6, and 7)[494], the absence of this protein may provide a permissive environment for BMPs to compete with activins for binding to ACVR2A, indirectly decreasing *Fshb* expression.

In Chapter 6, I discuss my results as they relate to current dogma and consider their relevance to humans. Though I used mice as a model in my thesis, my work was motivated by my desire to identify novel therapeutic strategies to treat human infertility.

6.1 GnRH regulation of FSH production

At the outset of my thesis, in Chapter 1, I described how FSH regulation by GnRH is poorly understood relative to LH. Though I demonstrated in Chapter 2 that HDACs are necessary for basal, activin-, and GnRH-induced *Fshb* expression[692], mechanisms of GnRH action remain unclear.

Based on my findings, a role for HDACs in FSH regulation should be investigated *in vivo*. A first approach could be to inject animals with HDAC inhibitors (such as TSA or entinostat)

systemically and follow changes in FSH production over time. However, a major caveat is that HDAC inhibitors will likely affect other organs, such as the gonads[850-852] or the brain[853,854]. Therefore, it would be challenging to associate changes in FSH production with direct modulation of *Fshb* expression in gonadotropes by HDAC inhibitors. These drugs are also associated with severe side effects[855-858], which may further confound the results.

Therefore, it would be more relevant to use genetic tools to establish a role for HDACs in *Fshb* expression in a cell autonomous manner. My study identified class I, but not class II, HDACs as critical regulators of *Fshb* expression *in vitro*[692]. This indicates a role for HDAC1, 2, 3, and/or 8[859]; since *Hdac8* has low expression levels in murine gonadotropes *in vivo*[267,268], future research should focus on HDAC1, 2, and 3. More specifically, floxed *Hdac1*[860,861], *Hdac2*[862], or *Hdac3*[863] can be crossed to GRIC mice to evaluate the function of each HDAC on gonadotrope function. However, given the functional overlap and compensation between HDACs[864-866], it may be necessary to generate gonadotrope-specific double or triple cKO mice. One would then assess gonadotropin expression levels in these animals under basal conditions or in response to exogenous GnRH, with the hypothesis that *Fshb* expression will be dramatically decreased in triple cKO animals.

An important limitation of these experiments (along with my own data) is that they do not indicate how HDACs regulate *Fshb* expression. Indeed, HDACs have pleiotropic functions[857], and changes in gene expression following HDAC inhibition might be a consequence of increased acetylation of non-histone proteins (such as transcription factors), histone tails (leading to chromatin de-compaction), or a combination of the two. The former can be assessed by protein isolation from control and *Hdac1*/*Hdac2*/*Hdac3* cKO pituitaries, followed by mass-spectrometry in order to identify candidate proteins that are differentially acetylated[867,868] in the absence of HDACs 1/2/3. However, given the small proportion of gonadotrope cells, and given the limited amount of material provided per murine pituitary gland, such an experiment might be challenging and onerous to conduct in mice.

On the other hand, changes in chromatin compaction can be assessed in a more straight-forward manner, with the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)[869]. One could use ATAC-seq to evaluate changes in chromatin compaction between purified

gonadotropes from control and *Hdac1*/*Hdac2*/ *Hdac3* cKO animals, injected with vehicle or exogenous GnRH. Such an approach would have at least two advantages. First, it could reveal HDAC- and/or GnRH-dependent changes in chromatin compaction at the level of the *Fshb* promoter or at novel sites, such as enhancer regions or promoters of other genes. Second, conducting ATAC-seq on purified gonadotropes would provide fundamental knowledge about this cell type that we currently lack.

Indeed, we know that the *Fshb* promoter is tightly compacted in αT3-1 and LβT2 cells[870-872]. This likely explains why *Fshb* is either not expressed (αT3-1 cells) or has low expression levels (LβT2 cells) in these cell lines. However, it is unclear if this compaction reflects the developmental timing at which the cell lines were developed[220,467-469,871,872], or if the *Fshb* promoter is similarly compacted in adult gonadotropes *in vivo*. Therefore, the ATAC-seq experiment described above could provide foundational knowledge about the dynamics of the chromatin structure around the *Fshb* promoter (and other promoters) following GnRH (or vehicle) stimulation, in the presence or absence of HDACs.

In sum, further studies conducted *in vivo* are required to clearly define the role of HDACs in *Fshb* expression, and how they might regulate GnRH-induced FSH production.

6.2 PR, progestagens, and gonadotropin production

I showed that gonadotrope-specific *Pgr* cKO females have blunted LH surges, but have normal fertility and GnRH self-priming[725]. My data therefore challenge three current notions of progesterone function in gonadotropes: 1) PR contributes to the activin-dependent FSH surge, 2) PR is required for GnRH self-priming in gonadotropes, and 3) the LH surge depends on GnRH self-priming.

6.2.1 PR regulation of FSH production

Though I did not assess serum FSH levels at the peak of the secondary surge (03h00)[873], FSH was comparable between controls and *Pgr* cKO females on late estrous morning (07h00). If FSH was impaired at any time point, then I should have observed smaller litter sizes in females.

That is, if FSH was reduced, folliculogenesis would have been attenuated. However, fertility was fully intact in these mice. Therefore, PR is dispensable for FSH production in mice.

These results were surprising, as a PR antagonist blocked the secondary FSH surge in rats[566-569]. Moreover, progesterone, acting via PR, stimulated the murine *Fshb* promoter in LβT2 cells[538,539]. It should be noted however that the glucocorticoid (GR) and androgen (AR) receptors can also bind the PR sites on the *Fshb* promoter[874]. Furthermore, the PR antagonist used to block the secondary FSH surge in rats[566-568], RU486, has antagonistic actions on GR[570]. GR also stimulated the murine *Fshb* promoter[539,668]. Therefore, it may be that *Pgr* cKO females showed no FSH production impairment due to compensation by GR. As murine gonadotropes express *Nr3c1* (encoding GR)[267,268], this possibility should be tested by crossing *Nr3c1* floxed mice[875,876] to GRIC mice in order to assess the cell autonomous roles of GR in gonadotrope cells. Such a study may also necessitate the simultaneous deletion of both *Pgr* and *Nr3c1*.

6.2.2. PR's role in GnRH self-priming

As mentioned in Chapter 3, GnRH self-priming describes the process through which sequential GnRH pulses greatly enhance LH release[549,556,558,559]. This process was first characterized in rats[560,770,877] and rat primary pituitary cultures[770,878,879]. It was later demonstrated that GnRH self-priming can be inhibited by RU486 in estradiol-primed pituitary cultures[556,557,767,880]. Of note, these effects did not require the presence of progesterone in the culture medium, suggesting that PR was acting in a ligand-independent fashion. Taken together, these data indicate that estradiol-induced PR activity contributes to GnRH self-priming, leading to enhanced LH secretion.

However, most of these results originated from rats or rat pituitary cultures. When murine pituitary cultures were treated with RU486, GnRH self-priming was unaffected[562]. This was perplexing, as pituitary cultures from *Pgr* global knockout females did not exhibit GnRH self-priming[562]. A caveat was that these females had never expressed *Pgr*, and we cannot rule out developmental effects. With the assumption that gonadotrope development was intact in these mice, how can we explain these species differences?

PR can exist as one of two isoforms; PR-A and PR-B. Rat, but not murine, gonadotropes produce higher levels of PR-A relative to PR-B[552]; RU486 acts as an antagonist on PR-A, but reportedly as a partial agonist on PR-B[881,882]. Then, perhaps the predominance of PR-A in rat gonadotropes explains why RU486 blocks GnRH-self priming in rat, but not in murine cells, which have equal amounts of PR-A and PR-B.

Nevertheless, GnRH self-priming was intact in gonadotrope-specific *Pgr* cKO females. This observation contradicts data obtained from global *Pgr* knockout mice. Several possibilities may explain this discrepancy, which were discussed in Chapter 3. Given that *Pgr* was selectively ablated in gonadotropes in my model, and that each female served as its own control in the GnRH self-priming experiment (see Chapter 3), my research indicates that PR is not required for GnRH self-priming in mice *in vivo*.

6.2.3. PR's role in the LH surge

Since the amplitude of the LH surge was blunted in gonadotrope-specific *Pgr* cKO females, it suggests that GnRH self-priming and amplification of the LH surge are independent and rely on two distinct regulatory pathways. A proof of concept for this idea is that GnRH self-priming is progesterone-independent, while the LH surge is progesterone-dependent (see more below).

While both murine and rat pituitary cultures can exhibit GnRH self-priming in the absence of progesterone[549,556,558,562], maximal amplitude of the LH surge requires progesterone in mice and rats. A typical method to induce a LH surge *in vivo* is to treat ovariectomized females with estrogens[561,883,884]; however, the amplitude of the surge is small compared to a natural LH surge (~4-fold lower)[561]. Only with injections of both estradiol and progesterone does the amplitude of the induced surge reach natural surge levels[561,884]. Thus, progesterone itself, likely through PR, contributes to surge amplitude. Given that GnRH self-priming is intact in the absence of PR, it begs for another mechanism of action of PR in the context of the LH surge.

It may be necessary to conduct RNA sequencing from purified gonadotropes isolated from control and gonadotrope-specific *Pgr* cKO females at the time of the pre-ovulatory surge. Such a study could lead to the identification of novel target genes that encode for proteins involved in the amplification of the LH surge. However, this approach holds several caveats and limitations.

First, the natural pre-ovulatory surge is difficult to predict in mice, making it challenging to euthanize animals at the appropriate time. One way to circumvent this would be to induce a surge in ovariectomized females treated with estradiol and progesterone[885]. Indeed, in this paradigm, the timing of the surge is predictable. Still, it may be necessary to investigate changes in gene expression at different time points prior to, during, and after the onset of the LH surge in control and gonadotrope-specific *Pgr* cKO females.

Second, it is not clear that the effects of PR at the time of the surge are caused by changes in gene expression. Indeed, liganded PR can induce MAPK phosphorylation[886], and MAPKs can in turn phosphorylate other substrates. In such a situation, it may be necessary to conduct mass-spectrometry on purified gonadotropes to identify proteins that are differentially post-translationaly modified[867,868] in *Pgr* cKO gonadotropes relative to controls. Again, a surge induction protocol might have to be used. However, as described in section 6.1, the limited amount of material provided by murine pituitaries might make such an experiment challenging.

Third, it may be that PR is required for the release of LH rather than for its production. This could be investigated by comparing the pituitary LH content of control and gonadotrope-specific *Pgr* cKO females following a surge induction protocol. A higher pituitary LH content in *Pgr* cKO females would suggest impaired LH secretion in the absence of PR, rather than impaired production.

Overall, my results demonstrate that PR is required for amplification of the LH surge, in a GnRH self-priming-independent manner. The precise mechanism(s) involved in PR action at the time of the preovulatory surge remain(s) to be described in future studies.

6.2.4 Progesterone regulation of gonadotropin production in humans

The stimulatory effects of progesterone on gonadotropin production have also been suggested in humans, though it is challenging to assess where (hypothalamus or pituitary gland) progesterone is acting. Estradiol-treated pre-menopausal women secreted FSH in response to exogenous progesterone[784,887], and progesterone increased GnRH-induced FSH secretion in these women[765]. Progesterone also increased LH levels in estradiol-primed monkeys[888,889], women[887,890], and transgender women[890]. This effect was not observed in estradiol-primed

castrated male monkeys[891] and transgender men[890], which suggests a role for progesterone in regulating LH secretion in a sex-specific manner. It should be noted though that one cohort of estradiol-primed orchidectomized men displayed increased FSH and LH in response to progesterone[892]. In sum, progesterone plays a permissive role for gonadotropin secretion in women, which appears to be impaired by a history of exposure to androgens.

Next, is PR playing a role in regulating gonadotropin levels in women? PR antagonists blocked the LH surge in women[887], and had differential effects on FSH depending on the stage of the menstrual cycle. In the early-mid follicular phase, PR antagonists either had no effects or mildly decreased FSH production[887,893], while in the late follicular phase, PR antagonists inhibited FSH secretion[887]. These data suggest a positive role for PR in regulating gonadotropin levels in women, though it is challenging to elucidate whether PR and/or progesterone act centrally or in the pituitary gland. Moreover, RU486 was used in most of these studies, and a role for GR cannot be ruled out.

In sum, progesterone can induce gonadotropin secretion in humans, more consistently so in women than in men. The exact mechanisms of action of progesterone and/or PR at the level of gonadotropes in humans remain to be elucidated.

6.3 TGFβ family regulation of FSH production

6.3.1 Activins: still relevant in FSH production or ligands of the past?

Based on data from primary pituitary cultures, 'basal' *Fshb* expression is dependent on an endogenous TGFβ family ligand that is inhibited by inhibins[543,710,894], follistatin[543,895], and SB431542[692,896-898] (an inhibitor of the type I receptors ALK4/5/7). It was assumed that this ligand was an activin, and activin B in particular, as *Inhbb* (which encodes activin B) but not *Inhba* (which encodes activin A) is expressed in the pituitary gland[267,268,700]. Moreover, an anti-activin B antibody suppressed FSH production in rats[116] and in rat pituitary cells[616]. In contrast, FSH production was not altered in murine pituitary cultures treated with the same antibody[836] and *Inhbb*-deficient mice had elevated FSH levels[633]. These data suggest that activin B might regulate FSH synthesis in rats, but not mice. Nevertheless, the data presented in Chapter 4 (type II

receptor knockouts) and previous observations in *Smad3/4* cKOs clearly demonstrate that one or more TGFβ ligands regulate FSH in mice *in vivo*. The identity of this ligand is not yet known.

Though there are 33 genes encoding TGFβ-superfamily members[899], only a handful of ligands are *bona fide* candidates. This is because clear criteria have been established and most family members fail to satisfy them. First, the ligand must be antagonized by inhibins[635,710]. Inhibins bind ACVR2A, ACVR2B, and BMPR2[805,900-903]. Therefore, ligands that bind AMHR2 and TGFBR2 can be excluded. Indeed, as noted in Chapter 4, these two receptors are not even expressed in gonadotropes[267,268,656]. Second, the ligand must be antagonized by follistatins[836,898,904,905]. Follistatin bioneutralizes activins, some GDFs (GDFs 8 and 11), and some BMPs (BMPs 2, 4, 6, 7, and 15)[906-909]. Third, the ligand must bind ACVR2A and ACVR2B, as I showed in Chapter 4. This limits the list to activins, some GDFs (GDFs 8 and 11), some BMPs (BMPs 2, 6, and 7), and nodal[587,720,844,910,911]. Activin A can be excluded since *Inhba* is not expressed in murine pituitaries and/or gonadotropes[267,268,700], and an activin A bioneutralizing antibody does not affect FSH production in murine pituitary cultures[836]. Activin B can be eliminated from contention for the reasons described above[633,836]. Nodal can also be excluded, as it is not bioneutralized by follistatin[912], and it relies on a co-receptor, Cripto[589,590], which is not expressed in gonadotropes[267,268]. The BMPs that bind ACVR2A and ACVR2B can similarly be eliminated from contention, as mice lacking the BMP type I receptors, ALK2 and ALK3, in gonadotropes do not exhibit reproductive anomalies[723]. In addition, ALK2 and ALK3 signal via SMADs 1, 5, and 8, but the relevant R-SMAD in murine gonadotropes is SMAD3[658,659,674]. This leaves GDF8 and/or GDF11 as the likely suspects.

GDF8 and GDF11 are structurally related to each other and to the activins. They are antagonized by follistatins and bind to ACVR2A and ACVR2B. They stimulate the SMAD2/3 pathway, though preferentially signal via the type I receptor ALK5[591-593,913]. The activins preferentially use ALK4[788]. GDF8/11 can also signal via ALK4 in certain contexts[591,592,914]. GDF11 may also signal via ALK7, similarly to activin B[593]. However, *Acvr1c* (which encodes ALK7) is not highly expressed in gonadotrope cells[267,268], and FSH production is normal in pituitary cultures from *Acvr1c*-null mice[660]. This suggests that ALK4 and/or ALK5 are the likely receptors to mediate the relevant TGFβ ligand's induction of FSH production *in vivo*.

Acvr1b (ALK4) and *Tgfbr1* (ALK5) knockout mice are embryonic lethal[915,916], precluding their use to assess FSH production *in vivo*. Given the existence of floxed animals for these alleles[916,917], it would be feasible to characterize mice in which *Acvr1b* and *Tgfbr1* are knocked out specifically in gonadotropes (using GRIC animals), alone or in combination. If FSH production is affected in *Tgfbr1* cKO animals, given that activins are unable to bind ALK5[572,579,661,918], it would further suggest that GDF8/11, and not activins, are the relevant ligands *in vivo*. In fact, should both *Acvr1b* and *Tgfbr1* need to be recombined to cause sterility, it would further suggest a role for GDFs in this system, as GDF8 and GDF11 can bind all four receptors (ALK4, ALK5, ACVR2A, and ACVR2B)[592]. Of the two, only *Gdf11* is expressed in murine pituitary gland[267,268]. Moreover, GDF8 knockout mice[919] have not been reported to exhibit reproductive phenotypes, to my knowledge. Nevertheless, genetic or pharmacological perturbations of GDF8 or GDF11 should be employed to determine whether either or both ligands regulate FSH production *in vivo*.

6.3.2 Necessity of TGFβ vs GnRH signaling in FSH production

Mice that lack *Gnrh1* expression (*hpg* mice) are FSH-deficient[166], as are *Smad4*/*Foxl2*[390,680], *Smad3*/*Smad4*[658], and now, *Acvr2a*/*Acvr2b* (Chapter 4) dcKO mice. While an argument could be made that GnRH, somehow, signals through FOXL2 or SMADs (though unlikely), the observation that GnRH cannot compensate for the absence of ACVR2A and ACVR2B is intriguing. This is made even more obvious in castrated *Acvr2a*/*Acvr2b* dcKO males, which have increased GnRH pulsatility (as indicated by the increase in *Lhb* expression and LH production) yet continue to have undetectable FSH.

Perhaps GnRH is only required to initiate gonadotrope activity, namely at the time of puberty[920,921]. This is unlikely, as GnRH antagonists decrease FSH secretion in adult rodents[710,728,922,923] and humans[924,925]. It may be that GnRH is required to promote activin-like signaling in gonadotropes. For example, it was suggested that fast-frequency pulses of GnRH induce *Fst* expression[926], and follistatin antagonizes (certain) TGFβ-ligands. Recall that GnRH preferentially stimulates FSH production at low-frequency pulses. It may also be that GnRH regulates the production of the relevant TGFβ ligand(s). TGFβ superfamily members are produced as precursor proteins that require proteolytic cleavage to become functional[927,928]. This

is mediated by proteases[928], that can be activated or inhibited by phosphorylation[929-932]. It is possible that the kinase cascade induced by GnRH signaling also leads to the phosphorylation of these proteases. However, given that the identity of the relevant TGFβ ligand(s) inducing *Fshb* expression *in vivo* is unclear (see above), it is challenging to hypothesize which protease(s) might be involved. Still, it would be worthwhile to investigate the effects of protease-specific inhibitors[933-935] on GnRH induction of *Fshb* expression to test the hypothesis that GnRH promotes the production of active TGFβ ligands.

6.3.3 TGFβ regulation of FSH production in humans

No mutations in *SMAD3*, *SMAD4*, *FOXL2*, *ACVR2A*, or *ACVR2B* are linked to decreased FSH levels in humans. It should be noted that loss-of-function mutations in these genes are typically heterozygous, and mice with a single functional *Foxl2*[681] or *Acvr2a* allele (see Chapter 4) in gonadotropes show no impairments in FSH production, or no indication of such. Moreover, human mutations in *SMAD3*, *SMAD4*, *FOXL2*, *ACVR2A*, or *ACVR2B* cause global functional alterations across tissues (as opposed to gonadotrope-specific cKO mouse models) and lead to severe phenotypes. *SMAD3* and *SMAD4* mutations are linked to cancers[936,937], *FOXL2* to blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES, a disorder characterized, in part, by premature ovarian failure)[938,939], and *ACVR2B* to cardiac defects[940,941]. Some women with BPES have both *FOXL2* alleles mutated, but some FOXL2 activity is usually preserved[942,943], making it difficult to correlate these mutations to FSH production. As for loss-of-function mutations in *ACVR2A*, they are associated with preeclampsia, a hypertensive disorder that occurs during pregnancy[944,945]. This indicates preserved fertility in these women, though again, *Acvr2a*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ female mice are fertile, and heterozygous males have no signs of hypogonadism relative to controls (see Chapter 4).

While activins potently induce the murine *Fshb* promoter in LβT2 cells (in promoter-reporter assays), these ligands only mildly activate the human *FSHB* promoter[666,672]. This may be explained by the fact that the proximal SMAD/FOXL2 binding sites in the murine *Fshb* promoter (GTCTAAACA) are not perfectly conserved in the human promoter (ATCTAAACA). These observations cast doubt on the relevance of activin signaling for *Fshb* expression in humans.

Given the challenges in conducting human studies, a mouse model expressing a human *FSHB* transgene (h*FSHB*) was developed[946]. Specifically, this transgene can rescue fertility in *Fshb* knockout mice[947], indicating that the human FSHβ subunit successfully dimerizes with the murine CGA subunit, and that this hybrid FSH is bioactive. Importantly, these data also indicate that the genetic sequence within the transgene is sufficient for gonadotrope-specific expression and transcriptional regulation. This model provides a unique tool to investigate the regulation of the h*FSHB* gene *in vivo*. Indeed, production of hybrid FSH is greatly reduced when these transgenic animals are crossed to *Acvr2a* global knockout animals[319]. This suggests that the hFSHB transgene is sensitive to TGFβ signaling, though the loss of ACVR2A throughout organs (as opposed to gonadotrope-specific loss of ACVR2A) may confound these results.

Given the lack of responsiveness of the human *FSHB* promoter in the context of promoter-reporter assays, we set out to elucidate whether this promoter depends on transcription factors described for the murine promoter, namely SMAD4 and FOXL2[390,680]. To test this, we generated gonadotrope-specific *Smad4* and *Foxl2* dcKO animals, which expressed the human *FSHB* transgene. These animals were hypogonadal, and had low pituitary murine *Fshb* and human *FSHB* expression, indicating that the human transgene could not be expressed in the absence of SMAD4 and FOXL2[948]. These data suggest that the TGFβ signaling pathway described in rodents may also be relevant in humans.

Finally, several correlative pieces of evidence indicate that TGFβ signaling regulates FSH production in humans. First, fetal human pituitaries in culture secrete FSH in response to activin, in a dose-dependent manner[543]. Second, inhibin levels negatively correlate with FSH levels in women, before and after the onset of menopause[949]. Third, postmenopausal women secrete less FSH following the injection of an ACVR2A-Fc fusion protein[807] or an antibody against ACVR2A[808]. These data indicate the potential for TGFβ signaling to be targeted in the context of IVF protocols and/or other infertility treatments.

Although there is evidence to suggest that human FSH production relies on TGFβ signaling, the specific ligands inducing *FSHB* expression remain to be identified. Indeed, as described in section 6.3.1, FSH production in mice and rats likely depends on different endogenous TGFβ factors. Therefore, the relevant ligand(s) also need(s) to be determined in

humans. It is difficult to hypothesize which ligand(s) may induce FSH secretion in humans. Therefore, it would be important to obtain single-cell RNA sequencing profiles from human pituitaries, in order to rule in or out potential ligands that are expressed (or not).

6.4 GATA2 and gremlin-1: a novel regulatory mechanism of *Fshb* expression?

As described in Chapter 1, several transcription factors have been hypothesized to mediate gonadotrope development. A notable difference between αT3-1 and LβT2 cells is that the latter expresses both *Fshb* and *Gata2*, whereas the former does not. Actions of GATA2 have been investigated in other contexts, notably in prostate cancer[950,951]. There, GATA2 acts as a pioneer factor, a transcription factor that can open chromatin, modifying the landscape of accessible DNA[952,953]. This is critical to allow other transcription factors to access promoters or other regulatory elements. Given the well-established role of GATA2 in promoting chromatin opening in prostate cancer cells, it was possible that GATA2 acted similarly in gonadotropes, providing these cells the unique ability to express *Fshb*. However, based on the residual FSH production in *Gata2* cKO and pitKO[276] males, and the absence of phenotype in gonad-intact cKO females, GATA2 is not absolutely essential for *Fshb* expression.

An important caveat of the *Gata2* cKO (Chapter 5) and pitKO[276] models is that recombination might have occurred too late in development. Indeed, *Gata2* mRNA can be detected as early as embryonic day (ED) 10.5 in murine pituitaries[258], while Cre activity only starts on ED 11.5[280] and 12.75[283] in pitKO and cKO gonadotropes, respectively. As such, it may be that recombination occurred too late to prevent potential pioneering effects, as these can be long-lasting[268,954]. However, αT3-1 cells that stably express *Gata2* do not express *Fshb* (unpublished data). This at least suggests that GATA2 is not sufficient to enable *Fshb* expression in this cell line.

By characterizing gonadotrope-specific *Gata2* cKO animals, I potentially identified a novel protein involved in the regulation of FSH production: gremlin-1. The expression of *Grem1* had been described to be male-specific in mouse pituitaries[719]. Not only did I validate these findings in Chapter 5, but I also demonstrated that: 1) *Grem1* expression was gonadotrope-specific in the pituitary (as suggested by single-cell RNA-seq data[267,268]), 2) GATA2 was necessary for *Grem1* expression, and 3) ovariectomized females expressed *Grem1*, which correlated with increased

Gata2 expression. Given the apparent increase in BMP-target gene expression in *Gata2* cKO male gonadotropes (and non-gonadotropes), it suggests that gremlin-1 functionally inhibited BMP signaling in these cells. In Chapter 5, I discussed potential mechanisms through which gremlin-1 may regulate *Fshb* expression. Though I did not prove that the decrease in *Grem1* was the cause of impaired FSH production, future research should investigate this possibility (see more below).

One way to establish a causal link between GATA2, gremlin-1, and FSH production would be to rescue gremlin-1 expression in *Gata2* cKO gonadotropes. More specifically, a certain strain of genetically engineered mice expresses *Grem1* from the *Rosa26* locus in Cre-positive cells[955]. These animals could be crossed to control and gonadotrope-specific *Gata2* cKO mice to generate mice which constitutively express *Grem1* in gonadotropes (hereafter "gonadotrope-specific *Grem1*-overexpressing animals") in the presence or absence of GATA2. The hypothesis is that FSH production will be normalized in gonadotrope-specific *Gata2* cKO, *Grem1*-overexpressing males. Moreover, sham-operated, gonadotrope-specific *Grem1* overexpressing females (controls and *Gata2* cKO) may show enhanced FSH production relative to females that do not overexpress *Grem1* in gonadotropes.

Another strategy to demonstrate that gremlin-1 regulates FSH production would be to specifically knock out *Grem1* in male gonadotropes using *Grem1*$^{fx/fx}$ mice[956] crossed to GRIC animals. The hypothesis is that gonad-intact, gonadotrope-specific *Grem1* cKO males, but not females, will show impaired FSH production. However, gonadectomized gonadotrope-specific *Grem1* cKOs of both sexes will display reduced FSH levels relative to controls.

The observation that *Grem1* is expressed in ovariectomized, but not gonad-intact, females is intriguing. Does gremlin-1 contribute to the rise in FSH production post-ovariectomy? Does its absence in gonad-intact females explain, at least in part, why female mice produce lower levels of FSH relative to males? Generating animals that constitutively express *Grem1* specifically in gonadotropes, or animals in which *Grem1* is recombined in that cell population would also provide answers to these questions.

Finally, future studies should also determine whether FSH regulation by gremlin-1 is a mouse-specific phenomenon. Indeed, rat gonadotropes do not appear to express *Grem1*[271]. Rather, *Grem1* expression seems restricted to thyrotropes in that species[271]. Still, rat thyrotropes

express *Gata2*[271], and it may be that gremlin-1 acts in a paracrine and autocrine manner in rats and mice, respectively. Furthermore, GATA2 is detectable in gonadotropin-positive pituitary adenomas in humans[957], suggesting that this transcription factor is also expressed in human gonadotropes.

In sum, the discovery that gremlin-1 may facilitate FSH production by gonadotropes requires further investigation on this protein *in vivo*.

Conclusions

The field of reproductive biology gained momentum in the late 20th-beginning of the 21st century, with the purification of GnRH in the early 70's, the first IVF baby in the late 70's, the isolation of inhibins and activins in the 80's, and the description of kisspeptin neurons in the early 2000's. As outlined at the outset of this thesis, while the reproductive axis is quite well understood, important outstanding questions remain. How does GnRH differentially regulate LH and FSH through a single receptor? What mediates the massive release of LH at the time of the preovulatory surge? How does TGFβ signaling induce FSH production *in vivo*? Which transcriptional factors provide gonadotropes the unique ability to express *Fshb* basally and in response to TGFβ signaling? Throughout this thesis, I was able to provide insight into each of these questions. However, novel challenges arose from my results. If the progesterone receptor is required for LH surge amplification, but not for GnRH self-priming, how does PR amplify the preovulatory LH surge? If not activins, which TGFβ ligand(s) induce FSH production *in vivo* in mice? Could gremlin-1 represent a novel target or drug to modulate FSH production by the pituitary gland? Answering these questions has the potential to unveil novel therapeutic strategies to effectively treat infertile couples, or to better understand the aetiology of their infertility.

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