MARINA AUGUSTO SILVEIRA

CONTROLE NEUROENDÓCRINO DA REPRODUÇÃO: FATORES QUE MODULAM A ATIVIDADE DE NEURÔNIOS GNRH E KISSPEPTINA

Tese apresentada ao Programa de Pós-Graduação em Ciências Morfofuncionais da Universidade de São Paulo, para a obtenção do título de Doutor em Ciências

São Paulo
2017

MARINA AUGUSTO SILVEIRA

NEURAL CONTROL OF REPRODUCTION: NEUROMODULATORS OF GNRH AND KISSPEPTIN NEURONS ACTIVITY

Thesis presented to the Graduate Program in Morphofunctional Science of the University of São Paulo to obtain the title of Ph. D. in Science

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **140** nas fls. **135** do livro **02** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a)) **Renata Frazão**, Coordenador (a) da Linha de pesquisa *"Vias neurais que regulam a fertilidade: Ação do hormônio prolactina em neurônios kiss1"* do qual participam o(s) aluno(s) **Marina Augusto da Silveira, Regina Pereira da Silva,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA) em **18.09.2012, com validade de 4 anos**.

São Paulo, 19 de setembro de 2012.

Prof. Dr. WOTHAN TAVARES DE LIMA
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São Paulo, 12 de setembro de 2016.

Prezado(a) Professor(a),

Informo que o projeto intitulado "***Vias neurais que regulam a fertilidade: Ação do hormônio prolactina em neurônios kiss1***", registrado sob o protocolo nº ***140/2012*** e aprovado em 18/09/2012 que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, foi **prorrogado até 18/09/2020**.

Diante desta prorrogação e da declaração de que não houve alteração da metodologia e das técnicas descritas na licença inicial para o uso de animais, autorizo a inclusão das espécies e quantidades descritas abaixo para continuidade ao referido projeto:

Espécie	Linhagem	Sexo	Idade/Peso	Quantidade por ano
Camundongo	Kiss1rhGFP	Fêmea	2-3 meses	1º: 30 2º: 30 3º: 30 4º: 30

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA-ICB deverá ser informada.

Cordialmente,

Prof. Dr. **Anderson de Sá Nunes**
Coordenador - CEUA-ICB/USP

Prof.(a) Dr.(a) **Renata Frazão**
Departamento de **Anatomia**
Instituto de Ciências Biomédicas - USP

DEDICATION

To my mother, my father, my sister, my grandma and my boyfriend for all the affection and support to get here.

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No achievement is made alone. During my years as a PhD student, I received the support of many people, who were fundamental to my growth, maturation and that gave me strength to never quit.

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EPIGRAFH

“The experience of life envolves everything.
Life is like this: it warms and it cools,
it tightens, and it loosens, quiet and restless.
What the life wants from us is courage.”
Guimarães Rosa

RESUMO

SILVEIRA, M. A. **Controle neuroendócrino da reprodução: fatores que modulam a atividade de neurônios GnRH e kisspeptina.** 2017. 101 f. Tese (Doutorado em Ciências Morfofuncionais) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2017.

Os neurônios GnRH e os neurônios kisspeptina representam as duas populações neuronais de maior importância no controle da reprodução. Os esteroides sexuais têm um papel essencial na modulação da rede de neurônios GnRH. Durante o ciclo reprodutivo feminino, o estradiol liga-se ao seu receptor expresso pelos neurônios kisspeptina e, exerce tanto *feeddback* negativo quanto positivo para modular a liberação de GnRH. Muitos estudos sobre os mecanismos neurobiológicos subjacentes ao *feedback* de estradiol foram feitos em camundongos ovariectomizados com reposição de estradiol (OVX+E). Neste modelo, a atividade do neurônio GnRH é dependente de estradiol e hora do dia, e o aumento da atividade do neurônio GnRH no final da tarde coincide com um pico dário de LH. A amplitude desse pico aparenta ser menor em OVX+E comparada com fêmeas em proestrus, talvez porque outros fatores ovarianos não sejam substituídos nesse modelo. Nós testamos a hipótese de que a atividade de neurônios GnRH é maior em proestrus comparada com o modelo OVX+E. Nossos resultados demonstraram que, embora a taxa de disparo dos neurônios GnRH seja similar entre os camundongos em proestrus e OVX+E, o padrão dos potenciais de disparo revelou uma mudança para maior duração dos "*bursts"* em camundongos em proestrus, enquanto os intervalos entre os potenciais de disparo foram mais curtos em camundongos OVX+E. O pico de LH em resposta a uma injeção de GnRH no início da tarde foi maior em camundongos em proestrous comparado com diestrous ou OVX+E. Estas observações sugerem que a menor amplitude do pico de LH observada no modelo OVX+E provavelmente não é devido à uma alteração na atividade do neurônio GnRH, mas devido à redução da sensibilidade pituitária, mudanças no padrão de potencial de disparo e / ou acoplamento excitação-secreção em neurônios GnRH. Prolactina é outro hormônio com impacto na modulação do eixo HPG. Estudos anteriores mostraram que os neurônios de kisspeptina são mediadores importantes dos efeitos da prolactina sobre a reprodução. Contudo, os mecanismos celulares recrutados pela prolactina para afetar os neurônios kisspeptina permanecem desconhecidos. Observamos que uma pequena porcentagem de neurônios kisspeptina no núcleo AVPV foi indiretamente despolarizada pela prolactina. Este efeito requer a via de sinalização PI3K. Não foram observados efeitos sobre a atividade dos neurônios kisspeptina do ARH, apesar de uma elevada percentagem de neurônios no ARH expressarem a fosforilação STAT5 induzida pela prolactina. Adicionalmente, foram gerados camundongos portadores de inativação de *Stat5a/b* em células kisspeptina. Estes mutantes exibiram um início precoce de ciclicidade estro, indicando que os fatores de transcrição STAT5 exercem um efeito inibitório sobre o início da puberdade. Este estudo foi importante para descrever novas percepções sobre os neurônios envolvidos no controle da reprodução.

Palavras-chave: GnRH. Kisspeptina. Estradiol. Patch-clamp. Prolactina.

ABSTRACT

SILVEIRA, M. A. **Neural control of reproduction: neuromodulators of GnRH and kisspeptin neurons activity.** 2017. 101 p. Ph. D. Thesis (Morphofunctional Sciences) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2017.

GnRH neurons and kisspeptin neurons represent the two neuronal populations of major importance in the control of the reproduction. Sexual steroids have an essential role in the modulation of GnRH neuron network. During the female reproductive cycle, estradiol binds to its receptor expressed by kisspeptin neurons and, exerts both negative and positive feedback at both the central level to alter GnRH release. Many studies of the neurobiological mechanisms underlying estradiol feedback have been done on ovariectomized, estradiol-replaced (OVX+E) mice. In this model, GnRH neuron activity depends on estradiol and time of day, and the activity increase in the late afternoon coincident with a daily LH surge. Amplitude of this surge appears lower than in proestrous mice, perhaps because other ovarian factors are not replaced. We hypothesized GnRH neuron activity is greater during the proestrous-preovulatory surge than the estradiol-induced surge. Our results demonstrated that although the overall GnRH neuron firing rate was similarly between proestrus and OVX+E mice, the patterning of action potentials revealed a shift towards longer burst duration in proestrous mice, whereas intervals between spikes were shorter in OVX+E mice. LH response to an early afternoon injection of GnRH was greater in proestrous than diestrous or OVX+E mice. These observations suggest the lower LH surge amplitude observed in the OVX+E model is likely not attributable to altered mean GnRH neuron activity, but due to reduced pituitary sensitivity, subtle shifts in action potential pattern, and/or excitation-secretion coupling in GnRH neurons. Prolactin is another hormone with impact in the modulation of the HPG axis. Previous studies have shown that kisspeptin neurons are important mediators of prolactin's effects on reproduction. However, the cellular mechanisms recruited by prolactin to affect kisspeptin neurons remain unknown. We observed that a small percentage of kisspeptin neurons in the AVPV nucleus was indirectly depolarized by prolactin. This effect required the PI3K signaling pathway. No effects on the activity of ARH kisspeptin neurons were observed, despite a high percentage of arcuate neurons expressing prolactin-induced STAT5 phosphorylation. Additionally, mice carrying *Stat5a/b* inactivation in kisspeptin cells were generated. These mutants exhibited an early onset of estrous cyclicity, indicating that STAT5 transcription factors exert an inhibitory effect on the timing of puberty. This study was important to describe new insight about the neurons involved in the control of reproduction.

Keyword: GnRH. Kisspeptin. Estradiol. Patch-clamp. Prolactin.

ABBREVIATIONS

3V	third ventricle
AMPA/KA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate;
AP-5	2-amino-5-phophonovalerate;
APs	action potentials;
ARH	arcuate nucleus of the hypothalamus;
AVPV	anteroventral periventricular nucleus of the hypothalamus;
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione;
DAB	3,3'-diaminobenzidine;
Egr1	early growth response protein 1;
ERα	estrogen receptor alpha;
FSH	follicle-stimulating hormone;
Fshb	follicle-*stimulating hormone* subunit *beta;*
GABA	ϒ-aminobutyric acid;
GnRH	gonadotropin-releasing hormone;
Gnrhr	gonadotropin-releasing hormone *receptor;*
GPR54	G-protein-coupled receptor Kiss1r;
HPG	hypothalamic-pituitary-gonadal;
hrGFP	humanized Renilla green fluorescent protein
Icv	intracerebroventricular;
Ih	hyperpolarization-activated non-specific cation currents;
IR	input resistance;
IsADP	sodium currents of the afterdepolarization;
JAK2	Janus kinase 2;
KPBS	0.02 M potassium PBS;
LH	luteinizing hormone;
Lh	luteinizing hormone subunit beta;
MK801	dizocilpine;
MPA	medial preoptic area;
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione;

NMDA	N-metil-D-aspartato;
OVX	ovariectomy;
OVX+E	ovariectomy with estradiol replacement;
PBS	phosphate-buffered saline;
PeN	rostral periventricular nucleus of the hypothalamus;
PI3K	phosphatidylinositol 3-kinase;
Ppia	peptidylprolyl isomerase A;
PRLRs	prolactin receptors;
PSCs	postsynaptic currents;
pSTAT5	phosphorylated form of signal transducer and activator of transcription-5;
pSTAT5-ir	pSTAT5 immunoreactivity;
RMP	resting membrane potential;
sIAHP-UCL	slow $[Ca^{2+}]_i$-activated afterhyperpolarization current;
STAT	signal transducer and activator of transcription;
TIDA neurons	tuberoinfundibular dopamine neurons;
TTX	tetrodotoxin.

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B - SILVEIRA M. A., et al., GnRH neuron activity and pituitary response in estradiol-induced vs proestrous luteinizing hormone surges in female mice. **Endocrinology**, v.158, p. 356-366., 2017. ..... *97*

C - PEDROSO, J. A. B., Changes in Leptin Signaling by SOCS3 modulate fasting-induced hyperphagia and weight regain in mice. **Endocrinology**. v. 157. P. 3901 – 3914., 2016..... *98*

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1 LITERATURE REVIEW

1.1 The hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis is essential in the regulation of reproduction and fertility. The fact that gonadal function is under control by the central nervous system was first established in 1955 (HARRIS, 1955). After several years, it was published that gonadotropin-releasing hormone (GnRH) present in the hypothalamus is released in the pituitary portal vessel (HAISENLEDER et al., 2008; WILDT et al., 1981) and stimulates the synthesis and release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (AMOSS et al., 1971; MATSUO et al., 1971). These gonadotropins activate gametogenesis and steroid synthesis by the gonads, and gonadal steroids modulate the GnRH neuron network and function through a central mechanism of negative feedback in both sexes and positive feedback exclusively in females (CHRISTIAN et al., 2009) (Figure 1). The HPG axis becomes active during the gestation. Studies made in sheep and monkeys suggest the establishment of the GnRH pulse generator during the fetal life (HUHTANIEMI et al., 1979; POLKOWSKA, 1995; TERASAWA; KEEN, et al., 1999). In different species, GnRH release is greater during postnatal life compared to the neonatal and juvenile period (HOMPES et al., 1982; POLING; KAUFFMAN, 2012). Interestingly, during the juvenile period, the GnRH release became quiescent (GRUMBACH, 2002; SCHMIDT; SCHWARZ, 2000; WINTER et al., 1975). Around the age of puberty onset, the GnRH suppression ends gradually and the GnRH generator is reactivated (URBANSKI; OJEDA, 1985; WATANABE; TERASAWA, 1989). The increase in the synthesis and release of GnRH will lead to the puberty onset in both males and females (BOURGUIGNON; FRANCHIMONT, 1984; TERASAWA et al., 1984; WATANABE; TERASAWA, 1989). In adults, the pulsatile GnRH release is driven by a neural timing mechanism in the hypothalamus, placing GnRH neurons in the position of the hypothalamic GnRH pulse generator (KARSCH, 1980; POHL; KNOBIL, 1982). Therefore, GnRH neurons of the medial preoptic area comprise the final common pathway for the central regulation of fertility.

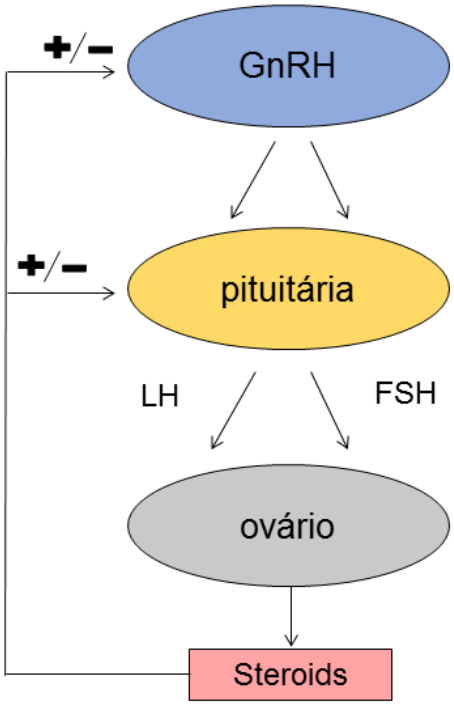

Figure 1 - *GnRH neurons form the final common pathway for the central regulation of reproduction.* Demonstrative scheme of the hypothalamus-pituitary-gonadal axis: GnRH neurons of the preoptic medial area stimulate the pituitary to release LH and FSH that will act in the ovary stimulating the production of sexual steroids. Estradiol will modulate GnRH neurons network through a mechanism of both positive and negative feedback upon GnRH neurons and pituitary.

1.2 GnRH neurons network are modulated by steroids

Estrogen inhibits GnRH and LH release through a mechanism of negative feedback resulting in the inhibition of LH release (CARATY et al., 1989; KARSCH et al., 1987). In rodents, estrogen levels are basal during estrus, but on late metestrus and diestrus begins to rise significantly (YOSHINAGA et al., 1969). During proestrus estradiol reaches peak concentrations and switches from negative to positive feedback action (CLARKE et al., 1987; TURGEON; BARRACLOUGH, 1977). The continuous increase of GnRH levels in pituitary portal blood serves as the final signal for LH surge (MOENTER et al., 1990; MOENTER et al., 1991; XIA et al., 1992), subsequently initiating ovulation (BRONSON; VOM SAAL, 1979; CLARKE et al., 1987; DÖCKE; DÖRNER, 1965; MOENTER et al., 1991) (Figure 2).

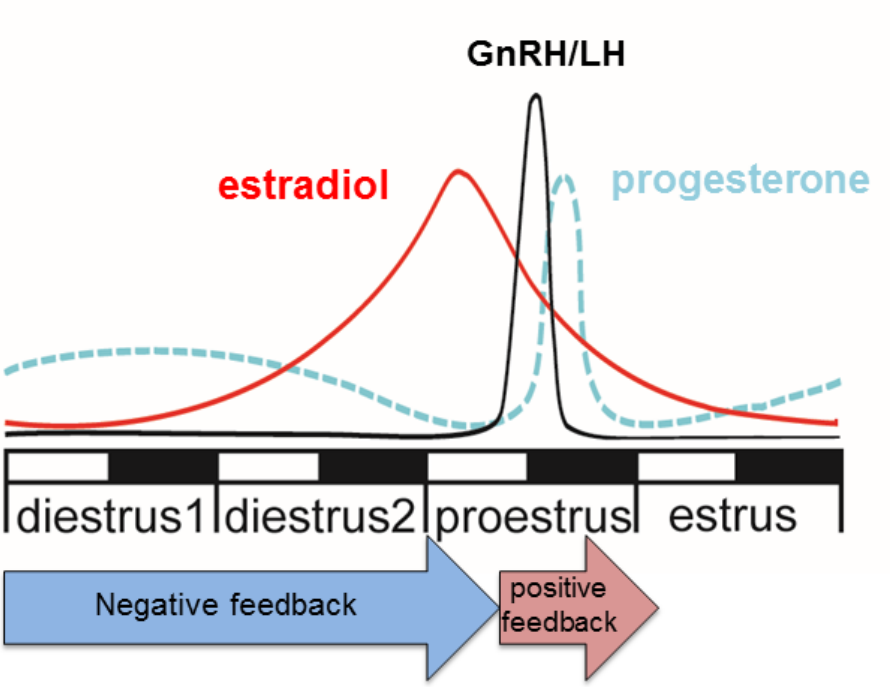

Figure 2 - adapted from Physiology of Reproduction (NEILL, 2005): *Estrogen is essential for the control of ovulation.* Estrogen inhibits LH release via negative feedback, but switch to positive feedback upon GnRH neurons and gonadotropes. The continuous increase in GnRH levels on pituitary serve as a signal for LH surge trigging ovulation.

Because of the importance of estrogen to modulate GnRH neurons network it is necessary to understand the neurobiological mechanisms underlying estrogen feedback. In this regard, different experimental models of positive feedback induced by ovariectomy (OVX) followed by replacement with high physiologic/ supraphysiologic levels of estradiol have been developed and used for many laboratories. The induction of positive feedback in these models can be made by injection of 17β-estradiol (WINTERMANTEL et al., 2006; ZHANG et al., 2013) or Silastic implants containing 17β-estradiol (CHRISTIAN et al., 2005; LEGAN; KARSCH, 1975; NORMAN, 1975). The OVX/OVX+E daily surge model avoids injections and was developed in 2005 by Christian et al., to facilitate studies about endogenous generation and timing of GnRH surges in the presence of a supraphysiologic level of estradiol in the circulation. In this model, the LH surge is induced for several days during positive feedback. Furthermore, both LH and GnRH neuron firing rate are suppressed in the AM during negative feedback and both are elevated in the PM during positive feedback. In contrast, in OVX mice, neither LH levels nor GnRH neuron firing activity exhibit time-

of-day differences. This suggests that increased GnRH neuron activity in the PM is estradiol-dependent (CHRISTIAN et al., 2005).

Because estradiol levels are constant in this model, these studies have helped us understand the estradiol and time-of-day dependence of several neurobiological variables that likely contribute to changes in GnRH neuron activity and release. It is important to point out, however, that although estrogen is critical for inducing the LH surge (DÖCKE; DÖRNER, 1965), it is not the only ovarian factor that may play a role in the surge. It was demonstrated in female mice, that estradiol treatment after OVX and adrenalectomization induces neuroprogesterone synthesis. Similar effects were observed in males castrated and adrenalectomized. This suggests that during the positive feedback, estradiol could potentially enhance neuroprogesterone synthesis that is involved in the LH surge (MICEVYCH et al., 2003; MICEVYCH; SINCHAK, 2011). It is important to point out that the modulation of GnRH neuron network by estrogen is made by steroid-sensitive afferent neurons once GnRH neurons do not express estrogen receptor alpha (ER-α) (HERBISON; PAPE, 2001; HRABOVSZKY et al., 2000; HRABOVSZKY et al., 2001).

1.3 Electrophysiological characteristics of GnRH neurons

Several inputs can change central mechanisms and alter fertility through the modulation of GnRH neuron activity and release. The first study that used electrophysiological approaches to record GnRH neurons in brain slices demonstrated that GnRH neurons were acutely hyperpolarized by estradiol in the guinea pig model (KELLY et al., 1984). One difficulty in carrying out such study at that time was the identification of GnRH neurons. The electrophysiological recording of GnRH neurons in brain slices became easier with the development of models in which promoter-driven reporter genes were applied to the identification of GnRH neurons in rats (KATO et al., 2003), mice (SKYNNER et al., 1999; SPERGEL et al., 1999; SUTER; SONG et al., 2000) and fish (KANDA et al., 2010; WAYNE et al., 2005). The majority of GnRH neurons recorded from brain slices fired spontaneous action potential in males and in females during natural cycle or induced positive feedback (NUNEMAKER et al., 2002; SIM et al., 2001; SPERGEL et al., 1999; SUTER; WUARIN, et al., 2000) and in cultured GnRH (ABE; TERASAWA, 2005; KUSANO et al., 1995). Most action potentials in these neurons are organized in bursts pattern suggesting hormone release (ABE; TERASAWA, 2005; DUTTON; DYBALL, 1979; KUEHL-KOVARIK et al., 2002; NUNEMAKER

et al., 2002; SUTER; WUARIN et al., 2000).Some studies suggest that the burst of action potentials in GnRH neurons can be related with the $[Ca^{2+}]_i$ transients. Simultaneous recordings of calcium dynamics and firing rate of GnRH, demonstrated that $[Ca^{2+}]_i$ transients could be correlated to each burst of action potentials (LEE et al., 2010). Calcium is essential in the regulation of a large variety of neuronal processes. Both extracellular and intracellular calcium will modulate the physiology of neurons and acute rises in the $[Ca^{2+}]_i$ transients will result in many effects on cellular physiology (BERRIDGE, 1998; BERRIDGE et al., 2000). Few studies have been done to measure the intracellular levels of calcium in GnRH neurons. Interestingly GnRH neurons present ability to generate longer spontaneous $[Ca^{2+}]_i$ transients compared to other neuronal classes (JASONI et al., 2010; LEE et al., 2010; TERASAWA; SCHANHOFER, et al., 1999).

GnRH neurons have high input resistance that in physiological conditions is near 1 gigaohm (Gohm) (DEFAZIO; MOENTER, 2002; KUEHL-KOVARIK et al., 2002; LEE et al., 2010; SUTER, SONG, et al., 2000; SUTER, WUARIN, et al., 2000; XU et al., 2008). This high resistance suggest that small current could have an effect in GnRH neuron membrane potential (MOENTER, 2010). Some currents have been identified as candidates involved in the bursts observed in GnRH neurons, these include the low threshold T-type calcium current (I_T) (ZHANG et al., 2009), the hyperpolarization-activated non-specific cation currents (I_h) (CHU et al., 2010), a calcium-dependent, afterhyperpolarization potassium current (I_{AHP}) (LEE et al., 2010) and a persistent sodium current (I_{NaP}) (OKA, 1996). GnRH neurons present I_h and once I_h is blocked the action potential firing and the properties of bursts are altered (CHU et al., 2010; ZHANG et al., 2007). The mRNAs of two I_h channels, HCN1 and HCN2, are highly expressed in mouse GnRH neurons, and estradiol upregulates the mRNA expression of HCN1 (BOSCH et al., 2013). The I_{NaP} and sodium currents of the afterdepolarization (I_{sADP}) are also present in GnRH neurons and may contribute to depolarization of the threshold and burst initiation in these cells (CHU et al., 2010; OKA, 1996). The slow calcium activated afterhyperpolarization current (sIAHP-UCL), another type of calcium-activated potassium current, has also been suggested to be involved in burst firing of GnRH neurons (LEE et al., 2010).

As previolusly said, GnRH neurons do not express ER-α (HERBISON; PAPE, 2001; HRABOVSZKY et al., 2000; HRABOVSZKY et al., 2001), so the steroid regulation of GnRH neurons happens through steroid-sensitive afferent neurons (WINTERMANTEL et al., 2006). GnRH neurons receive spontaneous synaptic from both glutamatergic and gamma-amino butyric acid (GABA)ergic inputs. In OVX+E rats, glutamate released in the preoptic area increases during the LH

surge (JARRY et al., 1995) and blockade of N-metil-D-aspartato (NMDA) or alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate (AMPA/KA) glutamate receptors blocks the LH surge. In OVX+E2 rats, NMDA and non-NMDA receptors appear to be necessary for the physiological mechanisms triggering the estradiol-induced LH surge (LÓPEZ et al., 1990). The utilization of a specific and potent noncompetitive NMDA receptor antagonist dizocilpine MK801 for blockade of NMDA receptors resulted in a significant attenuation of the proestrous LH surge, demonstrating that the preovulatory gonadotropin surge in the female rat is dependent on NMDA neurotransmission (BRANN; MAHESH, 1991). A study focused on AMPA receptors using intracerebroventricular (Icv) administration of the selective AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) found attenuation on steroid-induced LH surge in the ovariectomized adult female rat, suggesting an important physiological role of AMPA receptors in the production of the steroid-induced LH surge (PING et al., 1997). Although these studies have demonstrated that glutamate functions are an important transmitter involved in the regulation of GnRH and LH secretion in females during positive feedback, these approaches have limitations: measurements of glutamate concentrations or agonists of its receptor do not provide information about the phenotype of the cells that are releasing or affected by this transmitter. This happens because measurements are required to be made over multi-cell regions and injection of glutamate or other drugs into the preoptic area or via Icv could affect several neurons besides GnRH neurons. During positive feedback, the glutamatergic transmission to GnRH neurons is not different between OVX and OVX+E, although estradiol decreased postsynaptic currents (PSC) frequency during negative feedback. This suggests that estradiol suppresses glutamatergic transmission to GnRH neurons during negative feedback (CHRISTIAN et al., 2009).

Besides glutamatergic transmission, GABA has long been reported as one of the main regulators of GnRH neurons. It is well established that GnRH neurons express the mRNA for both $GABA_A$ (PETERSEN et al., 1993) and $GABA_B$ receptors (SLIWOWSKA et al., 2006) and receive direct afferent inputs from GABAergic neurons (LERANTH et al., 1985). GABAergic transmission is regulated by estradiol and all GnRH neurons receive GABAergic transmission (CHU et al., 2009). Interesting, in the daily surge model was observed that during negative feedback estradiol decreases GABA transmission to GnRH neurons, but during positive feedback this transmission is increased. GABAergic PSCs amplitude increases during the positive feedback, suggesting that the efficacy of $GABA_A$ receptor ($GABA_AR$) activation is elevated with high levels of estradiol (CHRISTIAN;

MOENTER, 2007). This suggests that estradiol is able to induce diurnal shifts in GABAergic transmission to modulate GnRH neuronal firing activity and hormone release in a time of day dependent manner. Electrophysiological evidences about the $GABA_AR$ activation to modulate GnRH neurons activity are still controversies (DEFAZIO et al., 2002; HAN et al., 2002; WATANABE et al., 2009). The suggestion that the activation of $GABA_AR$ excites GnRH neurons is supported by the fact that in GnRH neurons the expression of Na-K-Cl co-transporters (NKCC1) is continued during the adult life resulting in higher chloride (Cl^-) concentrations with depolarized equilibrium potential of GABA (DEFAZIO et al., 2002). In most neurons, the intracellular Cl^- concentration is elevated during the embryonic period and first week of postnatal life, resulting in excitatory effect of GABA. In general, the level of intracellular Cl^- is reduced resulting in the inhibitory effect of GABA (LEINEKUGEL et al., 1999). This effect is observed due to an increase in K-Cl co-transporters (KCC) expression, that extrudes Cl^- from the cell (KAILA et al., 2014), concomitant with a reduction in NKCC1, responsible for the accumulate of Cl^- and raised intracellular Cl^- levels (RIVERA et al., 1999). Interesting, in GnRH neurons this developmental pattern is not followed with a result in high function of NKCC1 and low expression of KCC2 (LOWE; GOLD, 1993; REISERT et al., 2005). Few other neurons, also follow this development pattern during adult life (MILLER; DACHEUX, 1983; ROHRBOUGH; SPITZER, 1996; SUNG et al., 2000).

1.4 Kisspeptin neurons are essential for reproduction control

Neurons expressing the neuromodulator kisspeptin co-express estrogen receptor alpha (ERα) which is essential to initiate the positive and negative feedback of GnRH release (SMITH; CUNNINGHAM et al., 2005). These neurons, here denominated as kisspeptin neurons, are the most important activator of GnRH neurons (HAN et al., 2005). Kisspeptin neurons excites GnRH neurons, stimulating GnRH release by acting on the G-protein-coupled receptor Kiss1r (also known as GPR54) expressed by GnRH neurons (HAN et al., 2005; IRWING et al., 2004) (Figure 3).

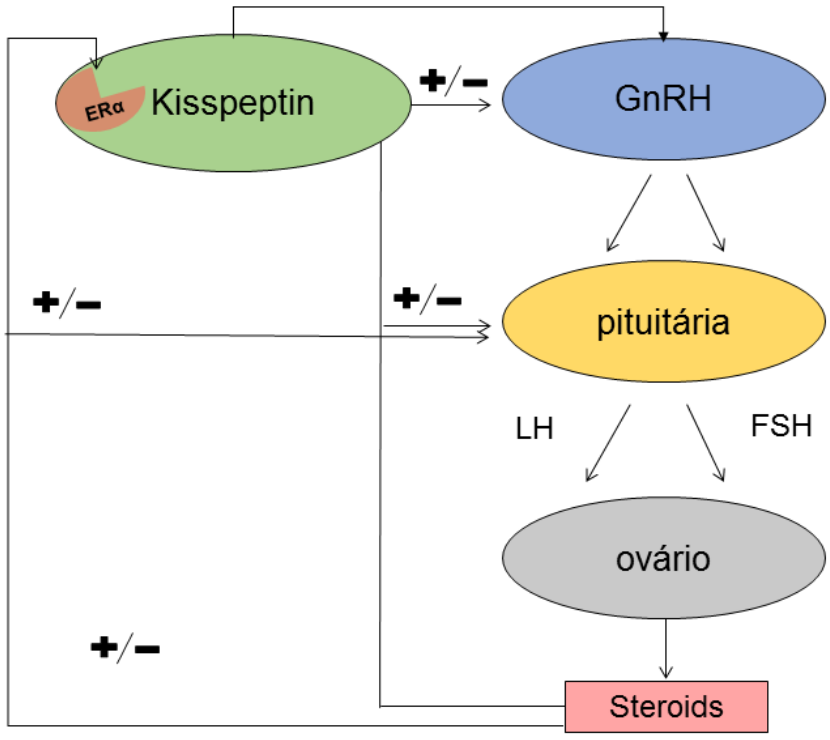

Figure 3 - *Kisspeptin modulates GnRH neurons*. Kisspeptins stimulate GnRH neurons, and GnRH secretion due to Kiss1r receptor activation. Released GnRH results in increased gonadotrophin secretion from the pituitary gland, which stimulates the synthesis and secretion of sex steroids by the gonads. Sexual steroids act on estrogen receptor alpha through a mechanism of both negative and positive feedback upon kisspeptin neurons.

The kisspeptin is the product of cells expressing the *Kiss1* gene, this gene is located on the long arm of chromosome 1 (1q32) and in 1996 was described by Lee et al., as a metastatic suppressor gene in malignant melanoma cells. The initial product of this gene is the kisspeptin-1, a 145-amino acid hydrophilic protein. After proteolytic cleavage followed by transfer of an NH 2 group to its carboxy terminal moiety, kisspeptin-54 is generated, in addition to other smaller fragments such as kisspeptin-10, kisspeptin-13 and kisspeptin-52 (KOTANI et al., 2001; LEE et al., 1996; OHTAKI et al., 2001; STAFFORD et al., 2002).

In humans and rodents, mutations in the *Kiss1* gene and its receptor induce infertility due to hypogonadotrophic hypogonadism. This pathology is characterized by incomplete or partial pubertal development due to defects in migration, synthesis, secretion or function of GnRH neurons, with consequent decrease of secretion of FSH and LH (DE ROUX et al., 2003; FUNES et al., 2003; LAPATTO et al., 2007; SEMINARA et al., 2003). Kisspeptin neurons, as well as GnRH neurons, are

essential for onset of puberty and reproductive functions. An increase in GnRH release from the hypothalamus, regulated by kisspeptin neurons, is one of the factors/signal for the initiation of puberty (DE ROUX et al., 2003; SEMINARA et al., 2003). Puberty is a complex phenomenon regulated by genetic and environmental factors. In both males and females, an elevated overall expression of *Kiss1* mRNA in the hypothalamus is observed around the age of puberty onset (NAVARRO et al., 2004). The ablation of Kiss1r results in a lack in the progress toward puberty and severe deficits in their reproductive function such as disrupted estrous cycle, thread-like uteri and small ovaries (D'ANGLEMONT DE TASSIGNY et al., 2007). It is important to point out, however, that the ablation of kisspeptin neurons, in a modified mice model, in early stages of life does not influence the puberty onset neither fertility (MAYER; BOEHM 2011). Otherwise, kisspeptin neuron ablation at 20 days of age resulted in estrous acyclicity and infertility. This data suggests that some compensatory mechanism early in the development can lead to the reproductive success in the absence of kisspeptin/GPR54 signaling, but this mechanism is absent in adult life (MAYER; BOEHM 2011).

The *Kiss1* gene is expressed in several tissues such as pancreas, liver, testes, small intestine (HAUGE-EVANS et al., 2006; OHTAKI et al., 2001), gonads (COLLEDGE, 2008; FAIRGRIEVE et al., 2016), and placenta (CARTWRIGHT; WILLIAMS, 2012). In the central nervous system, brain regions, such as, the anteroventral periventricular nucleus (AVPV), the anterior periventricular nucleus (PeN) arcuate (ARH) nucleus (GOTTSCH et al., 2004), the antero-dorsal preoptic nucleus, the medial nucleus of the amygdala, and the cerebral cortex also contain cells expressing the mRNA that encodes the *Kiss1* gene, (CRAVO et al., 2011; GOTTSCH et al., 2004; LEE et al., 1999). The AVPV, PeN and ARH Kisspeptin neurons are well known as an essential component in the HPG axis (here the nucleus AVPV and PeN will be reported together as AVPV). Interesting, circulating levels of estradiol modulate the expression of the *Kiss1* gene in AVPV and ARH (SMITH; ACOHIDO, et al., 2006; SMITH; DUNGAN, et al., 2005). In the AVPV, the expression of *Kiss1* decreases when circulating levels of estradiol are low, and increases significantly when estradiol levels are elevated. In the ARH, the opposite effect is observed, expression of *Kiss1* gene increases when circulating levels of estradiol are low and decreases with estradiol replacement (SMITH; CLIFTON, et al., 2006). Therefore, kisspeptin neurons of the AVPV are known to be involved in the control of positive feedback that regulates the GnRH/LH surge as occurs in proestrus. In the ARH nucleus, kisspeptin

neurons are known to be involved in negative feedback control in gonadotrophin secretion during most of the estrous cycle (SMITH, CUNNINGHAM, et al., 2005).

Most of the kisspeptin neurons in the AVPV expresses GAD67 and therefore are potentially GABAergic. In the ARH, most of the kisspeptin neurons are glutamatergic, however 50% also coexpress GAD67 (CRAVO et al., 2011). Additionally, in the ARH nucleus, kisspeptin neurons co-express a subpopulation of neuropeptides: neurokinin B and dynorphin, which are also implicated in GnRH pulse generation (BURKE et al., 2006). The neurons in this subpopulation are called KNDy (Kisspeptin-NKB-Dyn) neurons and they has been described in different species: sheep (GOODMAN et al., 2007), rats (BURKE et al., 2006), mice (NAVARRO et al., 2009) goats (WAKABAYASHI et al., 2010) and humans (RANCE; YOUNG, 1991).

1.5 Electrophysiological characteristics of kisspeptin neurons

The biophysical properties of AVPV and ARH kisspeptin neurons have been previously described in mice and guinea pigs (DE CROFT et al., 2012; DUCRET et al., 2010; FRAZÃO et al., 2013; GOTTSCH et al., 2011; QIU et al., 2011). Kisspeptin neurons are the presynaptic pacemaker neurons, which modulate GnRH neurons. The first study regarding electrical characteristics of neurons located in the AVPV demonstrated that unidentified cells located within this nucleus exhibit estrous cycle-dependent firing and respond to both glutamate and GABA (DUCRET et al., 2010). AVPV kisspeptin neurons of mice, express I_h currents, T-type Ca^{2+} currents that contribute to burst firing, and persistent I_{NaP}, that is able to amplify the depolarization induced by T-type calcium channels (ZHANG et al., 2013). Similar to GnRH neurons, the understanding about the electrophysiological properties of kisspeptin neurons became more efficient with the development of transgenic mice models in which reporter fluorescent proteins allow the visualization of kisspeptin cells (CRAVO et al., 2011; CRAVO et al., 2013; GOTTSCH et al., 2011; MAYER; BOEHM, 2011). Additionally, using the *Kiss1*-Cre mice model it is possible to induce site-specific recombination events to manipulate the expression of other genes via the Cre-LoxP system (CRAVO et al., 2011; DUBOIS et al., 2015). Most of AVPV kisspeptin neurons, of males and females, exhibit a bimodal RMP influenced by KATP channels, once most of them are quiescent at rest, and a small percentage presents overshooting action potentials (APs) (FRAZÃO et al., 2013). The firing rate AVPV kisspeptin neurons is at high frequency (PIET et al., 2013; WANG et al., 2016) and their activity is

greater within elevated levels of estrogen during proestrus and OVX+E (FRAZÃO et al., 2013; WANG et al., 2016). Interesting, the cycle-dependent effects of AVPV kisspeptin neuron seems to be associated to estradiol, but not progesterone. The additional reposition with progesterone in OVX+E mice does not change overall excitability and burst events in AVPV kisspeptin neurons (WANG et al., 2016). ARH kisspeptin neurons are also composed of firing and quiescent cells, but unlike AVPV neurons, the range of RMPs did not follow a bimodal distribution (FRAZÃO et al., 2013; GOTTSCH et al., 2011). In opposition to what has been described in the AVPV kisspeptin neurons, in the ARH estradiol does not seem to affect the membrane potential of kisspeptin neurons, although estradiol decreased the steady-state capacitance of these neurons (FRAZÃO et al., 2013). Additionally, currents I_h and I_t have been reported in ARH kisspeptin neurons of guinea pig and mice (GOTTSCH et al., 2011; QIU et al., 2011). These currents seem to be involved in the burst firing activated through glutamatergic inputs in ARH kisspeptin neurons (GOTTSCH et al., 2011).

As previously mentioned, GABAergic transmission has been suggested as one of the most important regulators of the HPG axis (DEFAZIO et al., 2002; HAN et al., 2002; HERBISON; MOENTER, 2011). Different studies suggest that AVPV and ARH kisspeptin neurons express $GABA_A$ and glutamate receptors. (DEFAZIO et al., 2014; FRAZÃO et al., 2013; PIET et al., 2013; QIU et al., 2011; ZHANG et al., 2013). Experiments using OVX+E female mice demonstrated that the amplitude and frequency of GnRH GABAergic PSCs is amplified by kisspeptin (PIELECKA-FORTUNA; MOENTER, 2010). Additionally, GABAergic transmission to AVPV kisspeptin neurons are modulated by time of the day and estradiol in OVX+E mice (DEFAZIO et al., 2014).

The advances in transgenic mice have allowed better and more sophisticated interrogation about the factors, which drives kisspeptin neurons membrane excitability and therefore the modulation of the HPG axis. The effect of some neuromodulator, such as, leptin, ghrelin, vasopressin signaling, in kisspeptin neurons has been previously described (CRAVO et al., 2013; FRAZÃO et al., 2014; PIET et al., 2015; QIU et al., 2011). Otherwise, more studies are necessary to investigate the role of other neuromodulators whose synthesis dysregulation can lead to reproductive dysfunction, such as the prolactin hormone.

1.6 Prolactin hormone and signaling

Prolactin is a polypeptide hormone synthesized and secreted by specialized cells from the anterior portion of the pituitary gland, the lactotrophs. The circulatory system will transport prolactin to act on target cells that express the prolactin receptor (PRLR). The secretion of prolactin is mediated through a mechanism of negative feedback in the hypothalamus, which tonically inhibits the secretion of prolactin by the adenohypophysis (DEMARIA et al., 1999). Dopamine released by the nerve terminals of dopaminergic neurons diffuses into capillaries of the pituitary gland, where it reaches the lactotrophs and attaches to its membrane-specific receptors D2, inhibiting the release of prolactin (KOGA et al., 1987). The increase in the release of prolactin by lactotrophs may occur by neutralizing the inhibitory effect of dopamine (DEMARIA et al., 1999). Prolactin production has also been described in few brain areas, such as, hypothalamus (FUXE et al., 1977), telencephalon, hippocampus, amygdala (DEVITO, 1988), caudate putamen (EMANUELE et al., 1987; HARLAN et al., 1989) and cerebellum (SEROOGY et al., 1988). However, the physiological importance of the local secretion it is not known.

Multiple isoforms of membrane-bound PRLRs have been identified, differing in the length and composition of their cytoplasmic tail. In rats, for example, the following three major PRLRs isoforms were identified: short (291 amino acids), intermediate (393 amino acids), and long (591 amino acids). In mice, one long and three short isoforms of the PRLRs have been described (BOLE-FEYSOT et al., 1998). When proalctin binds to its receptor different pathways can be activated to induce prolactin biological effects. The receptor activation results in a rapid phosphorylation of Janus kinase 2 (JAK2 - Janus family of tyrosine kinase type 2) that leads to the phosphorylation of tyrosine residues. The phosphorylated tyrosines in the receptor-JAK2 complex form binding sites for members of a family of cytoplasmic proteins known as signal transducers and transcription activators (STATs). In several tissues, different isoforms of STATs have been reported to be involved in the prolactin signal transduction. The PRLR is expressed in several brain areas, for example, amygdala, hippocampus, hypothalamus, preoptic area and thalamus (BAKOWSKA; MORRELL, 1997). Besides the nervous system, PRLR is also expressed in the heart, mammary gland, ovary, liver, thymus, pancreas, spleen, kidney, adrenal gland, skeletal muscle and skin. The biological actions of prolactin are broad, among them the maintenance of homeostasis, cell proliferation, body growth, metabolic and reproductive control (NAKAGAWA et al., 1985). There are eight members in the STAT family, and three of them, STAT1, STAT3 and STAT5, are involved in the activation of

PRLRs (DARNELL et al., 1994; GAO et al., 1996; GOUILLEUX et al., 1994). Among these three cytoplasmic proteins, the most known in PRLRs mediated transduction is STAT5, which has two isoforms: STAT5a and STAT5b, encoded by two different *Stat5a* and *Stat5b* genes (AZAM et al., 1995; LIU et al., 1995). However, both isoforms possess Tyr-694 tyrosine, which is phosphorylated by JAK2. Subsequently, STATs separate from the receptor-JAK2 complex, homodimerize or heterodimerize with other STATs and translocate to the nucleus, where they activate the gene transcription pathway (GOUILLEUX et al., 1994). Therefore, the identification of the phosphorylation of STAT5 (pSTAT5) through immunohistochemistry techniques can be used as a marker of neurons that express PRLRs and were activated by PRL action (BROWN et al., 2010). The phosphotyrosine residues of the activated PRLR may also serve as a docking site for others adapter proteins which can lead to the activation of different signaling pathways, such as the mitogen-activated protein kinase (MAPK) cascade or the phosphatidylinositol 3-kinase (PI3K) cascade (BOLE-FEYSOT et al., 1998; FREEMAN et al., 2000). In addition, it has been demonstrated that PRLRs activation is also involved in rapid acute effects that lead to changes in membrane excitability. For example, prolactin acutely induces rapid effects on the membrane excitability of neurons (BROWN et al., 2012; BUONFIGLIO et al., 2015; LYONS et al., 2012; MOSS et al., 1985; ROMANÒ et al., 2013;). Such effects occur because PRLRs activation can activate fast-acting signaling mechanisms, such as the PI3K pathway, tyrosine kinase-dependent K^+ channels or the production of intracellular messengers that open voltage-independent Ca^{2+} channels, which in turn allows for ionic changes across the cell membrane (FREEMAN et al., 2000; LYONS et al., 2012).

1.7 Actions of prolactin in the reproductive system

Elevated levels of prolactin cause reproductive dysfunctions, representing a frequent cause of infertility in both males and females (GLEZER; BRONSTEIN, 2015; NEWEY et al., 2013; SHIBLI-RAHHAL; SCHLECHTE, 2011; VILAR et al., 2014). Prolactin suppresses the GnRH mRNA levels and the release of GnRH, LH and FSH in both humans and animal models (BOHNET et al., 1976; FOX et al., 1987; GRATTAN et al., 2007; MATSUZAKI et al., 1994; SONIGO et al., 2012). Despite the effects of prolactin on GnRH system, only a small percentage of GnRH neurons expresses PRLRs mRNA, prolactin-induced pSTAT5 or phosphorylated cAMP response element-binding protein. In

addition, prolactin exerts no direct effect on GnRH neurons' firing rate (BROWN et al., 2012). Therefore, prolactin likely acts through interneurons to modulate the HPG axis.

To better understand how hyperprolactinemia affects reproduction, several studies have been done to investigate possible prolactin-target neurons that may modulate the HPG axis (ARAUJO-LOPES et al., 2014; BROWN et al., 2010; BROWN et al., 2015; KOKAY et al., 2011; LIU et al., 2014; SONIGO et al., 2012). Kisspeptin neurons have been suggested as potential candidates to mediate the effects of prolactin on reproduction. Evidence for this possibility was provided by studies showing that most of kisspeptin neurons express the PRLRs mRNA (KOKAY et al., 2011; LI et al., 2011) and those receptors are functional since an acute prolactin stimulus induces pSTAT5 expression in most of kisspeptin neurons (ARAUJO-LOPES et al., 2014; BROWN et al., 2014). In OVX+E mice model, most of the neurons that express the Kiss1 gene located in the AVPV co-express the PRLRs (DE CROFT et al., 2012; KOKAY et al., 2011). Furthermore, it is known that chronic treatment with prolactin changes the estrous cycle of mice, induces reduction of Kiss1 gene mRNA in both AVPV and ARH nuclei, suppresses serum LH levels and suppresses the kisspeptin synthesis resulting in the loss of stimulation of GnRH neurons (SONIGO et al., 2012). Although hyperprolactinemia is one of the main causes of infertility in both females and males, the mechanisms by which this pathology inhibits the reproductive axis leading to infertility it is not completely understood.

2 OBJECTIVES

The goal of this study was to bring contribution to the literature about the two most important neurons involved in the regulation of the HGP axis: GnRH neurons and kisspeptin neurons. Given the importance of sexual steroids to modulate GnRH neurons network, we first evaluated (chapter 1) the neurobiological changes during the natural cycle (diestrous and proestrous) compared to a model of induced positive feedback (OVX+E) to characterize:

- The amplitude of the LH surge;
- Firing rate and pattern of GnRH neurons;
- Pituitary response to exogenous GnRH.

In the second chapter, we evaluate if kisspeptin cells could represent the neuronal pathway by which prolactin modulates the HPG axis. To answer this question, we performed anatomical and electrophysiological approaches to investigate:

- Whether prolactin can affect the membrane excitability of kisspeptin neurons**;**
- If the ablation of STAT5 expression in kisspeptin cells may represent a key transcription factor which determine the timing of puberty and the reproduction.

CHAPTER I - GnRH neuron and pituitary response in estradiol-induced vs. proestrous luteinizing hormone surges in female mice

1.1 INTRODUCTION

GnRH neurons of the medial preoptic area and hypothalamus comprise the final common pathway for the central regulation of fertility. GnRH controls the synthesis (HAISENLEDER et al., 2008; WILDT et al., 1981;) and release of the pituitary gonadotropins, LH and FSH, which activate gonadal steroidogenesis and gametogenesis. For most of the female reproductive cycle, estradiol exerts negative feedback on GnRH release, resulting in inhibition of LH release (CARATY et al., 1989; KARSCH et al., 1987). At the end of the follicular phase (proestrus in rodents), estradiol switches from negative to positive feedback action. This induces a surge of GnRH release and increases pituitary responsiveness to GnRH (CLARKE; CUMMINS, 1984; TURGEON; BARRACLOUGH, 1977), ultimately leading to the LH surge and ovulation (BRONSON; VOM SAAL, 1979; DÖCKE; DÖRNER, 1965; MOENTER et al., 1990; MOENTER et al., 1991; SARKAR et al., 1976).

To better understand the neurobiologic mechanisms underlying estradiol feedback, different experimental models of positive feedback induced by OVX followed by replacement with high physiologic/ supraphysiologic levels of estrogen have been used (ZHANG et al., 2013). These replacement regimens include daily subcutaneous injection of 17ß-estradiol benzoate for 5 to 6 days after OVX, insertion of Silastic implants containing 17ß-estradiol designed to produce a slightly supraphysiologic circulating estradiol level at the time of OVX followed by injection of 17b-estradiol benzoate 6 days later (WINTERMANTEL et al., 2006), and implantation of Silastic capsules that produce constant high physiologic levels of 17b-estradiol. The latter avoids injections, which produce varying levels of estrogen throughout the day; nocturnal rodents treated in this manner exhibit daily LH surges peaking at lights out (CHRISTIAN et al., 2005; LEGAN; KARSCH, 1975; NORMAN, 1975). These daily transitions from estradiol negative to positive feedback occur despite a constant estradiol level; changes in circulating levels of this steroid thus do not contribute to the change in feedback status. In mice prepared in this daily surge model (OVX+E), both GnRH neuron firing rate and LH are suppressed in the morning during negative feedback and both are elevated at night during positive feedback compared with OVX controls, which do not exhibit time of day–dependent shifts (CHRISTIAN et al., 2005).

The steady estradiol levels in the daily LH surge model facilitate understanding of how estradiol and time of day contribute to changes in GnRH neuron activity and release. It is important to point out, however, that although estradiol is critical for inducing the LH surge (DÖCKE; DÖRNER, 1965), it is not the only ovarian steroid that changes in an estrous cycle dependent manner (WALMER et al., 1992). Progesterone levels are also dynamic, and there is evidence for a role for progesterone in mediating estrogen positive feedback (MICEVYCH et al., 2003; MICEVYCH; SINCHAK, 2011). Of interest in this regard, the amplitude of the daily estradiol induced LH surge appears to be lower than that previously reported for the proestrous surge in mice (BRONSON; VOM SAAL, 1979). Numerous variables, including strain, LH assay reference standards and methods, housing conditions, and diet, differ among these studies, precluding a direct comparison with the exception of one recent study in which OVX+E implant followed by estradiol injection produced lower amplitude surges than observed on proestrus (CZIESELSKY et al., 2016).

No studies that we are aware of have directly compared models at both the pituitary and hypothalamic levels. Because many of the electrophysiologic studies of GnRH neurons have been performed in the daily OVX+E GnRH/LH surge model using constant in vivo physiologic estradiol treatment, it is important to determine if the neurobiologic changes induced were similar to those that occur on proestrus. We thus investigated the LH surge, GnRH neuron firing rate and pattern, and pituitary response to GnRH generated by this daily surge OVX+E model vs that in diestrous and proestrous mice to test the hypotheses that the hormonal changes of proestrus induce greater positive feedback responses at both the pituitary and hypothalamus.

1.2 MATERIALS AND METHODS

1.2.1 Animals

Female GnRH-GFP mice (SUTER; SONG, et al., 2000) on a C57Bl6/J or CBB6F1 background and aged 59-137 days were used. All mice were provided with water and Harlan 2916 chow *ad libitum* and were held on a 14L:10D light cycle with lights on at 0400 Eastern Standard Time (EST). For studies during the estrous cycle, vaginal cytology was monitored for at least a week before experiments to determine estrous cycle stage; mice were studied on diestrus or proestrus. For studies with controlled estradiol feedback (OVX+E), mice were ovariectomized and received a subcutaneous Silastic implant that contained 0.625 µg 17ß-estradiol in sesame oil in the scapular region; surgery was done under isoflurane general anesthesia with bupivacaine as a local analgesic. Studies were performed 2-3 days after surgery. The Institutional Animal Care and Use Committee of the University of Michigan approved all procedures.

1.2.2 Slice preparation and cell identification.

Chemicals were purchased from Sigma Chemical Company unless noted. All solutions were bubbled with 95% O_2/5% CO_2 throughout the experiments and for at least 30 minutes before exposure to tissue. At 1600-1630 EST, near the expected onset of the LH surge, the brain was rapidly removed and placed in ice-cold sucrose saline solution containing (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 $NaHPO_4$, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300 µm) slices were cut with a Leica VT1200S (Leica Biosystems). Slices were incubated in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ (pH 7.4) for 30 min at room temperature (21- 23 C) and then transferred to 100% ACSF for additional 30-180 min at room temperature before recording. For recording, slices were placed into a chamber continuously perfused with ACSF at a rate of 3ml/min with oxygenated ACSF heated to 31 ± 1 C with an inline-heating unit (Warner Instruments). GFP-positive GnRH neurons were identified by brief illumination at 488 nm on an Olympus BX51WI microscope. Recorded cells were mapped to an atlas (PAXINOS; FRANKLIN, 2001) to determine if any trends based on anatomical location emerged; no such trends were apparent in these data sets. Recordings were performed 1-4 h after brain slice preparation; no difference in firing patters was

evident based on time after brain slice preparation. No more than two cells per animal were included for analysis, and at least six animals were tested per parameter.

Extracellular recording. Recording micropipettes were pulled from borosilicate capillary glass (type 7052, 1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Inc.) using a Flaming/Brown P-97 puller (Sutter Instruments) to obtain pipettes with a resistance of 2-3 MΩ when filled with the appropriate pipette solution. Recordings were made with an EPC-8 with ITC-18 interface or one channel of an EPC-10 dual patch clamp amplifier and Patchmaster software (HEKA Elektronik) running on a Macintosh computer.

Targeted extracellular recordings were used to record long-term (1 hr) firing activity. This method maintains internal milieu and has minimal impact on the firing rate of neurons (ALCAMI et al., 2012; NUNEMAKER et al., 2002; 2003). Recording pipettes were filled with HEPES-buffered solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 2.5 CaCl2, 1.3 MgCl2, and 3.5 KCl, and low-resistance (diestrus: 12 ± 4 MΩ; proestrus: 13 ± 3 MΩ; OVX+E: 11 ± 4 MΩ) seals were formed between the pipette and neuron after first exposing the pipette to the slice tissue in the absence of positive pressure. Recordings were made in voltage-clamp mode with a 0 mV pipette holding potential. Signals were acquired at 20 kHz and filtered at 10 kHz. Resistance of the loose seal was checked frequently during first 5 min of recordings to ensure a stable baseline, and also between each 10-min recording period; data were not used if seal resistance changed >30% or was >25 MΩ.

A*nalysis of extracellular recordings* Action currents (events), the membrane currents associated with action potential firing, were detected off-line using custom programs in Igor Pro 6.31 (Wavemetrics). Mean firing rate (Hz) was calculated by dividing the total number of events by the duration of recording. Data were binned at 60-sec intervals and were transferred to Excel (Microsoft) for evaluation of % quiescence (1-min bins containing <1 event). In addition to overall activity, the pattern of action potentials and grouping into bursts can potentially affect neurosecretion (CAZALIS et al., 1985; DUTTON; DYBALL, 1979). Action potential grouping (bursts) was detected using software that systematically adjusted the maximum time between events (burst window) for inclusion in a burst from 0.01 sec to 1.5 sec in 10 msec intervals. Burst windows of 0.01, 0.15, 0.21, 0.5, 1.0 and 1.5sec were chosen for comparison. The shortest window, 0.01 s, encompasses intraburst intervals in typically bursting neurons of the cortex and hippocampus (CHEN et al., 2009). The 0.21sec burst window was defined based on whole-cell current-clamp recordings of GnRH neurons;

this was the longest time between action potentials in which there was a continuous depolarization towards threshold for the next action potential (CHU et al., 2010). Because the whole-cell configuration alters intracellular milieu and thus may affect action potential generation, additional burst windows were included that span the typical duration of action-potential-induced increases in intracellular calcium levels in cell types for which this is well characterized (KERR et al., 2005; KOMIYAMA et al., 2010), and the afterdepolarization of GnRH neurons, during which increased firing can occur (1.0-1.5 sec (CHU; MOENTER, 2006)). For each burst window analyzed, the percentage of spikes in bursts was calculated and the distribution of burst duration was compared using the Kolmogorov-Smirnov test; because this test is valid only for two distributions and our primary interest was comparisons between OVX+E and proestrous mice, these groups were chosen for comparison. Interspike interval was also examined by comparing the mean and distribution of the $\log_{10}$ of this parameter over entire recordings (NOWAK et al., 2003; ROMANÒ et al., 2013).

1.2.3 LH surge in proestrous and OVX+E mice

Vaginal cytology of ovary-intact mice was determined for ≥ 10 days prior to sampling to confirm normal estrous cyclicity. Trunk blood was collected within 30 minutes of lights out from proestrous mice (2 trials, n = 3,6 total) or OVX+E mice on day 2 post-surgery (three trials, n = 8,4,3). For all samples, serum was separated by centrifugation and stored at -20 C until assay.

1.2.4 Pituitary responsiveness to exogenous GnRH

Pituitary responsiveness to GnRH in the different animal models was evaluated in the afternoon, before onset of either the proestrus or E2-induced LH surge (GnRH injections 1300-1400 EST). A baseline blood sample (~14 µl) was obtained from the tail tip. Mice were then injected with GnRH (Bachem H4005, 150 ng/kg in 0.9% saline; diestrus n = 6, proestrus n = 10, OVX+E on day 2 post surgery n = 8) (GLANOWSKA et al., 2014). Trunk blood was collected 15 minutes after GnRH injection. Serum from baseline and trunk blood samples was separated and stored at -20 C until assay for LH. Uterine mass was determined to confirm proestrous (uterine mass > 100 mg) and diestrous (uterine mass < 80 mg) stages. Pituitaries were snap frozen in liquid nitrogen and maintained at -80

C until lysis. Pituitaries were lysed in 350 µl of buffer containing (in mM): 20 HEPES, 150 KCl, 10 MgCl2, 2.5 DTT, plus 1X EDTA-free protease inhibitor (Roche), and 0.5% v/v RNase inhibitor (Protector RNase Inhibitor; Roche).

1.2.5 LH assays

Serum LH was measured in by the University of Virginia Center for Research and Reproduction Ligand Assay and Analysis Core. For trunk blood (Figure 1) LH was measured in singlicate by a sensitive two-site sandwich immunoassay (FALLEST et al., 1995; HAAVISTO et al., 1993) using monoclonal antibodies against bovine LH (no. 581B7) and against the human LH-beta subunit (no. 5303: Medix Kauniainen, Finland) as described previous (HAAVISTO AM et al., 1993). Pituitary content was measured in duplicate in pituitary lysate diluted 1:20 in assay buffer prior to assay using the two-site sandwich assay. The tracer antibody, (no. 518B7) is kindly provided by Dr. Janet Roser (MATTERI et al., 1987), (Department of Animal Science, University of California, Davis) and iodinated by the chloramine T method and purified on Sephadex G-50 columns. The capture antibody (no. 5303) is biotinylated and immobilized on avidin-coated polystyrene beads (7 mm; Epitope Diagnostics, Inc., San Diego, CA). Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program) is used as standard. The assay has a sensitivity of 0.04 ng/ml, the intraassay CV 4.5% and the interassay CV 8.3%.

For pituitary response (Figure 4A), serum was diluted 1:10 in assay buffer (0.2% BSA, 0.05% Tween-20 in PBS) and LH determined using ultra-sensitive ELISA based on Steyn et al. (STEYN et al., 2013). The capture monoclonal antibody (anti-bovine LH beta subunit, 518B7) is provided by Janet Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) is provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) is purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) is used as the assay standard. The Limit of Quantitation (Functional Sensitivity) was defined as the lowest concentration that demonstrates accuracy within 20% of expected values and intra-assay coefficient of variation (%CV) <20%, and was determined by serial dilutions of a defined sample pool. Intra-assay and inter-assay %CVs were 6.5% and 8.6%, respectively; functional sensitivity was 0.16 ng/ml.

*1.2.6 Pituitary RNA extraction and real time PCR***.**

Total RNA was isolated including on-column DNasing (RNeasy, Qiagen) from 100 µl pituitary lysate and 2 µl of lysate were used to measure total protein (BCA assay, Pierce). Pituitary RNA (100 ng) and a standard curve of pooled mouse pituitary RNA 400ng-98 pg [1:4 dilution] was reverse transcribed as described (GLANOWSKA et al., 2014). Pituitary cDNA (1ng/µl final concentration for samples; 1 pg/µl-4 ng/µl for standards) was assayed in duplicate for: *Cga, Lhb, Fshb, Gnrhr, Egr1, Actin,* and *Ppia* mRNAs by hydrolysis probe-based qPCR chemistry (aka Taqman). All primers and probes were purchased from Integrated DNA Technologies (IDT); Table 1 shows primer and probe sequences, and IDT Prime Time assay number. PCR was conducted using Applied Biosystems Gene Expression Mastermix (ThermoFisher). Normalized relative expression of transcripts was determined by the ΔΔCt method (BUSTIN, 2002), the average of *Actin* and *Ppia* expression were used for normalization.

1.2.7 Statistical analysis.

Statistical analyses were performed using Prism 7 (GraphPad Software). The number of cells or mice per group is indicated by n. Data are reported as individual values with mean ± SEM. Data distribution was tested using Shapiro-Wilk normality test. Data distribution and experimental design were used to select appropriate statistical comparisons, which are specified for each data set in the results. The null hypothesis was rejected if $P < 0.05$.

1.3 RESULTS

1.3.1 LH surge amplitude is lower in OVX+E compared to proestrus mice

The OVX/OVX+E daily surge model presents a constant physiological level of estradiol in the circulation that induces a daily LH surge for several days and facilitates studies of generation and timing of GnRH surges (CHRISTIAN et al., 2005). During the estrous cycle of rodents, estradiol positive feedback occurs on proestrus (BLAKE, 1976). Comparison of the LH surge amplitude between the estradiol-induced daily surge model (n = 15) and proestrous mice (n = 9) under the same husbandry and assay conditions reveals the amplitude of the daily LH surge is lower than the proestrous surge (Figure 1, $P < 0.001$, Students t test with Welch's correction).

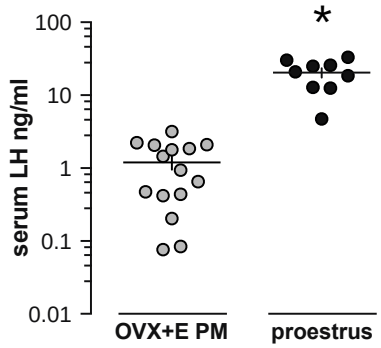

Figure 1 - *The estradiol-induced LH surge is lower amplitude than the preovulatory proestrous surge.* Serum LH measured within 30 min of lights out in OVX+E mice (grey circles, n = 15) and proestrous mice (black circle, n = 14). Note log scale; each symbol represents one animal; mean ± SEM are shown by the horizontal and vertical lines, respectively. *$P <$ 0.001 calculated by Student's t-test with Welch's correction.

1.3.2 Overall firing rate is similar in GnRH neurons from OVX+E and proestrous mice

To determine if changes in GnRH neuron activity are associated with the difference in LH surge amplitude between proestrous and OVX+E mice, firing rate was monitored using targeted single-unit extracellular recordings made in late afternoon from GFP-identified GnRH neurons in brain slices from females in diestrus (negative feedback, n = 14), proestrus (positive feedback n = 17), or OVX+E (estradiol-induced positive feedback, n = 11). Figure 2A shows representative firing patterns during one-hour recordings. There was no difference in mean firing rate of GnRH neurons between OVX+E (n = 11) and proestrous mice (n = 17), but both of these groups had a higher firing rate than cells from diestrous mice (n = 14, Figure 2B, Kruskal-Wallis/Dunn's, both $P < 0.03$).

Similarly, the percent of quiescent 1-min bins (defined as ≤1 event/minute) was greater ($P < 0.02$) in both OVX+E (38%) and proestrous (37%) mice than diestrous mice (74%, Kruskal-Wallis/Dunn's, Figure 2C). Thus, the overall pattern of GnRH activity does not account for the difference in LH surge amplitude.

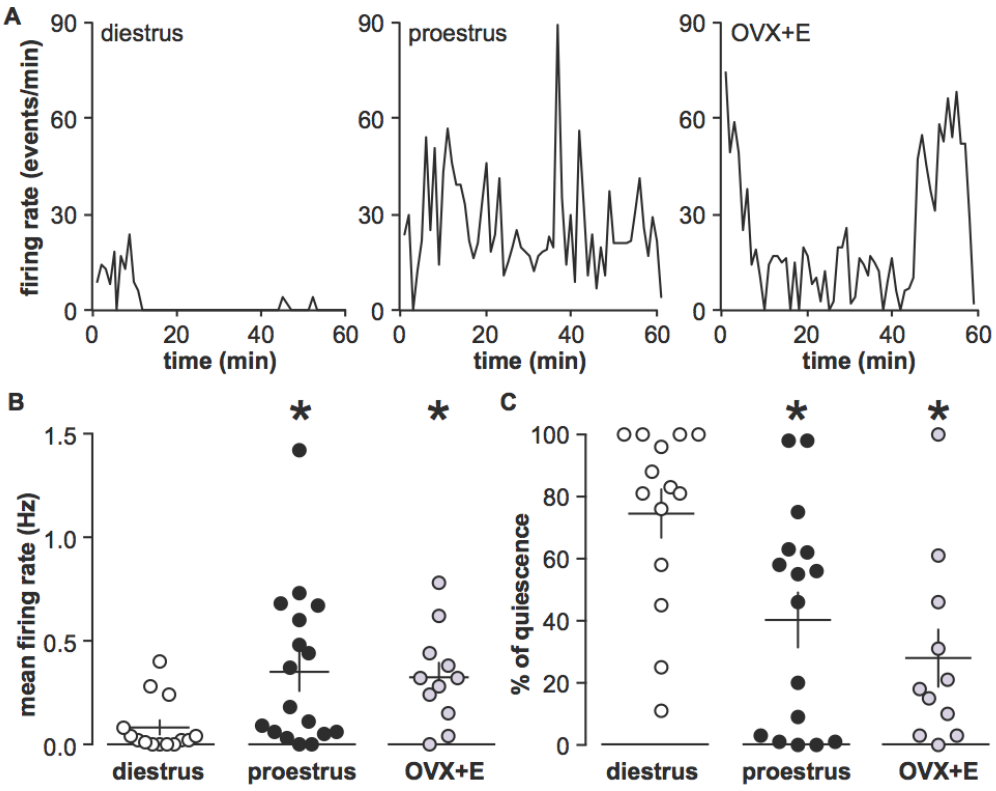

Figure 2 - *GnRH neuron activity is elevated and similar during the estradiol-induced daily surge and preovulatory proestrous surge. (A)* Representative firing patterns for GnRH neuron recorded on diestrus (left), proestrus (middle) and OVX+E mice (right). *(B)* Individual values for mean firing rate and percentage of quiescence in GnRH neurons from diestrous (open circles), proestrous (black circles) and OVX+E (gray circles) mice. Each symbol represents one mouse; mean ± SEM are shown by the horizontal and vertical lines, respectively. * $P < 0.05$ calculated by Kruskal-Wallis/Dunn's test.

1.3.3 Action potential spike patterning in GnRH neurons from OVX+E and proestrous mice

Overall firing activity provides an initial screen of neuronal activity, but the activity likely associated with hormone release occurs over much shorter time windows referred to as burst firing. To better understand the pattern of action currents in GnRH neurons burst window of the first burst

(i.e., minimum interspike interval) and percentage of total spikes contained within bursts were compared (Figure 3A, B). Burst duration was then analyzed for selected burst windows (Figure 3C-G). The burst window in which the first burst was detected was longer in GnRH neurons from proestrous than OVX+E mice (Figure 3A), but no difference was observed between these groups in the percent of spikes contained within bursts at any burst window studied (Figure 3B, * $P < 0.05$, Kruskal-Wallis/Dunn's). In contrast, at burst windows of 0.15 and 0.21 sec, the % of spikes in bursts is lower in cells from diestrous mice. At a burst window of 0.01sec, typical of intraburst spike frequency in other neurons (CHEN et al., 2009), no bursts were observed in GnRH neurons from any groups (Figure 3B), consistent with previous reports in GnRH neurons (CHU et al., 2010; CHU et al., 2012; GASKINS; MOENTER, 2012; LEE et al., 2010). Burst duration was not different between GnRH neurons from OVX+E and proestrous mice except at the longest window examined, 1.5 sec, in which burst duration was longer in proestrous mice ($P < 0.05$, Kolmogorov-Smirnov test). Interspike interval was shifted to shorter durations in OVX+E compared to proestrous mice (Figure 3H, * $P < 0.05$ Kolmogorov-Smirnov test).

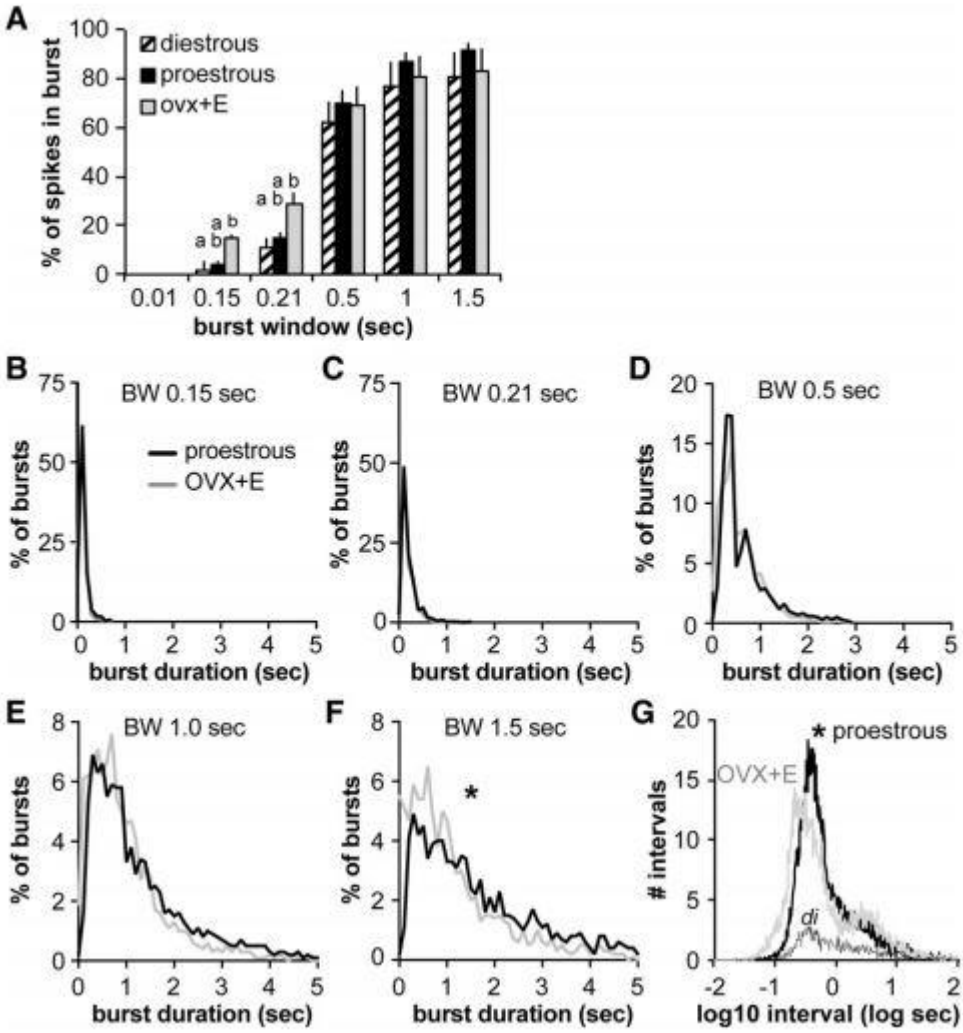

Figure 3 - *GnRH neuron burst duration is increased on proestrus compared with OVX+E mice.* (A) Mean 6 standard error of mean percentage of spikes in bursts at different burst windows (BWs) in GnRH neurons from diestrous (hatched), proestrous (black), and OVX+E (gray) mice. Different lower case letters indicate $P < 0.05$ (Kruskal-Wallis/Dunn). (B–F) Histograms of the percentage of bursts of different duration in cells from proestrous (black line) and OVX+E (gray line) mice for burst windows indicated. (G) Log10 interval histogram in proestrous (black), OVX+E (gray), and diestrus (di, thin blackline) mice. *$P < 0.05$ proestrous vs OVX+E mice, Kolmogorov-Smirnov test.

1.3.4 Pituitary responsiveness to exogenous GnRH is greater in proestrous mice

The observed reduction in LH surge amplitude (Figure 1) could be due to reduced pituitary response in the daily surge model. To test this hypothesis, we evaluated pituitary responsiveness to exogenous GnRH in diestrous (n = 6), proestrous (n = 10) and OVX+E (n = 8) mice during the early

afternoon before surge onset in the positive feedback models. Serum LH was monitored before and 15 min after IP injection of 150 ng/kg GnRH (Figure 4). No difference was observed in LH levels before GnRH injection. All three groups responded with an increase in serum LH, but LH values in proestrous mice were greater than the other two groups during the post injection sample (two-way repeated-measures ANOVA/Holm-Sidak, $P < 0.05$). Uterine mass not different between proestrus and OVX+E mice but was greater than diestrous mice (Figure 4B, one-way ANOVA/ Tukey's, $P < 0.05$). This suggests estradiol elevation was present and similar in both surge models (SHIM et al., 2000).

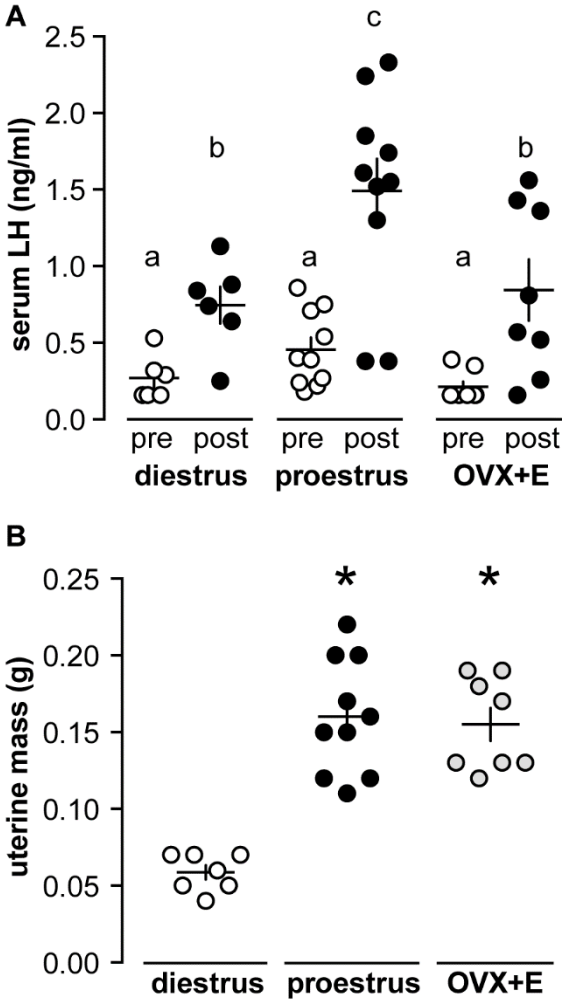

Figure 4 - *Pituitary response is lower in the daily estradiol-induced surge model. (A)* Serum LH before (pre, open circles) and 15 min after (post, black circles) IP injection 150 ng/kg GnRH in diestrus, proestrus and OVX+E mice. Each symbol represents one animal; mean ± SEM are shown by the horizontal and vertical lines, respectively. Different lower case letters indicate $P < 0.05$ calculated by two-way repeated-measures ANOVA/Holm-Sidak. *(B)* Uterine mass of diestrous (open circles), proestrous (black circles) and OVX+E (gray circles) mice. *$P < 0.05$ calculated by one-way ANOVA/Tukey's.

The greater LH response in proestrous mice could be attributable to differences in pituitary gene expression or LH content. To begin to test this, quantitative RT PCR was used to evaluate the steady-state mRNA levels of specific genes. No difference in steady-state mRNA for *Gnrhr* or *Egr1*, an immediate early gene downstream of GnRH receptor signaling and required for *Lhb* expression (YUEN et al., 2002) was observed among groups (Figure 5A,B, one-way ANOVA/Tukey's, $P > 0.05$). *Lhb* and Cga mRNA were greater ($P < 0.05$) in diestrous compared to proestrous and OVX+E, which were not different from one another (*Lhb*, Figure 5C, *Cga* diestrus 1.9 ± 0.2, proestrus 0.9 ± 0.1, OVX+E 1.1 ± 0.1, Kruskal-Wallis/Dunn's). For *Cga*, one point was removed from both the diestrous and proestrous data sets based on positive Grubb's outlier tests. Despite reduced mRNA in proestrous and OVX+E mice, pituitary LH content was not different among groups (diestrus 33 ± 7, proestrous 52 ± 39, OVX+E 41 ± 35 pg LH/µg protein, one-way ANOVA/Tukey's, $P > 0.05$). Expression of *Fshb* was greater in OVX+E compared both to diestrous and proestrous mice (Figure 5D, Kruskal-Wallis/Dunn's, $P < 0.05$).

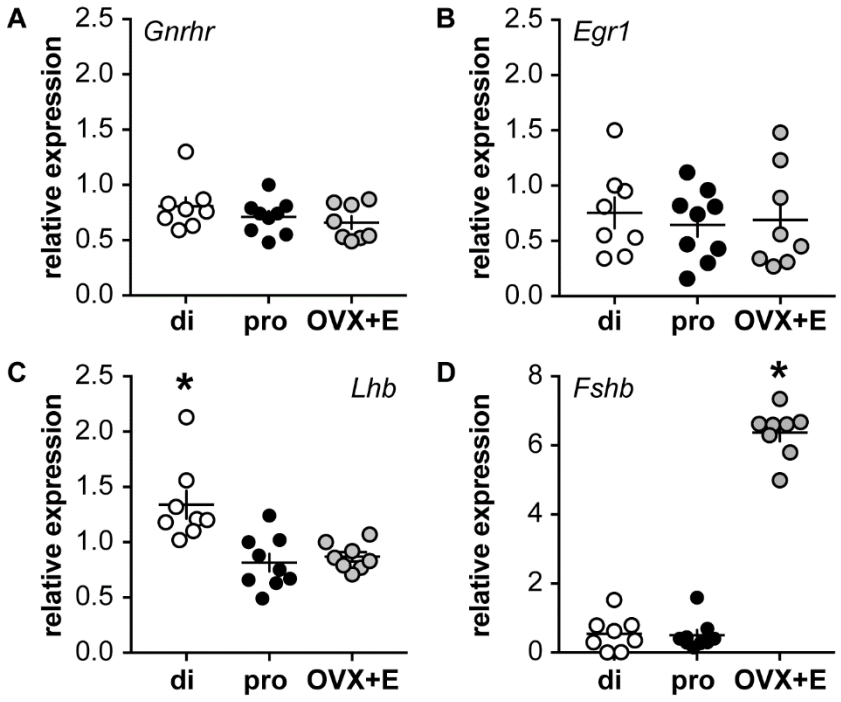

Figure 5 *- Relative steady-state mRNA levels of selected pituitary genes, normalized to mean expression of actin and ppia. (A)* GnRHR, *(B)* Egr1, *(C)* LHb and *(D)* FSHb in pituitaries from diestrous (open circles), proestrous (black circles) and OVX+E (gray circles) mice. Each symbol represents one mouse; the horizontal and vertical lines show mean ± SEM, respectively. Not change in scale of y axis in *(D)* * $P < 0.05$ calculated by one-way ANOVA/Tukey's.

1.4 DISCUSSION

Preovulatory GnRH and LH surges are essential for successful reproduction in most species. The GnRH surge is initiated by high physiologic estradiol levels due to a switch in feedback action of this steroid from negative to positive feedback upon both the GnRH network and pituitary. The importance of estradiol in the control of ovulation led to the development of models in which estradiol levels were modified in ovariectomized animals to induce the positive feedback response while reducing other ovarian variables (CHRISTIAN et al., 2005; LEGAN; KARSCH, 1975; NORMAN, 1975; WINTERMANTEL et al., 2006; ZHANG et al., 2013). These models have facilitated studies on the dependence and contributions of estradiol to central neuronal and pituitary changes underlying surge generation. Here we showed that the amplitude of the LH surge in an OVX+E mouse model (CHRISTIAN et al., 2005) is lower than that during proestrus. Our findings suggest this may be due to a reduced response in the pituitary in combination with subtle shifts in action potential patterning, rather than differences in the mean firing rate of GnRH neurons.

The present study revealed that despite a higher amplitude LH surge, the increase in mean GnRH firing rate over one hour was not different between proestrous and estradiol-induced surges. This suggests replacement of estradiol alone is able to recapitulate many of the positive feedback mechanisms induced by peripheral sex steroids to increase GnRH neuron activity. The present data support and extend previous studies of the firing rate of GnRH neuron during the natural cycle, specifically a higher firing rate on proestrus compared to metestrus (FARKAS et al., 2013). In contrast, a recent study showed a greater firing rate of GnRH neuron on diestrus than proestrus (PIET et al., 2016). This latter observation may be attributable to a short duration of recordings (5 min versus 1 hour in the present study) or differences in the timing of slice preparation, which was 4-5 hours before expected onset of the LH surge in the former study and just before expected onset of the LH surge in the present study.

Bursts of action potentials are related to neuroendocrine secretion (CAZALIS et al., 1985; DUTTON; DYBALL, 1979;). Burst firing in GnRH neurons tends to be lower frequency (CHU et al., 2010; CHU et al., 2012; GASKINS; MOENTER, 2012; LEE et al., 2010) than other cells in which burst firing has been studied (CHEN et al., 2009; LLINÁS; JAHNSEN, 1982; WANG et al., 2016). Of interest in this regard, detailed analysis of the firing pattern of GnRH neurons revealed burst duration is increased in proestrous compared to OVX+E mice when longer burst windows are

examined. Longer bursts of action potential, as observed on proestrus, could thus lead to increased GnRH release possibly by maintaining increased cytoplasmic calcium levels needed for vesicle fusion. Few measurements of intracellular calcium have been done in GnRH neurons and limitations of the sampling rate or physical properties of the calcium indicator preclude exact conclusion of the duration of calcium elevation, but these events appear to be prolonged in GnRH neurons (LEE et al., 2010; TERASAWA; SCHANHOFER, et al., 1999). Of interest, the afterdepolarization potential (ADP) of GnRH neurons, which is estradiol-sensitive, has a similar duration to the long burst window analysis that revealed a difference in burst duration (CHU; MOENTER, 2006; WANG et al., 2008). Interspike interval has also been used to classify bursting cells (NOWAK et al., 2003; ROMANÒ et al., 2013). Intervals were shifted to shorter durations in OVX+E vs. proestrous mice. This may indicate a shorter action potential refractory period in the former but this did not generate longer burst durations. Thus, although both groups present similar overall firing frequency, the short-term patterning observed on proestrus might lead to a greater GnRH release, which could contribute to the increased LH surge amplitude observed in this group.

Estradiol positive feedback acts not only centrally to alter GnRH release, but at the pituitary to alter responsiveness to GnRH (CLARKE; CUMMINS, 1984; TURGEON; BARRACLOUGH, 1977). During the early afternoon, before onset of the LH surge, pituitary responsiveness to exogenous GnRH was increased in proestrous mice compared to OVX+E and diestrous mice, which were similar to one another. These observations suggest that the increase amplitude of the LH surge in proestrous mice model may be in part due a difference in pituitary rather than central mechanisms (CLARKE; CUMMINS, 1984; SHUPNIK, 1996; WILDT et al., 1981). There was no difference in steady state levels of Gnrhr mRNA. Differential translation of this mRNA pool, subcellular receptor localization and signaling are alternative mechanisms that could account for lower amplitude LH surges in OVX+E mice. With regard to the latter, there was also no difference in expression of Egr1, an immediate early gene induced by GnRH receptor signaling (YUEN et al., 2002). Other signaling pathways could be different among the groups, for example the gonadotropin-inhibitory hormone pathway, which reduces LH release in response to GnRH (CICCONE et al., 2004; KRIEGSFELD et al., 2006; TSUTSUI et al., 2000). There was also no difference in pituitary LH content among groups, despite expected suppression of Lhb and Cga mRNA by higher estradiol levels (BURGER et al., 2004; NILSON et al., 1983) in proestrous and OVX+E mice based on uterine mass. The high levels of Fshb levels in OVX+E compared to diestrous and proestrous mice is likely attributable to reduced

levels of inhibin after OVX (DALKIN et al., 1993). None of these changes in steady-state mRNA seem poised to contribute to a difference in LH surge amplitude between the two models.

Although the similarity of overall GnRH neuron activity between OVX+E and proestrous mice is consistent with a primary role for estradiol in surge induction, it is important to consider other steroid hormone changes during the cycle (WALMER et al., 1992). Centrally produced progesterone has been reported to enhance estradiol-induced LH surges (MICEVYCH et al., 2003; MICEVYCH; SINCHAK, 2011). A role for ovarian progesterone is perhaps more likely to affect continuation of, rather than initiation of positive feedback as serum levels of this hormone increase after onset of the LH surge (WALMER et al., 1992). Expression of progesterone receptor is required for positive feedback, although these actions may be ligand-independent (CHAPPELL et al., 1999; CHAPPELL; LEVINE, 2000). Of interest with regard to action of peripheral progesterone, activity of kisspeptin neurons of the AVPV, a region hypothesized to be critical for induction of positive feedback (SIMERLY; SWANSON, 1987), is not different between OVX+E and OVX+E mice also treated with progesterone (WANG et al., 2016), again suggesting primary neuronal activity changes are mainly an estradiol effect.

In sum, despite a marked difference in LH surge amplitude between proestrous and OVX+E mice, GnRH neuron firing was fairly similar between these models, with subtle shifts towards patterns that may induce increased hormone release on proestrus. Pituitary response was also enhanced on proestrus and the combination of these changes may contribute to increased LH release. While it is not possible to rule out differences in parameters that were not examined (e.g., specific biophysical properties of GnRH neurons, other factors such as GnIH), these data suggest the OVX+E model recapitulates many aspects of the proestrous surge. A recent study demonstrated more consistent induction of LH surges with this type of constant estradiol implant model (DROR et al., 2013). This reliability in combination with similarity to proestrous surges make these models useful for studying the mechanisms of estradiol negative and positive feedback. Moreover, steroid replacement is the only choice for investigating feedback in genetic models that do not exhibit reproductive cycles, such as kisspeptin knockouts and vasopressin knockouts (D'ANGLEMONT DE TASSIGNY et al., 2007; MILLER et al., 2006).

CHAPTER 2 - Stat5 signaling in kisspeptin cells regulates the timing of puberty

2.1 INTRODUCTION

Cytokines play a pivotal role in reproduction by regulating the HPG axis. For example, leptin signaling in the hypothalamus is required for the onset of puberty, as well as to maintain reproduction (AHIMA et al., 1996; DONATO; CRAVO; FRAZÃO; ELIAS, 2011; DONATO; CRAVO; FRAZÃO; GAUTRON, et al., 2011). Leptin and several other cytokines possess receptors that depend on an association with the JAK2 to induce the transduction of their intracellular effects. This class of cytokine receptors recruits transcription factors from the family STAT, as well as signaling pathways that can induce rapid non-genomic effects, such as the PI3K pathway. Thus, the recruitment of STAT proteins controls the transcription of target genes, whereas the activation of the PI3K signaling pathway may mediate acute changes in neuronal electrical activity (BOLE-FEYSOT et al., 1998; FREEMAN et al., 2000; FURIGO et al., 2016).

One particular cytokine that has interesting effects on the reproductive system is prolactin. Hyperprolactinemia causes reproductive dysfunctions, representing a frequent cause of infertility in both males and females (GLEZER; BRONSTEIN, 2015; SHIBLI-RAHHAL; SCHLECHTE, 2011; NEWEY et al., 2013; VILAR et al., 2014). Several studies have reported that prolactin may modulate the reproductive axis through its actions on kisspepitin neurons (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; SJOEHOLM et al., 2011). The *Kiss1* gene encodes neuropeptides known as kisspeptins, which play a critical role in reproduction (DE ROUX et al., 2003; FUNES et al., 2003; SEMINARA et al., 2003). In rodents, kisspeptin neurons are mainly located in the AVPV, PeN and ARH of the hypothalamus (GOTTSCH et al., 2004). Most of the kisspeptin neurons express PRLRs and are directly responsive to prolactin (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; KOKAY et al., 2011; LI et al., 2011; SJOEHOLM et al., 2011). Because the Kiss1/Kiss1r system is a key regulator of reproduction (DE ROUX et al., 2003; SEMINARA et al., 2003) and kisspeptin neurons are direct targets of prolactin (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; KOKAY et al., 2011; SJOEHOLM et al., 2011; SONIGO et al., 2012), the effects of prolactin on the reproductive axis are thought to be mediated by these neurons. Consistent with this hypothesis, systemic or intracerebroventricular infusion of prolactin suppresses hypothalamic expression of

kisspeptin and plasma levels of LH (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; SONIGO et al., 2012). Notably, peripheral kisspeptin administration can restore gonadotropin secretion and ovarian cyclicity in hyperprolactinemic female mice (SONIGO et al., 2012). Thus, prolactin signaling in kisspeptin neurons exerts important effects on reproductive function.

While the physiological effects of cytokines have been studied for many years, little information is available concerning the molecular mechanisms by which cytokines modulate reproduction. Although it is known that kisspeptin neurons are direct targets of prolactin, the mechanisms of action of prolactin in the regulation of reproduction via these neurons remain largely unknown. Therefore, our first aim was to investigate whether prolactin can affect the membrane excitability of kisspeptin neurons, which would indicate the activation of rapid, non-genomic signaling pathways. In addition, we ablated STAT5 specifically in kisspeptin neurons to investigate whether this key transcription factor regulates the timing of puberty and the reproduction. The experiments proposed in this study can help to unravel important information about the cellular mechanisms recruited by cytokines to affect reproduction.

2.2 MATERIALS AND METHODS

2.2.1 Animals

The Kiss1-humanized Renilla green fluorescent protein (Kiss1-hrGFP; strain C57BL/6-Tg(Kiss1-hrGFP) KG26Cfe/J, Jackson Laboratories) and Kiss1-Cre mouse models (strain C57BL/6-Tg(Kiss1-Cre) J2-4Cfe/J, Jackson Laboratories, Bar Harbor, ME, USA) were generated as previously described (CRAVO et al., 2011; CRAVO et al., 2013). Female Kiss1-Cre mice were crossed with mice carrying loxP-flanked *Stat5a*/*b* alleles (strain B6.129S6-*Stat5a/Stat5b*^{tm2Mam}/Mmjax) (CUI et al., 2004; LEE et al., 2008). Heterozygous offspring from these matings were then crossed to homozygous *Stat5a*/b mice, generating mice that were homozygous for *stat5a*/b alleles and that either expressed Cre in a *Kiss1*-dependent fashion (Kiss1/STAT5KO) or did not express Cre (control).

The mice were weaned at 3 weeks of age and genotyped via PCR using DNA extracted from the tail tip of the mice (REDExtract-N-Amp™ Tissue PCR Kit, Sigma). The mice were housed in the animal care facility of the Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo in an environment with controlled light (12 h on/12 h off; lights on at 6:00 am) and temperature (21–23 C). All experiments and procedures were performed in accordance with the guidelines established by the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Committee on the Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo.

2.2.2 Electrophysiological recordings

To examine the acute effects of prolactin on the membrane excitability of kisspeptin neurons, whole-cell patch-clamp recordings were performed in female Kiss1-hrGFP mice (8-12 weeks). The estrous cycle was monitored daily, in the morning, by the observation of vaginal smears. After detecting regular estrous cyclicity, we selected females in diestrus for electrophysiological experiments. The mice were decapitated, and the entire brain was removed. After removal, the brains were immediately submerged in ice-cold, carbogen-saturated (95% O_2 and 5% CO_2) ACSF (124 mM NaCl, 2.8 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM glucose and 2.5 mM $CaCl_2$). Coronal sections from a hypothalamic block (250 µM) were cut with a Leica VT1000S Vibratome and then incubated in oxygenated ACSF at room temperature for at least 1 h before being

transferred to the recording chamber. The slices were transferred to the recording chamber and allowed to equilibrate for 10 - 20 min before recording. The slices were bathed in oxygenated ACSF (30 °C) at a flow rate of ~2 mL/min. The pipette solution for whole-cell recording contained the following: 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 mM (Mg)-ATP, pH 7.3. Epifluorescence was briefly used to target the fluorescent cells; at this time, the light source was switched to infrared differential interference contrast imaging to obtain the whole-cell recording (Leica DM6000 FS equipped with a fixed stage and a Leica DFC360 FX high-speed monochrome fluorescence digital camera). Kisspeptin neurons in the AVPV, PeN and ARH were recorded. During the recordings, the neurons were maintained in hypothalamic slice preparations, and the data analyses were performed as previously described (FRAZÃO et al., 2013; WILLIAMS et al., 2010). The electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices); low-pass filtered at 2 - 4 kHz, and analyzed offline on a PC using pCLAMP software (Molecular Devices). The recording electrodes had resistances of 5 - 7 MΩ when filled with the K-gluconate internal solution. The input resistance (IR) was assessed by measuring the voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse (500 ms of -10 to -50 pA). The membrane potential values were compensated to account for the junction potential (-8 mV). A bath solution containing prolactin from ovine pituitary (250 nM, Sigma) was perfused for approximately 5 min. The action potentials (APs) firing rate was determined by comparing the average firing rates 2 minutes immediately before and 2 minutes during the application of prolactin to the bath. For some experiments, the changes in resting membrane potential (RMP) in response to prolactin were monitored in the presence of tetrodotoxin (TTX, 1 µM) and synaptic blockers (6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at 10 µM, 2-amino-5-phosphonovalerate (AP-5) at 50 µM, and picrotoxin at 50 µM), LY294002 (10 µM) or wortmannin (100 nM). TTX and LY294002 were obtained from Alomone Labs; picrotoxin, CNQX, AP-5 and wortmannin were purchased from Tocris Bioscience.

2.2.3 Histological determination of prolactin-responsive kisspeptin neurons

Kiss1-hr-GFP female mice (14-16 weeks of age) in diestrus received an i.p. injection of ovine prolactin (10 µg/g, Sigma) or vehicle (phosphate-buffered saline, PBS) between 11:00 am and 12:00 pm, followed by perfusion 120 min later. For tissue collection, the mice were deeply anesthetized and

perfused transcardially with saline and then with a 10% buffered formalin solution. The brains were collected and post-fixed in the same fixative for 1-2 h and cryoprotected overnight at 4 C in 0.1 M PBS containing 20% sucrose, pH 7.4. The brains were cut (30 μm sections) in the frontal plane on a freezing microtome. Four series were collected and stored at -20 C in cryoprotectant until processing for immunohistochemistry to detect pSTAT5-immunoreactivity (pSTAT5-ir).

2.2.4 Immunohistochemistry and image production

The brain sections from mice treated with prolactin or vehicle were rinsed in 0.02 M potassium PBS, pH 7.4 (KPBS), followed by pretreatment with water containing 1% hydrogen peroxide and 1% sodium hydroxide (solution pH >13) for 20 min. After rinsing with KPBS, the sections were incubated in 0.3% glycine and 0.03% lauryl sulfate for 10 min each. Next, the sections were blocked in 3% normal donkey serum for 1 h and incubated with an anti-pSTAT5^{Tyr694} primary antibody (1:1000; Cell Signaling; #9351) for 48 h. Subsequently, the sections were incubated with biotin-conjugated donkey anti-rabbit IgG (1:1000, Jackson Laboratories) for 1 h and then with an avidin–biotin complex (1:500, Vector Labs, Burlingame) for 1 h. The peroxidase reaction was performed using 0.05% 3,3'-diaminobenzidine (DAB), 0.25% nickel sulfate and 0.03% hydrogen peroxide. After rinsing with KPBS, the sections were further incubated overnight with the primary antibody (anti-hrGFP: 1:2000, Agilent Technologies). The sections were then incubated with biotin-conjugated donkey anti-rabbit (1:1000, Jackson Laboratories), followed by the avidin-biotin complex and DAB as the chromogen. The sections were mounted onto gelatin-coated slides and coverslipped using DPX mounting medium.

The quantification of the single- or dual-labeled neurons and the percentage of colocalization were determined in the AVPV (relative to bregma: 0.26), PeN (relative to bregma: 0.02) and two rostrocaudal levels of the ARH (relative to bregma: -1.94 and -2.30). The approximate bregma coordinates of each rostrocaudal level were obtained according to the mouse brain atlas (PAXINOS; FRANKLIN, 2001). The cells were counted on one side of a defined level of each nucleus, as previously described (CRAVO et al., 2013). The photomicrographs of brain sections were acquired using a Zeiss Axiocam HRc camera connected to a Zeiss Axioimager A1 microscope (Zeiss, Munich, Germany). The images were digitized using the Axiovision software (Zeiss). Photoshop image-

editing software was used to combine the photomicrographs into plates. Only the sharpness, contrast and brightness were adjusted.

2.2.5 Sexual maturation in Kiss1/STAT5KO mice

To evaluate the importance of STAT5 signaling in kisspeptin neurons during sexual maturation, mice with the *Stat5a/b* genes inactivated specifically in kisspeptin cells were created (Kiss1/STAT5KO). Sexual maturation was assessed by determining the age at vaginal opening, the first occurrence of vaginal cornification in the vaginal lavage (first estrus) and the onset of cyclicity, which is the first occurrence of an estrous cycle of typical duration (4 to 7 days in mice), as previously described (BOHLEN et al., 2016; NELSON et al., 1990). These parameters were assessed daily. The body weights were recorded at each specific sexual maturation stage.

2.2.6 Fertility and serum dosages

Kiss1/STAT5KO and control female mice were paired with sexually experienced wild-type C57BL/6 males. The number of days until giving birth and the number of pups per litter were determined. To assess the serum prolactin and LH levels, blood samples were collected immediately after the decapitation of adult Kiss1/STAT5KO and control female mice in diestrus (16-20 weeks of age). Additionally, to assess leptin levels, blood samples were collected from female mice at 47 days of age. Mice were sacrificed in the morning (10:00 am -12:00 pm). Mouse prolactin concentrations were determined using an ELISA kit according to the manufacturer's instructions (Sigma), as was the serum leptin concentration (Crystal Chem). LH levels were determined by double-antibody radioimmunoassay using a kit provided by the National Hormone and Peptide Program (Harbor-University of California at Los Angeles, USA), as previously described (AQUINO et al., 2016; TRASLAVIÑA; FRANCI, 2011). All samples were assayed using the same radioimmunoassay. The lower limit of detection and intra-assay coefficient of variation were 0.16 ng/mL and 1.8%, respectively.

2.2.7 Relative Gene Expression

STAT5a and STAT5b mRNA levels were assessed in AVPV and ARH regions to confirm the conditional deletion. Adult Kiss1/STAT5KO and control female mice (16-20 weeks of age) were anesthetized and decapitated, and the entire brain was removed. Brain coronal sections (250 µm) were cut by using a vibratome. AVPV micropunches were obtained using an 18-gauge needle. A single midline punch was taken from a coronal section, obtained at 0.26 to 0.02 mm relative to bregma according to the mouse brain atlas (PAXINOS; FRANKLIN, 2001). Additionally, a coronal section of 500 µm was cut (bregma -1.70 to -2.18), and bilateral punches using an 18-gauge needle were performed to collect the ARH. Total RNA from the punches was extracted using the PicoPure RNA isolation kit according to the manufacturer's instructions (Thermo Fisher Scientific). An assessment of RNA quantity was performed using an Epoch Microplate Spectrophotometer (BioTek). Reverse transcription was performed using 0.2 µg of total RNA from the AVPV punches and 0.5 µg of total RNA from ARH punches; SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) and random primers p(dN)6 (Roche Applied Science) were used. Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). The following primers were used: STAT5a sense: cgctggactccatgcttctc; STAT5a antisense: gacgtgggctcctcacactga; STAT5b sense: ggactccgtccttgataccg; and STAT5b antisense: tccatcgtgtcttccagatcg. The thermal cycling conditions were set at 50 ºC for 2 min and 95 ºC for 10 min initially, followed by 15 s at 95 ºC (melting step) and 1 min at 60 ºC (annealing/extension step) for 40 cycles. The relative mRNA was quantified by calculating $2^{-\Delta\Delta}$Ct. The data were normalized to cyclophilin A mRNA levels (sense: tatctgcactgccaagactgagt; antisense: cttcttgctggtcttgccattcc) and reported as fold changes compared with the values obtained from the respective control group (normalized to 1.0). The average cycle threshold (Ct) values for STAT5a were 26.9 and 25.4 for the AVPV and ARH micropunches, respectively. The average Ct values for STAT5b were 21.2 and 21.3 for the AVPV and ARH micropunches, respectively.

2.2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. The data are expressed as the mean ± standard error of the means (SEM). The comparisons between groups were performed using unpaired two-tailed Student's *t* tests. For electrophysiological studies, we used repeated-measures ANOVA and the Newman-Keuls multiple comparison test to compare the data before, during and after drug application. A P value < 0.05 was considered to be statistically significant.

2.3 RESULTS

2.3.1 The membrane excitability of AVPV kisspeptin neurons is indirectly affected by prolactin via PI3K prolactin pathway

To determine whether prolactin activates fast-acting signaling pathways that can induce acute changes in the membrane excitability of kisspeptin neurons, we used hypothalamic slice preparations from adult Kiss1-hrGFP mice and whole-cell patch-clamp recordings. The hrGFP expression allowed us to specifically identify kisspeptin neurons in the AVPV (Figure 1A, B), as previously reported (CRAVO et al., 2013). In current-clamp mode, neurons were recorded under zero current injection (I = 0) in the whole-cell patch-clamp configuration. Importantly, because the biophysical properties of the AVPV and PeN recorded neurons were similar, as previously described (FRAZÃO et al., 2013), the data obtained from these populations are reported together (as AVPV). Approximately 70% of the recorded AVPV kisspeptin neurons were quiescent at rest and showed a hyperpolarized RMP when compared with the remaining 30% of the recorded cells (data not shown). These results are consistent with earlier studies (FRAZÃO et al., 2013). The average RMP of all AVPV kisspeptin neurons was -65.4 ± 1.6 mV (range: -47 mV to -79 mV, n = 22 cells from 14 mice). Of the total recorded cells, approximately 20% of the AVPV kisspeptin neurons presented spontaneous APs at rest (1.3 ± 0.2 Hz, n = 5 from 22 recorded cells). As an initial control, we first performed a set of experiments in which only vehicle solution was applied to the bath. As expected, the vehicle solution (PBS) had no effect on membrane excitability (n = 10 cells from 6 mice; Table 1; Figure 1C). On the other hand, just a few minutes after the addition of prolactin to the bath, approximately 20% of the AVPV kisspeptin neurons were depolarized (4 of 22 recorded neurons from 15 mice; Table 1; Figure 1D). The prolactin-activated cells showed a +5.0 ± 1.0 mV change in the RMP (Table 1; $P < 0.005$). The AVPV kisspeptin neurons that depolarized exhibited a significant increase in the whole-cell IR (Table 1; $P = 0.03$). After prolactin washout, the RMP and IR were restored to baseline values (Table 1). The remaining recorded neurons (18 of 22 neurons) were unaffected by prolactin (Table 1). Notably, prolactin did not affect the APs firing rate of the recorded AVPV kisspeptin neurons (after prolactin: 1.5 ± 0.4 Hz; $P = 0.7$).

Previous reports indicated that prolactin could modulate the membrane excitability of tuberoinfundibular dopamine (TIDA) neurons through the PI3K signaling pathway (LYONS et al., 2012). To determine whether PI3K mediates prolactin-induced changes in the membrane excitability

of AVPV kisspeptin neurons, brain slices were pretreated with selective PI3K inhibitors, wortmannin and LY294002. When pretreated with wortmannin, prolactin failed to depolarize AVPV kisspeptin neurons (Table 1). Similarly, the PI3K inhibitor LY294002 blocked prolactin-induced effects in the AVPV kisspeptin neurons membrane excitability (Table 1; Figure 1E). These results suggest that PI3K signaling is required for the prolactin-induced depolarization of AVPV kisspeptin neurons.

To investigate whether the observed effects were dependent on AP-mediated synaptic transmission, brain slices were pretreated with TTX and synaptic blockers. When prolactin was administered in the presence of TTX and synaptic blockers, no effect was observed on the membrane excitability of AVPV kisspeptin neurons (Figure 1F). No differences were observed in the average RMP (n = 20 cells from 11 mice; Table 1; $P = 0.2$) or in the IR (Table 1; $P = 0.2$). The lack of responsiveness to prolactin in the presence of TTX indicates that other populations of prolactin-responsive cells are mediating prolactin-induced changes in the membrane excitability of AVPV kisspeptin neurons.

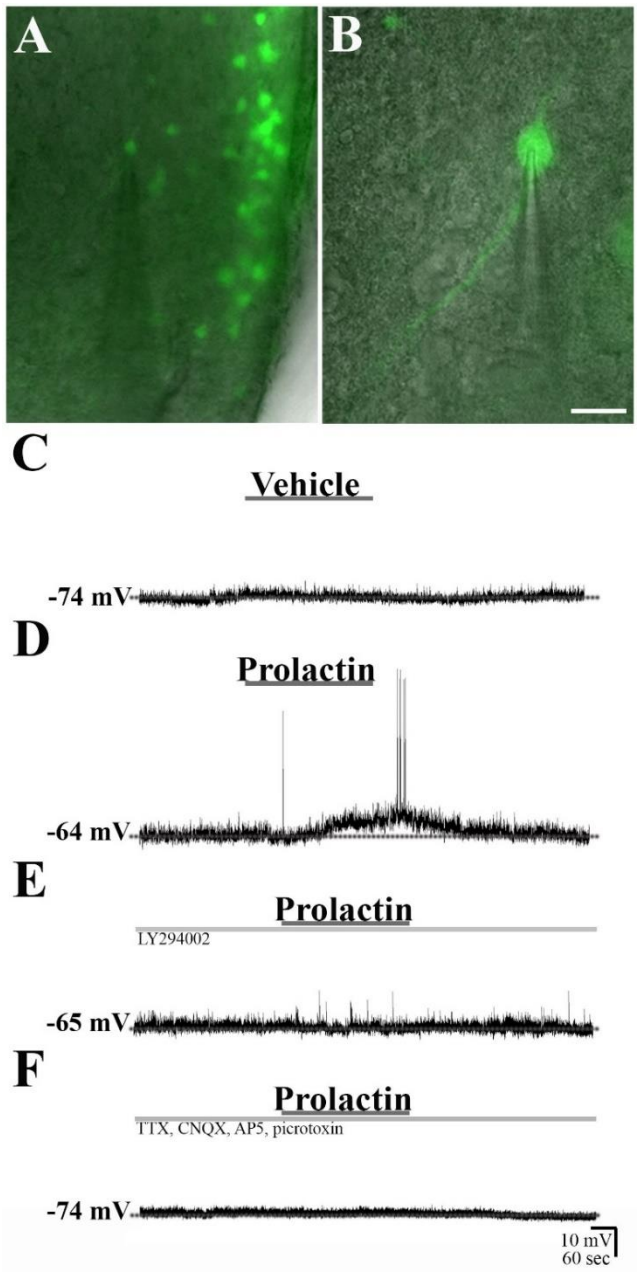

Figure 1 - *Prolactin indirectly depolarizes AVPV kisspeptin neurons via PI3K signaling pathway.* Identification of an AVPV neuron targeted for whole-cell patch-clamp recording, at low (A) and high (B) magnification. (C, D) Representative current-clamp recordings demonstrate that vehicle administration to the bath induced no effect on membrane excitability, while prolactin depolarized the membrane potential of AVPV kisspeptin neurons. (E, F) Representative current-clamp recordings demonstrating the absence of prolactin-induced changes in the RMP of AVPV kisspeptin neurons in brain slices pretreated with selective PI3K inhibitor LY294002 (10 µM) or pretreated with TTX (1 µM) and synaptic blockers (20 µM CNQX, 50 µM AP-5 and 50 µM picrotoxin). The dashed lines indicate the RMP. Scale bar: 20 µm.

Table 1 - Prolactin effects in the kisspeptin neurons membrane excitability.

AVPV	*Baseline*	*Drug effect*	*Washout*
Vehicle (n =10)			
RMP (mV)	-66.4 ± 2.9	-66.8 ± 3.1	-67.4 ± 3.3
Input resistance (GΩ)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Prolactin - responsive cells (n = 4 of 22)			
RMP (mV)	-63.7 ± 5.8	58.7 ± 6.3 **	-64.0 ± 5.5
Input resistance (GΩ)	0.7 ± 0.1	0.9 ± 0.1 *	0.7 ± 0.1
Prolactin - unresponsive cells (n = 18 of 22)			
RMP (mV)	-65.8 ± 1.6	-65.6 ± 1.5	-66.7 ± 1.5
Input resistance (GΩ)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Wortmannin + Prolactin (n = 20)			
RMP (mV)	-69. 6 ± 2.1	-69.8 ± 2.1	-69.8 ± 2.1
Input resistance (GΩ)	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
LY294002 + Prolactin (n = 20)			
RMP (mV)	-69.3 ± 2.5	-69.05 ± 2.6	-69.8 ± 2.7
Input resistance (GΩ)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
TTX, Synaptic blockers + Prolactin (n = 20)			
RMP (mV)	-69.3 ± 1.8	-69.7 ± 1.8	-69.1 ± 1.6
Input resistance (GΩ)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
ARH			
Prolactin - unresponsive cells (n = 20)			
RMP (mV)	-55.1 ± 1.9	-55.4 ± 1.9	-54.7 ± 2.0
Input resistance (GΩ)	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1

Mean ± SE, ** $P < 0.05$; * $P < 0.5$.

2.3.2 Membrane excitability of the ARH kisspeptin neurons is not affected by prolactin

Next, we evaluated whether prolactin could also induce changes in the membrane excitability of ARH kisspeptin neurons. Consistent with the literature (GOTTSCH et al., 2011; FRAZÃO et al., 2013), the majority of ARH kisspeptin neurons are quiescent at rest (≅ 65% of all recorded cells, data not shown). The average RMP of all ARH kisspeptin neurons was -55.10 ± 1.9 mV (range: -44 mV to -72 mV, n = 20 cells from 12 mice), and 35% of the recorded neurons presented spontaneous APs at rest (1.4 ± 0.4 Hz, n = 7 from 20 recorded cells). Interestingly, prolactin did not affect the membrane excitability of ARH kisspeptin neurons. The addition of prolactin to the bath induced no effect on the average RMP (Table 1; Figure 2; $P = 0.14$), APs firing rate (after prolactin: 1.8 ± 0.4 Hz; n = 7; $P = 0.4$) or IR of ARH kisspeptin neurons (Table 1, $P = 0.4$). These results indicate that prolactin does not induce acute effects on the membrane excitability of ARH kisspeptin neurons.

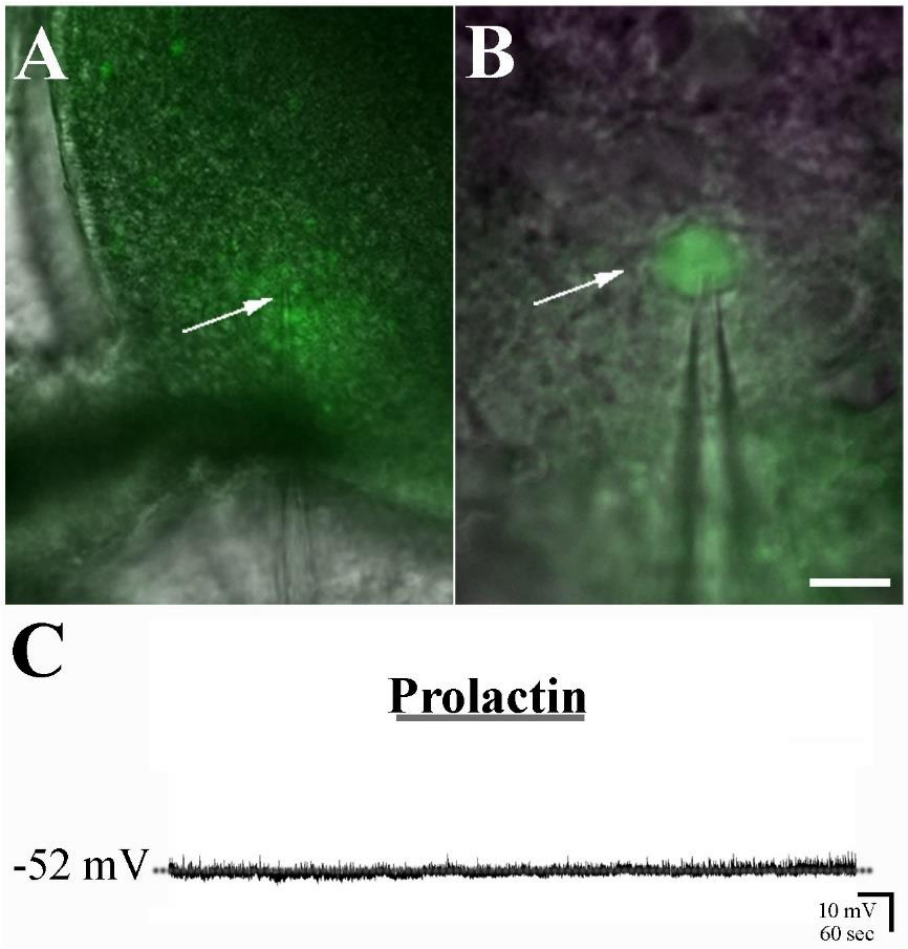

Figure 2 - *The membrane excitability of ARH kisspeptin neurons is not modulated by prolactin***.** (A-B) Identification of a recorded neuron within the arcuate nucleus (ARH), at low (A) and high (B) magnification. (C) Representative current-clamp recording demonstrating that prolactin induces no effect on the membrane excitability of ARH kisspeptin neuron. The dashed lines indicate the RMP. Scale bars: 20 μm.

2.3.3Prolactin induces STAT5 phosphorylation in a large percentage of kisspeptin neurons

Although we found no evidence that prolactin induces direct, acute effects on the membrane excitability of kisspeptin neurons, previous studies reported that many kisspeptin neurons are responsive to prolactin (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; KOKAY et al., 2011; LI et al., 2011; SJOEHOLM et al., 2011). Therefore, in the present study, we used Kiss1-hrGFP female mice as a model to allow the visualization of kisspeptin neurons (CRAVO et al., 2011) and determined the percentage of neurons expressing pSTAT5 after an acute prolactin stimulus. As a control, we assessed vehicle-treated mice, and virtually no pSTAT5-positive nuclei were observed in kisspeptin neurons (Figure 3A, B). In contrast, prolactin stimulus induced pSTAT5 expression in

approximately 32% of the AVPV kisspeptin neurons (19.0 ± 6.5 of 56.7 ± 8.2 hrGFP-positive cells, n = 4, Figure 3C) and in 13% of the PeN kisspeptin neurons (4.7 ± 1.6 of 35.3 ± 5.8 cells, n = 4). Even higher percentages of colocalization were observed in the ARH; in the rostral ARH, 74% of kisspeptin neurons exhibited pSTAT5 after a prolactin stimulus (22.2 ± 3.1 of 29.5 ± 1.5 cells, n = 4), while in the caudal ARH, 68% of kisspeptin neurons co-expressed pSTAT5 (40.2 ± 6.5 of 59.0 ± 4.2 cells, n = 4, Figure 3D).

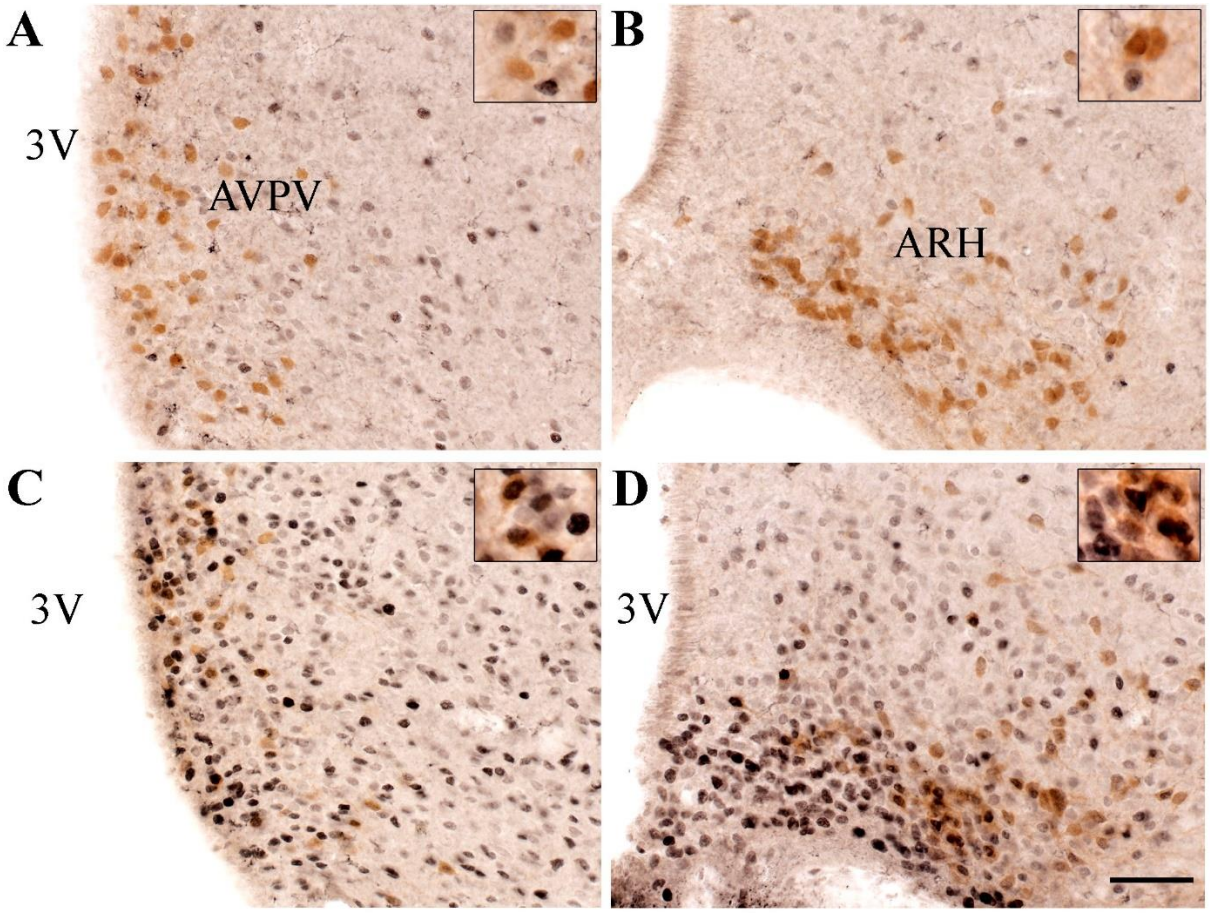

Figure 3 - *A high percentage of kisspeptin neurons are responsive to prolactin when using prolactin-induced pSTAT5 as a marker.* Brightfield photomicrographs of coronal sections of mouse brain, showing the coexpression of the humanized Renilla green fluorescent protein (hrGFP, light brown cytoplasmic staining) and prolactin-induced pSTAT5-ir (black nuclear staining) in vehicle-treated (A, B) or prolactin-treated mice (C, D). The insets highlight the hrGFP and pSTAT5-ir coexpression in the anteroventral periventricular nucleus (AVPV, n = 4; A, C) and in the arcuate nucleus (ARH, n = 4; B, D). Abbreviation: 3V, third ventricle. Scale bars A-D: 50 µm, inset: 25 µm.

2.3.4 Inactivation of the STAT5 signaling pathway in kisspeptin cells leads to an early onset of estrous cyclicity

Since our findings indicated that prolactin preferentially recruits STAT5 signaling in kisspeptin neurons rather than fast-acting pathways, we hypothesized that the STAT5 signaling pathway may play an important role on kisspeptin neuronal properties and consequently on the reproductive axis. Therefore, to determine the importance of the STAT5 signaling pathway in kisspeptin cells, we generated mice carrying a conditional deletion of *Stat5a/b* genes only in kisspeptin cells (Kiss1/STAT5KO). Initially, the sexual maturation of the mice was determined by the daily observation of the age of vaginal opening, first estrus and the onset of estrous cyclicity. No statistically significant differences were observed in the age of vaginal opening or first estrus when comparing Kiss1/STAT5KO and control mice (Figure 4A, B, D, E). However, the onset of estrous cyclicity was significantly advanced in Kiss1/STAT5KO females compared to control mice (Figure 4G, H; $P = 0.02$). For example, when 60% of Kiss1/STAT5KO mice had already displayed the onset of cyclicity, fewer than 20% of the age-matched control mice had reached the same stage of sexual maturation (Figure 4G). Importantly, at all sexual maturation stages, no differences in body weight were observed when comparing Kiss1/STAT5KO mice to the control females (Figure 4C, F, I; $P > 0.05$). To evaluate whether changes in circulating leptin levels could account for these differences, blood samples were obtained from Kiss1/STAT5KO and control female mice at 47 days of life, which was the average age of the onset of estrous cyclicity. The mean serum leptin levels were similar between groups (control mice, 0.37 ± 0.2 ng/mL, n = 6; Kiss1/STAT5KO, 0.39 ± 0.1 ng/mL, n = 5; $P = 0.95$). Therefore, our findings suggest that STAT5 signaling in kisspeptin cells exerts an inhibitory effect on the onset of cyclicity in female mice.

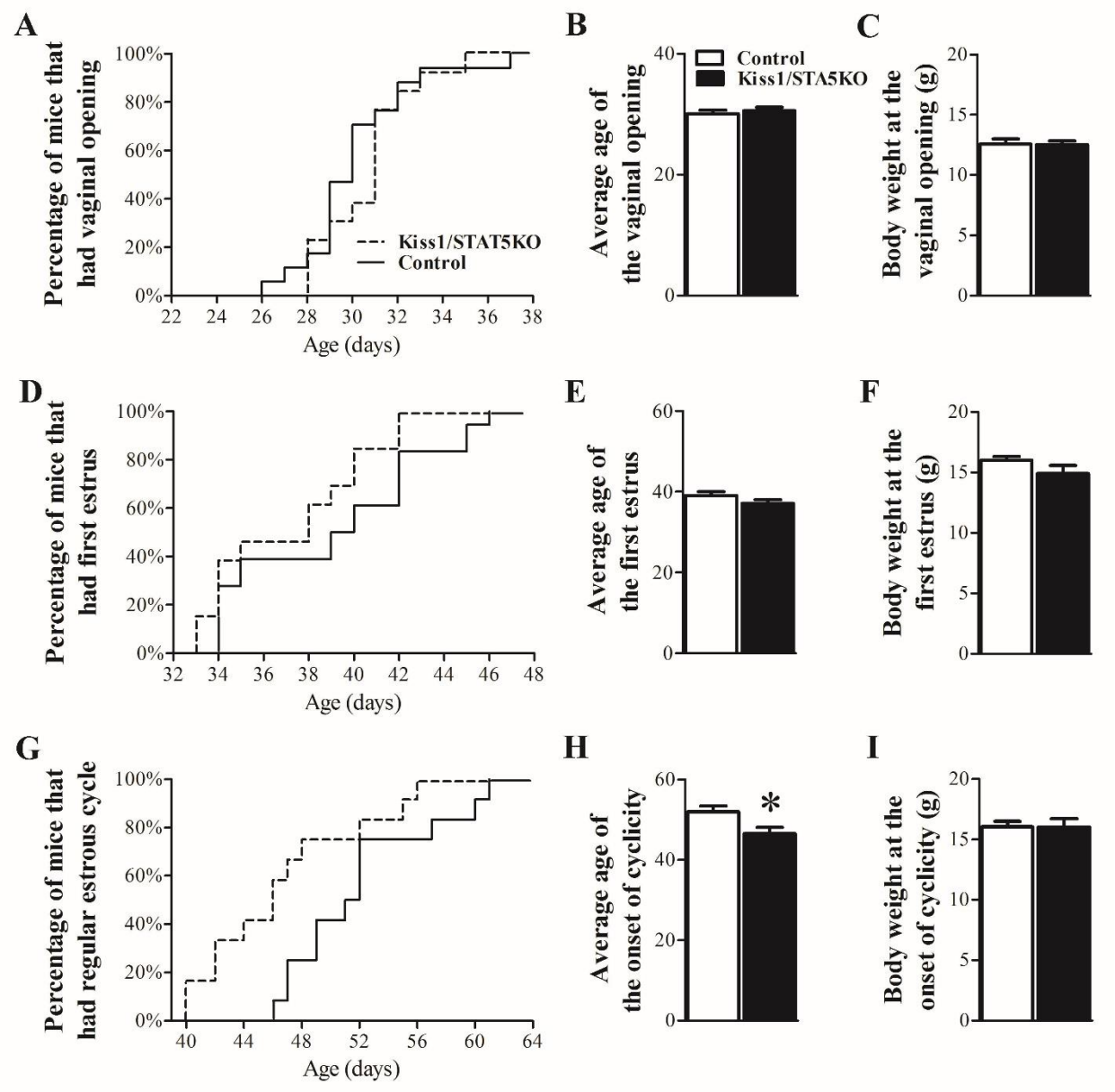

Figure 4 - *Lack of Stat5a/b expression in kisspeptin cells leads to an early onset of estrous cyclicity.* (A, D, G) Graphs showing the percentage of control (n = 17) and Kiss1/STAT5KO (n = 13) females that exhibited vaginal opening (A), first estrus (D) and the first occurrence of an estrous cycle of typical duration (G). (B, E, H) Average time required for control and Kiss1/STAT5KO females to exhibit vaginal opening (B), first estrus (E) and the onset of cyclicity (H). (C, F, I). Body weights at different stages of sexual maturation. *, significantly different from the control group ($P < 0.05$).

We further investigated whether conditional STAT5 deletion could influence fertility in adult female mice. We observed that the number of days until giving birth was similar between Kiss1/STAT5KO and control female mice (Figure 5A; $P = 0.3$). Additionally, the Kiss1/STAT5KO

and control dams produced similar numbers of pups per litter (Figure 5B; $P = 0.4$), and the Kiss1/STAT5KO dams showed no apparent problems in supporting their litters during lactation (data not shown). Similarly, when prolactin and serum LH levels were assessed in adult Kiss1/STAT5KO and control mice in diestrus, similar values were obtained between groups (Figure 5C, D). Thus, STAT5 signaling in kisspeptin cells regulates the timing of puberty in female mice, but it is not required for fertility in adulthood.

The conditional deletion was confirmed by assessing STAT5a and STAT5b mRNA levels in the AVPV and ARH punches of Kiss1/STAT5KO and control mice. The gene expression analysis demonstrated a significant reduction in the levels of STAT5a (40%, $P = 0.03$) and STAT5b mRNA (40%, $P = 0.02$) in the AVPV of Kiss1/STAT5KO female mice (Figure 5E). Additionally, a 30% non-significant reduction in STAT5a mRNA ($P = 0.3$) and a 40% reduction in STAT5b mRNA ($P = 0.01$) were also observed in the ARH of Kiss1/STAT5KO mice when compared to the control mice (Figure 5F).

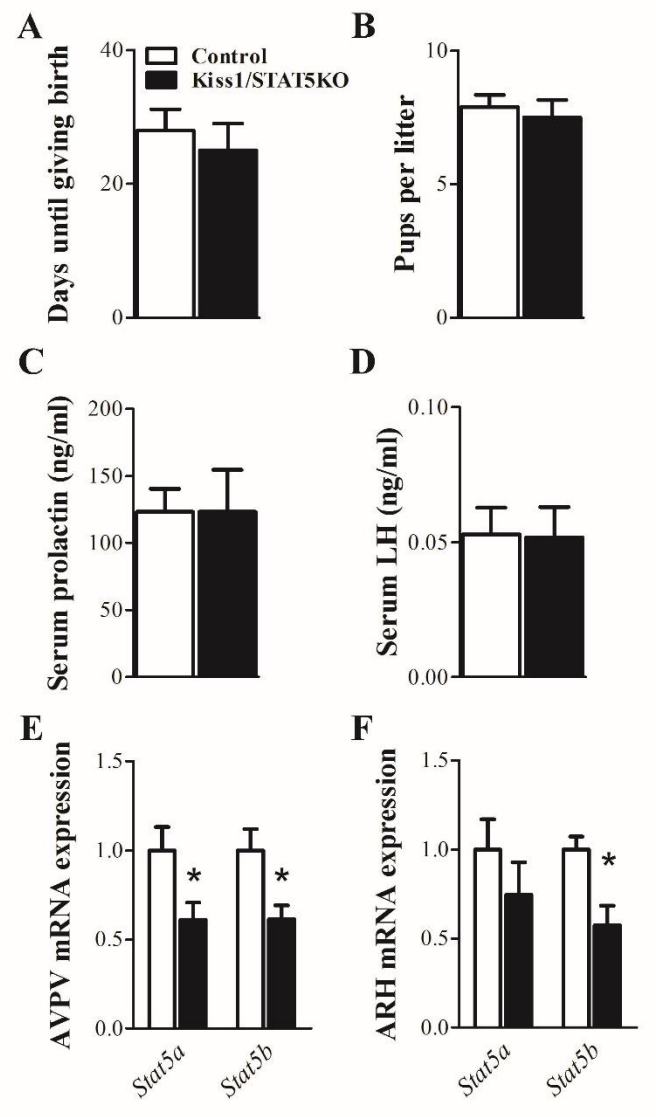

Figure 5 - *Fertility is preserved in Kiss1/STAT5KO female mice***.** (A, B) Comparison of days until giving birth and the number of pups generated per litter of control (n = 8 litters) and Kiss1/STAT5KO female mice (n = 7 litters). Adult Kiss1/STAT5KO mice showed no deficit of serum prolactin (C, n = 5-8/group) or LH levels (D, n = 6-7/group) compared to control female mice. (E, F) Bar graphs showing the expression of Stat5a and Stat5b mRNA in the anteroventral periventricular (AVPV) punches and in the arcuate nucleus (ARH) punches collected from control or Kiss1/STAT5KO female mice (n = 5-6/group). *, significantly different from the control group ($P < 0.05$).

2.4 DISCUSSION

In the present study, we investigated whether prolactin recruits fast, non-genomic signaling pathways to modulate the membrane excitability of kisspeptin neurons. Additionally, we evaluated the importance of STAT5 expression in kisspeptin neurons for the sexual maturation and fertility. We found that only a small percentage of AVPV kisspeptin neurons were excited by prolactin. Importantly, these effects required the PI3K signaling pathway but were blocked by TTX and synaptic blockers, indicating that prolactin relies on other neuronal populations to indirectly affect kisspeptin cells. Although the majority of ARH kisspeptin neurons expressed prolactin-induced pSTAT5, prolactin did not influence the membrane excitability of these cells. Notably, STAT5 signaling in kisspeptin neurons exerts a negative influence on the onset of estrous cyclicity because STAT5 ablation in kisspeptin neurons can accelerate this indicator of sexual maturation. However, STAT5 expression in kisspeptin neurons is not required for maintaining fertility in adulthood.

Several studies have highlighted the potential of prolactin to modulate the HPG axis. Prolactin suppresses the mRNA expression of GnRH and the release of GnRH, LH and FSH in both humans and animal models (ANDERSON et al., 2008; BOHNET et al., 1976; COHEN-BECKER et al., 1986; FOX et al., 1987; GRATTAN et al., 2007; MATSUZAKI et al., 1994; PARK et al., 1993; SELMANOFF et al., 1991; SONIGO et al., 2012). Despite the effects of prolactin on the GnRH system, only a small percentage of GnRH neurons express PRLR mRNA, prolactin-induced pSTAT5 or phosphorylated cAMP response element-binding protein (BROWN et al., 2012; GRATTAN et al., 2007). In addition, prolactin exerts no direct effect on the firing rate of GnRH neurons (BROWN et al., 2012). Therefore, prolactin likely acts through interneurons to modulate the HPG axis. Kisspeptin neurons have been suggested as potential candidates that could mediate the effects of prolactin on reproduction. Evidence for this possibility was provided by studies showing that most of kisspeptin neurons express PRLR mRNA (KOKAY et al., 2011; LI et al., 2011) and these receptors are functional, given that an acute prolactin stimulus induces pSTAT5 expression (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; SJOEHOLM et al., 2011). In female mice in diestrus, 60% of AVPV kisspeptin neurons are prolactin-responsive, while 30% of AVPV kisspeptin neurons express pSTAT5 in lactating female mice (BROWN et al., 2014). In the ARH, prolactin induces pSTAT5 in 70-80% of kisspeptin neurons of primiparous, ovariectomized or lactating rats (ARAUJO-LOPES et al., 2014; SJOEHOLM et al., 2011). Since in the present study prolactin induced pSTAT5 in

approximately 30% of kisspeptin neurons of the AVPV and in 70% of the ARH, our results are in accordance with the range found in the literature, especially when considering the multiple approaches used to identify kisspeptin neurons between studies, which may lead to significant differences.

Prolactin has been shown to differentially recruit transcriptional and/or electrophysiological responses in different populations of hypothalamic neurons to modulate several biological functions (BROWN et al., 2012; BUONFIGLIO et al., 2015; FREEMAN et al., 2000; LYONS et al., 2012; MOSS et al., 1985; ROMANÒ et al., 2013). For example, prolactin enhances the discharge and spike duration of TIDA neurons, so increased serum prolactin can promote dopamine release to limit its own secretion (LYONS et al., 2012; ROMANÒ et al., 2013). However, whole-body STAT5b knockout mice have greatly elevated serum prolactin concentrations, indicating that STAT5b is also required to mediate the negative feedback action of prolactin on TIDA neurons (GRATTAN et al., 2001). Prolactin also induces either STAT5 or fast-acting signaling pathways in preoptic area neurons, an important cell population that modulates maternal behaviors (BUONFIGLIO et al., 2015; DOBOLYI et al., 2014). However, unlike TIDA neurons, brain STAT5 signaling is not required for maternal care, which suggests that prolactin's effects on maternal behavior may be predominantly regulated by changes in neuronal membrane excitability (BUONFIGLIO et al., 2015). Our findings indicate that the cellular mechanisms recruited by prolactin to modulate kisspeptin neurons may be distinct from the pattern observed in either TIDA neurons or preoptic neurons. In kisspeptin neurons, prolactin acts predominantly via the STAT5 pathway, and no direct effect of prolactin on membrane excitability was found, although 20% of AVPV kisspeptin neurons were indirectly depolarized by prolactin. Notably, these indirect effects depend on the PI3K signaling pathway. However, although the PI3K inhibitors used in the present study are selective at the concentrations employed in the current study, they have also been shown to inhibit multiple protein kinases (CROSS et al., 1995; DAVIES et al., 2000; NAKANISHI et al., 1995). Therefore, we cannot discard the hypothesis that other signaling pathways may contribute to the observed effect of prolactin on AVPV kisspeptin neurons membrane excitability. Interestingly, the disruption of the PI3K pathway in kisspeptin neurons reduced the fertility of female mice, although no changes in pubertal development, LH levels, or estrous cyclicity were observed in these mutants (BEYMER et al., 2014). Therefore, although the PI3K signaling pathway may directly or indirectly regulate kisspeptin neurons, our findings indicate that prolactin relies largely on the transcription factor STAT5 to modulate kisspeptin neurons.

Nevertheless, STAT5 is recruited by different cytokines (GRIMLEY et al., 1999). Consequently, the observed effects on the timing of puberty of the Kiss1/STAT5KO mice may be mediated by the absence of signaling of other cytokines besides prolactin. In fact, the leptin receptor-induced activation of the STAT5 pathway controls estrous cycling (PATTERSON et al., 2012). However, leptin signaling in kisspeptin neurons arises only after pubertal development and leptin receptor inactivation in kisspeptin cells did not affect reproduction (CRAVO et al., 2013; DONATO; CRAVO; FRAZÃO; GAUTRON, et al., 2011). Additionally, STAT5 inactivation in leptin receptor-expressing cells did not affect the timing of puberty (SINGIREDDY et al., 2013). Therefore, future studies will be required to determine whether the effects caused by STAT5 deletion in kisspeptin neurons are specific for prolactin signaling or involve other cytokines.

Brown et al. (2012) demonstrated that only a small percentage (5%) of unidentified neurons located in the AVPV were excited in response to the acute administration of prolactin. The slightly higher responsiveness observed in our study (20%) was probably caused by the fact that we could specifically record from kisspeptin neurons. Additionally, since prolactin excitatory effect on AVPV kisspeptin neurons was completely blocked by co-treatment with TTX and synaptic blockers, these results suggest that prolactin's effects on AVPV kisspeptin neurons depend on another class of neurons that postsynaptically modulate the membrane excitability of AVPV kisspeptin neurons. Prolactin-responsive cells are observed in several nuclei of the preoptic region, including the AVPV itself and also the rostromedial preoptic area, the median preoptic nucleus and medial preoptic nucleus, as well as in other brain nuclei that send projections to kisspeptin neurons (BROWN et al., 2010; BUONFIGLIO et al., 2015; DONATO, CRAVO, FRAZÃO, GAUTRON, et al., 2011; FURIGO et al., 2014; NAGAISHI et al., 2014; SALAIS-LÓPEZ et al., 2017). Interestingly, approximately 85% of the cells that express PRLRs in the preoptic area exhibit markers of GABAergic neurons (KOKAY et al., 2011). The excitatory effects of prolactin on AVPV kisspeptin neurons contrast with the classical inhibitory tone of the GABAergic transmission. Previous studies have shown that GABA hyperpolarizes AVPV kisspeptin neurons (DEFAZIO et al., 2014) and selective activation of $GABA_A$ receptors decreases kisspeptin-induced gonadotropin secretion (GARCÍA-GALIANO et al., 2012). Accordingly, $GABA_A$ receptor antagonist stimulates kisspeptin release in the medial basal hypothalamus of prepubertal monkeys (KURIAN et al., 2012). However, the GABA effect on kisspeptin neurons may vary depending on the sexual maturation stage, the time of day or estradiol availability (DEFAZIO et al., 2014; KURIAN et al., 2012). Therefore, the identity

and physiological functions of this particular population of prolactin-responsive neurons that activates AVPV kisspeptin neurons needs to be further determined.

Prolactin induces dopamine secretion in brain slices of female mice in diestrus (ROMANÒ et al., 2013). Therefore, we cannot discard the hypothesis that prolactin-induced dopamine release may modulate or block a possible effect of this hormone on the membrane excitability of kisspeptin neurons. ARH kisspeptin neurons, at least in ewes, express the D2 dopamine receptor and may thus be dopamine-responsive (GOODMAN et al., 2012). Additionally, kisspeptins act as a component of the feedback circuit that stimulates prolactin secretion through the inhibition of TIDA neurons in an estrogen-dependent manner (RIBEIRO et al., 2015). During lactation, dopamine secretion is suppressed to allow physiological hyperprolactinemia (ROMANÒ et al., 2013). Therefore, in the future, it will be interesting to investigate the effects of dopamine on the activity of kisspeptin neurons and whether the lack of dopamine would be a permissive factor for prolactin to act on the membrane excitability of kisspeptin neurons.

Prolactin seems to exert an inhibitory effect on the kisspeptin system because Kiss1 mRNA expression is suppressed by physiological (during lactation) or pharmacological hyperprolactinemia, which, in turn, suppresses LH secretion (ARAUJO-LOPES et al., 2014; BROWN et al., 2014; LADYMAN; WOODSIDE, 2014; SONIGO et al., 2012; TRUE et al., 2011; YAMADA et al., 2007). In accordance with the inhibitory role played by prolactin on reproduction, Kiss1-specific STAT5 knockout mice exhibited an early onset of estrous cyclicity. Body weight is an important factor that can interfere with the timing of sexual maturation in both humans and animal models (BOHLEN et al., 2016; DUNGER et al., 2006; FRISCH; MCARTHUR, 1974; KAPLOWITZ et al., 2001; MARTOS-MORENO et al., 2010; ROA; TENA-SEMPERE, 2014). Since no differences in body weight or leptin levels were noted during the development of Kiss1/STAT5KO mice, the early onset of estrous cyclicity seems to be an effect caused by conditional STAT5 deletion. As previously reported (NELSON et al., 1990), genetic determinants of onset of cyclicity may differ from those of vaginal opening and first cornification. Vaginal opening and first estrus are dependent on an increase in plasma estradiol, while estrous cyclicity is dependent on a preovulatory increase in plasma estradiol, as well as on the ability of the HPG axis to respond to the LH surge and induce ovulation (NELSON et al., 1990). Therefore, it is plausible to think that kisspeptin-conditional STAT5 deletion influences the onset of estrous cyclicity but not necessarily other events during puberty. Since Kiss1

expression increases in the AVPV, but not in the ARH, during pubertal transition (CLARKSON; HERBISON, 2006; NAVARRO et al., 2012), we can speculate that the lack of STAT5 in AVPV kisspeptin neurons during pubertal transition would be a permissive factor for the observed effects inducing an early onset of estrous cyclicity. Additionally, because the mutant mice are fertile, we cannot exclude the hypothesis that compensatory mechanisms during development may account for the lack of a reproductive phenotype in adult Kiss1/STAT5KO mice. The significant reduction in STAT5 mRNA observed in the microdissected AVPV and ARH areas indicates a significant recombination in the expected brain areas. Because the microdissected areas contain several other classes of neurons that express STAT5, aside from kisspeptin cells (e.g., dopaminergic, leptin receptor-expressing and GABAergic neurons) (BROWN et al., 2016; BUONFIGLIO et al., 2015; FURIGO et al., 2014; NAGAISHI et al., 2014), some mRNA expression was expected in mutant mice.

Additionally, STAT5 is essential for the development of functional corpora lutea in the rodent ovary, as demonstrated by the infertility displayed by prolactin, PRLR or whole-body STAT5a/b knockout mice (HORSEMAN et al., 1997; ORMANDY et al., 1997; TEGLUND et al., 1998). Ovarian cells express the Kiss1 gene, and kisspeptin immunoreactivity has been identified in various cell types, including the corpora lutea (CASTELLANO et al., 2006; MERHI et al., 2016). Therefore, we cannot rule out the hypothesis that the anticipation of the onset of estrous cyclicity observed in Kiss1/STAT5KO mice involves direct effects on the ovaries. However, whether kisspeptin cells in the ovaries co-express PRLRs requires further investigation. Finally, similar to brain-specific STAT5a/b knockout mice, STAT5 kisspeptin-conditional deletion did not disrupt the fertility of adult mice (BUONFIGLIO et al., 2015; LEE et al., 2008).

5 CONCLUSIONS

The HGP axis is the key regulator of reproduction. The understanding of the activity and biophysical properties of the neurons involved in this neuronal pathway is extremely important. This study contributed with new insights about the field; otherwise, future researches are necessary to increase our knowledge about the neuronal control of reproduction.

About the neurobiological characteristics induced during the positive feedback in the OVX+E mice model compared to the natural cycle, our data suggested that OVX+E model recapitulates many aspects of the proestrous surge to increase GnRH activity. The lower LH amplitude observed in the OVX+E could be due to differences in the burst patterns and a lower pituitary response. The similarity between OVX+E and proestrous to increase GnRH activity, confirm the importance of this model to recapitulated most of the positive feedback, triggering the LH surge.

Regarding main effects of prolactin on kisspeptin neurons, our findings suggest that prolactin relies predominantly on STAT5, rather than fast acting signaling pathways, to directly modulate kisspeptin neurons. Additionally, we demonstrated that STAT5 signaling in kisspeptin cells controls the timing of puberty, although it is not required to maintain fertility in adulthood. Thus, the second part of our study brings suggestions about the importance of cytokines in the modulation of the HPG axis and open the door to further studies to be carried out in this field.

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ATTACHMENTS

A – SILVEIRA, M. A. STAT5 signaling in kisspeptina cells regulates the timing of puberty. **Mol Cell Endocrinol**.,2017. In press.

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STAT5 signaling in kisspeptin cells regulates the timing of puberty

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ABSTRACT

Previous studies have shown that kisspeptin neurons are important mediators of prolactin's effects on reproduction. However, the cellular mechanisms recruited by prolactin to affect kisspeptin neurons remain unknown. Using whole-cell patch-clamp recordings of brain slices from kisspeptin reporter mice, we observed that 20% of kisspeptin neurons in the anteroventral periventricular nucleus was indirectly depolarized by prolactin via an unknown population of prolactin responsive neurons. This effect required the phosphatidylinositol 3-kinase signaling pathway. No effects on the activity of arcuate kisspeptin neurons were observed, despite a high percentage (70%) of arcuate neurons expressing prolactin-induced STAT5 phosphorylation. To determine whether STAT5 expression in kisspeptin cells regulates reproduction, mice carrying *Stat5a/b* inactivation specifically in kisspeptin cells were generated. These mutants exhibited an early onset of estrous cyclicity, indicating that STAT5 transcription factors exert an inhibitory effect on the timing of puberty.

B - SILVEIRA M. A., et al., GnRH neuron activity and pituitary response in estradiol-induced vs proestrous luteinizing hormone surges in female mice. **Endocrinology**, v.158, p. 356-366., 2017.

RESEARCH ARTICLE

GnRH Neuron Activity and Pituitary Response in Estradiol-Induced vs Proestrous Luteinizing Hormone Surges in Female Mice

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During the female reproductive cycle, estradiol exerts negative and positive feedback at both the central level to alter gonadotropin-releasing hormone (GnRH) release and at the pituitary to affect response to GnRH. Many studies of the neurobiologic mechanisms underlying estradiol feedback have been done on ovariectomized, estradiol-replaced (OVX+E) mice. In this model, GnRH neuron activity depends on estradiol and time of day, increasing in estradiol-treated mice in the late afternoon, coincident with a daily luteinizing hormone (LH) surge. Amplitude of this surge appears lower than in proestrous mice, perhaps because other ovarian factors are not replaced. We hypothesized GnRH neuron activity is greater during the proestrous-preovulatory surge than the estradiol-induced surge. GnRH neuron activity was monitored by extracellular recordings from fluorescently tagged GnRH neurons in brain slices in the late afternoon from diestrous, proestrous, and OVX+E mice. Mean GnRH neuron firing rate was low on diestrus; firing rate was similarly increased in proestrous and OVX+E mice. Bursts of action potentials have been associated with hormone release in neuroendocrine systems. Examination of the patterning of action potentials revealed a shift toward longer burst duration in proestrous mice, whereas intervals between spikes were shorter in OVX+E mice. LH response to an early afternoon injection of GnRH was greater in proestrous than diestrous or OVX+E mice. These observations suggest the lower LH surge amplitude observed in the OVX+E model is likely not attributable to altered mean GnRH neuron activity, but because of reduced pituitary sensitivity, subtle shifts in action potential pattern, and/or excitation-secretion coupling in GnRH neurons. (***Endocrinology*** **158: 356–366, 2017)**

C - PEDROSO, J. A. B., Changes in Leptin Signaling by SOCS3 modulate fasting-induced hyperphagia and weight regain in mice. **Endocrinology**. v. 157. P. 3901 – 3914., 2016.

Changes in Leptin Signaling by SOCS3 Modulate Fasting-Induced Hyperphagia and Weight Regain in Mice

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Weight regain frequently follows interventions that reduce body weight, leading to a failure in long-term obesity treatment. Inhibitory proteins of the leptin signaling pathway, such as the suppressor of cytokine signaling 3 (SOCS3), have been studied in conditions that predispose animals to obesity. However, whether SOCS3 modulates postrestriction hyperphagia and weight regain remains unknown. Mice lacking SOCS3 protein specifically in leptin receptor (LepR)-expressing cells (LepR SOCS3 knockout [KO]) were generated and studied in fasting and refeeding conditions. LepR SOCS3 KO mice exhibited increased leptin sensitivity in the hypothalamus. Notably, LepR SOCS3 KO males and females showed attenuated food intake and weight regain after 48 hours of fasting. Postrestriction hyperleptinemia was also prevented in LepR SOCS3 KO mice. Next, we studied possible mechanisms and neural circuits involved in the SOCS3 effects. SOCS3 deletion did not prevent fasting- or refeeding-induced c-Fos expression in the arcuate nucleus of the hypothalamus (ARH) nor fasting-induced increased excitability of ARH LepR-expressing cells. On the other hand, SOCS3 ablation reduced the mRNA levels of hypothalamic orexigenic neuropeptides during fasting (neuropeptide Y, agouti-related protein, orexin, and melanin-concentrating hormone). In summary, our findings suggest that increased leptin sensitivity contributes to the maintenance of a reduced body weight after food deprivation. In addition, the attenuated postrestriction food intake observed in mutant mice was not explained by fasting-induced changes in the activity of ARH neurons but exclusively by a lower transcription of orexigenic neuropeptides during fasting. These results indicate a partial dissociation between the regulation of neuronal activity and gene expression in ARH LepR-expressing cells. (***Endocrinology*** **157: 3901–3914, 2016)**

D – BOHLEN, T. M., Fatness rather than leptin sensitivity determines the timing of puberty in female mice. **Mol Cell Endocrinol**. v. 423, p. 11-21, 2016.

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Fatness rather than leptin sensitivity determines the timing of puberty in female mice

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ABSTRACT

Leptin is a permissive factor for the onset of puberty. However, changes in adiposity frequently influence leptin sensitivity. Thus, the objective of the present study was to investigate how changes in body weight, fatness, leptin levels and leptin sensitivity interact to control the timing of puberty in female mice. Pre-pubertal obesity, induced by raising C57BL/6 mice in small litters, led to an early puberty onset. Inactivation of *Socs3* gene in the brain or exclusively in leptin receptor-expressing cells reduced the body weight and leptin levels at pubertal onset, and increased leptin sensitivity. Notably, these female mice exhibited significant delays in vaginal opening, first estrus and onset of estrus cyclicity. In conclusion, our findings suggest that increased leptin sensitivity did not play an important role in favoring pubertal onset in female mice. Rather, changes in pubertal body weight, fatness and/or leptin levels were more important in influencing the timing of puberty.

E- BUONFIGLIO, D. C. et al. Neuronal STAT5 signaling is required for maintaining lactation but not for postpartum maternal behaviors in mice. **Horm Behav.,** v. 71, p. 60-68, 2015.

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Neuronal STAT5 signaling is required for maintaining lactation but not for postpartum maternal behaviors in mice

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ABSTRACT

Prolactin and placental lactogens control mammary development and lactation as well as play an important role in maternal behaviors. However, the molecular mechanisms in the brain responsible for this regulation remain largely unknown. Therefore, the present study investigated whether Signal Transducer and Activator of Transcription 5 (STAT5) signaling in the brain, the key transcriptional factor recruited by prolactin receptor and other hormones, is required for postpartum maternal behavior, maintenance of lactation and offspring growth. Neuronal ablation of STAT5 impaired the control of prolactin secretion and reduced the hypothalamic expression of suppressors of cytokine signaling (i.e., SOCS3 and CISH). In addition, neuronal STAT5 deletion attenuated the hyperphagia commonly observed during lactation by decreasing the hypothalamic expression of orexigenic neurotransmitters such as the neuropeptide Y and agouti-related protein. The lower food intake of lactating neuron-specific STAT5 knockout females resulted in reduced milk production and offspring growth. Unexpectedly, postpartum maternal behavior expression was not impaired in neuron-specific STAT5 knockout females. On the contrary, the latency to retrieve and group the pups into the nest was reduced in mutant dams. Finally, we demonstrated that approximately 30% of recorded neurons in the medial preoptic area were acutely depolarized by prolactin suggesting that fast STAT5-independent signaling pathways may be involved in the regulation of maternal behaviors. Overall, our results revealed important information about the molecular mechanisms recruited by hormones to orchestrate the activation of neural circuitries engaged in the induction of maternal care.

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Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation

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Abstract

Alzheimer's disease (AD) is associated with peripheral metabolic disorders. Clinical/epidemiological data indicate increased risk of diabetes in AD patients. Here, we show that intracerebroventricular infusion of AD-associated Aβ oligomers (AβOs) in mice triggered peripheral glucose intolerance, a phenomenon further verified in two transgenic mouse models of AD. Systemically injected AβOs failed to induce glucose intolerance, suggesting AβOs target brain regions involved in peripheral metabolic control. Accordingly, we show that AβOs affected hypothalamic neurons in culture, inducing eukaryotic translation initiation factor 2α phosphorylation (eIF2α-P). AβOs further induced eIF2α-P and activated pro-inflammatory IKKβ/NF-κB signaling in the hypothalamus of mice and macaques. AβOs failed to trigger peripheral glucose intolerance in tumor necrosis factor-α (TNF-α) receptor 1 knockout mice. Pharmacological inhibition of brain inflammation and endoplasmic reticulum stress prevented glucose intolerance in mice, indicating that AβOs act via a central route to affect peripheral glucose homeostasis. While the hypothalamus has been largely ignored in the AD field, our findings indicate that AβOs affect this brain region and reveal novel shared molecular mechanisms between hypothalamic dysfunction in metabolic disorders and AD.

Keywords Alzheimer's disease; ER stress; hypothalamus; inflammation; insulin resistance

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Introduction

Increasing evidence suggests an association between metabolic disorders, notably type 2 diabetes (T2D), and Alzheimer's disease (AD) (Craft, 2007; De Felice, 2013). Clinical and epidemiological studies indicate that diabetic patients have increased risk of developing AD (Ott *et al*, 1999; Sims-Robinson *et al*, 2010; Wang *et al*, 2012) and AD brains exhibit defective insulin signaling (Moloney *et al*, 2010; Bomfim *et al*, 2012; Craft, 2012; Talbot *et al*, 2012). Recent studies have shown that soluble amyloid-β peptide oligomers (AβOs), toxins that build up in AD brains and have been proposed to be major players in synapse failure in AD (reviewed in Ferreira & Klein, 2011; Selkoe, 2011; Mucke & Selkoe, 2012), are linked to impaired hippocampal insulin signaling. AβOs were found to cause internalization and cellular redistribution of insulin receptors, to block downstream hippocampal insulin signaling (De Felice *et al*, 2009; Ma *et al*, 2009; Bomfim *et al*, 2012) and to cause hippocampal endoplasmic reticulum (ER) stress (Lourenco *et al*, 2013), establishing molecular parallels between AD and T2D. Hyperinsulinemic/hyperglycemic individuals and mice show increased plasma and brain levels of Aβ (Ho *et al*, 2004; Takeda *et al*, 2010; Zhang *et al*, 2012), suggesting that altered peripheral metabolic homeostasis