Normal Reproductive function in male mice lacking pituitary kisspeptin receptor.

**Yaping Ma^{2,6#}, Olubusayo Awe^{3#}, Sally Radivick⁴, Xiaofeng Yang¹, Sara Divall⁵,
Andrew Wolfe², Sheng Wu^{1, 2, 3*}**

¹Department of Cardiovascular Sciences/Center for Metabolic Disease Research,
Temple University School of Medicine, Philadelphia, PA, 19140

²Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore,
MD, 21087

³Department of Cellular and Molecular Physiology, Johns Hopkins University School
of Medicine, Baltimore, MD, 21287

⁴Department of Pediatrics, Rutgers University Medical School, New Brunswick, NJ
08901

⁵Department of Pediatrics, University of Washington, Seattle's Children's Hospital,
Seattle, WA 98145-5005

Co-first author

*Correspondence and name of person to whom reprint requests should be addressed:

Sheng Wu

3500 North Broad Street / MERB 456

Philadelphia, PA, 19140

215-7073704

Email : sheng.wu@temple.edu

Key words: kisspeptin receptor; GnRH; pituitary; reproduction;

Abstract

The anterior pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulate gonadal development, gametogenesis and the secretion of the gonadal steroid hormones. The gonadotroph is primarily regulated by hypothalamic secretion of gonadotropin-releasing hormone (GnRH) from neurons of the rostral hypothalamus and is mediated by GnRH receptor signaling. Kisspeptin (KISS1)/kisspeptin receptor (KISS1R) signaling in GnRH neurons plays an essential role in reproductive function. As the kisspeptin receptor is present in the pituitary, kisspeptin signaling via the *Kiss1r* may regulate reproductive function at the level of pituitary. Using Cre/Lox technology, we deleted the *Kiss1r* gene in pituitary gonadotropes (PKiRKO). PKiRKO male and females have normal genital development, puberty onset, and fertility. Females have normal LH, FSH and estradiol while males had significantly increased basal serum FSH levels with no differences in basal serum LH, or testosterone levels. Overall, these findings indicate that the pituitary KISS1R does not play a role in male reproduction.

Introduction

Pubertal onset is marked by an increase in the frequency and amplitude of gonadotropin-releasing hormone (GnRH) pulses by the hypothalamus, which is followed by increased secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by the anterior pituitary (1-4). Kisspeptin and its G-protein coupled receptor (KISS1R/GPR54) have a critical role in regulating the

hypothalamic-pituitary-gonadal axis in all mammalian systems (5-8). Loss of function mutations of *Kiss1r* are associated with lack of puberty onset and hypogonadotropic hypogonadism in humans and rodents, whereas gain-of-function mutations produce precocious puberty in humans (8-12). In rodents, *Kiss1r* is highly expressed in hypothalamus and its role in regulating the reproductive hormone axis is firmly established (13, 14). Conditional knock-out studies have clearly defined a role for *Kiss1r* expression in the GnRH neuron for reproductive development and fertility (15, 16). Besides the hypothalamus, pituitary and gonads, *Kiss1r* is also detected in placenta, liver, pancreas and intestine.

GnRH neurons located in the hypothalamus have long been thought to be the final neuroendocrine output regulating the reproductive axis. The pituitary incorporates diverse cues and signals to affect reproductive output including estradiol (17), testosterone (18), progesterone (19) and inhibin (20), metabolic hormones such as insulin (21, 22), and pituitary derived activin (23). Hypothalamic regulatory factors such as corticotropin-releasing hormone (CRH) (24), dopamine (25), and pituitary adenylate cyclase-activating peptide (PACAP) (26) also affect pituitary function, suggesting that the pituitary may be accessible to KISS1, to activate KISS1R signaling cascades.

Kisspeptin increases gonadotropin gene expression in mouse primary pituitary cells in culture (14). *Kiss1r* expression is enhanced in the pituitary of female mice during the estradiol-induced LH surge (27), suggesting a possible role for kisspeptin as part of the constellation of regulatory inputs to the pituitary required for the

preovulatory release of gonadotropins. We sought to examine the physiological role of pituitary kisspeptin receptor using a novel kisspeptin receptor knockout mouse model (PKiRKO mouse). We find that pituitary kisspeptin signaling is not necessary for reproductive function.

Materials and Methods

Generation of Gonadotrope-specific Kiss1R knockout mice (PKiRKO)

To generate pituitary Kiss1 receptor (Kiss1R) knockout (PKiRKO) mice, we crossed *Kiss1r* heterozygous (fl/wt) female mice (16) with α GSU transgenic (α Cre^{+/-}) male (22, 28) mice. The α GSU transgenic (α Cre^{+/-}) effectively deletes floxed genes specifically in pituitary gonadotrophs. F1: female mice (Kiss1R fl/wt; α Cre⁺) and male mice (Kiss1R fl/fl; α Cre⁻) were crossed to produce PKiRKO mice (Kiss1R fl/fl; α Cre⁺). Litter mates (*Kiss1r* fl/wt; α Cre⁻ and *Kiss1r* fl/fl; α Cre⁻) were used as controls (referred as WT). DNA was extracted as described previously (29). Genotyping primers were designed to detect the presence of the floxed allele, WT allele, or knockout allele of Kiss1r: P1 sense (located in exon 1) and P3 antisense (located in exon 3). Genomic DNA obtained from extra-pituitary organs (eg, tail), primers P1 and P3 amplify a 2096-bp amplicon to indicate the floxed Kiss1r allele and 1882-bp band to indicate the WT allele. Genomic DNA obtained from the pituitary using primers P1 and P3 will amplify a 1120-bp amplicon if the sequence between the LoxP sites is excised indicating a KO allele.

Animals

Adult male and female mice (>2 months old) were used in this study. All animal studies were carried out in accordance with National Institutes of Health guidelines on animal care regulations and were approved by the Animal Care and Use Committee of the Johns Hopkins University. Mice were maintained under constant conditions of light and temperature (14: 10 h light/dark cycle; 22 C) and were fed a normal chow and water ad libitum.

Quantitative Real-Time PCR (qPCR)

RNA was extracted from the pituitary, liver, ovaries, uterus, testes, epididymis, adipose tissue and from two hypothalamic fragments encompassing the arcuate and the anteroventral periventricular nucleus (AVPV) (30), using TRIzol reagent (Ambion life technologies, Carlsbad CA, USA), according to the protocol provided by the supplier. 1µg of RNA was reverse transcribed to cDNA using an iScript cDNA kit (Bio-Rad Laboratories). Real-time qPCR was performed to determine the presence and relative expression levels of *Kiss1r* mRNA in the various tissues. Real-time qPCR was performed in duplicate using SYBR Green Master Mix (Bio-Rad Laboratories) and the CFX Connect qPCR machine (Bio-Rad Laboratories). For each primer set, PCR efficiency was determined by measuring a 10-fold serial dilutions of cDNA and reactions having 95% and 105% PCR efficiency were included in subsequent analyses. Relative differences in cDNA concentration between WT and PKiRKO mice were then calculated using the comparative threshold cycle (C_t) method. To compare the difference of *Kiss1r* expression in the same tissue between WT and PKiRKO, a ΔC_t was calculated to normalize for internal control using the equation: $C_t(\text{Kiss1R}) - C_t$

(18S). $\Delta\Delta C_t$ was calculated: ΔC_t (PKiRKO) - ΔC_t (WT). Relative Kiss1R mRNA levels were then calculated using the equation fold difference = $2^{\Delta\Delta C_t}$.

Pubertal onset and assessment

Preputial separation (PPS) in males was assessed daily beginning after postnatal day 21 by determining whether the prepuce could be manually retracted with gentle pressure. PPS is testosterone dependent and thus is an indicator of activation of the reproductive axis in males. Puberty in rodents is dependent on weight (31); hence, the weights of PKiRKO and wild type were assessed once a week in prepuberty (day 21) through adulthood (day 49). Anogenital distance is testosterone dependent and was determined at 8 weeks of age. Wet testicular weights were determined in freshly dissected mice at two months of age. In females, age at vaginal opening and first estrus are two markers of puberty onset and were assessed for daily in mice after 21 days of life until achieved. Vaginal smears were obtained daily over a period of 14 consecutive days in 6- to 11-week-old mice and cellular morphology examined under microscope.

Hormone assays

Blood samples were collected from submandibular vein (28) between 9:00 and 10:00 AM and basal levels of serum LH and FSH were measured. LH and FSH were measured using a Milliplex MAP immunoassay (Mouse Pituitary panel; Millipore, Massachusetts) on a Luminex 200IS platform (Luminex Corporation). The assay detection limit for LH was 0.012 ng/mL and for FSH was 0.061ng/mL. The intra-assay coefficients of variation (CV) for LH and FSH were between 5 % and 9%.

Serum E₂ levels were measured with a mouse/rat E₂ kit from Calbiotech (El Cajon, CA). The sensitivity for E₂ was 3 pg/mL. Testosterone levels were measured by radioimmunoassay by the University of Virginia Ligand Assay Core (Charlottesville, Virginia).

Fertility assessment

To determine whether male PKiRKO mice were fertile relative to controls, 3 WT and 4 PKiRKO male mice were housed individually with proven fertile WT female mice for 14 consecutive days and then were separated. If a pregnancy ensued, one week after female gave birth, a new male was inserted. Males were rotated among the proven females. To assess fertility in females, a similar strategy was used. Females were housed individually with proven fertile WT male mice for 14 consecutive days and then separated. If a pregnancy ensued, one week after female gave birth, a new male was inserted, rotating among the 7 proven males. The duration of the fertility study was 4 months with 4 rotations for each mouse.

Data Analysis

Data were analyzed by two tailed unpaired student t tests using Prism software (GraphPad Software, Inc, La Jolla, CA). All results are expressed as mean $\pm$ SEM (standard error of the mean). $P < 0.05$ was defined as statistically significant.

Results

Generation of pituitary specific *Kiss1r* knockout mice

PKiRKO (Kiss1R^{fl/fl}; α Cre⁺) mice were generated by Cre recombinase mediated excision of exon 2 of the *Kiss1r*, resulting in an attenuated *Kiss1r* gene in

pituitary, as shown in the schematic diagram of Figure 1A. The PCR product indicates the homozygous floxed-*Kiss1r* alleles (2096 bp) and WT alleles (1882 bp) (Figure 1 B); both bands are present in the heterozygous floxed-*Kiss1r* mouse schematized by Novaira HJ *et al.* (16). Shown in Figure C Lane 1 is a WT α Cre- male mice, followed by two α Cre⁺ male mice in lanes 2 and 3.

Pituitary and tissue *Kiss1r* mRNA expression in PKiRKO mice

The levels of *Kiss1r* mRNA and or protein expression was assessed by qPCR and immunostaining. qPCR demonstrated a reduction in *Kiss1r* mRNA by 88% (Figure 1D) and 64% (Figure 1E) in the pituitary of male and female PKiRKO mice (n=8), respectively, compared with WT mice (n=8). In contrast, no change in *Kiss1r* mRNA was observed in other tissues including the hypothalamus, adipose tissue, liver, or gonads ($P > 0.05$, n =4) (Figure 1 D&E).

Pubertal onset and reproductive function are normal in PKiRKO mice

Body weight has been demonstrated to be highly associated with age of puberty (32). Body weight was not different between male or female PKiRKO and WT mice (figure 2A). In males, PPS (preputial separation) is an androgen dependent process that serves as an external sign of male puberty onset (31). No significant difference was observed age of PPS between the WT and PKiRKO groups (Figure 2B), or achieved anogenital distance (figure 2C). The age of vaginal opening and first estrus was not different between PKiRKO and WT groups (Figure 2D). In sexually mature mice, gonad weight is a marker of reproductive tissue function. Gonad weights were normal between PKiRKO and WT male and female mice (Figure 3 A

and B). Estrous cycling pattern was similar between PKiRKO and WT mice (Figure 3C); percent time spent at each cycle stage was not significantly different. Testes or ovary histology was not different between PKiRKO and WT mice (data not shown). The number of sperm in the seminiferous tubules was not statistically different between WT and PKiRKO mice (data not shown).

Morning serum samples were obtained from non-breeding, post pubertal mice and serum LH and FSH hormone levels were determined. Basal serum LH in PKiRKO males or females was not significantly different relative to WT mice while FSH was significantly lower in PKiRKO males (Figure 3D). Testosterone and estradiol were not significantly different between WT and PKiRKO mice (Figure 3E and F).

Normal fertility in PKiRKO mice

Fertility was examined in WT and PKiRKO mice using a rotating mating protocol. PKiRKO male and female mice demonstrated a normal ability to produce offspring, as shown in Figure 4 A-C. Female WT mice bore their first litter with a similar latency after introduction to PKiRKO males or WT males [22.0 ± 0.8 d, $n=4$ (PKiRKO) vs 22.3 ± 0.6 d, $n=3$ (WT); $P>0.05$] (Fig. 4A). Female WT mice had the same number of litters with PKiRKO and WT sires [3.8 ± 0.5 , $n=4$ (PKiRKO) vs 3.7 ± 0.6 , $n=3$ (WT); $P>0.05$] (Fig. 4B) and a similar number of pups per litter [7.4 ± 1.4 , $n=4$ (PKiRKO) vs 9.3 ± 1.1 , $n=3$ (WT); $P>0.05$] (Fig. 4C). The same pattern was shown in males.

Discussion

Kisspeptin is a potent activator of the GnRH neuron, and thus is a critical

regulator of hypothalamic control of reproduction. The role of the KISS1/KISS1R signaling system has widened as investigators have identified extra-hypothalamic roles in tissues such as the liver, uterus, testes and ovary (33). Expression of KISS1R in the pituitary gonadotroph (34) points to the pituitary as a possible target for kisspeptin action. The functional significance of pituitary KISS1R is not well defined and may be the target of either centrally or peripherally derived KISS1. We sought to assess the role of KISS1 signaling at the level of the pituitary in mice by the development of a pituitary *Kiss1* knockout model (PKiRKO). We demonstrate that near complete reduction of pituitary KISSR gene and protein expression in does not affect reproductive development or function.

KISS1R is expressed on mouse pituitary gonadotrophs (FIG 2) which supports studies in rat (43) and sheep, (44) demonstrating co-expression of LH and KISS1R in the pituitary. Investigators have observed KISS1 induction of LH secretion in cultured primary rat and primate pituitary cells (35,36) and up-regulation of gonadotropin gene β -subunits, LH β and FSH β gene expression in L β T2 cells (14), although others have seen no direct effect of KISS1 on LH or FSH secretion (37,38). Other studies suggest that KISS1 may act directly on pituitary gonadotropes to stimulate LH release (39-41) or to increase gonadotropin or *Gnrhr* gene expression (42) which were observed *in vitro* using cell lines or primary culture. Our data indicate that these pituitary kisspeptin receptor signaling pathways may not play a role in reproduction. The PKiRKO male mice had a significantly lower morning FSH level (Figure 3). This lower FSH did not seem to affect reproductive function, as the

PKiRKO mice also had normal testosterone, testes cell structure and gross sperm production (Figure 3 and data not shown). We achieved 88% reduction in kisspeptin gene expression in the pituitaries of male mice and 64% in female mice so it is possible that the reproductive phenotype of the PKiRKO mice would be more pronounced if a greater reduction was achieved. The gonadotroph comprises a minority of the pituitary cell population so kisspeptin receptor expression in other pituitary cells may account for the observed expression. The α GSU transgenic Cre mouse effectively deletes floxed genes specifically in pituitary gonadotrophs indicating that the observed kisspeptin receptor in the pituitary is likely not present in the gonadotroph.

The LH and FSH response to peripheral and central administration of kisspeptin is used to assess GnRH neuron activity. The LH and FSH of humans and mice with disorders of GnRH neuron causing hypogonadism do not rise after peripheral kisspeptin administration. These data agree with our results that there does not appear to be a direct role of kisspeptin on pituitary regulation of LH, FSH, and reproductive function; kisspeptin does not directly affect pituitary function and LH/FSH physiology in males. Kisspeptin may become available to probe GnRH neuron activity in humans to differentiate between delayed puberty and hypogonadotropic hypogonadism. Kisspeptin action is specifically on the GnRH neuron indicating that this test will be specific to probe for GnRH neuron function.

Overall this study suggests that absence of kisspeptin signaling in pituitary gonadotrophs does not affect reproductive function or fertility in male or female mice.

The functional significance of kisspeptin receptor expression in the pituitary remains unclear.

Figure legends

Figure 1 Development of the PKiRKO mouse. A. schematic diagrams of constructs used to generate PKiRKO mice. Mice bearing LoxP sites flanking exon 2 of the *Kiss1r* were crossed with transgenic mice expressing Cre recombinase specifically in Gonadotrophs (α GSU). B. Genotyping by PCR analysis of the genomic DNA produced a 2096 bp amplicon in mice with a floxed allele and an 1882 bp amplicon in kisspeptin fl/fl mice, also shown are both bands present in the heterozygous floxed-*Kiss1r*. C. Lane 1- α Cre⁺ transgene negative. Lane 2 & 3- α Cre⁺ transgene positive mice. Amplicon size is 700 bp. D. Quantitative RT-PCR analysis of *Kiss1r* mRNA extracted from male and female mouse tissues. *Kiss1r* was significantly reduced (87.6% for male, 63.7% for female) in the pituitary of PKiRKO (KO) mice compared with that in wild type (WT) mice, but no difference in *Kiss1r* expression was observed in other tissues. Data are means $\pm$ SEM (n=8 for pituitary, n=4 for other tissues).

Figure 2. PKiRKO mice have normal puberty onset

A. Body weight change over time was not different between PKiRKO (n=15) and WT (n=15) male mice or female PKiRKO (n=15) and WT (n=15) mice. B. No significant difference was observed in age of preputial separation (PPS) in males. (28.1 $\pm$ 2.2 [WT] vs 27.8 $\pm$ 2.3 [PKiRKO]) WT (n=15) PKiRKO (n=15). C. Anogenital distance was not different between WT and PKiRKO male mice (n=6 each group). D. No difference was observed in age at vaginal opening (30.2 $\pm$ 2.6 [WT] vs. 28.9 $\pm$ 3.9[PKiRKO]) or first estrus (35.6 $\pm$ 2.4 [WT] vs 33.3 $\pm$ 3.2 [PKiRKO]).

Figure 3. PKiRKO mice have normal reproductive function

A. Testes weight was not significantly different between PKiRKO and WT male mice (n=6). B. Ovary weight was not significantly different between PKiRKO and WT female mice (n=6) C. Vaginal cytology was examined daily over a period of 14 consecutive days in 6- to 11-week-old mice. WT and PKiRKO groups both exhibited regular estrous cycles the percentage time spent at each cycle stage did not differ between groups. N= 6 each group. D. Morning LH (left Y axis and FSH (right Y axis) in male (left panel) and female (right panel) mice. n=6, P>0.05) E. Testosterone levels are not different between PKiRKO and WT mice (n=6 each group). NS= No significant difference.

Figure 4. PKiRKO Mice Have Normal Fertility.

A. After introduction with WT female (left panel) or male (right panel) respectively, the day of first litter was recorded. Values are mean $\pm$ S.E.M. NS, no significant. B.

Total numbers of litters per male (left panel) or female (right panel) was not significantly different between WT and PKiRKO mice. Number of pups per litter was also not significantly different between WT and PKiRKO mice (male, left panel; female right panel).

References

- [D1] Fink G. Neuroendocrine regulation of pituitary function: general principles. *Neuroendocrinology in Physiology and Medicine*, edited by Conn PM, Freeman ME. Totowa, NJ: Humana, 2000, p. 107–134.
- [D2] Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. *Kisspeptins and Reproduction: Physiological Roles and Regulatory Mechanisms*. *Physiol Rev* 92: 1235–1316, 2012; doi:10.1152/physrev.00037.2010
- [D3] Tena-Sempere M, Huhtaniemi I. Gonadotropins and gonadotropin receptors. *Reproductive Medicine: Molecular, Cellular and Genetic Fundamentals*, edited by Fauser BCJM. New York: Parthenon, 2003, p. 225–244.
- [D4] Bliss, S. P., Navratil, A. M., Xie, J., & Roberson, M. S. (2010). GnRH signaling, the gonadotrope and endocrine control of fertility. *Frontiers in Neuroendocrinology*, 31(3), 322–340. <http://doi.org/10.1016/j.yfrne.2010.04.002>
- [D5] Funes S, Hedrick JA, Vassileva G, et al. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun*. 2003;312:1357–1363
- [D6] Messenger S, Chatzidaki EE, Ma D, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA*. 2005;102:1761–1766
- [D7] Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse, *Endocrinology*, 2004, vol. 145 (pg. 4073-4077)
- [D8] Smith JT, Clarke IJ. Kisspeptin expression in the brain: catalyst for the initiation of puberty. *Rev Endocr Metab Disord*. 2007;8:1–9
- [D9] Seminara SB (2005) Metastin and its G protein-coupled receptor, GPR54: critical pathway modulating GnRH secretion. *Front Neuroendocrinol* 26:131–138
- [D10] de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA* 100:10972–10976
- [D11] Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC (2008) A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med* 358:709–715
- [D12] Silveira LG, Noel SD, Silveira-Neto AP, Abreu AP, Brito VN, Santos MG, Bianco SD, Kuohung W, Xu S, Gryngarten M, Escobar ME, Arnhold IJ, Mendonca BB, Kaiser UB, Latronico AC (2010) Mutations of the KISS1 gene in disorders of puberty. *JCEM* 95:2276–2280

- [D13] Dror T, Franks J, Kauffman AS. Analysis of Multiple Positive Feedback Paradigms Demonstrates a Complete Absence of LH Surges and GnRH Activation in Mice Lacking Kisspeptin Signaling. *Biology of Reproduction*. 2013;88(6):146. doi:10.1095/biolreprod.113.108555.
- [D14] Witham EA, Meadows JD, Hoffmann HM, et al Kisspeptin regulates gonadotropin genes via immediate early gene induction in pituitary gonadotropes. *Mol Endocrinol*, 2013, 27(8):1283-1294.
- [D15] Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol*. 2009;21:673–682
- [D16] Novaira HJ1, Sonko ML, Hoffman G, et al. Disrupted kisspeptin signaling in GnRH neurons leads to hypogonadotrophic hypogonadism. *Mol Endocrinol*, 2014, 28(2):225-238.
- [D17] Singh SP, Wolfe A, Ng Y, DiVall SA, Buggs C, Levine JE, Wondisford FE, Radovick, S. Impaired estrogen feedback and infertility in female mice with pituitary-specific deletion of estrogen receptor alpha (ESR1). *Biol Reprod* 81: 488–496, 2009.
- [D18] Ramaswamy S, Seminara SB, Pohl CR, DiPietro MJ, Crowley WF Jr, Plant TM. Effect of continuous intravenous administration of human metastatin 45–54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (*Macaca mulatta*). *Endocrinology* 148: 3364–3370, 2007.
- [D19] Girmus RL, Wise ME. Progesterone directly inhibits pituitary luteinizing hormone secretion in an estradiol-dependent manner. *Biol Reprod*. 1992;46:710–714.
- [D20] McLachlan RI, Robertson DM, de Kretser D, Burger HG. Inhibin--a non-steroidal regulator of pituitary follicle stimulating hormone. *Baillieres Clin Endocrinol Metab*. 1987;1:89–112
- [D21] Williams TC, Berelowitz M, Berk MA, Frohman LA. Differential effects of insulin- and proinsulin-induced hypoglycemia on pituitary hormone and catecholamine secretion *Diabetes Care*. 1987 May-Jun;10(3):278-85.
- [D22] Brothers KJ, Wu S, DiVall SA, Messmer MR, Kahn CR, Miller RS, et al. Rescue of obesity-induced infertility in female mice due to a pituitary-specific knockout of the insulin receptor. *Cell Metab* 2010; 12:295–305. pmid:20816095
- [D23] Bilezikjian LM, Vale WW. The local control of the pituitary by activin signaling and modulation. *Open Neuroendocrinol J* (2011) 4:90–101.10.2174/1876528901104010090
- [D24] Hauger R. L., and Aguilera G.. 1993. Regulation of pituitary corticotropin releasing hormone (CRH) receptors by CRH: interaction with vasopressin. *Endocrinology* 133:1708–1714.
- [D25] Rosario Pivonello, Diego Ferone, Wouter W. de Herder, Johan M. Kros, Maria Laura Del Basso De Caro, Marica Arvigo, Lucio Annunziato, Gaetano Lombardi, Annamaria Colao, Leo J. Hofland, Steven W. J. Lamberts; Dopamine Receptor Expression and Function in Corticotroph Pituitary Tumors, *The Journal of*

- Clinical Endocrinology & Metabolism*, Volume 89, Issue 5, 1 May 2004, Pages 2452–2462, <https://doi.org/10.1210/jc.2003-030837>
- [D26] Cyclic adenosine 3',5'-monophosphate/protein kinase A and mitogen-activated protein kinase 3/1 pathways are involved in adenylate cyclase-activating polypeptide 1-induced common alpha-glycoprotein subunit gene (Cga) expression in mouse pituitary gonadotroph LbetaT2 cells. Harada T, Kanasaki H, Mutiara S, Oride A, Miyazaki K *Biol Reprod*. 2007 Oct; 77(4):707-16.
- [D27] Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* 2008; 28: 8691-8697.
- [D28] Naik K, Pittman It, Wolfe A, Miller RS, Radovick S, Wondisford FE. A novel technique for temporally regulated cell type-specific Cre expression and recombination in the pituitary gonadotroph. *J Mol Endocrinol*. 2006;37:63–69.
- [D29] Wolfe A, Divall S, Singh SP, Nikrodhanond AA, Baria AT, Le WW, Hoffman GE, Radovick SJ. Temporal and spatial regulation of CRE recombinase expression in gonadotrophin-releasing hormone neurones in the mouse. *Neuroendocrinol*. 2008 Jul; 20(7):909-16
- [D30] Quennell JH, Howell CS, Roa J, Augustine RA, Grattan DR, Anderson GM. Leptin deficiency and diet-induced obesity reduce hypothalamic kisspeptin expression in mice. *Endocrinology* (2011) 152:1541–50. doi:10.1210/en.2010-1100
- [D31] Evaluation of the male pubertal onset assay to detect testosterone and steroid biosynthesis inhibitors in CD rats. M. S. Marty, J. W. Crissman, E. W. Carney *Toxicol Sci*. 2001 Apr; 60(2): 285–295.
- [D32] Baker ER. Body weight and the initiation of puberty. *Clin Obstet Gynecol*. 1985;28(3):573–9.
- [D33] Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere M. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? *Endocrinology* 147: 4852–4862, 2006.
- [D34] Gutierrez-Pascual E, Martinez-Fuentes AJ, Pinilla L, Tena-Sempere M, Malagon MM, Castano JP. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinising hormone and growth hormone secretion. *Neuroendocrinol* 19: 521–530, 2007.
- [D35] Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P et al. (2005) Characterization of the potent LH releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology* 146,156–163.
- [D36] R.M. Luque, et al. Kisspeptin regulates gonadotroph and somatotroph function in nonhuman primate pituitary via common and distinct signaling mechanisms *Endocrinology*, 152 (3) (2011), pp. 957-966
- [D37] Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T. Peripheral administration of metastatin induces marked gonadotropin release and ovulation in

- the rat. *Biochem Biophys Res Commun* 320: 383–388, 2004.
- [D38] Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. *J Neuroendocrinol* 16: 850–858, 2004.
- [D39] Gutierrez-Pascual E, Martinez-Fuentes AJ, Pinilla L et al. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinizing hormone and growth hormone secretion. *J Neuroendocrinol*, 2007, 19:521-530.
- [D40] Luque RM, Cordoba-Chacon J, Gahete MD et al. Kisspeptin regulates gonadotroph and somatotroph function in nonhuman primate pituitary via common and distinct signaling mechanisms. *Endocrinology*, 2011, 152:957-966.
- [D41] Castellano JM, Navarro VM, Fernandez-Fernandez R et al. Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. *Mol Cell Endocrinol*, 2006, 257-258:75-83.
- [D42] Naor Z, Clayton RN, Catt KJ. Characterization of gonadotropin releasing hormone receptors in cultured rat pituitary cells. *Endocrinology*, 1980, 107:1144–1152.
- [D43] Richard N, Galmiche G, Corvaisier S, Caraty A, Kottler ML. KiSS-1 and GPR54 genes are co-expressed in rat gonadotrophs and differentially regulated in vivo by oestradiol and gonadotrophin-releasing hormone. *J Neuroendocrinol*, 2008, 20(3):381-393.
- [D44] Backholer K, Smith J, Clarke IJ. Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe. *Endocrinology* 150: 5488–5497, 2009.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

A

B

C