Dissecting the ontogeny and functional relevance of altered GABAergic circuitry in polycystic ovary syndrome (PCOS)

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Abstract

Polycystic ovary syndrome (PCOS) is the most common cause of female infertility worldwide, yet this prevalent endocrine disorder remains poorly understood. Although classically considered an ovarian disease, altered brain wiring may play a central role in the pathogenesis of PCOS. Neuroendocrine derangements in PCOS include elevated luteinizing hormone (LH) pulse secretion, which likely results from high gonadotropin-releasing hormone (GnRH) pulse frequency and mirrors defects within the GnRH neuronal network. Enhanced GABA actions in GnRH neurons have been proposed to be a culprit of altered GnRH/LH secretion and underlie some of the pathological features of the disorder. Pre-clinical and clinical evidence support the idea that prenatal androgen excess may program an abnormal GABA-to-GnRH neuron circuit to develop PCOS during adult life. In this regard, the present study aimed to answer fundamental questions about the ontogeny, rescue and biological function of altered GABAergic innervation to GnRH neurons.

This PhD project used a prenatally androgenized (PNA) mouse model that recapitulates the cardinal features of PCOS and mimics the elevated LH pulse frequency of the disease. Initial studies aimed to address whether GnRH neuronal network changes observed in PNA mice were programmed through early androgen exposure or driven by adult androgen excess. Control and PNA GnRH-GFP transgenic mice were evaluated at postnatal day (PND) 25, prior to pubertal onset. Confocal imaging and analysis of GABA inputs onto GnRH neurons revealed that GABAergic contact was significantly increased in prepubertal PNA mice ($P < 0.05$). In addition, circulating testosterone levels at PND 25 were not different between PNA and control groups, suggesting that brain circuit abnormalities were not dependent upon early manifestation of androgen excess in a PCOS-like condition.
Serial blood sampling defined the developmental timing of androgen excess in PNA mice, showing that circulating testosterone levels rise significantly during early adulthood (PND 50 and PND 60) in PNA animals when compared to controls ($P < 0.01$). All hyperandrogenic PNA mice presented disruption of estrous cyclicity, displaying significant arrest in the metestrous stage and a complete absence of the proestrous stage ($P < 0.0001$), indicating anovulatory cycles. To determine whether neuroendocrine derangements of LH regulation would persist after the removal of hyperandrogenic ovaries, the same cohort of mice were ovariectomized (OVX) and serial blood sampling was performed to investigate LH pulse dynamics. Although LH pulse frequency was similar between the groups, OVX PNA mice exhibited greater LH pulse amplitude ($P < 0.0001$) and magnitude of LH release than controls ($P < 0.001$), implying that defects within the GnRH network remained in the absence of hyperandrogenic ovaries and suggesting that the primary pathology of PCOS is in the brain.

Compelling clinical evidence indicates that long-term androgen receptor (AR) blockade with flutamide (Flut) is able to restore both the sensitivity of the GnRH pulse generator and menstrual cyclicity in PCOS women. This PhD project tested the hypothesis that these improvements may be the result from plastic changes in the brain that rescue normal GABAergic wiring to GnRH neurons. Control and PNA mice were treated with Flut (25 mg/kg/day) or an oil vehicle from PND 40 to PND 60. GABA inputs to GnRH neurons were assessed as previously performed for prepubertal animals and confirmed that oil-treated adult PNA mice display enhanced GABAergic contact on GnRH neurons ($P < 0.01$). Remarkably, Flut treatment was able to decrease and rescue normal GABA-to-GnRH neuron circuit features in PNA mice ($F_{1,21}^{treatment} = 41.8; P < 0.0001$). Results also showed that estrous cyclicity of PNA mice improved considerably during Flut treatment. Evaluation of ovarian morphology treatment showed that AR signaling blockade
improved preovulatory follicle recruitment and restored normal features of the granulosa and theca cell layers in these follicles.

Previous neuroanatomical work indicates that increased GABAergic wiring to GnRH neurons in PNA mice originates largely from the arcuate nucleus (ARN) of the hypothalamus. In this PhD project, I investigated the functional role of GABA neurons originating in the ARN in regulating LH secretion using in vivo optogenetics. Selective targeting and expression of channelrhodopsin-2 E123T accelerated variant (ChETA) in the ARN GABA neurons was achieved using vesicular GABA transporter (VGAT)-Cre mice. ARN GABA neurons were activated by delivering blue light pulses (5ms) at 2 and 20 Hz during 10 minutes in diestrus female, male and PNA mice. Optogenetic activation at 20 Hz elicited robust LH release similarly in male and diestrus female ($P < 0.05$), whereas 2-Hz stimulation failed to evoke changes in LH levels. Interestingly, 20-Hz light stimulation in PNA mice induced smaller changes in LH levels when compared to male and diestrus female groups ($P < 0.05$). These data suggest that altered LH release in PNA mice might reflect a decreased pituitary LH releasable pool due to a formerly high GnRH pulse frequency stimulation in the PCOS-like condition.

Together, these findings support the idea that a prenatal androgen insult can program altered GABAergic brain circuits early in development, prior to pubertal onset, and might be the culprit for developing subsequent androgen excess during early adulthood. This PhD thesis also highlights that abnormal GABA-to-GnRH neuron circuit remains plastic in adult PNA mice; and that the specific GABAergic pathway from ARN GABA neuron is biologically relevant to modulate LH secretion. These data support the important role of ARN GABA neurons in the regulation of the GnRH neuron biology in healthy fertility and in the pathophysiology of PCOS.
Acknowledgements

Stay prepared to work hard, accept constructive criticism, be willing to take continuous failures and tell the world about your passion for science. These were a few of the things colleagues told me that I should consider getting through a PhD. However, they forgot to mention that the people around us are essential for the completion of this daring journey. Foremost, I would like to express immense gratitude to my supervisor and friend Dr Rebecca Campbell. Her great help and guidance predated my arrival to the laboratory, since I was hesitant about leaving everything behind to pursue a dream. I am profoundly grateful for her encouragement, patience, support and motivation for science! I hope that this journey of sharing knowledge, coffee and laughter will continue after this PhD.

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To all of you,

My sincere gratitude.
Dedication

In memory of my beloved father who told me I could beat the odds of our reality, travel the world, encounter new cultures and keep my love for biology.

Every new step that I take, you will never be forgotten.
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<tbody>
<tr>
<td>3V</td>
<td>Third Ventricle of the brain</td>
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<tr>
<td>3β-diol</td>
<td>3β-Androstanediol (a.k.a. 5α-androstane-3β,17β-diol)</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-Associated Virus</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related Peptide</td>
</tr>
<tr>
<td>AHA</td>
<td>Anterior Hypothalamic Area</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARN</td>
<td>Arcuate Nucleus</td>
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<tr>
<td>AVPV</td>
<td>Anteroventral Periventricular nucleus</td>
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<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAH</td>
<td>Congenital Adrenal Hyperplasia</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>ChETA</td>
<td>Channelrhodopsin-2 E123T Accelerated variant</td>
</tr>
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<td>Channelrhodopsin-2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>Cl⁻ᵢ</td>
<td>Intracellular chloride ion</td>
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<tr>
<td>CREB</td>
<td>cAMP Response Element-Binding protein</td>
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<td>DAT</td>
<td>Dopamine Transporter</td>
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<td>EGFR</td>
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<td>ERBB</td>
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<td>FSH-R</td>
<td>Follicle-Stimulating Hormone-Receptor</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>GIRQ</td>
<td>G Protein activated Inwardly Potassium (K\textsuperscript{+}) Channels</td>
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<td>GnRH</td>
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<tr>
<td>LHCGR</td>
<td>Luteinizing Hormone/Choriogonadotropin Receptor</td>
</tr>
<tr>
<td>MBH</td>
<td>Mediobasal Hypothalamus</td>
</tr>
<tr>
<td>ME</td>
<td>Median Eminence</td>
</tr>
<tr>
<td>mPOA</td>
<td>medial Preoptic Area</td>
</tr>
<tr>
<td>mPSCs</td>
<td>miniature Postsynaptic Currents</td>
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<tr>
<td>MS</td>
<td>Medial Septum</td>
</tr>
<tr>
<td>MUA</td>
<td>Multiunit Activity</td>
</tr>
<tr>
<td>NK3</td>
<td>Neurokinin Receptor 3 (a.k.a. Tachykinin receptor 3)</td>
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<tr>
<td>NKB</td>
<td>Neurokinin B</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
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<tr>
<td>OVLT</td>
<td>Organum vasculosum of the lamina terminalis</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
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<tr>
<td>PgRMC1</td>
<td>Progesterone Receptor Membrane Component 1</td>
</tr>
</tbody>
</table>
PNA  Prenatally Androgenized mice
PND  Postnatal Day
PPA  Postpubertally Androgenized mice
PR   Progesterone Receptor
PRKO Progesterone Receptor Knockout
PRV  Pseudorabies Virus
PSCs Postsynaptic Currents
rPOA rostral Preoptic Area
RT-PCR Reverse Transcription-Polymerase Chain Reaction
TH  Tyrosine Hydroxylase
THADA Thyroid Adenoma-Associated protein
VGAT Vesicular GABA Transporter
VNO  Vomeronasal Organ
YAP1 Yes-Associated Protein 1
Chapter One:

Literature Review
1.1 Introduction

Polycystic ovary syndrome (PCOS) is the main endocrine disorder leading to female infertility, affecting 5 to 20% of women in the reproductive age in New Zealand and around the world (Farquhar et al., 1994; Lizneva, Suturina, et al., 2016). Although PCOS is a major concern for health care and fertility management, critical knowledge of its etiology and pathophysiological mechanisms remain unclear. Three cardinal features characterize PCOS: polycystic ovaries, menstrual irregularities and excess androgen production known as hyperandrogenemia (HA) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Other comorbidities such as a metabolic syndrome including diabetes mellitus type 2, and depression are also associated with PCOS (Milsom et al., 2013; Azziz et al., 2016). The heterogeneity of disease presentation has hindered better understanding of PCOS pathological mechanisms and the advance of new therapeutic interventions.

Despite great efforts of clinical and basic research, the definitive origins of PCOS are still obscure making it target of treatments mainly based on the postpubertal manifestation of the disease. Genetic, epigenetic and environmental factors are proposed to trigger and be engaged in the broad-spectrum of the disorder. Among these factors, elevated maternal testosterone levels are highly associated with the development of PCOS in daughters (Sir-Petermann et al., 2009), implying that prenatal androgen excess may induce a biological program for the adult manifestation of the disease. Although PCOS has been classically considered an ovarian disorder (Hughesdon, 1982; Dumesic and Richards, 2013), increasing evidence has shown that disturbances in brain circuits controlling gonadotropin-releasing hormone (GnRH) neurons underlie the pathogenesis of PCOS.
Neuroendocrine impairments leading to abnormal luteinizing hormone (LH) secretion are common in women with PCOS. In particular, high LH pulse frequency secretion is a hallmark for the majority of PCOS patients (Pastor et al., 1998). This is likely to reflect altered gonadal steroid hormone-mediated negative feedback mechanisms within the GnRH neuronal network, which is composed by neuronal afferents that regulate the GnRH neuron activity (Chhabra et al., 2005; Blank, McCartney and Marshall, 2006). The components of the GnRH neuronal network which guarantee proper GnRH neuron activity are diverse and not entirely defined (Herbison, 2014).

Among steroid-sensitive afferent neurons in this brain network, gamma-aminobutyric acid (GABA) neurons are recognized to be crucial players for the regulation of GnRH neuron activity. GABAergic transmission and innervation to GnRH neurons has been found to be increased in a prenatally androgenized (PNA) mouse model of PCOS which exhibits the cardinal features of the disease (Sullivan and Moenter, 2004; Moore et al., 2015). Recently, our research group discovered that GABA neurons residing in the arcuate nucleus (ARN) of the hypothalamus provide enhanced innervation to GnRH neurons in PNA mice associated with neuroendocrine and reproductive impairments (Moore et al., 2015). Therefore, this novel neuronal pathway might be biologically relevant for the control of GnRH/LH secretion. Yet it remains to be determined whether ARN GABA neurons have a functional role in normal and PCOS-like condition.

This introduction will first review the literature regarding the general features and pathophysiological mechanisms of PCOS. It will also highlight evidence from clinical and PCOS animal models that attempt to understand the role of prenatal androgen exposure as a possible mechanism driving the development of the disease. In particular, this introduction will review both classical and more recent findings focusing on the impact of prenatal androgens in programing the GnRH neuronal network for the manifestation of PCOS during adult life. An overview of the hypothalamic-pituitary-gonadal (HPG) axis underpinning the functional aspects of GnRH neurons
and GnRH/LH pulsatile secretion is also discussed in this section. Finally, this introduction reviews key aspects of GABA actions in GnRH neurons and discuss the idea that abnormal GABAergic inputs to GnRH neurons may underlie the development and pathogenesis of PCOS.
1.2 Polycystic Ovary Syndrome (PCOS)

1.2.1 PCOS: diagnosis & pathophysiology

The original description from Stein and Leventhal’s work described PCOS as an association among ovarian cysts, amenorrhea, hirsutism and obesity (Stein and Leventhal, 1935). However, it was not until 1990 that an official definition of PCOS was firstly established during a sponsored workshop by the United States of America (USA) National Institutes of Health (NIH) (Zawadzki and Dunaif, 1992). From that meeting, PCOS was defined as the clinical and/or biochemical identification of hyperandrogenemia (HA) with chronic oligo-anovulation. A second and broader criteria emerged after a meeting in 2003 sponsored by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) held in Rotterdam, Netherlands. As a consensus, the Rotterdam criteria stated that PCOS should be diagnosed upon the presence of two of the three symptoms: menstrual irregularities, HA and polycystic ovaries after ultrasonography report confirmation (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004).

In accordance with the Rotterdam criteria four possible PCOS phenotypes can be identified from the combination of the three main symptoms (Lizneva, Suturina, et al., 2016) (Table 1.1). This heterogeneity is suggestive of different etiological backgrounds yet the mechanisms underlying the PCOS pathogenesis remain unclear. Although this phenotypic approach was established in order to facilitate the identification of PCOS in clinical practice, different research groups have reinstated PCOS as a primarily hyperandrogenic disorder (Azziz et al., 2009). Therefore, the identification of polycystic ovaries with chronic oligo-ovulation (phenotype D in Table 1.1) would not be defined as PCOS according to the diagnostic criteria of the Androgen
Excess PCOS Society 2006 meeting (Azziz et al., 2006). In addition, PCOS is commonly associated with metabolic syndrome, cardiovascular diseases, cancer of the reproductive tract and psychosocial diseases such as depression (for a more detailed review, see Dumesic et al., 2015), making this disorder a major concern in health care management. This section covers the diagnosis and pathophysiological mechanisms of PCOS cardinal features.

<table>
<thead>
<tr>
<th>Features</th>
<th>Phenotype A</th>
<th>Phenotype B</th>
<th>Phenotype C</th>
<th>Phenotype D</th>
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<tr>
<td>HA</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Polycystic ovaries</td>
<td>X</td>
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<td>Ovulatory dysfunction</td>
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**Table 1.1. Defining the possible phenotypic presentations of PCOS.** Current diagnostic criteria for PCOS may lead to confusion during clinical assessment due to the heterogeneous manifestation of this disease. Lizneva and colleagues proposed a four-phenotype classification of PCOS, which can be used during clinical practice and when investigating the pathophysiological mechanisms of this disorder. HA = hyperandrogenemia. (Adapted from Lizneva et al.2016).

1.2.1.1 *Androgen excess in PCOS*

Among the three cardinal features of PCOS, androgen excess is a hallmark for the disease and it is considered to be the culprit for menstrual irregularities and associated metabolic traits (Azziz et al., 2009). Studies have shown that around 60% of women with PCOS display clinical evidence of HA, which includes hirsutism, acne and androgenic alopecia; and around 60-80% display the biochemical confirmation of increased androgen plasma levels (Conway, Honour and Jacobs, 1989; Chang et al., 2005). Clinical assessment and grading of hirsutism usually applies the Ferriman-Gallwey scoring screening, which evaluates the pattern of body hair growth in post-pubertal women (Ferriman and Gallwey, 1961; Azziz et al., 2009). The measurement of total
testosterone levels and the exclusion of other possible causes of androgen excess such as androgen-secreting adrenal tumors and congenital adrenal hyperplasia (CAH) are recommended during clinical screening (Balen et al., 2016). The careful assessment of dermatological signs of hyperandrogenism should be considered as it might be dependent on ethnicity or as secondary to increased body weight (Williamson et al., 2001; Ollila et al., 2017). According to clinical reports, the calculation of bioavailable/free testosterone should be taken as the primary diagnostic tool during clinical assessment and be sufficient to diagnose hyperandrogenism (Balen et al., 2016).

Hirsutism in adolescent girls may be an early sign of androgen excess and for the manifestation of PCOS during reproductive age. It may indicate that androgen actions in the HPG axis might trigger and perpetuate the reproductive impairment of the syndrome later in life. The Ferriman-Gallwey score system might not be the best tool to identify hirsute girls during early menarche as it mainly considers subjects above 18 years old of age (Ferriman and Gallwey, 1961; Ibáñez et al., 2017). Alternatively, clinical studies may include the assessment of pubertal breast stage, which uses the Tanner scale (Marshall and Tanner, 1969), along with hormone measurements to assess and confirm developmental stage and hyperandrogenism (McCartney et al., 2009). The Endocrine Society guidelines indicates that chronic oligomenorrhea and the biochemical confirmation of hyperandrogenism can be applied to diagnose PCOS adolescent girls (Legro et al., 2013). Although there are some compelling evidence showing that HA is an appropriate marker for PCOS in adolescent girls (Ibáñez et al., 2014; Villarroel et al., 2015), elevated androgen levels associated with irregular menses may be transient and reflect an incomplete maturation of the HPG axis (Ibáñez et al., 2017). Therefore, a decisive criteria to determine whether HA in adolescence is critical for the development of PCOS remains unclear.
1.2.1.2 Ovarian dysfunction

Ovarian follicular arrest, cystic-like fluid-filled structures and increased stromal content in the ovaries are common traits of polycystic ovaries (Webber et al., 2003; Conway et al., 2014). According to the Rotterdam criteria, polycystic ovaries are identified by ultrasound diagnostic imaging showing the presence of ≥ 12 follicles of 2-9 mm diameter (as average value from both ovaries) and increased ovarian volume (more than 10 cm³) (Jonard et al., 2003; Balen et al., 2016).

A clinical report, which evaluated cortical biopsies of polycystic and normal ovaries, revealed that PCOS women exhibit an abnormal increase of the number of growing follicles (Webber et al., 2003). The same report also demonstrated that the mean proportion of early preantral follicles (including primordial and primary) in anovulatory PCOS patients is six times higher than normal women.

Dysregulation of the early follicular development is highly associated with androgen excess, suggesting that elevated androgen actions establish the full spectrum of ovarian dysfunction (Jonard and Dewailly, 2004). Ovaries are considered to be the main source of androgen excess in PCOS (Gilling-Smith et al., 1994). Androgen synthesis occurs in the ovarian theca cell layer following the activation of LH receptor (LHCGR) during normal follicular development (Tsang, Armstrong, and Whitfield 1980). An overview of the ovarian follicular development is important to understand how androgen excess may disrupt folliculogenesis. The initial steps are independent of the actions of gonadotropins and controlled by transcriptional and autocrine/paracrine factors (Richards and Pangas, 2010). Follicle-stimulating hormone (FSH) from the pituitary gland acts in the ovary in synergy with intra-ovarian signals, such as anti-müllerian hormone (AMH) and growth factors to promote small follicle growth. A cyclic recruitment is initiated and during later stages of folliculogenesis, grown antral follicles secrete activins and estrogens, which in turn, increase the
actions of FSH in the ovaries. The selection of dominant follicles is triggered in the final steps of folliculogenesis through synchronized actions of FSH and LH in the granulosa and theca cell layer, respectively, enhancing steroidogenesis. Therefore, from the initial cohort of recruited small follicles just one or a few dominant follicles, depending on the species, are selected to ovulate (Goodman and Goodman, 2009). The ovary itself is a target for androgen actions, which influence follicular growth, atresia attenuation and FSH-mediated actions on granulosa cells (Figure 1.1) (Prizant, Gleicher and Sen, 2014; Sen et al., 2014).

**Figure 1.1. Androgen-mediated actions in the ovary.** The theca cells are the main source of androgen production in the ovaries. Androgen actions throughout the ovarian follicular development has received more attention in recent years. Androgens are crucial for the transition from small follicles to antral follicles, although it is unclear whether they are able to regulate the follicle recruitment from primordial to primary type and whether they involved in ovulation. Androgen receptor (AR) is expressed in the theca cells (in blue), granulosa cells (in orange) and oocyte (in pink). AR signaling activation inhibits pro-apoptotic factors impeding atresia to occur in excess and increases the expression of follicle-stimulating hormone (FSH) receptor in the granulosa cells. (Figure adapted from Prizant et al., 2014).
Different studies have shown that androgen receptor (AR) is expressed in the oocytes, theca cells and granulosa cells of both humans and rodents (Chadha et al., 1994; Hirai et al., 1994; Lenie and Smitz, 2009). In the mouse, AR signaling in the granulosa cells has been recognized to be crucial for the normal expression of FSH receptor (FSH-R), prevent follicular atresia and promote follicular growth (Sen et al., 2014). Although essential to normal folliculogenesis, genetic studies knocking out AR from granulosa cells (GCARKO), revealed that AR signaling in these cells is not required for the presentation of ovarian dysfunction in a PCOS mouse model, which androgen excess is induced during adulthood (Caldwell et al., 2017). Likewise, AR signaling in the theca cells seems to have minimal impact on folliculogenesis or fertility in mice (Ma et al., 2017). Specific deletion of AR in oocytes using a Cre-recombinase technology driven by growth differentiation factor-9cre (oocyte-specific) generates fertile mice with normal folliculogenesis. Together, these findings suggest that androgen actions in granulosa cells are important for normal folliculogenesis; however, androgen-mediated PCOS ovarian dysfunction might be the result of upstream dysregulations in the HPG axis.

1.2.1.3 Menstrual irregularities

Menstrual irregularities indicate the occurrence of oligo- or anovulatory events and are a common feature of PCOS (Fauser et al., 2012). Oligomenorrhea is identified by a menstrual cycle that last more than 35 days or occurs less than eight times a year. Amenorrhea is diagnosed when menstrual cycles are longer than 199 days (Broekmans et al., 2006). In the clinics, plasma progesterone may also be used as marker for oligo-anovulation and blood levels should be below 3-4 ng/mL during the midluteal phase of the menstrual cycle (Legro et al., 2007; Dumesic et al., 2015). Clinical studies show that PCOS women may also present suboptimal FSH levels or elevated LH:FSH ratio, which are associated with anovulatory infertility (Manieri et al., 1992; Tarlatzis et al., 1995). Large
longitudinal cohort study showed that infertility was 15-fold higher in PCOS women whereas only 16% of non-PCOS subjects noted infertility (Joham et al., 2015). In addition, results from a 31 years follow-up study showed that 66% of PCOS women reported history of infertility in opposition to a significant lower number of 6% in the control group (Wild et al., 2000). Therefore, PCOS is a highly prevalent endocrine disorder leading to sub- and infertility making it a major concern for health management.

1.2.1.4 PCOS & Metabolic syndrome

PCOS diagnostic criteria does not include metabolic syndrome, although the disorder is commonly associated with obesity, insulin resistance (IR) and type 2 diabetes mellitus (Jayasena and Franks, 2014; Ollila et al., 2017). Hyperinsulinemia and IR are known to exacerbate androgen excess and irregular cycles in obese and also non-obese PCOS women when compared to body weight matched subjects (Stepto et al., 2013). The molecular mechanisms of IR in PCOS are likely to be different from those observed in non-PCOS insulin resistant diabetic women (Book and Dunaif, 1999). Possible causes of IR in PCOS include abnormalities in the insulin receptor activity and enhanced cytokine actions leading to impaired insulin signaling (Escobar-Morreale, Luque-Ramírez and González, 2011). Reproductive tissues such as ovaries and pituitary are more resistant to hyperinsulinemia in the presence of elevated androgen levels in mice (Andrisse et al., 2017). Specifically, insulin stimulates the activity of ovarian P450scC enzyme increasing androgen production (Li et al., 2013) and increases the secretion of FSH and LH from culture rat pituitary (Adashi, Hsueh and Yen, 1981). Therefore, IR seems to be tissue-specific in PCOS and androgens might sustain insulin stimulatory actions in reproductive tissues (Figure 1.2).
Figure 1.2. Pathogenesis of PCOS and the relationship between hypothalamic-pituitary-gonadal (HPG) dysfunction, androgen excess and metabolic features of the disease. Genetic/epigenetic predisposition to increased androgen production by the polycystic ovaries is implicated in disrupting neuroendocrine mechanisms controlling LH secretion. Menstrual irregularities and distressing effects of hyperandrogenemia such as hirsutism are commonly present in association with metabolic traits of PCOS. Elevated androgen levels can upregulate insulin-mediated pathway increasing luteinizing hormone (LH) secretion and induce insulin resistance in other peripheral tissues. Insulin resistance and hyperinsulinemia aggravate this scenario as insulin stimulate androgen synthesis. (Figure adapted from Jayasena and Franks, 2014).

Body weight is not a determinant factor for the development of IR in PCOS as lean PCOS girls can present hyperinsulinemia before pubertal onset (Sir-Petermann et al., 2009), indicating that impaired insulin sensitivity might be part of PCOS pathogenesis. However, reports suggest
that IR and additional effect of western diet may maintain the increase testosterone production by the ovaries and anovulation (Jayasena and Franks, 2014). In some reported cases, pharmacological intervention that leads to increased insulin sensitivity such as metformin treatment is able to improve menstrual cyclicity, HA and fertility (Yilmaz et al., 2005; Gambineri et al., 2006). The importance of weight loss to improve menstrual cycles in PCOS patients seems to be overemphasized (Sirmans and Pate, 2013) showing a small effect of ~5% in improvement of menstrual cyclicity after weight loss as reported by the Androgen Excess and PCOS Society (Moran et al., 2009).

It is important to highlight that the association of increased body weight, elevated androgen levels and decreased reproductive function does not always imply causation. Rosenfield and Bordini discussed several studies demonstrating that obesity and androgen levels may affect independently the female reproductive function by a biphasic effect on the gonadotropin secretion (Rosenfield and Bordini, 2010). This means that the too high or too low either relative adipose mass or androgen levels negatively impact LH secretion independently. Hence, in PCOS patients who exhibit elevated LH levels, the metabolic dysfunction can aggravate the disease symptoms but might not be causing the syndrome. Considering that around 30-50% of PCOS women do not show the metabolic syndrome (DeUgarte, Bartolucci and Azziz, 2005), it might suggest that distinct molecular targets might underlie the pathogenesis of either obese or non-obese PCOS phenotypes. Therefore, the next section will cover some of the genetic and developmental determinants which might be involved in the pathogenesis of PCOS.
1.2.2 Pathogenesis & developmental aspects of PCOS

1.2.2.1 Genetic determinants & the genesis of PCOS

Heritable alleles and their association with environmental factors may drive the phenotypic presentation of PCOS. Early reports pointed to PCOS as an autosomal dominant inheritance disorder with around 40-50% of prevalence between first-degree familial relatives (Hague et al., 1988). However, this autosomal dominant inheritance was questioned after a twin study performed in an Australian monozygotic and dizygotic twin cohorts concluding that there was a clear difference in the genetic basis between hyperandrogenic and non-hyperandrogenic PCOS women (Jahanfar et al., 1995). Thus, it is unlikely that PCOS is a result from a single autosomal genetic determinant. A larger twin study performed in a Dutch cohort using quantitative estimation of the genetic influence found a strong genetic contribution with a heritability rate of 79% for the prevalence of PCOS according to the Rotterdam criteria (Vink et al., 2006).

Genome-wide association studies (GWAS) attempt to provide genetic associations that may have mechanistic values to understand the development of PCOS. Two initial large GWAS analyzed risk loci for the developing PCOS in Han Chinese population and, together, they elucidated eleven candidate genes (Chen et al., 2011; Shi et al., 2012). Relevant PCOS association signals were found in the thyroid adenoma associated (THADA) gene intronic region, a gene associated with thyroid cancers, energy balance and risk for type 2 diabetes (Kloth et al., 2011; de Melo et al., 2015; Moraru et al., 2017); DENND1A loci, a gene that encodes connecdenn 1 and involved in trafficking of endosomes and involved in androgen production (Tee et al., 2016); and variants in the gene of FSH receptor (FSH-R), which is critical for folliculogenesis and fertility (Simoni et al., 2008).
A recently published comprehensive GWAS elucidated more aspects of the genetic genesis of PCOS and its neuroendocrine correlates (Day et al., 2015). This study evaluated more than 5,000 Caucasian European women and used dense imputation of genotypes, which identify association signals and draw mechanistic interpretations from the genetic data. They discovered six genetic loci associated with PCOS: YAP1 (a transcriptional factor involved in cancer), THADA, RAD50 (encodes a DNA double-strand break repair protein), KRR1 (a ribosome assembly factor), FSHB (β-subunit of FSH) and epidermal growth factor receptor (EGFR) family members such as ERBB4 and ERBB3. This study also presented that polymorphisms which may lead to altered FSH secretion and signaling may participate in the neuroendocrine genesis of PCOS, once this was positively correlated with elevated LH:FSH ratio in their cohort. Notably, EGFR family members are involved not just in LH-mediated steroidogenesis (Park et al., 2004) and ovulation but also they have strong influence on brain circuitry development (Namba et al., 2017). Using Mendelian randomization analysis to infer causal roles, they identified that body weight, insulin resistance and low levels of sex hormone-binding globulin (SHBG) are risk factors for the development of PCOS. A strong and positive association between the risk of having PCOS and showing delayed menopause was found, suggesting an attenuation of ovarian ageing in these subjects (Day et al., 2015). This is linked with earlier hypotheses proposing that a genetic predisposition to delayed reproductive senescence in PCOS would support the evolutionary paradox of being a highly prevalent disease that impairs female reproductive competence (Abbott, Dumesic and Franks, 2002; Mulders et al., 2004; Casarini and Brigante, 2014). In addition to the genetic basis of the disorder, in utero environmental factors may endeavor a better understanding of PCOS development.
Chapter One

1.2.2.2 Developmental programing: role of intrauterine androgenization in the pathogenesis of PCOS

Although there is a consensus that PCOS is not caused by one single determinant, there is a strong association between exposure to prenatal androgen excess and the presentation of the cardinal features of the disease after pubertal onset. In utero androgen excess in humans caused by congenital adrenal hyperplasia (CAH) and maternal Cushing’s disease, both which expose female fetuses to high androgen levels, leads the clinical manifestation of PCOS in adulthood (Hague et al., 1990; Barnes et al., 1994). Likewise, daughters of PCOS women, who have higher circulating testosterone levels during pregnancy than normal subjects, show signs of ovarian and metabolic dysfunction during pubertal development and an increased risk of developing PCOS in adulthood (Crisosto et al., 2007; Sir-Petermann et al., 2009). Placental tissue of mothers with PCOS exhibit higher activity of 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD-1), which increases androstenedione levels, and lowers the activity of P450 aromatase, which is critical in converting androgens to estrogens (Maliqueo et al., 2013). Consequently, altered placental steroidogenesis in PCOS could sustain elevated intrauterine androgen levels during pregnancy and affect the development of female offspring. Together, these data point toward a prenatal androgen programing as potential mechanism to drive PCOS features later.

The idea of a PCOS in utero origin propose two mains hypotheses on how the developmental programing generates the reproductive impairment of PCOS. One explanation makes the ovarian dysfunction as the starting point of the disease. Thus, androgen imprinting in the ovaries triggers an abnormal secretion of testosterone following the onset of puberty (Gilling-Smith et al., 1994; Rosenfield and Bordini, 2010; Zhang et al., 2014). In turn, high testosterone plasma levels might modify the hypothalamic-pituitary-gonadal (HPG) axis functioning to the full
presentation of PCOS symptoms. A second explanation places the brain as the primary site in the PCOS pathogenesis. During perinatal life, hypothalamic circuits, which are involved in the control of reproductive and metabolic function, are highly responsive to sex steroid hormone modulation (Phoenix et al., 1959; McCarthy, Nugent and Lenz, 2017). Accordingly, prenatal androgen actions may alter the organization of components within the GnRH neuronal network and ultimately drive abnormal GnRH/LH pulsatility and ovulatory dysfunction (Blank, Mccartney and Marshall, 2006; Moore and Campbell, 2016). It is important to note that one explanation does not exclude the occurrence of the other and that both could happen concomitantly in PCOS. To investigate these different hypotheses, PCOS animal models, which can be generated with prenatal androgen exposure, have been broadly used in basic research.

1.2.2.3  *Prenatally androgenized (PNA) PCOS animal models*

Clinical studies are mostly aimed at controlling PCOS symptoms and are limited in terms of access to human tissues and hypothesis-driven investigation of pathological mechanisms. Therefore, the use of PCOS animal models has provided important evidence about the pathophysiology of the disease. Most of these models employ prenatal or early postnatal androgen exposure, which are able to recapitulate the cardinal traits of PCOS. Although the number of androgen exposure protocols has increased in recent years, the present literature review focuses on the most commonly used prenatally androgenized (PNA) models as they have provided substantial evidence about the PCOS-like development in non-human primates, sheep and rodents.

1.2.2.3.1  *Non-human primates*

Considering the shared characteristics among primates, the rhesus monkey (*Macaca mulatta*) is an ideal model to study PCOS. Similar to humans, monkeys are a precocious and monovulatory
species, and show complete intrauterine ovarian development. Prenatal androgen exposure was firstly proposed to be studied in non-human primates following evidence that elevated intrauterine androgen levels in CAH induce ovulatory dysfunction, HA and LH hypersecretion in female offspring (D H Abbott et al., 1998). Pregnant rhesus monkeys exposed to testosterone, during gestational days (GD) 40 to GD 60 (pregnancy ~ 165 days) generated female offspring that recapitulate the main PCOS features such as anovulation, HA, enhanced LH secretion and polycystic ovaries (Dumesic et al., 1997; D H Abbott et al., 1998; Eisner et al., 2002). Importantly, female rhesus monkeys can be naturally hyperandrogenic and develop PCOS-like features. A recent study revealed that the prevalence of PCOS-like monkeys within a laboratory population was close to findings in humans (~15%), and hyperandrogenic rhesus monkeys exhibit high serum concentration of LH and anti-müllerian hormone (AMH) positively associated with infertility (Abbott et al., 2017). Therefore, neuroendocrine, ovarian and associated metabolic features of PCOS can be recapitulated in the non-primate model. However, the time consuming generation of these animals, high costs and poor availability of transgenic lines have limited their broad use in basic research.

1.2.2.3.2 Sheep

Sheep are monovulatory animals similar to humans and provide a valuable model to study PCOS. Early (GD 30-90) or mid (GD 69-90) gestation prenatal testosterone exposure is able to recapitulate anovulation, cystic-like ovarian morphology and HA (Birch et al., 2003). The PNA sheep model exhibits elevated LH pulse frequency secretion and increased response to intermittent treatment of exogenous GnRH (Manikkam et al., 2008), recapitulating the neuroendocrine impairment of PCOS. PNA sheep also display disruption of the LH surge, and impaired estradiol- and progesterone-negative feedback on GnRH/LH release (Padmanabhan and Veiga-Lopez, 2013a).
Although the model provides a good opportunity to study the cardinal features of PCOS, a high degree of virilization of the external genitalia is observed in PNA female sheep (Roland et al., 2010), which raises the question whether this is modeling a masculinization of the brain or PCOS. The relatively high maintenance costs, longer gestational length (~150 days), seasonal aspects of breeding and lack the of specific transgenic animals presents hurdles for broader use of this model (for more details about this model, see review Veiga-lopez et al., 2009).

1.2.2.3.3 Rodents

Prenatal exposure during late pregnancy with testosterone or dihydrotestosterone (DHT), a non-aromatizable androgen, evokes PCOS-like features in rats and mice. In rats, a small dose of 1 mg/day during gestational days (GD) 21-23 is able to increase LH secretion frequency, attenuate progesterone receptor expression in the POA and eliminate the LH surge in adulthood (Foecking et al., 2005). By increasing the prenatal testosterone or DHT dose to 3 mg/day during the same period, PNA rats present higher testosterone levels, increased LH frequency and amplitude, irregular estrous cyclicity and ovarian dysfunction (Wu et al., 2010). A prenatal dose of 5 mg/day of testosterone during the same gestational period evokes the PCOS cardinal features and metabolic associated traits such as increased body weight, hyperinsulinemia and dyslipidemia in adulthood (Demissie et al., 2008). Therefore, these findings suggest that a differential intrauterine androgen milieu might be key to the heterogeneity manifestation of PCOS. PNA female rats also display anxiety-like behaviors that are associated with disrupted AR signaling in the amygdala (Hu et al., 2015); indicating PNA rats as suitable models to investigate PCOS associated neurological diseases such as anxiety and depression.

Likewise, mice and transgenic mouse technology has offered important genetic tools to understand and define PCOS pathogenesis. Prenatal exposure with DHT in mice at a dose of 250
μg/day during late pregnancy (GD 16-18) generates adult female mice displaying elevated testosterone plasma levels, ovarian dysfunction and impaired steroid-mediated negative feedback mechanisms such as in the human condition (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Neuroendocrine features of PCOS are also present in PNA mice such as high LH pulse frequency secretion (Moore, Prescott and Campbell, 2013). This neuroendocrine derangement is associated with an increase of dendritic spine density in GnRH neurons during adulthood (Moore, Prescott and Campbell, 2013) suggestive of enhanced afferent synaptic inputs onto these neurons. Aberrations within the hypothalamic circuits controlling GnRH/LH secretion are also observed in PNA mice (Moore, Prescott and Campbell, 2013; Moore et al., 2015), which will be further discussed in section 1.4.4. PNA mice are lean without significant and present mild metabolic phenotype including increased fasting glucose levels, early form of islet dysfunction in the pancreas and adipocyte hypertrophy (Roland et al., 2010; Caldwell et al., 2014). Modeling lean PCOS provides a unique opportunity to dissect out specific factors that might contribute to the reproductive impairment in PCOS without the secondary influence of increased body weight or hyperinsulinemia.
1.3 The control of reproduction & the female brain

The control of female reproduction in mammals is ultimately dependent upon neural circuits in the brain. Located in the hypothalamus, gonadotropin-releasing hormone (GnRH) neurons provide the final output from the brain with pulsatile release of GnRH into the hypophyseal portal system signaling to the pituitary gland. In the pituitary, GnRH promotes the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the systemic blood stream, which levels are tightly regulated during the female reproductive cycle. These two gonadotropins act downstream in the ovaries to coordinate ovarian follicle development, ovulation and the production of ovarian steroid hormones, such as estrogens and progesterone. Completing a closed-loop system, steroid hormones act back in the hypothalamus to regulate the GnRH neuron activity through a steroid-sensitive neuronal network. Therefore, the gonadal feedback information to the brain is crucial for the normal reproductive function (Goodman and Goodman, 2009; Herbison, 2014).

Internal and external clues can modulate the activity of GnRH neurons and impact the entire hypothalamic-pituitary-gonadal (HPG) axis. To guarantee fertility and balance it out with energy expenditure, neuroendocrine systems sense various cues and transmit the information to GnRH neurons. In a pathological state, however, an inappropriate regulation of GnRH neurons generates an abnormal secretion of gonadotropins, which negatively affects fertility. This is the case of PCOS, in which ~75% of patients with the disorder exhibit elevated LH pulsatile secretion, an enhanced response to exogenous gonadotropin stimulation and an increased LH:FSH ratio in the blood (Rebar et al., 1976; Taylor et al., 1997; Patel et al., 2004). Thus, PCOS is considered a neuroendocrine disorder associated with ovarian dysfunction and menstrual irregularities. The present section provides an overview of the intrinsic features of the GnRH neuron network and
discusses evidence supporting the notion that changes within this system may establish the development of PCOS.

1.3.1 The GnRH system & the HPG axis

1.3.1.1 Developmental, morphological & intriguing features of GnRH neurons

When compared to other brain cells, GnRH neurons display peculiar characteristics from the time they are born to their final locus within the basal forebrain. The ontogenesis of GnRH neurons and the olfactory system are remarkably associated. In mice, GnRH neurons are born in the medial part of the olfactory placode of the developing nose known as vomeronasal organ (VNO) (Schwanzel-Fukuda and Pfaff, 1989). Neural crest cells from the VNO give rise to GnRH neurons, which later migrate along the nervus terminalis, the vomeronasal nerve and then to the olfactory nerve through the cribriform plate and into the developing forebrain (Wray, Grant and Gainer, 1989; Forni et al., 2011). In humans, GnRH-expressing cells are firstly detected in the basal lamina of the VNO, similar to rodents, and migration into the forebrain begins around the 48th day of gestation (Casoni et al., 2016).

Various signals influence GnRH neuron migration such as cell adhesion molecules, neurotransmitters and growth factors (Wierman, Kiseljak-vassiliades and Tobet, 2011). Hypophysiotropic GnRH neurons, representing 50-70% of the total population, reach their final location in a scattered distribution throughout the anterior portion of the hypothalamus of rodents (Jennes, Stumpf and Sheedy, 1985; Silverman, Jhamandas and Renaud, 1987). Cell bodies of hypophysiotropic GnRH neurons are mainly located in the medial septum (MS), rostral preoptic area (rPOA), organum vasculosum of the lamina terminalis (OVLT), and anterior hypothalamic
area (AHA) (Skynner et al., 1999), with GnRH neuron fibres extending toward the median eminence (ME) and hypophyseal portal system (Figure 1.3).

Figure 1.3. GnRH neuron distribution in the mouse brain. (A) View of the location of the hypothalamus (blue dashed lines) in the human and the mouse brain, both in a sagittal view. (B) This image shows magnified sagittal view of a schematic mouse brain showing the location of GnRH neurons (green dots) and brain coronal sections from GnRH-green fluorescent protein (GFP) mice. The GnRH neurons are found in a scattered distribution throughout the hypothalamus in the (a,b)medial septum (MS), (c)rostral preoptic area (rPOA), (c)organum vasculosum of the lamina terminalis (OVLT) and (d)anterior hypothalamic area (AHA). GnRH neuron fibres reach the hypophyseal portal vessels through
(e) median eminence (ME) to secrete GnRH pulses into the hypophyseal portal system to the pituitary gland (pit). Scale bar =100µm; fx: fornix, ac: anterior commissure, cc: corpus callosum. (Figure adapted and used with the permission of Dr Rebecca E. Campbell).

The GnRH neuron population constitutes a heterogeneous group of cells with intriguing morphological and electrophysiological characteristics. The majority of adult GnRH neurons show a unipolar or bipolar morphology, which incorporates the cell body, and one or two dendrites (Jennes, Stumpf and Sheedy, 1985). Using transgenic mouse technology to drive the expression of green fluorescence protein (GFP) in GnRH neurons, the morphological features of GnRH-GFP neurons can be visualized, allowing further investigation of their cellular structure and electrophysiological features (Spergel et al., 1999). Using biocytin, a low molecular-weight dye, to fill GnRH-GFP neurons in acute mouse brain slices, it was revealed that these neurons display a long dendrite that extents over 1000 µm from the soma and is decorated with spines and filopodia (Campbell, Han and Herbison, 2005). At different levels of the hypothalamus, such as MS, OVLT and rPOA, GnRH neurons are found to project in different directions and fairly often display a “hair-pin” loop toward the ME (Herde and Herbison, 2015).

The soma of GnRH neurons is decorated with somatic spines, which are neuronal structures that mainly harbour excitatory inputs (Harris and Weinberg, 2012). With electron microscopy resolution, at the level of the cell body, GnRH neurons can be classified as “smooth” or “spiny” cells according to the density of neuronal spines and cytoplasmic protrusions (Jennes, Stumpf and Sheedy, 1985). This spiny type of GnRH neurons was later associated with enhanced activity as most of these cells express c-Fos, a neuronal marker of increased neuronal activity, at the time of the preovulatory LH surge in mice (Chan et al., 2011). The remodelling of somatic spines might
be dependent on ovarian steroid signaling since ovariectomy is able to significantly reduce the spine density whereas estradiol treatment mimicking positive feedback increases the number of spines in activated (c-Fos-positive cells) GnRH neurons (Chan et al., 2011). Pathological conditions are associated with altered spine density in GnRH neuron. In normal female mice, c-Fos-expressing GnRH neurons (surged) exhibit an increased density of somatic spines when compared with non-surged GnRH neurons following LH surge protocol (Moore, Prescott and Campbell, 2013). Conversely, in PNA mice, these cellular remodelling is absent as both surged and non-surged GnRH neurons display high number of somatic spines in GnRH neurons similarly (Moore, Prescott and Campbell, 2013). This finding suggests that a greater proportion of GnRH neurons might be in hyperactive state in PNA mice compared to normal females.

The GnRH neuron dendrite is also decorated with dendritic spines as observed with electron microscopy (Jennes, Stumpf and Sheedy, 1985) or by using biocytin filling of GnRH neurons (Campbell, Han and Herbison, 2005). Although dendritic spines are mainly located in the proximal dendrite (< 50 µm from the soma), they can also be found in distal segments of the neuron in the mouse (Herde and Herbison, 2015). For the majority of neurons in the forebrain, actions potentials are usually initiated in the proximal segments of axons, which possess a high density of voltage-activated ion channels to trigger neuron firing (Scholz et al., 1993; Hu et al., 2009). On-cell recording in GnRH neuron dendrites coupled with whole-cell path clamp at the cell body revealed that their dendrites bear the spike initiation site and are capable of propagating spikes (Roberts, Hemond and Suter, 2008). Further characterization using calcium (Ca^{2+}) imaging and electrical recordings in brain slices of GnRH-GFP mice confirmed the dendrites as the neuronal structure responsible for the generation of action potentials (Iremonger and Herbison, 2012).
Morphological studies presented that around 2/3 of axons of GnRH neurons are originated from dendritic segments (Herde and Herbison, 2015). Additionally, the same work showed that the detection of Ankyrin G, a marker of action potential initiation site, is mostly present at dendrites and ~14% of the GnRH neurons displayed Ankyrin G in axons. Together, these reports elucidate that GnRH neuron axons are not the most common site generating neuronal spiking, which seems to be dictated by dendrites. Remarkably, reports using a series of morphological and electrophysiological tools showed that GnRH neuron dendrites possess an exquisite hybrid structure, capable of receiving and propagating action potentials referred as dendron (Herde et al., 2013; Iremonger and Herbison, 2015). In addition, biocytin cell filling technique in mouse brain slices revealed that GnRH neuron dendrites intertwine, show dendro-dendritic associations and receive shared synapses from afferent neurons (Campbell et al., 2009). Together, these findings show that GnRH neurons are outfitted with a long and active dendrite (or dendron), which can receive and integrate a vast network of inputs (Figure 1.4).

Axonal projections of GnRH neurons are located in the OVLT and ME and the majority of axons rise from distal dendrites (Herde and Herbison, 2015). In the ME, GnRH neuron processes branch out from dendritic trees toward the output zone, which contacts fenestrated vessels due to the absence of blood brain barrier (BBB) (Pelletier et al., 1974; Silverman and Desnoyers, 1976; Herde et al., 2011). Glial interactions occur with GnRH neurons in the ME to control their axonal access to the hypophyseal portal system. Tanycytes, a specialized ependymal cell located at the base of the third ventricle (3V) of the brain, regulates the retraction or ensheathment of GnRH neuron terminals through semaphoring7A signaling, which in turn, is dependent on ovarian steroid hormone modulation (Parkash et al., 2015). Therefore, glia-GnRH neuron interaction might be important to control the hypophysiotropic demands of different patterns of GnRH secretion throughout the female reproductive cycle (Figure 1.4).
Figure 1.4. Morphological features of GnRH neurons. This scheme shows a model presented by Campbell, R.E. to decipher the morphological features of GnRH neurons in the different regions of the hypothalamus. GnRH neuron cell bodies are mostly found throughout the hypothalamus being distributed in a scattered way in the medial septum (MS; neurons in blue), in the rostral preoptic area (rPOA; neurons in pink) and anterior hypothalamic (AHA; neurons in yellow). GnRH neuron processes are mainly found in the organum vasculosum of the lamina terminalis (OVLT) and in the median eminence (ME). (A) GnRH neurons can be classified into “spiny” and “smooth” according to the presence or absence, respectively, of neuronal spines which decorate the soma and dendrites. (B) GnRH neuron dendrites can extend over 1000 µm from the MS/rPOA toward the ME. Dendrites are the main site of action potential initiation (green glowing line) and have been recently referred as dendrons. (C) Around 20% of GnRH neurons project dendritic trees, which branch out in more than two processes and axonal-like structures in the OVLT. (D) GnRH neuron dendrites also intertwine, show dendro-dendritic associations and receive shared synapses from afferent neurons. (E) Axons raise from dendrons and are mostly found in the external layer of the ME. (F) GnRH neuron dendrites often project in different directions and fairly often display a “hair-pin” loop toward the ME.
1.3.1.2 Functional aspects of GnRH neurons & gonadotropin secretion

Reproductive function is dependent upon pulsatile secretion of GnRH, which coordinates and drives the secretion of gonadotropins. Seminal studies in non-human primates revealed that the secretion pattern of LH is pulsatile (Dierschke et al., 1970), and that continuous administration of GnRH silences gonadotropin secretion (Belchetz et al., 1978). Using a push-pull technique in the portal vasculature in ovariectomized sheep, researchers were able to measure and show that GnRH and LH secretion are paired at a pulse relation rate of 1:1 ratio (Clarke and Cummins, 1982) (Figure 1.5). In contrast, FSH secretion does not show a direct correlation with or dependence upon GnRH pulses as demonstrated in gonadectomised ewes which had a hypothalamic-pituitary disconnection and were treated with exogenous GnRH (Clarke et al., 1986). Although FSH secretion is uncoupled from GnRH pulses, FSH synthesis is dependent upon GnRH receptor (GnRH-R) signaling in gonadotropes through a constitutive secretory pathway (Thompson and Kaiser, 2014).
Figure 1.5. The pulse profile of GnRH and LH peptides in the hypophyseal portal and systemic blood of ovariectomized ewes. The pattern of GnRH and LH release showed a 1:1 relationship with the detection of one GnRH/LH pulse per hour in the absence of negative feedback from ovarian steroid hormones. The arrows show larger GnRH pulses, which correlate with LH pulses and point out small magnitude pulses that are not paired with LH release. GnRH levels were obtained with a push-pull perfusion technique in the basal hypothalamus in conscious sheep. LH levels were concomitantly measure through cannulation of jugular vein (Figure adaptated from Clarke and Cummins, 1982).

The pulse relation rate of 1:1 of GnRH:LH ratio determines the LH pulse frequency secretion and, in females, whereby measurements of LH pulse frequency in the systemic blood is used as a proxy for GnRH pulse frequency in the hypophyseal portal blood (Clarke and Cummins, 1982). The amplitude of LH secretion is also determined by the GnRH pulse frequency but in an
inversely proportional manner. This means that low-frequency of GnRH pulses induce high LH pulse amplitude, and high-frequency GnRH pulses generates a reduced LH pulse amplitude (Clarke and Cummins, 1985). These findings suggest that the releasable pool of LH-containing secretory granules in the pituitary, which underpins the pulse amplitude, is determined by frequency of GnRH stimulation in the gland. The GnRH pulsatile release drives two distinguished types of LH secretion: LH pulses in both sexes and a pre-ovulatory LH surge, which is only present in females. During the transition from follicular phase to the luteal phase of the female reproductive cycle GnRH/LH pulse frequency increases significantly culminating in the detection of a LH surge release, which is critical for ovulation (Smith, Freeman and Neill, 1975; Karsch et al., 1997; Czieselsky et al., 2016).

Intrinsic pulsatile electrical pattern can be detected in GnRH neurons in certain circumstances. Ex vivo studies using cell cultures of embryonic olfactory placode explants or immortalized cell cultures (termed GT1-7 cells), found that GnRH neurons display intrinsic burst firing (Funabashi et al., 2001; Nunemaker et al., 2001), synchronized Ca$^{2+}$ oscillations (Terasawa, Schanhofer, et al., 1999) and pulsatile GnRH release (Terasawa, Keen, et al., 1999). Although ex vivo preparations are useful to understand the innate mechanisms of GnRH neuron activity, they do not possess the complexity of an entire adult neuronal network. To date, intrinsic pulsatile firing activity for the majority of adult GnRH neurons recorded in vivo has not been shown. Considering that GnRH neurons share more synapses with non-GnRH neuron than with themselves (Campbell et al., 2009), it is possible that synchronized pulsatile GnRH secretion might be the result of the action of upstream afferent neurons. Therefore, extrinsic factors might be fundamental for the functioning of the GnRH neuron network, as seen in other brain cells such as in oxytocin neurons, which allow to coordinate burst firing and peptide release (Ludwig and Leng, 2006).
Attempts to unveil the endogenous pattern of GnRH neuron electrical activity and make associations with GnRH release have used electrophysiological recordings of GnRH-GFP neurons in brain slices (Spergel et al., 1999; Suter et al., 2000). Reports showed that some GnRH neurons exhibit sustained bursting activity with bursting interval values that can vary from seconds to several minutes (Nunemaker, DeFazio and Moenter, 2002; K. Lee et al., 2010). However, GnRH interval of hormone release has been found to be longer, from minutes to hours, making it difficult to understand how the cellular electrical behavior is coupled with the episodic release of GnRH. In vivo recordings in GnRH-GFP mice reported that 86% of the GnRH neurons displayed spontaneous activity; however, only a small portion of these active neurons (~15%) exhibited burst firing (Constantin, Iremonger and Herbison, 2013). The requirement of a burst firing pattern to induce pulsatile release of GnRH/LH was recently addressed using an optogenetic approach in mice (Campos and Herbison, 2014). This study demonstrated that a continuous optical stimulation of 10 Hz over 2 min, which activated a small number of GnRH neurons (~5%), can induce pulse-like LH secretion in vivo. In contrast, a burst pattern of optogenetic stimulation did not elicit any LH release. Therefore, these data suggest that burst firing activity of GnRH neurons is not required to promote GnRH/LH pulse secretion. Together, these findings strengthen the idea that the control of synchronous pulsatile activity of GnRH neurons is upstream from GnRH neurons within their neuronal network.

1.3.1.3 Gonadal steroid hormone feedback regulation in the female HPG axis

For the majority of vertebrates, the variation of circulating gonadotropins and ovarian steroid hormones levels reflect the functioning of the HPG axis and define the female reproductive cycle (Figure 1.5A). In humans, as in rodents, low and tonic gonadotropins levels and increasing concentrations of estradiol determine the follicular phase (Smith, Freeman and Neill, 1975).
Estrogens are potent downregulators of the HPG axis exerting a negative feedback signal to ultimately decrease GnRH/LH secretion through direct actions in the GnRH neuronal network and the pituitary gland (Karsch et al., 1997; Goodman and Goodman, 2009). However, rising levels of estrogens induce a switch from negative to positive feedback at the end of the follicular phase (Yamaji et al., 1971), which increases the activity of GnRH neurons culminating in a LH surge in females (Figure 1.5B). The LH surge is critical for the occurrence of ovulation and subsequent rise in progesterone secretion from the corpus luteum in the ovary (Zeleznik and Plant, 2015).

Figure 1.5: The female reproductive cycle & HPG axis in mammals. (A) Diagram showing the female reproductive cycle. The follicular phase of the female reproductive cycle is characterized by low levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Rising levels of estrogens take place from the mid follicular phase due to their elevated production by developing ovarian follicles. FSH biosynthesis and secretion is increased by activins, produced by the ovaries, during late follicular phase. FSH actions in the ovary are important for the selection of preovulatory follicles and expression of the LH receptor. Following the rising levels of estrogen, LH secretion is robustly increased, described as a surge, and is critical for ovulation (dashed purple line). In humans, two days around the ovulation are defined as fertile period (yellow box). LH actions in the ovaries induce luteinization and progesterone synthesis, which is found at high concentration in the bloodstream. (B) Feedback mechanisms in the HPG axis. Gonadal steroid hormones such
as estrogens and progesterone decrease the HPG axis activity via negative feedback regulation. At the level of the brain, these hormones act on steroid-sensitive afferent neurons to ultimately reduce the activity of GnRH neurons. At the pituitary gland, steroid hormones are able to attenuate the biosynthesis and secretion of FSH and LH. However, rising estrogens induce a switch from negative to positive feedback leading to an increase in GnRH neuron activity and subsequent LH secretion.

Ovarian steroid hormones are able to modulate GnRH/LH pulsatile release through different mechanisms in the brain. Investigating the negative feedback in gonadectomized ewes, evaluation of the hypophyseal portal blood revealed that estrogen has a direct effect in the brain by suppressing GnRH pulsatile secretion in a dose-dependent manner (Evans et al., 1994). Direct negative feedback in the pituitary gland can be observed in postmenopausal women treated with graded doses of exogenous GnRH and estrogen after depletion of endogenous GnRH (Shaw et al., 2010). Interesting to note, this negative effect is stronger on FSH release when compared to LH, demonstrating a differential sensitivity to estrogen signaling in gonadotropes. The absence of gonadal steroid hormones drive a significant increase of GnRH pulses as it was demonstrated in intact and in gonadectomized male rats using a push-pull perfusion in hypophyseal portal blood (Levine and Duffy, 1988). In mice, recent advances in methods to measure pulsatile LH release replicated the enhanced LH pulse frequency induced by gonadectomy in both sexes, and the LH levels during the female estrous cycle, which follow similar pattern of other species (Czieselsky et al., 2016).

1.3.1.3.1 Estrogen signaling

Estrogens exert their biological regulation through the activation of classical and non-classical pathways. To date, both classical and non-canonical estrogen receptors have been identified to
mediate estrogenic effects. These include the classical estrogen receptor (ER) α and β, which can act as transcriptional factors or trigger rapid signaling when located at the cell membrane; splice variant ERαΔ4; ER-X and a G-protein coupled estrogen receptor (GPER, previously named GPR30) (see reviews Kelly and Rønnekleiv, 2012; Micevych, Mermelstein and Sinchak, 2017).

This literature review will focus on estrogen signaling pathways in the brain which are critical to assure proper GnRH/LH secretion and fertility.

GnRH neurons express ERβ but do not contain ERα (Herbison and Pape, 2001), which indicated possible direct estrogenic actions in these neurons to control fertility. However, pharmacological blockade of ERβ in mice do not alter estrous cyclicity or feedback mechanisms (Cheong et al., 2014). The role of ERβ activation in GnRH neurons remains poorly understood, although some evidence suggest that this receptor is involved in the regulation of intracellular pathways such as the cAMP response element-binding protein (CREB) in these cells (Abrahám et al., 2003; Kwakowsky, Herbison and Ábrahám, 2012). Both global and neuron-specific deletion of ERα in mice leads to the absence of estrogen-mediated positive feedback, ovarian dysfunction and infertility (Winternantel et al., 2006). In contrast, mutant mice with global loss of ERβ are fertile and able to mount a typical LH surge. In addition, by using inducible tamoxifen-based Cre-LoxP approach, the deletion of ERα in adult neurons results infertile mice which fails to respond to estrogen-mediated negative feedback (Cheong et al., 2014). Therefore, ERα-expressing afferent neurons are critical for reproduction but not direct estrogen actions in GnRH neurons.

1.3.1.3.2 Progesterone signaling

Progesterone signalling is also required for normal feedback control in the hypothalamus, providing strong restraint of GnRH/LH secretion during the luteal phase of female reproductive cycle (McCartney et al., 2002). Progesterone receptor knockout mice (PRKO) show chronic
anovulation, uterine hyperplasia, impairment of sexual behaviour and infertility (Lydon et al., 1996). Additionally, PRKO mice exhibit 2-fold higher basal LH levels when compared to wild-type animals, suggesting an impairment in negative feedback regulation of the HPG axis (Chappell et al., 1997). Although PRKO mice display normal estrogen-mediated negative feedback, PRKO females do not display a LH surge or show GnRH-induced self-priming, indicating that progesterone and estrogen signalling act in synergy to induce the gonadotropin surge (Chappell et al., 1999). Progesterone is known to potentiate positive feedback allowing the proper magnitude of LH surge following estrogenic priming in different species, such as in rodents (Kalra and Kalra, 1983; Leite et al., 2016), non-human primates (Schenken et al., 1985) and humans (Hutchens et al., 2016).

Similar to ERα, GnRH neurons do not express the classical nuclear PRs, proposing that progesterone modulation of the HPG axis also occurs via the steroid-sensitive afferent neuronal network of GnRH neurons (Skinner, Caraty and Allingham, 2001). Interestingly, progesterone can signal directly onto GnRH neurons through non-classical membrane receptors such as the progesterone receptor membrane component 1 (PgRMC1) (Bashour and Wray, 2012). Using calcium imaging in primary cultures from mouse brain explants, researchers discovered that activation of PgRMC1 rapidly attenuate GnRH neuron activity (Bashour and Wray, 2012).

1.3.1.3.3 Brain regions involved in the regulation of feedback mechanisms

Neuroanatomical studies have elucidated two important sites for the regulation of the feedback mechanisms: the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARN) (Smith, Cunningham, et al., 2005; Smith, Dungan, et al., 2005). Genetic-induced retrograde tracing with pseudorabies virus (PRV) in mice defined that the majority of ERα-expressing primary afferents to GnRH neurons reside in the AVPV and ARN (Wintemantel et al., 2006). Neuron-
specific ERα knockout mice do not display ERα expression in the AVPV, which is associated with total loss of c-fos expression in GnRH neurons and lack of LH release at the time of the LH surge (Wintermantel et al., 2006). Within the AVPV, neurons expressing the peptide kisspeptin are considered to be the primary mediators of positive feedback (Herbison, 2016). Kisspeptin neurons and kisspeptin signalling in GnRH neurons are critical for normal reproductive function and onset of puberty as demonstrated in humans (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012) and rodents (Funes et al., 2003; Seminara et al., 2003; Gottsch et al., 2004). A more detailed description of this important afferent neuronal group is addressed in the following section.

The excitability of GnRH neurons is highly regulated by neurotransmitter and neuropeptide transmission from the AVPV. Using parahorizontal brain slice preparation, which preserves the rPOA and AVPV connections in the mouse brain, researchers were able to show that AVPV neurons project functional monosynaptic connections to the majority of recorded GnRH neurons (Liu et al., 2011). This work showed that AVPV neurons transmit GABA and glutamate fast synaptic currents with low frequency stimulations (< 1 Hz), and the majority of monosynaptic transmission is GABAergic. They also found that female mice present lower stimulus threshold required to synaptically activate a GnRH neuron than males and higher frequencies (5–10Hz) relied upon kisspeptin signaling in both sexes (Liu et al., 2011). AVPV neurons are known to show a peculiar dual-phenotype expressing both vesicular GABA transporter (VGAT) and vesicular glutamate transporter 2 (VGLUT2), markers of GABA and glutamate neurons, respectively (Ottem et al., 2004). Therefore, it does not come as surprise that conditional knockout of ERα of either glutamate or GABA neurons in the limbic brain ablates the normal LH surge in mice (Cheong et al., 2015).
The ARN is considered an important hypothalamic site for the regulation of GnRH/LH pulses. Early studies, placing estrogen microimplants in the caudal part of the mediobasal hypothalamus (MBH) in sheep, demonstrated a significant reduction of LH pulses following estrogen infusion (Caraty \textit{et al.}, 1998). The ARN, which resides within the MBH, receives a robust dopaminergic innervation from the retrochiasmatic region of the hypothalamus A15 and controls the GnRH/LH pulse frequency in ewes (Thiéry \textit{et al.}, 1995). Genetic retrograde tracing with PRV, also revealed that ERα-expressing neurons in the ARN make synaptic contact with GnRH neurons, suggesting that ARN neurons could directly regulate GnRH neuron activity (Wintermantel \textit{et al.}, 2006). Electrical recordings of guinea pig brain slices revealed that estradiol treatment significantly decreased the activity of GnRH neuron fibers in the MBH and non-GnRH neurons residing in the ARN (Kelly, Ronneklev and Eskay, 1984). These findings indicate that estrogen acts as a strong downregulator of GnRH neurons through steroid-sensitive afferents residing in the ARN.

Progesterone-sensitive neurons in the ARN are proposed to regulate the pulsatile release of GnRH/LH. Progesterone-treated female rats display the expected restrain of the LH secretion, however, intra-ARN administration of RU486, a PR antagonist, increases LH pulse frequency (He \textit{et al.}, 2017). PNA mice, a PCOS mouse model, displays an increased LH pulse frequency that is associated with decreased expression of PR in the ARN (Moore \textit{et al.}, 2015). This indicates that PR signaling in the ARN is required for the normal functioning of the GnRH pulse generator. Although the nature of those neurons remains to be defined, GABA actions in the ARN seems to be critical to reduce GnRH/LH pulsatility in ewes (Jackson and Kuehl, 2002). These evidence suggest that progesterone actions in the ARN are key for the control of steroid-mediated negative feedback in the hypothalamus and disturbances in this region drive abnormal LH pulse secretion.
1.3.1.4  *The GnRH neuronal network*

GnRH receive a range of homeostatic cues through a diverse network of afferent neurons. The interaction of all these transynaptic inputs modulate GnRH neuron activity and to ultimately change GnRH/LH pulse secretion in order to orchestrate the control of fertility (Herbison, 2014). Although early morphological findings using electronic microscopy speculated that GnRH neurons displayed few synaptic inputs (Jennes, Stumpf and Sheedy, 1985), a genetic study using microarray profiling showed that GnRH neurons express at least 50 different receptors of neurotransmitters and neuropeptides (Todman, Han and Herbison, 2005). Multiple pharmacological studies investigating the effect of agonist and antagonist drugs of a variety of neurochemical molecules in the hypothalamic tissue or into the 3V, revealed that GnRH neuron activity is modulated by a vast list of signals (Campbell, 2007) (*Figure 1.7*).
Figure 1.7. An overview of the GnRH neuronal network. Schematic view of gonadotropin releasing hormone (GnRH) neuron and its main afferent neurons. The figure shows some of the recognized neuronal inputs on GnRH neurons based on morphological, pharmacological, electrophysiological and genetic studies. The transynaptic effect of each modulator is shown as activator (+) or inhibitor (-), although differences in technical approach can vary among studies showing contradictory outcomes (e.g.: GABA, serotonin). The brain regions that are known to allocate some of these inputs are represented as full drawn neuron such as RP3V and ARN, for kisspeptin neurons, dorsal raphe, for serotonin neurons, and locus coeruleus, for norepinephrine neurons. A more detailed description of the GnRH neuronal network can be found in Herbison, 2014. (Figure adapted from Campbell, 2007.)
Although the GnRH neuronal network includes many different neuronal phenotypes and the interactions within this network is complex (Dudas and Merchenthaler, 2006; Herbison, 2014), the present literature review introduces the three major afferent neuronal components considered to be critical for fertility: glutamate, kisspeptin and GABA neurons.

1.3.1.4.1 Glutamate neurons
Glutamatergic fibres are found in close apposition with GnRH neuron soma and terminals (Eyigor and Jennes, 1996; Kawakami et al., 1998) and glutamate is recognized to excite GnRH neurons (Maffucci and Gore, 2009). Glutamate can directly signal to GnRH neurons through the kainate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate receptor (NMDA) (Spergel et al., 1999; Kuehl-Kovarik et al., 2002; Suter, 2004). An early study reported that glutamatergic inputs on GnRH neurons play major role in the onset of puberty as daily injections of NMDA antagonists in prepubertal female rats are able to delay onset of puberty (Urbanski and Ojeda, 1990). Additional experiments found that infusion of NMDA antagonists blocked the estradiol-mediated LH surge in prepubertal ovariectomized rats (Urbanski and Ojeda, 1990). During the proestrus phase of the female rat estrous cycle, there is an increase of Ca$^{2+}$-dependent glutamatergic transmission via AMPA receptors and blocking this synaptic transmission decreases the LH surge (Tada et al., 2013). Therefore, glutamate transmission might be involved in the pubertal onset and the capacity to mount the LH surge in females.

Glutamate is also implicated in mediating estrogen-induced positive feedback in females. In proestrus phase of the estrous cycle, the administration of NMDA agonist increases GnRH mRNA levels in the rPOA of female rats (Gore et al., 2000). Likewise, the blockade of NMDA receptors attenuates gnrh gene expression and prevents the occurrence of the LH surge in female
rats (Suzuki, Nishihara and Takahashi, 1995). Estrogen actions decrease glutamatergic transmission to GnRH neurons during negative feedback by suppressing glutamate postsynaptic currents (PSCs) in female mice (Christian, Pielecka-Fortuna and Moenter, 2009), suggesting that a downregulation of the glutamatergic tone is needed for negative feedback. The requirement of estrogen signalling in glutamate neurons was elucidated recently through the deletion of ERα from glutamate neurons using Cre-LoxP technology in mice (Cheong et al., 2015). The researchers showed that ERα deletion in glutamate neurons generates infertile female mice with high basal LH levels, disrupted estrous cyclicity and impaired positive and negative feedback. Nevertheless, it is needed to note a caveat in these findings as glutamatergic neurons co-express other essential peptides for reproduction such as kisspeptin (Cheong et al., 2015), meaning that the ERα deletion from glutamate neurons has broad effects. So far, these evidence supports that glutamatergic inputs within the GnRH neuronal network are critical for HPG axis activation, regulation of GnRH levels and modulation of estrogen-mediated feedback mechanisms.

1.3.1.4.2 Kisspeptin neurons
The critical role of kisspeptin in reproduction was recognized after two independent clinical reports showing that mutations in the kisspeptin receptor GPR54 led to hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003). Basic research studies ultimately showed that mutations leading to the loss of kisspeptin production or its receptor in GnRH neurons causes infertility in mice, recapitulating the human condition (d’Anglemont de Tassigny et al., 2007; Lapatto et al., 2007; Kirilov et al., 2013). Kisspeptin is mainly found in neurons of two hypothalamic regions, the AVPV and the ARN (Smith, Cunningham, et al., 2005; Smith, Dungan, et al., 2005; Clarkson and Herbison, 2006; Franceschini et al., 2006). Neuroanatomical studies using viral tracing technique in mice support the idea that AVPV kisspeptin neurons project to both
the GnRH neuron soma and dendrites, whereas ARN kisspeptin neurons project solely to the GnRH neuron terminal regions (Hoong Yip et al., 2015).

Estrogen differently modulates the expression of the Kiss1 gene in the AVPV and ARN kisspeptin neuronal populations in females. The AVPV kisspeptin neurons show significant decrease of Kiss1 mRNA levels in gonadectomized female mice, while estrogen replacement significantly increases the levels of Kiss1 mRNA (Smith, Cunningham, et al., 2005). ERαKO mice do not exhibit the increase of kisspeptin content in AVPV following estradiol treatment to induce LH surge suggesting that a direct estrogentic effect controlling kisspeptin in this region (Smith, Cunningham, et al., 2005). Compared to female mice, males display a significant reduction of the number of kisspeptin neurons, which is dependent of testosterone actions during perinatal period (Clarkson et al., 2014). Additionally, an injection of kisspeptin antisera into the POA blocks the preovulatory LH surge and disrupts estrous cyclicity in female rats (Kinoshita et al., 2005). Studies also showed that c-FOS-positive kisspeptin neurons are found in the AVPV in mice at times of the LH surge, and the number of c-FOS kisspeptin neurons is strongly correlated with the number of c-FOS-positive GnRH neurons (Clarkson et al., 2008). Therefore, these findings suggest that, to date, RP3V kisspeptin neurons are the strong candidates to mediate positive feedback actions in the GnRH neuronal network.

In contrast to the findings for RP3V kisspeptin neurons, estrogen treatment decreases Kiss1 mRNA levels and castration has an opposite effect by increasing Kiss1 mRNA levels leading to the assumption that ARN kisspeptin neurons could be the site of estrogen-mediated negative feedback (Smith, Cunningham, et al., 2005; Smith, Dungan, et al., 2005; Smith et al., 2007). However, a controversial evidence challenged this view by showing that kisspeptin cell-specific deletion of ERα in OVX mice treated with estradiol regime normally suppress LH levels (Dubois
et al., 2015) and perhaps other hypothalamic neuronal populations are important to rely estrogen-mediated negative feedback. ARN kisspeptin neurons also express the tachykinin neurokinin B (NKB) and its receptor NK3. Both NKB and NK3 are critical to fertility since genetic studies showed that mutant patients for both genes exhibit hypogonadotropic hypogonadism (Topaloglu et al., 2009). ARN kisspeptin neurons also express dynorphin and its kappa opioid receptor (KOR), which inhibitory effects and were known to decrease LH secretion in female rats (Schulz et al., 1981). Therefore, the ARN neurons, which now carry the name of KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons, are hypothesized to be the core of GnRH pulse generation to ultimately dictate the pace of GnRH pulses (Navarro et al., 2009; Lehman, Coolen and Goodman, 2010; Goodman, Hileman, Casey C. Nestor, et al., 2013).

More recently, activation of ARN kisspeptin neurons with optogenetics revealed that specific activation of this population elicit pulse-like LH release (Han et al., 2015). This effect is found to be more pronounced in ovariectomized female mice than in diestrus females suggesting that ovarian steroid hormones are downregulators of the ARN kisspeptin neurons (Han et al., 2015). Likewise, optogenetic inhibition of ARN kisspeptin neurons decreases LH pulse frequency in ovariectomized female mice (Clarkson et al., 2017). These KNDy neurons also show an increase in burst firing in brain slices of castrated male mice, while estrogen treatment decrease it to the levels of intact animals (Vanacker et al., 2017). In summary, the current idea in the field is that ARN kisspeptin neurons are the central regulators of GnRH/LH pulses and gonadal steroid-mediated negative feedback.

1.3.1.4.3 **GABA neurons**

Among neurotransmitters, GABAergic neurons are a major source of inputs onto GnRH neurons, and virtually all GnRH neurons receive GABAergic innervation (Decavel and Van den Pol, 1990;
The role of GABAergic signalling in GnRH neurons has been extensively investigated since seminal work showing that injection of GABA into the third ventricle of the brain stimulated LH secretion without changing circulating FSH levels in male rats (Ondo, 1974). Following study demonstrated similar effect of GABA actions leading to LH release in female rats (Vijayan and McCann, 1978). Conversely, later investigations showed that bilateral injection of GABA into the medial preoptic area (mPOA) blocks the LH surge in female rats (Herbison and Dyer, 1991). Additionally, intravenous injection of muscimol, a GABA type A receptor (GABA\(_{\text{A}}\)R) agonist, decreases the basal multiunit activity (MUA) volley of neurons in the ARN, whereas bicuculline, GABA\(_{\text{A}}\)R antagonist, increases GnRH neuron activity and LH secretion (Hiruma, Sano and Kimura, 1994). In support of an inhibiting role for GABA on GnRH neurons, a decline in GABA release in the POA precedes the LH surge in rodents (Jarry, Perschl and Wuttke, 1988).

Introducing a detour in the field, electrophysiological studies using brain slices of GnRH-GFP mice defined a clear depolarizing effect of GABA and GABA\(_{\text{A}}\)R in GnRH neurons (DeFazio et al., 2002). Although these findings diverged from earlier reports, it is important to note that different experimental approaches could explain the divergent outcomes. Differences in GABA actions have been seen in \textit{in vitro} versus \textit{in vivo} techniques (Herbison and Moenter, 2011; Constantin et al., 2012; Constantin, Iremonger and Herbison, 2013). Furthermore, the origin of GABAergic inputs and the expression of neuropeptides within the GABA neurons might be important factors determining the response of GnRH neurons since GABA can facilitate or attenuate the transmission of other neuromodulators (Roberts, Hemond and Suter, 2008; Glanowska and Moenter, 2011; DeFazio, Elias and Moenter, 2014). Considering the extensive research on this subject, a more detailed literature about the importance of GABA-to-GnRH neuron circuit in reproduction is presented in the following review.
1.4 The GABA-to-GnRH neuron network

In the hypothalamus, the neurotransmitter GABA is present in around 50% of all synaptic inputs, and frequently colocalized with different neuropeptides (Decavel and Van den Pol, 1990). GABAergic transmission to GnRH neurons changes in different physiological conditions to guarantee reliable GnRH secretion (Leranth et al., 1985; Sim et al., 2000; Sullivan and Moenter, 2005). Therefore, it is not surprising that GABAergic signalling affects GnRH neurons at all stages of life, from developmental cell migration, through pubertal development adult regulation of fertility. A summary of the main findings featuring these aspects are discussed in the present section.

1.4.1 The GABA system & the developing GnRH neurons

Two homologous enzymes catalyse the synthesis of GABA, glutamate decarboxylase (GAD) 65 and GAD67. In the course of mouse embryonic development, GAD67 is expressed in the nonsensory epithelium/vomeronasal organ epithelium region, in which GnRH progenitor cells arise, while GAD65 is expressed mainly in the sensory proliferative zones (Vastagh et al., 2015). During early development a small population of GnRH neurons (~ 30%) produce GABA but cease the neurotransmitter production after the first day of postnatal development in rodents (Tobet et al., 1996). The overexpression of GAD67 in GnRH neurons leads to a misguided migration of GnRH cells inside the forebrain leading to disrupted of estrous cyclicity and fertility in adult mouse (Heger et al., 2003). According to these data, we can infer that GABA actions on immature GnRH neurons and the downregulation of the GABAergic signalling tone during development are crucial for a normal neuronal migration into the hypothalamus.
Downstream effects of the two main GABA receptors, GABA\textsubscript{A}R and GABA\textsubscript{B}R, also play a role in GnRH neuron migration. A pharmacological study in mice showed that treatment with GABA\textsubscript{A}R agonist muscimol during the 10\textsuperscript{th} to the 15\textsuperscript{th} day of pregnancy inhibits GnRH neuron migration and attenuates GnRH neuron fibre extension (Bless \textit{et al.}, 2000). In the same work, they reported that antagonizing GABA\textsubscript{A}R with bicuculline leads to an incorrect distribution of GnRH neurons in the basal forebrain. In addition, time-lapse analysis in mouse hypothalamic explants showed that GABA\textsubscript{A}R signalling is important to slowdown GnRH neuron migration in order to refine their directional movement and final organization in the hypothalamus (Casoni \textit{et al.}, 2012). Therefore, GABA\textsubscript{A}R-mediated actions in developing GnRH neurons define how these neurons travel from the nasal placode to the forebrain, extending their projections towards important target regions such as the ME.

1.4.2 GABA receptors & in the adult GnRH neuron

Two distinct classes of receptors, defined by their molecular nature and downstream cellular cascades, are the main targets of GABA. Firstly, GABA\textsubscript{A}R, a ligand-gated ion channel, and secondly the GABA\textsubscript{B}R, a metabotropic transmembrane receptor. Both GABA receptors are found in postsynaptic sites and GABA\textsubscript{B}R is also found at the presynaptic membrane (Bowery, 1989; Otis, Staley and Mody, 1991). GABA\textsubscript{A}R is composed by five protein subunits that can belong to 16 different molecular categories (α1–6, β1–3, γ1–3, δ, ε, θ, π), and functions as a selective channel for chloride ions (Cl\(^-\)) (Olsen and Sieghart, 2009). Distinctively, GABA\textsubscript{B}R is associated with G\(\alpha\)\_i/o proteins and regulates mainly inwardly rectifying potassium (K\(^+\)) channels (GIRK), voltage-gated Ca\(^{2+}\) channels and adenylyl cyclase-mediated pathways (Kandel \textit{et al.}, 2014). Notably, GnRH neurons expresses both GABA\textsubscript{A}R (Todman, Han and Herbison, 2005) and GABA\textsubscript{B}R (Zhang \textit{et al.}, 2009).
1.4.2.1 GABA$_A$R-mediated actions in GnRH neurons

Using the expression of LacZ reporter technology to identify GnRH neurons in mice, a report showed that GnRH neurons undergo region-dependent changes in the GABA$_A$R subunit composition from birth to adult life (Simonian et al., 2000). These changes in GABA$_A$R subunit expression over the course of postnatal development may affect neuronal migration and electrical behavior (Jung et al., 1998). Specifically, with post hoc identification of GnRH neurons by single cell RT-PCR, a great heterogeneity was found for $\alpha$ and $\beta$ subunits of GABA$_A$R in juvenile male and female mice (Sim et al., 2000). Although the combinations $\alpha 5\beta 1$, $\alpha 5\gamma 2$, $\beta 1\gamma 2$ and $\alpha 5\beta 1\gamma 2$ are unchanged during postnatal development, the expression of $\alpha 2$, $\alpha 4$ and $\beta 3$ decreases toward adulthood, while a marked elevation of $\gamma 2$ expression takes place in adult GnRH neurons (Sim et al., 2000). The relevance of $\gamma 2$ expression remains unclear since the genetic deletion of this subunit in GnRH neurons with a Cre-LoxP strategy has minimal impact on the reproductive function of female mice (Kiho Lee et al., 2010).

In most mature forebrain neurons, the activation of GABA$_A$R leads to inhibition of neuronal activity. GABA$_A$R are Cl$^-$ channels known for their inhibitory effects through the increase of Cl$^-$ conductance which hyperpolarizes the neuronal membrane (Baumann, Baur and Sigel, 2003). The main factor dictating the neuronal response to GABA$_A$R activation and channel opening in neurons is the intracellular Cl$^-$ concentration, which is dependent upon the expression of Na$^+$–K$^+$–2Cl$^-$ co-transporters (NKCCs) and K$^+$–Cl$^-$ co-transporters (KCCs) (Ben-Ari, 2002). In most instances, immature neurons show higher expression of NKCC1, which leads to the accumulation of intracellular Cl$^-$. This process generates a more depolarising reversal potential for Cl$^-$ when compared to the resting membrane potential and increases neuronal excitation when GABA binds to GABA$_A$R. During the development of many neurons in the brain, there is a molecular switch
leading to a decreased expression NKCC1 and an increase of the expression of KCC2, which ultimately drives an outward flux of Cl⁻ and a decline in intracellular Cl⁻ concentration (Herbison and Moenter, 2011). Accordingly, in this case the resulting effect of GABA biding to the GABAAR is a hyperpolarization of the neuronal membrane (Figure 1.8).

![Diagram of GABAAR effects](image)

**Figure 1.8. Depolarizing and hyperpolarizing GABAAR effects in neurons.** Intracellular Cl⁻ concentration drives the direction of the neuronal response by GABA binding to GABAAR. The expression of Cl⁻ cotransporters in neurons dictates the intracellular chloride concentration. NKCC1 is able to increase Cl⁻ inward flux, which brings the membrane potential to the depolarized reversal potential for Cl⁻ and, consequently, setting a depolarized membrane potential for GABA (green outlined box). In contrast, KCC2 can trigger Cl⁻ outward flux, leading to a significant decrease of intracellular Cl⁻ concentration and hyperpolarization of the neuronal membrane (red outlined box). The balance of NKCC1 and KCC2 seems to be a definitive characteristic by which GnRH neurons may respond differently to GABAAR activation when compared to other mature neurons in the forebrain. (Figure modified from Herbison and Moenter, 2011).
In contrast to most of mature neurons, adult GnRH neurons maintain a Cl⁻ homeostasis similar to immature neurons due to high expression of Cl⁻ accumulator proteins, such as NKCC1 anion exchanger 2 (AE2), and low levels of KCC2 (DeFazio et al., 2002; Taylor-Burds, Cheng and Wray, 2015). Early studies using GnRH-secreting immortalized neurons (GT1-7) report that GABAAR activation induces increased intracellular Ca²⁺ concentration and high frequency of Ca²⁺ oscillations (Hales, Sanderson and Charles, 1994). An influential study from DeFazio and colleagues, using GnRH-GFP mice, showed that activation of GABAAR leads to depolarization of GnRH neurons, independently of age, sex or gonadal steroid levels in female mice (DeFazio et al., 2002). In addition, important in vivo electrophysiological findings revealed that GnRH neuron firing and the different firing patterns that these neurons display are dependent upon GABAAR signaling (Constantin, Iremonger and Herbison, 2013).

1.4.2.2 GABA_BR-mediated actions in GnRH neurons

The role of GABA_BR has not been as extensively investigated as GABA_AR activation effects on GnRH neurons. Heretodimerization of R1 and R2 subunits allow the functioning of GABA_BR and, as mentioned above, this receptor mainly regulates neuronal activity by postsynaptic activation of GIRK channels or presynaptic inhibition of Ca²⁺ channels (Bowery, 1993). Electrophysiological recordings coupled with single cell RT-PCR revealed that R1 and R2 subunits are expressed in ~93% and 56% of GnRH neurons, respectively, in adult mice (Zhang et al., 2009). Constitutive loss of GABA_BR R1 generates mice with normal pubertal onset and basal LH levels, but disrupted estrous cyclicity and a significant decrease in female fertility (Catalano et al., 2005). The absence of functional GABA_BR in mice leads elevated GnRH secretion pulse frequency in hypothalamic explants from females but not males (Catalano et al., 2010). Together, these data indicate that GABA_BR is required for normal GnRH pulses and fertility in females.
Pharmacological in vitro studies elucidated the functional role of GABA<sub>B</sub>R activation in GnRH neurons. The administration of baclofen, a GABA<sub>B</sub>R agonist drug, induces a marked postsynaptic hyperpolarization of GnRH neurons through the activation of GIRK channels in a concentration-dependent manner (Zhang et al., 2009; Liu and Herbison, 2011). As mentioned in previous sections, with the development of a parahorizontally slice preparation in GnRH-GFP mice brains, which preserves the rPOA and AVPV regions, study showed that inputs from the AVPV are a source of GABAergic inputs onto GABA<sub>B</sub>R in GnRH neurons (Liu and Herbison, 2011). Interestingly, using this brain slice preparation, just 22% of GnRH neurons from males exhibit GABA<sub>B</sub>R inhibitory postsynaptic currents (iPSCs) whereas a larger number of neurons (70%) in diestrus females show the same response (Liu and Herbison, 2011). When looking at the effect of GABA<sub>B</sub>R activation in different GnRH neurons according to their location in the mouse brain, baclofen induces a strong decrease of spontaneous firing in neurons from the rPOA or AHA (Constantin et al., 2012). These studies suggest that GABA<sub>B</sub>R signaling is required in arresting the GnRH neuronal activity leading to a decrease in GnRH/LH pulses mainly in females.

1.4.3 GABAergic transmission in GnRH neurons

In mammals, the majority of synaptic inputs on GnRH neurons are GABAergic, defining a pivotal role of this neurotransmitter to regulate GnRH neuronal activity (Jennes, Stumpf and Sheedy, 1985; Jansen et al., 2003; Hrabovszky et al., 2012). Initially, GABAergic transmission to GnRH neurons was reported to be via phasic fast activation of GABA<sub>A</sub>R making GABA actions the main neurotransmitter component regulating the resting membrane of these neurons (Spergel et al., 1999; Sullivan, DeFazio and Moenter, 2003). Further studies using mouse brain slice preparation and the combination of different GABA<sub>A</sub>R blockers (gabazine ≤1µM, which blocks phasic currents; and bicuculline 20µM, which blocks all GABA currents), revealed that the majority of
GnRH neurons (> 90%) exhibit tonic GABA$_A$R-mediated currents and it is mandatory for GnRH neuron excitability (Bhattarai et al., 2011). This tonic GABAergic transmission leads to the hyperpolarization of GnRH neurons, it is mediated by extrasynaptic expression GABA$_A$R activation and partially controlled by the activity of glial and neuronal GABA transporters (Bhattarai et al., 2011). Even though both receptors can mediate GABAergic transmission, GABA$_A$R postsynaptic currents (PSCs) are the main contributors for information processing in GnRH neurons.

The expression of GABA$_A$R and GABA$_B$R might be differently positioned throughout the extent of GnRH neuron soma and dendrites. The full length of GnRH neurons seems to receive GABAergic contact, which has been investigated using transgenic GnRH-GFP mice and labelling of vesicular GABA transporter in order to visualize GABA inputs (Campbell, Han and Herbison, 2005; Moore et al., 2015). Keeping in mind that GnRH neurons can generate action potentials in dendrites, the integration of afferent inputs has been investigated in animal models and with computational modelling. A study comparing coronal (preserving the rPOA region) and thick near-horizontal (preserving the AHA region) mouse brain slices showed that stimulatory responses from GABA$_A$R are mainly observed in the coronal sections, whereas GABA$_B$R-mediated inhibitory actions are present in both slice preparations (Constantin et al., 2012). Therefore, the differential expression of GABA$_A$R and GABA$_B$R may drive a net excitation or a net inhibition in GnRH neurons (↑GABA$_A$R:↓GABA$_B$R expression in the cell body and proximal dendrites; and ↓GABA$_A$R:↑GABA$_B$R in the distal dendrite toward the ME).

Changes in miniature PSCs (mPSCs), which relate to action-potential independent neurotransmitter release, can be modulated by the gonadal steroid hormone milieu. These changes in GABA mPSCs in GnRH neurons are due to modifications in the rate of action potential
independent release of GABA with little effect observed on action potential-driven release (Sullivan and Moenter, 2005; Penatti et al., 2010; Pielecka-Fortuna and Moenter, 2010). This indicates that gonadal steroid actions are likely to modulate GABAergic transmission by changing the anatomical contact of GABA inputs to GnRH neurons rather than changing the activity of these GABAergic cells (Herbison and Moenter, 2011). These findings can be illustrated in the pathological scenario of a PCOS mouse model that presents impairment of negative feedback. As referenced in previous sections, PNA mice display chronic androgen excess, which is associated with increased GABAergic contact on GnRH neurons (Moore et al., 2015).

1.4.4 PCOS & GABA-to-GnRH circuit abnormalities

Evidence from clinical and pre-clinical models of PCOS indicate that PCOS is a neuroendocrine disorder. As reviewed in previous sections, the majority of PCOS patients (~70%) show increased LH secretion with a decreased sensitivity to ovarian steroids to decrease the circulating gonadotropin levels in blood (Manieri et al., 1992; Berga, Guzick and Winters, 1993; Pastor et al., 1998). This suggests that an impairment of negative feedback mechanism that is established in the context of androgen excess in PCOS drives an overactivation of GnRH neurons. It is suspected that increased GnRH neuronal activity leads to high GnRH pulsatile release causing enhanced LH pulse frequency secretion from the pituitary gland. Overstimulation of theca cells in the ovary by LH increases androgen production and disrupts ovarian follicular development. At the same time, androgen actions in the periphery leads to the development of PCOS symptoms such as hirsutism and acne, and influence the development of associated disorders, for instance, the metabolic syndrome (Dumesic et al., 2016; Moore and Campbell, 2017) (Figure 1.9).
Figure 1.9. An overview of the hypothalamic-pituitary-gonadal (HPG) axis in the pathophysiology of PCOS. (A) A neuroendocrine disorder: Clinical and basic science studies have considered PCOS as a neuroendocrine disorder according to its clinical manifestation. Impairment of steroid-mediated negative feedback can induce high frequency luteinizing hormone (LH) secretion, which is prevalent in PCOS patients and observed in animal models of the disease. Enhanced LH actions in the ovaries will increase ovarian androgen production and ovarian dysfunction. Evidence suggests that inappropriately high levels of androgens indirectly establish impaired negative feedback through steroid-sensitive hormones in the brain, which control gonadotropin-releasing hormone (GnRH)
neuron activity. Supporting evidence has shown that altered GABAergic drive and innervation onto GnRH neurons may underlie some of these pathological and ontogenetic features of PCOS (Figure adapted from Moore and Campbell, 2017). (B) PCOS women present increased LH secretion: Representative traces of pulsatile LH secretion from (i) normal woman (eumenorrheic) and (ii) PCOS woman (anovulatory hyperandrogenic) studied during 24-h of the follicular phase of menstrual cycle. The X axis shows clock time and the Y axis shows circulating LH levels in the blood. (Graphical data adapted from Berga, Guzick and Winters, 1993).

The evidence that androgen excess is highly associated with menstrual irregularities and ovarian dysfunction (Hatch et al., 1981; Fauser et al., 2012) indicates that, although GnRH neurons do not express the classical AR, the GnRH neuronal network can detect elevated levels of androgens in the blood and impact female fertility. Evidence from animal studies demonstrates that GABA neurons are able to sense circulating androgen levels and transmit this information to GnRH neurons. Studies using mouse brain slices showed that DHT, which has potent androgenic actions, enhances GABA\(_{\text{AR}}\)-mediated PSCs to GnRH neurons (Sullivan and Moenter, 2004) by increasing the number of synaptic contact between GABA neurons and GnRH neurons (Moore et al., 2015). Although progesterone is able to decrease GnRH neuron firing through GABAergic transmission, DHT disrupts progesterone actions and elevates neuronal excitation (Sullivan and Moenter, 2005; Pielecka, Quaynor and Moenter, 2006). Therefore, GABA neurons within the GnRH neuronal network might be important to convey feedback signals from steroid hormones and regulate GnRH neuron activity. Furthermore, chronic androgen excess, which is observed in PCOS women and animal models, may establish and maintain GABA-to-GnRH neuron circuitry abnormalities. It remains to be determined the ontogeny of the brain circuit abnormalities and whether GABAergic contact on GnRH neurons can be attenuated by AR signaling blockade.
A decreased sensitivity to progesterone administration to lower LH levels is also typical in PCOS women suggesting an impairment in negative feedback mechanisms (Pastor et al., 1998). PNA mice also display a desensitization of the GnRH pulse generator by showing a decreased post-castration response to inhibitory effects of progesterone (Moore et al., 2015). This correlates with a dramatic decrease of progesterone receptor (PR) expression in GABA neurons located in the ARN (Moore et al., 2015). Our research group discovered a novel pathway of ARN GABA neurons contacting GnRH neurons in the rPOA with additional evidence that ARN GABAergic wiring onto GnRH neurons was robustly increased in PNA mice (Moore et al., 2015). These morphological findings suggested that decreased PR expression in ARN GABA neurons might lead to an overactivation of GnRH neurons and, therefore, high LH pulse frequency, which underlie the pathophysiology of PCOS. It remains unknown when these changes in GABAergic wiring occur in relation to the pubertal onset, and whether these circuit abnormalities are dependent on circulating androgen levels during adulthood in PNA mice. The evidence that enhanced GABAergic contact on GnRH neurons might lead to abnormal LH secretion leads to the hypothesis that ARN GABA-to-GnRH neuron is a functional circuit, although further experimental work is required to unveil the biological relevance of this novel pathway.
1.5 Thesis rational and aims

PCOS is a highly prevalent endocrine disorder leading to female infertility, yet it is poorly understood. Impairment of the neuroendocrine regulation within the GnRH neuronal network may underlie the pathogenesis of PCOS, suggesting that brain circuit abnormalities are the culprit for the manifestation of the disease. Androgen excess during intrauterine life may induce a biological program in neural circuits which control GnRH neuron activity to manifest later in adult reproductive life. Accordingly, PCOS can be model by prenatal androgen exposure, which generates female offspring that present PCOS-like reproductive impairment as we observe in PNA mice. Transgenic technology and brain circuit tracing made possible to identify that GABAergic transmission and contact on GnRH neurons are enhanced in PNA and that is associated with high LH secretion, impaired negative feedback, and irregular reproductive cycles. My thesis aimed to address two main questions. Firstly, I interrogated the developmental timing of brain circuit abnormalities in relation to the development of PCOS and its dependency of androgen receptor signaling. Secondly, I aimed to investigate the functional relevance of the ARN GABA-to-GnRH neuron circuit for LH secretion in PCOS-like and reproductively fit conditions.

Therefore, my PhD specific objectives included:

1) Determine whether altered GABAergic wiring is established before the onset of puberty.
2) Examine the developmental timing of androgen excess (testosterone blood levels) in PNA mice.
3) Investigate the effect of long-term androgen signaling blockade on GABAergic contact on GnRH neurons in PNA mice.
4) Interrogate the functional role ARN GABA neurons in the regulation of LH secretion in mice.

5) Dissect the effect of activating ARN GABA neurons on LH secretion in PNA mice.

1.6 Hypothesis and objectives of the study

Firstly, I hypothesized that GABA-to-GnRH neuron circuit abnormalities take place before the onset of puberty in PNA mice. During the initial steps of my PhD project, I aimed to determine the ontogeny of brain circuit abnormalities that may underlie the pathogenesis of PCOS. Hence, I applied immunolabeling to identify GABAergic contact on GnRH-GFP neurons in PNA mice during prepubertal stage at postnatal day (PND) 25 in comparison to normal females (Chapter 3). Secondly, I hypothesized that brain circuit abnormalities are programmed by prenatal androgen insult but not from postnatal actions of androgen excess or the activation of hyperandrogenic ovaries during puberty. Therefore, I further characterized the time of increased testosterone levels in PNA mice and the requirement of hyperandrogenic ovaries during adulthood (Chapter 4). The third objective of my PhD project tests the hypothesis that long-term androgen receptor signaling blockade can reverse GABA-to-GnRH neuron circuit abnormalities and restore reproductive cycles in PNA mice (Chapter 5). Despite anatomical evidence that enhanced GABAergic wiring on GnRH neurons may be critical for the pathogenesis and maintenance of reproductive impairment, it remains unclear whether the activation of specific ARN GABA neurons has a functional role on GnRH/LH secretion. Finally, I hypothesized that the ARN GABA-to-GnRH neuron circuit is biologically functional to trigger LH secretion and that the activation of this circuit drives abnormal LH release in PNA mice (Chapters 6).
Chapter Two:

General Materials and Methods
Chapter Two

2.1 Animals

All protocols were approved by the University of Otago Animal Ethics Committee (Dunedin, New Zealand) under the protocol approval number 07/15. All mice (*Mus musculus*) were bred and housed at University of Otago - Hercus Taieri Resource Unit (Dunedin, New Zealand). Animals were housed with *ad libitum* access to water and food, under 12h: 12h light-dark cycles (lights on at 0700h) and at 21°C. All wild-type and transgenic mouse lines had a genetic background of C57BL/6(B6) inbred strain. Transgenic lines used in this study were GnRH-GFP mice (Spergel *et al.*, 1999), vesicular GABA transporter (VGAT)-cre (Vong *et al.*, 2011) and GnRH-cre (Wintermantel *et al.*, 2006). To confirm the genotype of transgenic mice lines, PCR was performed by Ms. Mel Prescott, the Associate Research Fellow in the Campbell Laboratory.

2.2 Mouse models of PCOS: Prenatally androgenized (PNA) mice

In order to generate PNA female mice, pregnant dams were treated with 250μg of dihydrotestosterone (DHT; s.c. 100 μL of solution) during late pregnancy (days 16, 17 and 18). Briefly, DHT was previously prepared by dissolving 25 mg of the solute (Sigma-Aldrich, St Louis, MO, USA) into 1 mL of absolute ethanol to make up a 10x stock solution. On the day of injection, one part of the DHT ethanol solution was diluted in nine parts of sesame oil to create 1x solution for use. Control groups were injected with 100 μL of vehicle sesame oil at the same period during late pregnancy (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Offspring were weaned at the day 21 and separated by sex into different cages until experimentation.
2.3 Estrous cycle monitoring

Daily monitoring of vaginal smears was performed from 0700h to 0900h with a looped wire to collect aqueous vaginal cytology smears on glass slides. Smears were left to dry and stained with toluidine blue (0.5% v/v) for one minute. Using a light microscope with 10X and 20X objectives, vaginal cytology was examined to identify estrous cycle stages. Briefly, proestrous smears presented nucleated clustered cells without any presence of leucocytes; the estrous smears displayed only anucleated cornified epithelial cells; the metestrous stage presented a combination of cornified, leucocytes and few nucleated cells; and finally, the diestrous stage was identified as having high number of leucocytes without any cornified cells (Caligioni, 2009).

2.4 Pharmacological treatment: Long-term AR signaling blockade with flutamide

Pharmacological treatment with flutamide (Flut), an androgen receptor (AR) antagonist, was performed in studies shown in Chapter 5. Female mice were injected daily with 50 μL of Flut (Flut; Sigma-Aldrich, St Louis, MO, USA) 25 mg/kg/day for 20-22 days (Chapter 5). Animals received injections from postnatal day (PND) 40 up to at least PND 60, after which mice were euthanized in diestrous stage of their cycle. Briefly, 0.10 g of Flut was diluted in 1 mL of absolute ethanol to make up a 10x stock solution in an Eppendorf tube. On a daily basis, one part of Flut ethanol solution was diluted in nine parts of sesame oil to create a 1x solution for use. Control vehicle mice received 50 μL of sesame oil during the same period. All calculations were based on mice with an average weight of 20g. In order to determine the effect of Flut treatment, all female mice had estrous cyclicity followed daily. The dose of Flut used here was determined based on previous optimization in our laboratory and studies elsewhere (S Luo et al., 1997; Sullivan and Moenter,
Chapter Two

2004; Renier et al., 2015). See more information about Flut treatment optimization in Appendix III.

2.5 Surgeries

2.5.1 Ovariectomy

For experiments that determined the effect of ovariectomy on LH secretion in control and PNA mice (Chapter 4), ovariectomy surgery was performed as approved by the University of Otago Animal Ethics Committee. Female mice were anesthetized with 2% isoflurane and bilateral excision of the ovaries. Experiments were performed with sterile instruments and aseptic conditions. Mice were given a s.c. injection of carprofen (5 mg/kg body weight) after anesthesia was confirmed. Mice were placed in the prone position on a heating pad and the area of incision was prepared by shaving the hair and applying hibitane solution 4% to the skin. A dorsal midline incision was made through the skin below the ribs and followed by two small internal incisions through the muscular layer of the body wall bilateral to the backbone. The ovaries were exteriorized and the distal uterine horn was clamped just below the ovary. The ovaries were excised and the uterine horn was gently moved back into the body cavity. The skin incision was sutured and closed with one wound staple. The surgical procedure had an average duration of six minutes and approved post-surgical care was performed.

2.5.2 Stereotaxic intracerebral injections

Mice were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus (Stoeling®, Digital Lab Standard). The area of incision was prepared by removal of hair from the head, between the ears, and application of hibitane solution 4% to the skin. The eyes were kept protected with
lubricant mineral oil to avoid keratitis during surgery. Experiments were performed with sterile instruments and aseptic conditions. Mice were given a s.c. injection of carprofen (5 mg/kg body weight) after anesthesia was confirmed. A small midline incision was made through the skin with scalpel blade and the connective membranes were removed from the skull with a sterile cotton bud for better visualization of skull landmarks. The top of the mouse skull was positioned in horizontal plane by aligning the nasal suture, bregma and lambda using a dissection microscope (20X; Leica®, Wetzlar, Germany). Bregma position was used as stereotaxic reference zero and X and Y coordinates were calculated according to the desired target. Anterior-posterior (A/P), medial-lateral (M/L) and dorsal-ventral (D/V) measures were based on the mouse brain atlas (Paxinos and Franklin, 2004).

Experiments that performed viral injections used coordinates for the arcuate nucleus (ARN) and median eminence (ME) of the hypothalamus. Optogenetic experiments used coordinates for the rostral preoptic area (rPOA) in order to place optical fiber in the vicinity of GnRH neurons (Table 2.1).

<table>
<thead>
<tr>
<th>Target</th>
<th>A/P</th>
<th>M/L</th>
<th>D/V</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARN bilateral</strong></td>
<td>- 0.9 mm</td>
<td>± 0.3 mm</td>
<td>- 6.0 mm (from the dura mater).</td>
</tr>
<tr>
<td><strong>ME bilateral</strong></td>
<td>- 1.0 mm</td>
<td>± 0.3 mm</td>
<td>- 6.2 mm (from the dura mater).</td>
</tr>
<tr>
<td>rPOA</td>
<td>+ 0.9 mm</td>
<td>0 (pulling the sagittal sinus to the lateral side while injecting)</td>
<td>4.2 mm (from the dura mater).</td>
</tr>
</tbody>
</table>

**Table 2.1. Coordinates for stereotaxic intracerebral injections.** Bregma position was used as stereotaxic reference zero and X and Y coordinates were calculated according to the target. Measurements were used with mice displaying 22-25g of body weight and adaptations of coordinates were made when animal weight were outside of this range.
A small hole in the skull was made with surgical drill marking the injection site without damaging the brain parenchyma. Customized bilateral Hamilton needles or an optical fiber (Chapter 6) were lowered with D/V coordinates from the level of the dura mater. When performing viral transfections, needles were left in the site for five minutes prior to the injection. The injection rate was 50 nL per minute and needles were left in the target area for ten minutes following injection. Slow withdrawal of needles was performed after viral injections and any local bleeding was contained with sterile cotton swaps and saline. The skin was closed with surgical sutures and mice were place in temperature-controlled cages for post-operative management according to approved procedures by University of Otago Animal Ethics Committee. When performing optogenetic experiments, optical fiber was placed in the brain target thirty to forty minutes before the optogenetic protocol was initiated in anesthetized mice. Mice were euthanized at the end of all protocols with a lethal dose of pentobarbital (i.p. 3 mg/mL) and perfused with 20 mL of 4% paraformaldehyde (4% PFA) solution.

2.6 Serial blood sample collection

Mice were habituated with daily handling to avoid further distress during blood sampling. Blood samples were collected from a small cut in the mouse tail-tip. When performing sampling to determine the developmental timing of testosterone levels (Chapter 4), ~ 50 µL of blood was collected into glass capillary tubes at the same time in the morning (0900h to 1000h). Blood samples were spun at 4 G for 10 min at room temperature, and plasma was collected and kept at -20 °C until the day of hormone measurement. When collecting blood to measure pulsatile LH secretion (Chapters 4 and 6), mice were kept in a quiet room in the presence of two experimenters. As previously reported (Steyn et al., 2013; Czieselsky et al., 2016), 3 µL of blood were collected.
every 6 min for 2 h. Whole blood was diluted in 57 µL of 0.1 M phosphate buffered saline-tween (PBS-T) pH 7.4, frozen in dry ice and stored at -20 ºC for subsequent hormone measurement.

2.7 Hormone measurements

2.7.1 Testosterone levels with enzyme-linked immunosorbent (ELISA)

Plasma testosterone levels were determined using a commercially available rat/mouse ELISA kit (LDN GmbH&Co.KG, Nordhorn, Germany). Briefly, the testosterone mouse ELISA is based on the principle of competitive binding. A defined amount of testosterone conjugated with horseradish peroxidase and the steroid from the sample compete for a binding site in the coated testosterone antiserum present in the plate. The assay was performed with a sensitivity of 0.05 ng/mL and the intra-assay coefficient of variation for testosterone was 11.4 %.

2.7.2 LH sandwich ELISA method

A well establish sandwich ELISA method was used to determine pulsatile LH secretion in mice (Steyn et al., 2013; Czieselsky et al., 2016). Briefly, a 96-well high affinity binding plate (Corning®) was coated with 50 µL per well with bovine LHβ518B7 monoclonal antibody (1:1,000 in 1X PBS). Following an overnight incubation, plates received 200 µL per well of blocking buffer solution (5 % skim milk power in 0.05% PBS-Tween) and were incubate for 2h in a humidified chamber on an orbital shaker at room temperature. After incubation, plates were washed with 0.05% PBS-Tween (3 x 3 min washes). A standard curve for the detection of LH concentration was generated using serial dilutions of mouse LH-RP reference peptide provided by A. F. Parlow (National Hormone ad Pituitary Program, Torrance, CA, USA). Standards, positive controls and samples were incubated in the plates for 2h in a humidified chamber on an orbital shaker at room
Chapter Two

2.8 Immunohistochemistry

2.8.1 Preparation of tissue perfusion and fixation

Mice were anesthetized with 100 µL of lethal dose injection of pentobarbital (i.p. 3 mg/mL) until animal lacked a pedal reflex upon pinching the paws. Animals were placed in the supine position and the abdominal cavity was opened and exposed. A larger incision was made to expose the heart. The apex of the heart, aiming the left ventricle, was inserted with a 23 gauge needle and 20 mL of paraformaldehyde 4% (PFA 4%) solution was slowly injected to perfuse the tissues. Following perfusion, brains and ovaries were dissected and post-fixed in the same PFA 4% fixative solution for 1 hour or overnight, respectively, at room temperature. Brains were placed in a sucrose
cryoprotectant solution at 4°C overnight. Coronal brain sections were cut into three series of 30-µm thick sections using a microtome (Leica®, Wetzlar, Germany). Brain sections were kept in cryoprotectant solution (pH = 7.6) at -20°C until the immunohistochemistry protocol was performed. Following post-fixation, ovaries were kept in a 70% ethanol solution until paraffin embedding. Ovarian sections of 5 µm-thickness were collected in 50-µm intervals from one randomly chosen ovary. All ovarian sections were stained with hematoxylin and eosin. A detailed list of chemical reagents for each solution is provided in Appendix I.

2.8.2 Dual-label immunofluorescence

Brain sections were washed three times (10 min each washing) in 0.05M Tris-buffered saline (TBS) to remove cryoprotectant from the tissue. Sections were incubated with blocking solution (2% normal serum of the species in which secondary antibody was made) for 30 min in order to reduce non-specific binding. Next, sections were incubated with primary antibodies in incubation solution for 48h at 4°C. After the incubation period, sections were washed in TBS and placed in secondary antibody incubation solution. The protocol used biotinylated secondary IgG raised against the species of the primary antibodies. Tissues were incubated in 1:200 dilution of secondary antibodies for 90 min at room temperature on an orbital shaker. Next, sections were incubated with fluorophore-conjugated streptavidin at a 1:200 dilution in incubation solution for 3h at room temperature. After incubation, sections were washed with TBS (four times; 10 min each washing), mounted on glass slides, air-dried and coverslipped with CC/mounting medium (Sigma-Aldrich, St Louis, MO, USA).
2.8.3 Controls for the immunohistochemistry protocol

Validations of primary antibodies used in the project were found in the scientific literature and are listed in appendix II. The absence of primary antibody during first incubation procedure served to test the specificity of primary antibodies labeling and the background produced by secondary antibodies alone.

2.9 Image acquisition and analysis

Confocal imaging was performed using a Nikon A1R Multi-photon confocal microscope with 488, 543 and 647 nm diode lasers. Brain sections were imaged using a Plan NeoFluor 20X or 40X oil objective (1.30 NA), taking Z-stacks of 0.5 μm steps (pinhole at 1 AU) and analyzed with software NIS-Elements AR 4.00.00 (Nikon® Instruments Inc.). Specific confocal image acquisitions procedures and 3D reconstruction are referenced in each chapter separately. The ovarian sections were imaged using light microscope (Olympus Bx-51; Olympus Optical, Hamburg, Germany) with bright-field mode and SPOT advance camera software (Diagnostic Instruments, Inc.) for image acquisition. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

2.10 Statistical analysis

Statistical analysis was performed with PRISM software 7.0 (GraphPad Software, La Jolla, San Diego, CA, USA). All statistical tests are present in the methods section within each chapter. All data are represented as mean ± SEM. Statistical significance was accepted when \( P < 0.05 \). Graphical representation to display significant different are shown as * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) and **** \( P < 0.0001 \).
Chapter Three:

Ontogeny of altered GABAergic innervation of GnRH neurons in PCOS
3.1 Introduction

The pathogenesis of polycystic ovary syndrome (PCOS) is likely to involve both genetic and *in utero* environmental factors. Although the etiology of PCOS is undefined and likely to be multivariate (Barber *et al.*, 2015; Azziz *et al.*, 2016), exposure to elevated androgens in early development is known to trigger reproductive and metabolic traits of PCOS during adult life in both humans (Hague *et al.*, 1990; Barnes *et al.*, 1994) and in a variety of animal models (Dumesic *et al.*, 1997; McNeilly and Duncan, 2013; Padmanabhan and Veiga-Lopez, 2013a). Clinical findings have found that daughters of PCOS mothers, who most likely have elevated androgen levels during pregnancy, are at higher risk of developing PCOS features from early to late puberty (Sir-Petermann *et al.*, 2009; Maliqueo *et al.*, 2012). This is also a significant association between women with congenital adrenal hyperplasia (CAH) and the development of PCOS. CAH women exhibit abnormal overfunction of adrenal gland leading to elevated prenatal androgen levels and, during adulthood, display PCOS symptoms such as polycystic ovaries, hyperandrogenism and elevated LH levels (Hague *et al.*, 1990; Barnes *et al.*, 1994).

Evidence suggests that inappropriately high levels of androgens indirectly establish impaired negative feedback in PCOS patients. Acute testosterone administration does not increase pulsatile LH levels in healthy women (Dunaif, 1986). Likewise, acute blockade of the androgen receptor (AR) antagonist does not restore normal LH secretion in women with PCOS (Eagleson *et al.*, 2000). However, long-term treatment with an AR antagonist is able to re-establish the ability of estradiol and progesterone to suppress LH pulse frequency (Eagleson *et al.*, 2000). This implies that chronic androgen excess does not directly drive LH hypersecretion, but instead interferes with the ability of estradiol and progesterone to relay negative feedback signals within the GnRH neuron network, and leads to regulate high GnRH/LH release.
Prenatally androgenized (PNA) mice recapitulate the clinical features of PCOS, including androgen excess, impaired sex hormone feedback and enhanced LH secretion (Sullivan and Moenter, 2004; Roland and Moenter, 2011; Moore, Prescott and Campbell, 2013). Considering that hypothalamic brain circuits are highly shaped by steroid actions during prenatal development (Nuñez, Alt and McCarthy, 2003; McCarthy, Nugent and Lenz, 2017), prenatal androgen excess may drive the neuroendocrine impairment within the gonadotropin releasing hormone (GnRH) neuronal network in a PCOS-like condition. PNA mice also enable the use of transgenic tools to investigate the impact of androgen actions in the brain. Evidence to date has identified that PCOS traits in PNA mice are coincident with modifications in the GnRH neuronal network. GnRH neurons have significantly enhanced GABAergic transmission (Sullivan and Moenter, 2004) due to an increased GABA innervation (Moore et al., 2015; Moore and Campbell, 2016). In support of these pre-clinical findings, PCOS women exhibit elevated GABA levels in the cerebrospinal fluid (Hu et al., 2011; Kawwass et al., 2017) and larger serum concentrations of positive modulators of GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) activity such as allopregnanolone (Hedström et al., 2015). Whereas GABA typically acts as an inhibitory neurotransmitter, GnRH neurons can respond to GABA with depolarization via GABA<sub>A</sub>R activation (DeFazio et al., 2002; Herbison and Moenter, 2011). These observations suggest that increased GABA signaling in PCOS may be responsible for driving the hyperactive GnRH/LH system, which in turn overstimulates androgen production by the ovaries. However, little is known about the temporal mechanisms mediating this vicious cycle of brain abnormalities and androgen excess in the development and pathophysiology of PCOS.

These findings have led to important questions about the development and maintenance of identified PCOS-associated brain circuit abnormalities. It is unclear whether altered brain innervation is driven directly by PNA or indirectly by hyperandrogenism in adulthood. The present Chapter aimed to test the hypothesis that GABAergic contact on GnRH neurons is already altered
before pubertal onset in PNA mice. Immunolabeling of vesicular GABA transporter (VGAT) appositions to GnRH neurons in GnRH-GFP mice was used to visualize GABA inputs on GnRH neurons and investigate the timing of PNA-induced brain wiring remodeling. I also aimed to determine whether GABA-to-GnRH neuron abnormalities require prepubertal androgen excess. Therefore, circulating testosterone plasma levels were measured on postnatal day 25, prior to the onset of puberty in control and PNA mice.

3.2 Methods

3.2.1 Animals

Prepubertal GnRH- GFP mice at postnatal day (PND) 25 were used in all of experiments. GnRH-GFP mice express green fluorescent protein (GFP) under the control of GnRH promoter (Spergel et al., 1999) and have been broadly used to investigate developmental and electrophysiological aspects of GnRH neurons (Suter et al., 2000; Constantin, Iremonger and Herbison, 2013; Moore et al., 2015). Mice were housed at the Hercus Resource Unit (University of Otago - Dunedin) under 12h: 12h light-dark cycles (lights on at 0700h) with ad libitum access to water and food. To generate PNA mice, we used established protocols as previously described in the General Methods Chapter and from studies elsewhere (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Briefly, time-mated pregnant dams were injected with 100 µL of dihydrotestosterone (250 µg) in oil, or oil alone to serve as a control, on gestational days 16, 17 and 18.
3.2.2 Blood sampling and hormone measurements

3.2.2.1 Blood collection and tissue preparation

At PND 25, control (N = 8) and PNA (N = 13) GnRH-GFP female mice were euthanized by an overdose of sodium pentobarbital (30 mg/mL). Blood was collected from the vena cava just prior to transcardial perfusion with buffered-parafomaldehyde 4% (0.1 M phosphate buffer-PFA 4%; pH 7.6). Brains were dissected, post-fixed for 1 h at room temperature, and saturated in 30% Tris-buffered sucrose at -4 °C until processed for immunohistochemistry. Fixed brain tissue was cut into three series of 30-µm-thick coronal sections using a freezing microtome (Leica®, Wetzlar, Germany).

3.2.2.2 ELISA

Testosterone plasma levels were determined using a commercially available ELISA kit (LDN GmbH&Co.KG, Nordhorn, Germany). This assay is a well-established mouse/rat ELISA with reported specificity and reproducibility (found at LDN Immunoassays and Services reference: AR E-8000). Intra-assay coefficient of variation for testosterone was 11.4 %. LH levels were determined using well-established ELISA method as previously reported (Steyn et al., 2013; Moore et al., 2015; Czieselsky et al., 2016). A mouse LH-RP reference was provided by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). The assay sensitivity of the LH ELISA was 0.002 ng/mL and intra-assay coefficient of variation was 6.2%.

3.2.3 Immunohistochemistry

Coronal brain sections throughout the rostral preoptic area (rPOA) and containing the organum vasculosum of lamina terminalis (OVLT) of the hypothalamus were identified according to the
mouse brain atlas (Paxinos and Franklin, 2004). Free-floating immunohistochemistry was performed as previously described (Cottrell et al., 2006; Moore et al., 2015). Primary antibodies were used in the respective concentrations: polyclonal chicken anti-GFP (1:2,500, Aves Labs Inc.) and polyclonal rabbit anti-VGAT (1:750, Synaptic Systems). Brain sections were incubated with both primary antibodies in cocktail with 2% normal goat serum for 48 h at -4 °C. AlexaFluor488 goat anti-chicken (1:200) and Streptavidin568 (1:200, Molecular Probes, Invitrogen) secondary antibodies were used. Primary antibody omission served as a negative control.

3.2.4 Confocal imaging acquisition and analysis

Confocal imaging was obtained with a Nikon A1R Multi-photon confocal microscope with 488 and 543 nm diode lasers. Brain sections were imaged using Plan NeoFluor 40 x oil objective (1.30 NA), taking Z-stacks of 0.5 μm (pinhole at 1 AU) and analyzed with software NIS-Elements AR 4.00.00 (Nikon® Instruments Inc.). GnRH neurons and VGAT-immunoreactive (ir) puncta were better visualized with 3x zoom function (Nikon® Instruments Inc.) Following double immunofluorescence (VGAT-ir/GFP-ir) protocol, quantification of VGAT appositions on GnRH neurons was performed in two representative sections from the rPOA/OVLT region of the hypothalamus of control (N = 4) and PNA (N = 5) mice. From each animal, 10 to 12 GnRH neurons were randomly selected across 2 sections and scanned for analysis. Unipolar and bipolar GnRH neurons were selected randomly, and multipolar GnRH neurons were excluded from this analysis. VGAT apposition density contacting GnRH neuron soma and along 75 μm of the dendritic length were performed as previously reported (Moore et al., 2015). Briefly, the number of VGAT contact was counted manually and the density is calculated by considering the number of appositions per dendritic length. Values were averaged for the 10-12 GnRH neurons in each animal and used for statistical analysis.
3.2.5 Statistical analysis

Statistical analysis was performed with PRISM software 7.0 (GraphPad Software, San Diego, CA, USA). Serum testosterone level values were first tested for normal distribution with D'Agostino-Pearson’s normality test. Further evaluation of prepubertal PND 25 testosterone levels between control and PNA mice was performed with a two-tailed Student’s t test. Analysis of VGAT appositions onto GnRH neurons from prepubertal control and PNA mice used a Mann-Whitney U test. Graphical representation to display significant different are shown as * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) and **** \( P < 0.0001 \).

3.3 Results

3.3.1 Circulating testosterone levels are normal prior to the onset of puberty in PNA mice

Confirming that PND 25 animals were prepubertal, all control and PNA mice exhibited a complete absence of vaginal opening and LH levels were undetectable (<0.04 ng/mL). In order to determine whether PNA treatment induces early androgen excess, testosterone plasma levels were measured before pubertal onset at PND 25 from control (N = 8) and PNA (N = 13) mice with ELISA. In addition, circulating testosterone levels were not different between PNA and control groups (PNA: 0.20 ± 0.01 vs. control: 0.22 ± 0.03 ng/mL; Student’s t test) (Figure 3.1).
**Figure 3.1. Prepubertal PNA mice have similar testosterone blood levels.**

Testosterone blood levels from control (N = 8) and PNA (N = 13) mice at PND 25. Two-tailed Student’s *t*-test.

### 3.3.2 Morphological criteria to identify GABAergic inputs on GnRH-GFP neurons

Morphological analysis was performed with confocal projections of 45 GnRH-GFP neurons from control (N = 4) and 51 GnRH-GFP neurons from PNA (N = 5) mice. In agreement with other studies (Jennes, Stumpf and Sheedy, 1985; Herde and Herbison, 2015), GnRH neurons were mainly found to be unipolar or bipolar. Dendrites of bipolar GnRH neurons were classified as primary or secondary dendrites based on previous reports (Herde and Herbison, 2015; Moore *et al.*, 2015). Briefly, thicker dendrites with larger cross-sectional area leaving from the point of origin at the soma were classified as primary dendrites. Typically, on the opposite pole of the cell from the primary, the secondary dendrite were identified as the thinner process, with smaller cross-sectional area when compared to primary dendrite (**Figure 3.2**).

GABAergic contact on GnRH neurons was identified with immunostaining of VGAT puncta in contact with the GnRH-GFP membrane. Confocal analysis showed that all scanned
GnRH neurons showed VGAT appositions at the soma and dendrites. Close appositions of VGAT immunoreactive (-ir) puncta to GFP-ir GnRH neurons in the rostral preoptic area (rPOA) of the hypothalamus, indicated putative GABA synaptic inputs (Edwards et al., 1997; Cottrell et al., 2006). The locations of putative GABA synapses were classified as onto spines, crucial membrane structures which harbor excitatory inputs (Harris et al., 2013; Oh et al., 2016), or onto the somatic or dendritic shaft membrane without spines, here classified as non-spiny membrane (Figure 3.2).

**Figure 3.2. GABA inputs on prepubertal GnRH neurons.** Representative example of confocal image acquisition of a prepubertal GnRH neuron from a control mouse. In PND 25 mice GnRH neurons were mostly unipolar or bipolar neurons. Overlap of red (VGAT) and green (GnRH-GFP) provides yellow puncta, which indicates putative contact. The image on the right depicts the neuronal compartments of bipolar GnRH neurons. The thicker dendrite (with largest cross-sectional area leaving the soma) is classified as primary dendrite and the
Chapter Three

A thinner dendrite is classified as the secondary dendrite (Scale bar = 10 µm). In the second column, high magnification images show putative GABA inputs onto GnRH neuron spines (blue arrow head) and onto the non-spiny membrane (white arrow heads). GABA inputs are evident on the distal (I) and proximal (II) primary dendrite, soma (III) and secondary dendrite (IV) (Scale bar = 2 µm).

3.3.3 GnRH neurons receive increased GABAergic innervation in prepubertal PNA female mice

As reported previously (Moore et al., 2015), the total density of VGAT-ir appositions to GnRH neurons within each neuronal compartment and the number of GnRH neuron spines were evaluated in control and PNA mice (Figure 3.3 A). At PND 25, the total density of VGAT appositions to GnRH neurons was significantly increased in PNA mice when compared to controls ($P < 0.05$; Mann-Whitney $U$ test; Figure 3.3 B). In addition, the densities of VGAT appositions to GnRH neurons in all three neuronal compartments were significantly elevated ($P < 0.05$; Figure 3.3 C). The total number of GABA inputs to the non-spiny membrane of the soma and dendrites were also significantly higher in prepubertal PNA mice than in controls ($P < 0.05$; Figure 3.3 D), and specifically at the level of the soma ($P < 0.05$) and primary dendrite ($P < 0.05$) (Figure 3.3 E). The total density of VGAT appositions to GnRH neuron spines was similar between groups ($P = 0.19$; Figure 3.3 F); however, a greater number of GABAergic inputs on GnRH neuron somatic spines was detected in prepubertal PNA mice when compared to controls ($P < 0.05$; Figure 3.3 G). The total number of GnRH neuron spines was evaluated and results showed that it was similar between groups (PNA: $0.24 \pm 0.01$ vs. control: $0.23 \pm 0.03$ spines/µm). These findings revealed that prenatal androgen exposure is sufficient to program circuitry changes in the female GnRH neuronal network prior to the postpubertal development of androgen excess and reproductive deficits in PNA mice.
Figure 3.3. Increased GABAergic contact to GnRH neurons is present in prepubertal PNA mice. (A) Examples of confocal images of control (N = 4 / 45 neurons) and PNA (N = 5 / 51 neurons) mice displaying GnRH-GFP neurons (green) and VGAT-ir (red puncta) in the rPOA (Scale bar = 10 µm). Overlap of red and green provides yellow puncta, which indicates putative contact. Projected confocal images show 1.15 µm-thick stack details of VGAT appositions at the level of GnRH neuron soma (I) and dendrite (II and III). White arrowheads point to putative GABA inputs onto the non-spiny GnRH neuron membrane and blue arrowheads show GABA inputs onto spines (Scale bar = 5 µm). Histograms depict total VGAT-ir apposition density on GnRH neurons (B) and per neuronal compartment (C). Total VGAT-ir apposition density onto the non-spiny GnRH neuron membrane (D), and per neuronal compartment (E). Total VGAT-ir apposition density onto GnRH neuron spines (F), and per neuronal compartment (G). Histogram values are represented as mean ± SEM with dot plot of individual values. *P < 0.05; Mann-Whitney U test.

3.4 Discussion

The present study aimed to unveil the ontogeny of altered brain wiring in relation to the onset of puberty in a PCOS-like condition as recapitulated in PNA mice. Clinical and pre-clinical studies have shown that prenatal androgen exposure results in impaired adult reproductive function during. However, it is unclear how this dysfunction develops and whether changes in the HPG axis occur before pubertal onset and the manifestation of hyperandrogenism. These present findings indicate that a short prenatal androgen insult induces an abnormal increase in GABAergic innervation to GnRH neurons prior to pubertal development. These data also suggest that GABA-to-GnRH neuron abnormalities might be independent of elevated circulating levels of testosterone in PNA mice. With increasing evidence that the brain might be the primary site of PCOS pathogenesis (Moore and Campbell, 2017), the present discovery suggests that in utero androgen actions lead to the installation of a biological program in the female brain to develop the disease during adulthood.
Clinical studies have shown that the placenta of PCOS women exhibit alterations such as increased activity of 3β-hydroxysteroid dehydrogenase type 1 and decreased P450 aromatase activity (Maliqueo et al., 2013) allowing the establishment of elevated androgen levels during late gestation (Sir-Petermann et al., 2002). In addition, altered androgen milieu caused by CAH, which generates a hyperandrogenic in utero condition, induces PCOS features later in life (Hague et al., 1990; Barnes et al., 1994). Therefore, androgen actions in the prenatal brain may induce the development of PCOS. Our laboratory and others have used PNA treatment to induce PCOS-like condition in mice, which enable us to evaluate the impaired reproductive function without major metabolic traits associated with the disease (Sullivan and Moenter, 2004; Roland et al., 2010; Moore, Prescott and Campbell, 2013). Although there is evidence that prepubertal androgen exposure in non-human primates promote hyperandrogenism and elevated LH secretion during early adulthood (McGee et al., 2012), our findings suggest that this might not be the case for PNA mice. Similar to clinical studies (Crisosto et al., 2007; Villarroel et al., 2015) and consortium update (Ibáñez et al., 2017), these findings suggest that androgen excess may occur during pubertal development and maturation of the HPG axis but not prior to pubertal onset.

Our research group previously reported that adult PNA mice exhibit enhanced steroid-sensitive GABAergic innervation onto GnRH neurons, which is associated with high LH pulse secretion and impaired ovarian steroid-mediated negative feedback (Moore, Prescott and Campbell, 2013; Moore and Campbell, 2016). In the same manner, PCOS women display similar neuroendocrine disturbances which reflect a desensitization of the pulse generator driving GnRH/LH secretion to steroid hormone feedback (Blank, Mccartney and Marshall, 2006). Importantly, a clinical study showed that hyperandrogenic girls with PCOS exhibit a reduced sensitivity to decrease LH pulsatile secretion, which is associated but not dependent of testosterone plasma levels (Blank et al., 2009). Therefore, the present results with previous findings suggest
that the origin of the disease precedes the pubertal development and the postpubertal rise of androgen levels may sustain the neuroendocrine and reproductive pathological features of PCOS.

Fertility is relied upon the proper functioning of GnRH neurons and the GnRH neuron activity is dependent upon synaptic transmission from afferent inputs such as GABA neurons. To identify putative GABA inputs on GnRH neurons, I have used immunohistochemical labeling of VGAT, which accumulates GABA into the presynaptic vesicles (McIntire et al., 1997). VGAT-ir could be identified in virtually all analyzed GnRH-GFP neurons, reaffirming that GABA is a major input to GnRH neurons (Cottrell et al., 2006; Hrabovszky et al., 2012). Although better resolution of GnRH morphological features might be achieved with other techniques such as biocytin filling (Campbell, Han and Herbison, 2005; Cottrell et al., 2006), using the present immunohistochemical labeling of VGAT enabled the examination of the whole population of GnRH neurons in the rPOA/OVLT region. High resolution confocal imaging made possible the visualization and quantification of VGAT contact onto GnRH neuron spines and onto GnRH neuron non-spiny membrane. Future studies may address the functional relevance of GABA inputs on GnRH during prepubertal life and whether altered GABA inputs in this developmental stage may directly alter GnRH neuron functioning in PNA mice. It is important to note that VGAT-expressing neurons are also glycinergic (Chaudhry et al., 1998), and additional studies could define in the future whether glycinergic inputs to GnRH neurons undergo remodeling in a PCOS-like condition.

Increased GABA innervation may play a role in GnRH/LH hyperactivity in the PCOS condition as GnRH neurons can respond to GABA with excitation (DeFazio et al., 2002; Herbison and Moenter, 2011). In vitro studies have shown that androgens enhance GnRH neuron activity through increase GABA transmission to these neurons in adult mice (Pielecka, Quaynor and Moenter, 2006). The idea of GnRH neurons being quiescent during prepubertal life was recently
addressed by researchers, who showed that prepubertal GnRH neurons are surprisingly active in mice (Dulka and Moenter, 2017). In addition, they observed that PNA treatment attenuates the activity of GnRH neurons leading to an abnormal firing rate pattern (Dulka and Moenter, 2017). Remarkably, the present results showed that an abnormal increase of GABAergic innervation onto GnRH neurons occurs during the prepubertal age (PND 25) in PNA mice, and it might contribute to the altered GnRH neuron activity. However, it seems contradictory that increased GABAergic innervation and lower GnRH neuron activity during prepubertal life may promote an elevated GnRH neuron activity during adulthood. Clinical evidence from cross-sectional study revealed that obese girls exhibit low levels of nocturnal LH pulse frequency associated with high free testosterone levels during early puberty (Tanner 1-2), whereas non-obese girls display the normal increase of nocturnal LH pulses (McCartney et al., 2009). Interestingly, an opposite outcome was found during late puberty, which obese girls (Tanner 3-5) displayed both higher LH pulse frequency and testosterone levels when compared to normal subjects (McCartney et al., 2009). These findings suggest that a biological programing controlling GnRH neuron activity during early puberty may induce a “neuroendocrine switch” altering the postpubertal GnRH/LH secretion.

During prenatal and early postnatal life, GABA actions via GABA\(_A\)R in immature neurons are mainly excitatory through a differential chloride (Cl\(^-\)) homeostasis; by which cells maintain high intracellular Cl\(^-\) concentration and a more depolarizing reversal potential for Cl\(^-\) than the resting membrane potential (Ben-Ari, 2002). Prepubertal PNA mice exhibited enhanced GABAergic contact on non-spiny membrane but also at somatic spines, which harbor excitatory inputs in neurons, suggesting an altered regulation of GnRH neuron activity in a PCOS-like condition. Excitatory actions of GABA during early development are critical to induce de novo synapse formation and increase the number of dendritic spines through the regulation of intracellular calcium (Ca\(^{2+}\)) signaling in the mouse brain (Kirmse et al., 2015; Oh et al., 2016). In
addition, juvenile GnRH neurons can be excited by activation of GABA$_A$R (DeFazio et al., 2002). Therefore, increased GABAergic innervation may drive abnormal synaptic pruning in GnRH neurons during prepubertal life, which also may dictate their postpubertal electrical behavior. However, it is still not clear whether GABAergic overdrive in prepubertal GnRH neurons is the definitive cause of a later manifestation of PCOS-like traits in PNA mice. Future experiments will define the direct effect and timing of elevated GABA contact on GnRH neuronal activity.

The present findings postulate that prenatal androgen exposure induces prepubertal abnormalities in brain wiring, which may trigger the neuroendocrine impairment of PCOS following the pubertal activation of the HPG axis. This new insight is critical to better understand the developmental origin of PCOS, which seems highly dependent on androgen excess in utero. In addition, these findings opened that opportunity to investigate the developmental timing of androgen excess in PNA mice and whether hyperandrogenic ovaries are required to maintain the impaired LH secretion (Chapter 4). Considering that GABA-to-GnRH neuron abnormalities seemed programmed in utero and hardwired before pubertal onset, I will also address whether the blockade of androgen actions can reverse these altered brain circuitry (Chapter 5).
Chapter Four:

Developmental timing and requirement of androgen excess in PNA mice
4.1 Introduction

Female androgen excess during reproductive life is a hallmark of polycystic ovary syndrome (PCOS), affecting 60-80% of PCOS patients worldwide (Azziz et al., 2009; Ibáñez et al., 2017). Evidence from PCOS animal model studies suggest that overstimulation of theca cells in the ovaries mediated by luteinizing hormone (LH) drives the excessive androgen production (Eisner et al., 2002). There is compelling evidence that the establishment of hyperandrogenic ovaries during adult life sustains the neuroendocrine impairment of PCOS by impairing the sex steroid-mediated negative feedback mechanism. This leads to continuous cycle that promotes increased androgen levels, menstrual irregularities and, ultimately, infertility (Pastor et al., 1998; Azziz et al., 2009). However, little is known about the temporal and dynamics of androgen excess during pubertal development in a PCOS.

Although not included for diagnostic screening of PCOS, neuroendocrine derangements generating abnormal luteinizing hormone (LH) secretion are present in the majority of PCOS women (Manieri et al., 1992; Berga, Guzick and Winters, 1993; Dumesic et al., 2015). PCOS patients are reported to exhibit elevated LH pulse frequency and/or amplitude associated with ovulatory dysfunction (Rebar et al., 1976; Taylor et al., 1997; Azziz et al., 2016). Chronically elevated LH levels may be the result of an intrinsic elevated GnRH pulse frequency. Clinical study showed that administration of estrogens and progestin in PCOS women did not decrease LH levels in the same magnitude as in normal women, although testosterone levels were normalized by the oral contraceptives (Daniels and Berga, 1997). These data suggest the idea that postpubertal androgen excess does not directly drive high LH secretion but instead sustains the impaired sex steroid-negative feedback.
Such as in the human condition, adult prenatally androgenized (PNA) mice present elevated circulating testosterone levels (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). PNA mice also display high LH pulse frequency and impaired estradiol- and progesterone-mediated negative feedback (Moore, Prescott and Campbell, 2013; Moore et al., 2015). Interestingly, PNA mice exhibit a smaller rise in LH levels following castration when compared to control animals (Moore, Prescott and Campbell, 2013). This may indicate a reduced GnRH/LH pulse responsiveness to the sex steroid hormone negative feedback withdraw in a PCOS-like condition. As mentioned in previous chapters, this desensitization of the GnRH pulse generator may be the result of changes in brain circuits within the GnRH neuronal network. The first findings of the present PhD project elucidated the time which brain wiring abnormalities are present that is before of the onset of puberty (Chapter 3). It was also clear that the installation of altered GABA-to-GnRH neuron circuit was not dependent on earlier rise of circulating testosterone levels in the blood. Together, this evidence suggests that in utero androgen excess imprinting of hypothalamic circuits controlling GnRH/LH pulsatility is a forerunner in PCOS pathophysiology, whereas defects in the classical negative feedback and ovarian dysfunction are secondary features.

The present study aimed to further investigate the developmental timing of androgen excess in PNA mice to establish when hyperandrogenism is present in relation to brain circuit abnormalities and pubertal onset. To test both hypothesis, assessment of circulating testosterone levels, estrous cyclicity and analysis of post-castration LH pulsatility were all performed in the same cohort of animals, which followed from peripubertal stage into adulthood. In addition, I hypothesized that short-term withdraw of androgen excess by the removal of hyperandrogenic ovaries for 7 days is not able to fully restore normal LH pulse secretion in PNA mice. The “ideal” experiment would be to compare the LH pulse secretion before and after ovariectomy and normal and PNA female mice. However, due to animal ethics constraints, herein, I present the results of
LH pulse secretion profile of ovariectomized normal and PNA mice, which were previously used to determine the timing of androgen excess in PNA mice.

4.2 Methods

4.2.1 Animals

Control and PNA C57BL/6(B6) mice were housed at the Hercus Resource Unit (University of Otago - Dunedin) under 12h: 12h light-dark cycles (lights on at 0700h) with *ad libitum* access to water and food. To generate PNA mice, we used established protocol as previously described (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Briefly, time-mated pregnant dams were injected with 100 µL of dihydrotestosterone (250 µg) in oil, or oil alone to serve as a control, on gestational days 16, 17 and 18. All protocols were approved by the University of Otago Animal Ethics Committee (Dunedin, New Zealand).

4.2.2 Experiment 1: Developmental timing of plasma testosterone and assessment of estrous cyclicity

At postnatal day (PND) 30, 40, 50 and 60, control (N = 10) and PNA (N = 9) mice were handled and blood collection from the tail-tip was performed from 0900h to 1000h. Blood samples were spun at 4 G for 10 min at room temperature and plasma was collected and kept at -20 ºC until the day of hormone measurement. From PND 60 to PND 80, estrous cyclicity was followed by daily vaginal smears and examination of the vaginal cytology (Caligioni, 2009) (Figure 4.1).
4.2.3 Experiment 2: Effect of ovariectomy on pulsatile LH secretion of control and PNA mice

Using the same cohort of animals from Experiment 1, control (N = 7) and PNA (N = 8) mice, underwent ovariectomy surgery (surgical approach described in detail in Chapter 2) (Figure 4.1). Animals were left to recover seven days post-surgery and a bleeding protocol to assess LH pulsatile secretion was performed as previously reported (Czieselsky et al., 2016). Briefly, mice were kept in quiet room and habituated with daily handling to avoid further distress during blood sampling. During bleeding, 3 µL of blood was collected every 6 min for 2 h. Whole blood was diluted in 57 µL of 0.1 M phosphate buffered saline-tween (PBS-T) pH 7.4, frozen in dry ice and stored at -20 ºC for subsequent hormone measurements.

**Figure 4.1. Experimental design to assess the timing of testosterone excess and LH pulse frequency following castration in PNA mice.** Control and PNA mice were followed from postnatal day (PND) 30 to PND 90. Blood samples were collected via hematocrit tube at PND 30, 40, 50 and 60. From PND 60 to PND 80, estrous cyclicity was followed by daily vaginal smears and examination of the vaginal cytology. On PND 83,
control and PNA mice were ovariectomized and left to recover for 7 days. On PND 90, LH pulse secretion was assessed using bleeding protocol.

### 4.2.4 Hormone measurements

Circulating testosterone levels were determined in blood collected in a single time point (PND 30, 40, 50 and 60) using 50μl of whole blood via hematocrit tube to obtain 10μl of plasma to be used in the assay. Testosterone plasma levels were determined using a commercially available mouse/rat ELISA kit (LDN GmbH&Co.KG, Nordhorn, Germany). Intra-assay coefficient of variation for testosterone was 10.3 % and inter-assay coefficient of variation was 11.0%.

LH levels were determined in serially blood samples using well-established ELISA method as previously reported (Steyn et al., 2013; Moore et al., 2015; Czieselsky et al., 2016). A mouse LH-RP reference was provided by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). The assay sensitivity of the LH ELISA was 0.002 ng/mL. The assay sensitivity of the LH ELISA was 0.04 ng/mL and intra-assay coefficient of variation was 4.3%.

### 4.2.5 Analysis of LH pulsatility

Post-castration LH levels measured by ELISA were plotted on XY-graph using PRISM software 7.0 (GraphPad Software, San Diego, CA, USA). LH pulses were identified using pulse peaks above 10% of the preceding values and examination of pulse shape as previously reported (Clarkson et al., 2017). The software DynPeak (Vidal et al., 2012) was used to confirm LH pulses and identify LH pulse peak values (Figure 4.2 A). Mean LH levels were calculated by averaging all LH values collected during the serial bleeding. Mean basal LH levels were determined by the average of the lowest points identified in the plotted graph for each animal (Figure 4.2 A). The pulse interval was
determined by the time from the lowest point prior to the LH pulse peak to the next lowest point after the peak (base to base) (Figure 4.2 B). LH pulse frequency was determined by counting the number of LH peak values identified as LH pulses within the 2-h bleeding session. The LH pulse amplitude was measured by the calculation of the LH pulse peak value minus the lowest LH value prior to the peak (Figure 4.2 C). As a measure of the integrated LH release to GnRH pulses, the area under the curve (AUC) was determined. The mean basal LH was set as the baseline for the plotted graph and PRISM software was able to calculate the AUC (Figure 4.2 D).

**Figure 4.2. Method for analysis of LH pulsatility.** Representative profile of pulsatile LH secretion from ovariectomized female mice. (A) LH pulse was determined by LH pulse peak value that were above 10% of the preceding value and examination of pulse shape (blue asterisks). High LH peak values either at the starting point (time 0min) or at the end point (time 120min) were not considered to be a LH pulse. Basal LH values were identified as the
lowest values plotted on the graph (red circles). (B) LH pulse interval was determined by the time (X; in min) between the lowest points before and after a LH pulse. All individual intervals (green double arrows) were averaged to determine the mean LH pulse interval. (C) LH pulse amplitude was calculated by taking each LH pulse peak value minus the lowest LH level prior to the peak (green double arrows). (D) The area under the curve (AUC; light blue shade) was determined by PRISM software using the mean basal LH level as the baseline (blue dashed line) for the calculation.

4.2.6 Statistical analysis

Statistical analysis was performed with PRISM software 7.0 (GraphPad Software, San Diego, CA, USA). Testosterone and LH level values were firstly tested for normal distribution with D’Agostino-Pearson’s normality test. Testosterone levels throughout pubertal development of control and PNA mice were compared using repeated-measures two-way ANOVA with Sidak’s multiple comparison post hoc test. Evaluation of the percentage of days spent in each day of the estrous cycle of either control or PNA mice was analyzed with one-way ANOVA with Tukey’s post hoc test. Comparisons between the groups to determine differences in the percentage of days spent in each stage of the estrous cycle were analyzed with two-way ANOVA with Sidak’s post hoc test. Pulsatile LH profile parameters were analyzed by comparing control and PNA mice using two-tailed Student’s t test. Statistical significance was accepted when \( P < 0.05 \). Graphical representation to display significant different are shown as * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) and **** \( P < 0.0001 \).
4.3 Results

4.3.1 Androgen excess occurs during early adulthood in PNA mice

To determine the developmental timeline of androgen excess in our PCOS-like mouse model, circulating levels of testosterone were measured throughout peripubertal development and into adulthood, from postnatal day (PND) 30 to PND 60. Testosterone levels were similar between groups at PND 30 (PNA: 0.11 ± 0.03 vs. control: 0.10 ± 0.02 ng/mL) and PND 40 (PNA: 0.14 ± 0.03 vs. control: 0.15 ± 0.03 ng/mL) corresponding to early and mid-pubertal stages (Figure 4.3).

In contrast, by early adulthood at PND 50, average circulating testosterone levels of PNA mice were significantly elevated compared to controls (0.24 ± 0.06 vs. 0.06 ± 0.01 ng/mL; \( P < 0.01 \)). Mean testosterone levels remained higher in adult PNA mice at PND 60 when compared to control mice (Figure 4.3; 0.24 ± 0.06 vs. 0.08 ± 0.01 ng/mL; \( P < 0.05 \); \( F_{3,51}(\text{interaction}) = 6.2; F_{1,17}(\text{phenotype}) = 7.3 \); repeated-measures two-way ANOVA with Sidak’s post hoc test;).
Figure 4.3. Temporal assessment of blood testosterone levels revealed that androgen excess occurs during early adulthood in PNA mice. Circulating testosterone levels from postnatal day (PND) 30 to PND 60 in control (N = 10) and PNA (N = 9) mice. Data are shown as dot plots for each time point and bars represent mean ± SEM. Dashed blue lines indicate testosterone levels of control mice and red full lines indicate PNA mice. Handling of mice occurred just at PND 30, 40, 50 and 60. Differences between the groups are reported as *$P < 0.05$; **$P < 0.01$ and differences within each groups are reported as # $P < 0.05$. Data analysis was performed with repeated measures two-way ANOVA with Sidak’s post hoc test.

Analysis of testosterone fluctuations over the pubertal transition into adulthood in control animals demonstrated peak testosterone levels occur at PND 40, with levels falling significantly into adulthood (PND 50-60) ($P < 0.05$). In contrast, in PNA mice, peak testosterone levels were significantly higher at PND 50, when compared with peripubertal levels at PND 30 ($P < 0.05$) (Figure 4.3). While control mice all exhibited relatively low circulating testosterone in adulthood,
testosterone levels in PNA mice were highly variable. These data demonstrate that a short prenatal androgen exposure in female mice induces the development of high circulating testosterone levels that begin after pubertal onset and continue into adulthood.

### 4.3.2 PNA mice exhibit disrupted estrous cyclicity during adulthood

Control (N = 10) and PNA (N = 9) mice Estrous cycles of both groups were followed from PND 60 to PND 80. Despite variable testosterone levels at PND 50 and 60 in PNA mice, vaginal cytology confirmed that all PNA animals displayed a significant disruption of estrous cyclicity with a complete absence of proestrous smears, indicating the absence of ovulation ([Figure 4.4](#)) in agreement with previous reports (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013).
Figure 4.4. PNA mice exhibit significant disruption of reproductive cycles during adult life. Estrous cycles from control (N = 10) and PNA (N = 9) from postnatal day (PND) 60 to PND 80. Vaginal smears and cytological evaluation were used to identify the estrous stages proestrus (P), diestrus (D), metestrus (M) and estrus (E). All mice here presented are the same animals used to measure testosterone blood levels from PND 30 to PND 60 (data in figure 4.3). The code above each panel indicates the original identification number of each mouse.
Estrous cycle was further investigated by analyzing the percentage of days spent in each cycle stage. Results showed that control mice spent a significant different time in each stage of the cycle (dierstrus: 29.0 ± 1.4; proestrus: 22.0 ± 1.1; estrus: 43.5 ± 2.1; metestrus: 5.5 ± 2.2%; F_{3, 36} = 79.73; P < 0.0001) (Figure 4.5 A). Conversely, PNA mice spent an increased and similar percentage of time in estrus and metestrus (estrus: 39.4 ± 3.6; metestrus: 45.0 ± 6.5%) when compared to diestrus and proestrus (dierstrus: 15.6 ± 4.7; proestrus: 0.0 ± 0.0%) (F_{3, 32} = 22.9; P < 0.0001) (Figure 4.5 A). Comparisons between the groups revealed that PNA mice exhibited a significant decreased in the percentage of time spent in diestrus (P < 0.05) and proestrus (P < 0.0001) when compared to controls (Figure 4.5 B). In contrast, PNA spent a significant increase in the amount of time spent in metestrus (P < 0.0001) in comparison with control mice, while no differences were identified for the estrous stage (Figure 4.5 B). Together, these data illustrate the direct evidence that postpubertal androgen excess during is associated with chronic anovulation during adult life by disrupting the normal profile of estrous cyclicity in PNA mice.
Figure 4.5. PNA mice exhibits significant disruption of estrous cycles. Estrous cyclicity of control (N = 10) and PNA (N = 9) mice from postnatal day (PND) 60 to PND 80. All mice belong to the same cohort of animals which were previously bled to assess development timing of circulating testosterone levels (data in figure 4.3). (A) Histograms show mean ± SEM percentage of days spent in each stage of the estrous cycle of either control or PNA mice. Different letters indicate significant differences (P < 0.05); one-way ANOVA with Tukey’s post hoc test. (B) Comparison between control and PNA mice analyzing the time spent in each stage of the estrous cycle. *P < 0.05; ****P < 0.0001; two-way ANOVA with Sidak’s post hoc test. (ND = not detected).
4.3.3 Deranged LH regulation persists after castration in PNA mice

The majority of androgen excess in PCOS patients and PNA animal models originate from the ovary (Eisner et al., 2002; McCartney et al., 2004). In addition, diestrous PNA mice display significant increase of LH pulse frequency and decrease of LH pulse amplitude associated with elevated testosterone levels (Moore et al., 2015). To investigate the requirement of hyperandrogenic ovaries to the neuroendocrine impairment in PNA mice, LH pulse secretion was determined in control (N = 7) and PNA (N = 8) mice 7 days post-ovariectomy (OVX) on PND 90. The LH profile of each group was assessed (Figure 4.6A) and analysis of pulsatile LH secretion was performed. Results showed that OVX control and OVX PNA mice exhibit similar values of mean LH and basal levels (Figure 4.6 B and C).

In addition, OVX control and OVX PNA mice presented similar LH pulse frequency (control: 7.00 ± 0.44 vs. PNA: 7.12 ± 0.35 No. of pulses/2h; Figure 4.6 D) and pulse duration (control: 17.15 ± 1.08 vs. PNA: 16.32 ± 0.59 min; Figure 4.6 E). However, OVX PNA mice exhibited a significant increased LH pulse amplitude compared to OVX control mice (PNA: 3.82 ± 0.15 vs. control: 2.70 ± 0.12 ng/mL; P < 0.0001; Figure 4.6 F). The integrated LH response to GnRH pulses, as determined by the area under the curve (AUC) based on previous report (Patel et al., 2004), was significantly greater in OVX PNA mice than OVX controls (PNA: 237.4 ± 10.9 vs. control: 181.4 ± 6.6; P < 0.001; Figure 4.6 F). These data suggest that PNA mouse ovaries are important for sustaining the postpubertal neuroendocrine impairment of GnRH/LH pulse frequency. However, in the absence of ovarian hyperandrogenism, altered gonadotropin regulation persists, suggesting an increased gonadotrope responsiveness to GnRH pulses, as illustrate by enhanced LH pulse amplitude and AUC following OVX.
Chapter Four

A

Control # 271.2

Control # 272.1

PNA # 268.1

PNA # 270.3

Time (min)

LH (ng/mL)

Time (min)

LH (ng/mL)

Time (min)

LH (ng/mL)

B

Mean LH

(ng/mL)

Control

PNA

C

Mean basal LH (ng/mL)

Control

PNA

D

LH pulse frequency (No. of pulses/2h)

Control

PNA

E

LH pulse interval (min)

Control

PNA

F

LH pulse amplitude (ng/mL)

Control

PNA

G

AUC

Control

PNA

****

***
Figure 4.6. Amplitude of LH secretion remains increased after the removal of hyperandrogenic ovaries in PNA mice. (A) Examples of pulsatile LH levels in the blood from control (N = 7) and PNA (N = 8) mice after 7 days after ovariectomy on postnatal day (PND) 90. Asterisks indicate LH pulse peaks. Control and PNA mice belong to the same cohort of mice, which were followed from PND 30 to PND 60 to assess circulating testosterone levels (data in figure 4.3). Histograms show mean LH levels (B), mean basal LH levels (C), LH pulse frequency (D), LH pulse duration (E), LH pulse amplitude (F), and area under the curve (AUC) (G). Values are shown as mean ± SEM. Unpaired two-tailed Student’s t test; ***P < 0.001; ****P < 0.0001.

4.4 Discussion

Prenatal programing androgen excess promotes ovarian hyperandrogenism and abnormally elevated LH pulse frequency during adulthood in PNA animal models. Firstly, the present chapter aimed to elucidate the developmental timing of androgen excess in PNA mice. Our data showed that androgen excess in PNA mice was evident after pubertal development into adulthood at PND 50 and PND 60. Follow-up experiments demonstrated a direct association of androgen excess during early adulthood with the disruption of reproductive cycles. In particular, PNA mice did not display proestrous stage and spent more time in estrus and metestrus compared to controls. Lastly, the requirement of ovarian androgen production to sustain altered LH secretion was tested by the removal of hyperandrogenic ovaries in PNA mice. The results of this study showed that OVX promoted similar increase in LH pulse frequency in both control and PNA mice. However, the LH pulse amplitude and the magnitude of LH response, as determined by the AUC, were significantly elevated in OVX PNA mice.
In chapter 3, our data showed that control and PNA mice exhibit similar circulating testosterone blood levels before the onset of puberty at PND 25. Serial measurements of blood testosterone levels in PNA mice indicated that circulating androgen excess does not develop until PND 50. As the manifestation of PCOS is linked to the onset of puberty, it could be the case that PNA mice might display pubertal onset after PND 50 due to androgen excess. However, it does not seem to be the case as all mice from this study displayed vaginal opening by PND 40. In addition, our PNA mice exhibit first estrous by ≤ PND 40 as shown by previous work from our laboratory (Moore, Prescott and Campbell, 2013). These findings together with the results from Chapter 3 suggest that androgen excess is likely to be a downstream outcome from altered GnRH/LH secretion as it is manifested following pubertal onset. In girls, prepubertal adrenarche correlates with a higher risk of developing PCOS and hyperandrogenic ovaries due to precocious exposure to increased androgen levels (Vuguin et al., 1999; Silfen et al., 2002). In contrast, rodent adrenal glands do not possess the molecular machinery necessary to synthesize androgens (van Weerden et al., 1992) implying that PNA-induced androgen excess develops from an ovarian source. Another important source of androgens is the brain itself, which express significant amount of P450 aromatase converting estrogens into testosterone in situ. Changes in brain androgen levels are more dramatic during perinatal period tissue levels remain low during postnatal life in female rodents (Konkle and McCarthy, 2011); however, it is unknown whether prenatal androgen excess may induce prepubertal high brain androgen levels in PNA mice. Adult PCOS show elevated levels of a 53% increase in testosterone levels, which is associated with high GABA in the cerebrospinal fluid (Kawwass et al., 2017). Therefore, remains to be investigated whether an abnormal in situ steroidogenesis may still occur after the removal of hyperandrogenic ovaries in PNA mice. Regardless of the source, precocious exposure to androgens appears promote the development of
hyperandrogenic ovaries and is suggestive of a developmental programming of disease progression.

Previous reports have shown PNA treatment results in disruption of estrous cycles and infertility (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). The present study showed a direct evidence between androgen excess taking place during early adulthood and irregular estrous cyclicity at reproductive age in PNA mice. Specifically, whereas normal female mice spent a varied amount of time in each estrous cycle stages, PNA mice lacked the detection of proestrus, an indicative of ovulation, and exhibited a significant longer time in estrus and metestrus. Female rodents typically present a short luteal phase and the metestrus stage which usually lasts few hours (Smith, Freeman and Neill, 1975). The vaginal cytology of metestrus stage is characterized by a pronounced number of cornified epithelial cells along with leucocytes. Neonatal female mice treated with testosterone display persistent vaginal cornification and leucocyte infiltration associated with ovarian dysfunction leading to total absence of corpora lutea during adulthood (Kimura, Nandi and Deome, 1967). Interestingly, adult neonatally testosterone-treated mice can also exhibit persistent vaginal cornification after ovariectomy, posing the possibility an ovarian-independent persistent metestrus (Kimura, Basu and Nandi, 1967). These evidence together with the present findings suggest that perinatal androgen excess may promote organizational upstream derangements in the HPG axis, which persists until postpubertal androgen excess occurs to trigger ovarian dysfunction and irregular cycles.

The present findings also elucidated the dynamics of pulsatile LH secretion pattern in the absence of hyperandrogenic ovaries in PNA mice. Previous work in our laboratory showed that PNA mice exhibit elevated LH pulse frequency and significant decrease of LH pulse amplitude without changes in the mean LH baseline (Moore et al., 2015). In that study, high LH pulse
frequency was associated with hyperandrogenemia and impaired progesterone-mediated negative feedback in the brain. In the absence of negative feedback by ovarian steroid hormones, LH mean levels and LH pulse frequency increase in OVX mice (Moore, Prescott and Campbell, 2013; Czieselsky et al., 2016). Our results suggest that PCOS-like ovaries may be required to sustain altered LH pulse frequency in PNA mice since OVX was able to show similar LH values between the groups. Alternatively, it is also possible that this result may represent a physiological limit by which a higher LH pulse frequency cannot be achieved even in PCOS-like condition. Due to ethical limitations with our protocol, I could not demonstrate that this cohort of PNA mice exhibited higher LH pulse frequency prior to OVX procedure. However, this is well documented in previous reports (Moore, Prescott and Campbell, 2013; Moore et al., 2015).

The altered profile of LH pulsatile secretion is considered a direct readout of abnormal GnRH pulses and pituitary LH responsiveness in PCOS (Rebar et al., 1976; Patel et al., 2004). In the absence of hyperandrogenic ovaries, the LH pulse amplitude and the magnitude of LH release, as determined by the area under the curve, were significantly elevated in OVX PNA mice. Previous results showed that intact PNA mice display decreased LH pulse amplitude (Moore et al., 2015), which may be due to a high GnRH frequency release that reduces the readily releasable LH pool in the pituitary gland. Although it is reported that high GnRH frequency induces lower LH amplitude (Clarke and Cummins, 1985), long-term GnRH self-priming effect in the pituitary enhances the gonadotrope responsiveness (Hoff, Lasley and Yen, 1979). In addition, PCOS women exhibit greater LH amplitude release following intravenous administration of GnRH when compared to normal subjects without changes in LH frequency (Patel et al., 2004). This indicates that the gonadotrope responsiveness was dictated by a pre-exposure to higher GnRH actions in the pituitary gland, which was likely established in a scenario of hyperandrogenism in adulthood.
These results suggest that altered gonadotropin secretion was programed due to pre-exposure to high GnRH pulses during adulthood and this remains after castration.

Clinical evidence suggest that the impaired negative feedback is indirectly established by androgen excess in PCOS. Long-term treatment with androgen receptor (AR) blocker is able to re-instate the ability of ovarian hormones to decrease LH pulse frequency in PCOS women (Eagleson et al., 2000). However, acute administration of testosterone does not enhance pulsatile LH secretion or change the LH sensitivity to exogenous GnRH in healthy women (Dunaif, 1986). This indicates that chronic androgen exposure might the culprit of altered regulation of GnRH neuron activity and gonadotropin secretion. Because the removal of hyperandrogenic ovaries did not fully restore LH secretion in our experiments, it could be possible that prenatal-mediated organizational androgen actions within the GnRH neuronal network remains after 7 days post-castration in PNA mice. Another possibility is that ovariectomy and the removal of ovarian steroid negative feedback might have a greater impact on control animals than PNA mice. In fact, after 3 days post-castration, control mice exhibit greater mean LH levels compared to PNA mice (Moore, Prescott and Campbell, 2013). Therefore, PNA treatment might promote profound organizational changes in brain circuits involved in the control of ovarian steroid-mediate negative feedback, which persists until adulthood (Figure 4.7).
Based on previous reports, intact PNA mice exhibit high LH pulse frequency and decreased LH pulse amplitude compared to diestrus control mice (Moore et al., 2015). High GnRH/LH pulse frequency, in detriment of an impaired negative feedback mechanism, might overstimulate androgen production by the ovaries in PNA mice. Consequently, hyperandrogenic ovaries allow the installation of postpubertal androgen excess, which in turn, sustain a continuous cycle of impaired reproductive function in the PCOS-like condition. (B) The present findings showed that following ovariectomy, LH pulse frequency is high and similar in both control and PNA mice. Conversely, PNA mice still displays altered gonadotropin secretion exhibiting elevated magnitude of LH pulse amplitude, suggesting that neuroendocrine impairments remained after the removal of hyperandrogenic ovaries. Red (PNA) and blue (control) dashed traces in the graph indicate pulsatile LH release over time.
In conclusion, these findings elucidated the developmental timing of androgen excess in PNA mice, which occurs during early adulthood and has an important predicative value of disruption of reproductive cycles. Prenatal androgen programing of abnormal LH secretion, which may a primary result from altered brain wiring within the GnRH neuronal network, cannot be fully rescued by the removal of hyperandrogenic ovaries. Considering that chronic androgen actions may sustain brain circuit abnormalities in PCOS, pharmacological target of androgen actions from early adulthood might provide a better therapeutic avenue for the treatment of PCOS.
Chapter Five:

Reversal of GABA-to-GnRH circuit abnormalities in PNA mice
5.1 Introduction

The results presented in this PhD thesis have demonstrated that a prenatal androgen insult programs an abnormal GABAergic innervation to GnRH neurons prior to pubertal onset in PNA mice (Chapter 3). These findings suggested that enhanced GABA inputs on GnRH neurons might contribute to altered GnRH neuron activity during prepubertal stages (Dulka and Moenter, 2017), which remains until adult life (Sullivan and Moenter, 2004). The present results also elucidated that androgen excess takes place from early adulthood in PNA mice that is associated with pronounced disruption of estrous cyclicity (Chapter 4). Additionally, a short-term absence of hyperandrogenic ovaries did not fully restore the neuroendocrine impairment in PNA mice (Chapter 4). Therefore, prenatal androgen programming might promote hard wired changes in the brain in PCOS, which manifests earlier in life and may drive an abnormal activation of the HPG axis and, subsequently, the manifestation of postpubertal androgen excess. However, it remains unclear whether prenatal androgen excess and postpubertal androgen excess may contribute differently for the pathophysiology of PCOS.

Findings from clinical studies support the idea that elevated prenatal testosterone levels are associated with the development of PCOS in the female progeny (Sir-Petermann et al., 2009; Maliqueo et al., 2013; Caanen et al., 2016). The ability to recapitulate the neuroendocrine impairment of PCOS in various PNA animal models (Roland and Moenter, 2014) indicates that androgen actions in the prenatal brain might promote organizational changes that perpetuates until reproductive maturation. The mammalian brain is highly sensitive to ovarian steroid actions during perinatal life, which is critical for appropriate organization of hypothalamic circuits controlling reproduction (McCarthy, 2010). Prenatal androgen actions in the developing brain of ewes reduced the number of GABAergic and glutamatergic inputs on GnRH neurons and decrease glia-GnRH
neuron interactions (Jansen et al., 2011). These changes in synaptic input number can also be observed in PNA mice with enhanced steroid-sensitive GABAergic innervation on GnRH neurons (Moore et al., 2015). Therefore, prenatal androgen excess may contribute for the *in utero* pathogenesis of PCOS by promoting changes in hypothalamic circuits which control GnRH neuron activity.

On the other hand, postpubertal androgen excess may provide activational effects on the prenatally programmed brain circuitry. Clinical studies show that short-term administration of androgen receptor (AR) antagonist does not restore normal LH secretion in PCOS women (Eagleson et al., 2000). However, long-term treatment with an AR antagonist is able to re-establish the ability of estradiol and progesterone to suppress LH pulse frequency (Eagleson et al., 2000). Considering that PCOS women presents a decreased hypothalamic sensitivity to progesterone-mediated negative feedback (Chhabra et al., 2005), hyperandrogenemia may contribute to the perpetuation of an abnormal hypothalamic sensitivity to feedback inhibition by sex steroids. In fact, AR signaling is known to interfere with progesterone-sensitive GABAergic transmission in GnRH neurons, which ultimately leads to a hyperactive state of GnRH neurons (Sullivan and Moenter, 2005). These findings along with the results present so far (Chapter 3), indicate that organizational and activational effects of androgens in the female brain may allow the perpetuation of altered GABA-to-GnRH neuron abnormalities underlying the neuroendocrine dysfunction in PCOS.

Based on the evidence that long-term AR antagonist treatment is able to rescue normal functioning of ovarian steroid-mediated negative feedback (Eagleson et al., 2000), the present study investigated whether this might be the result of the reversal of GABA-to-GnRH neuron abnormalities. Specifically, I hypothesized that AR signaling blockade from late puberty (PND 40)
to adulthood (PND 60) is able to reverse altered GABA-to-GnRH neuron wiring, which in turn may change the associated PCOS reproductive phenotype in PNA mice.

5.2 Methods

5.2.1 Animals

Female GnRH-GFP mice (Spergel et al., 1999) were housed with ad libitum access to water and food, and under 12:12 h light-dark cycle (lights on at 0700h). To generate PNA mice, we used established protocol as previously described (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Briefly, time-mated pregnant dams were injected with 100 µL of dihydrotestosterone (250 µg) in oil, or oil alone to serve as a control, on gestational days 16, 17 and 18. All protocols were approved by the University of Otago Animal Ethics Committee (Dunedin, New Zealand).

5.2.2 Androgen receptor signaling blockade protocol

Control and PNA GnRH-GFP mice received daily 50 µL injections of either Flut (Sigma; s.c. 25 mg/kg/day) or a vehicle control (sesame oil) for 20 days from PND 40 to PND 60. The dose of Flut was based on previous studies in our laboratory and in a small pilot study performed at the beginning of my PhD project (for more details see Appendix III). Groups were identified as control+oil (N = 6), control+Flut (N = 7), PNA+oil (N = 5) and PNA+Flut (N = 7). All mice were monitored daily for estrous cyclicity through vaginal smears. Following at least 20 days of treatment, animals were euthanized when in the diestrus stage of their reproductive cycle. Mice were euthanized with an overdose of sodium pentobarbital (30 mg/mL) followed by transcardial perfusion with buffered-paraformaldehyde 4% (0.1 M phosphate buffer-PFA 4%; pH 7.6). Brains
were dissected and post-fixed for 1 h at room temperature following saturation in a 30% sucrose made up in Tris buffer saline (TBS) and stored at -4 °C. Ovaries were post-fixed for 24 h at room temperature and transferred to ethanol 70% solution at -4 °C until the day of inclusion in paraffin.

5.2.3 Immunohistochemistry

Fixed brain tissue was cut into 3 sets of 30-µm-thick coronal sections using a freezing microtome. Coronal sections covering the rostral preoptic area (rPOA) and organum vasculosum of lamina terminalis (OVLT) of the hypothalamus were chosen according to the mouse brain atlas (Paxinos and Franklin, 2004). Free-floating immunohistochemistry was performed as previously described in Chapter 3 and elsewhere (Cottrell et al., 2006; Moore et al., 2015) on one set of sections (including every third brain section). Primary antibodies were used in the following concentrations: polyclonal chicken anti-GFP (1:2,500, Aves Labs Inc.) and polyclonal rabbit anti-vesicular GABA transporter (VGAT) (1:750, Synaptic Systems). Brain sections were incubated with a primary antibody cocktail and 2% normal goat serum for 48 h at -4 °C. AlexaFluor488 goat anti-chicken (1:200) and Streptavidin568 (1:200, Molecular Probes, Invitrogen) were used as secondary antibodies. Primary antibody omission served as a negative control of the procedure.

5.2.4 Confocal imaging acquisition and morphological analysis

Confocal imaging was obtained with a Nikon A1R Multi-photon confocal microscope with 488 and 543 nm diode lasers. Brain sections were imaged using Plan NeoFluor 40 x oil objective (1.30 NA), taking Z-stacks of 0.5 µm (pinhole at 1 AU) and analyzed with software NIS-Elements AR 4.00.00 (Nikon® Instruments Inc.). Following double immunofluorescent labeling (VGAT-ir/GFP-ir), VGAT appositions to GnRH neurons were quantified in two representative sections.
from the rPOA/OVLT region of the hypothalamus of treated control and PNA groups. Ten to twelve GnRH neurons from each animal were randomly selected and scanned for analysis. VGAT apposition density was quantified at the level of GnRH neuron soma and along 75 µm of the primary and secondary dendrites as previously reported (Moore et al., 2015).

In order to evaluate GABA inputs on GnRH neurons, confocal imaging and analysis were performed as previously shown in Chapter 3 and elsewhere (Moore et al., 2015). Briefly, close appositions of VGAT immunoreactive (-ir) puncta to GFP-ir GnRH neurons indicated putative GABA synaptic input (Edwards et al., 1997; Cottrell et al., 2006) and were quantified with confocal microscopy. The dendrites of bipolar GnRH neurons were classified as either primary, the thicker dendrite (larger sectional area) at the point of origin from the soma, or secondary, the thinner dendrite, according to reported morphological criteria (Herde and Herbison, 2015). The locations of putative GABA synapses were classified as onto spines, crucial membrane structures which harbor excitatory inputs (Harris et al., 2013; Oh et al., 2016), or onto the somatic or dendritic shaft membrane without spines, here referenced as non-spiny membrane.

5.2.5 Morphological preparation and analysis of mouse ovaries

Ovaries were collected from diestrus control and diestrus PNA mice and one ovary was randomly chosen for morphological analysis as previously reported (Moore, Prescott and Campbell, 2013). Briefly, ovarian sections of 5 µm-thickness were collected every 50-µm interval and stained with hematoxylin and eosin. The number and type of ovarian follicles were determined throughout sections from the whole ovary as formerly described by others (Myers et al., 2004). Ovarian sections were visualized with light microscope (Olympus Bx-51; Olympus Optical, Hamburg, Germany) using 10X and 20X objectives. Ovarian sections were imaged with a 4X objective to
quantify the area of the section. Follicle wall thickness was determined in the largest preovulatory follicle from the ovarian section with the largest radius. The total follicle wall area and the area made up of granulosa or theca cell layers were defined using ImageJ software (National Institutes of Health, Bethesda, Maryland) and the percentage of the layer thickness in relation to the total follicle area was calculated.

5.2.6 Statistical analysis

Statistical analysis was performed with PRISM software 7.0 (GraphPad Software, La Jolla, CA, USA). Adult control and PNA mice were compared using two-way ANOVA with a Tukey’s post hoc test when analyzing GABA inputs on GnRH neurons and ovarian morphology features. A two-way ANOVA with a Bonferroni’s post hoc test was used to compare prepubertal and adult groups. All data are represented as mean ± SEM. Statistical significance was accepted when $P < 0.05$. Graphical representation to display significant different are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ AND **** $P < 0.0001$. 
5.3 Results

5.3.1 Long-term AR blockade reverses GnRH neuron circuit abnormalities in adult PNA mice

Control and PNA mice were treated with Flut (25 mg/kg/day) or an oil vehicle from PND 40 to PND 60 to determine the density of VGAT contact to GnRH neurons. By PND 40, all mice presented vaginal opening as a marker of pubertal onset. GABAergic contacts were assessed in 61 GnRH neurons from control+oil (N = 6), 72 GnRH neurons control+Flut (N = 7), 53 GnRH neurons from PNA+oil (N = 5), and 72 GnRH neurons from PNA+Flut (N = 7). Virtually all analyzed GnRH neurons presented VGAT appositions in the rPOA/OVLT region. Projected confocal images were analyzed using the same approach as for prepubertal mice in Chapter 3 (Figure 5.1).
Figure 5.1. Projected confocal images of GABA inputs on GnRH neurons in adult control and PNA mice. (A) Examples of confocal images of diestrous control and PNA mice showing GnRH-GFP neurons (green) and VGAT-ir contacts representing GABA inputs (red puncta) in the rPOA. Analysis proceeded with 10-12 GnRH-GFP neurons from each animal. Each column shows a representative GnRH neuron from control+oil (N = 6 / 61
neurons), control+Flut (N = 7 / 72 neurons), PNA+oil (N = 5 / 53 neurons) and PNA+Flut (N = 7 / 72 neurons) groups. White arrowheads indicate putative GABAergic inputs to GnRH neurons (scale bar = 10 μm). (B) Projected 3D reconstruction of a GnRH-GFP neuron from an adult PNA mouse illustrating VGAT-ir puncta contacts with white arrowheads indicating VGAT contact onto non-spiny GnRH neuron membrane and blue arrowheads indicating inputs onto somatic and dendritic spines. Rotated and zoomed images of VGAT contact with a somatic spine (I), front and back views of dendritic spines in the distal dendrite covered with GABA inputs (II and III), and VGAT contacts onto the proximal dendrite of a GnRH neuron (IV).

As reported previously (Moore et al., 2015), PNA mice receiving an oil vehicle (PNA+oil) exhibited significantly increased VGAT appositions to GnRH neurons at the soma (P < 0.0001), primary dendrite (P < 0.0001) and secondary dendrite (P < 0.001) compared to control mice (Figure 5.2 A). Remarkably, AR blockade with Flut robustly decreased the total density of GABA inputs to GnRH neurons in PNA mice to levels found in control+oil and control+Flut groups (Figure 5.2 A). VGAT contacts onto the non-spiny membrane of PNA+oil was also increased at the cell body (P < 0.0001) and primary dendrite (P < 0.001), whereas PNA+Flut group exhibited similar VGAT apposition density to both control groups (Figure 5.2 B). The density of VGAT appositions onto the GnRH neuron non-spiny membrane of secondary dendrites was not different among the groups (F1, 21 (treatment) = 0.70; P = 0.41) (Figure 5.2 B). Greater increases of GABA inputs onto the GnRH neuron spines were observed at the three neuronal compartments in PNA+oil group, while PNA+Flut, control+oil and control+Flut groups displayed lower values (Figure 5.2 C). These findings suggest that blockade of AR signaling can induce plasticity within the GABA-to-GnRH neuron circuit and reverse circuit abnormalities to control levels.

Spine density, used as a general index of synaptic input, was also remodeled by long-term AR blockade in PNA mice. GnRH neuron spine density in PNA+Flut was significantly decreased when compared to the PNA+oil group (soma: P < 0.05; primary dendrite: P < 0.01; secondary
dendrite: \( P < 0.01 \) (Figure 5.2 D). When comparisons were made among the four groups, spine density was not statistically different between control+oil and PNA+oil groups, although PNA+oil mice displayed greater density than control+Flut group at the soma (\( P < 0.01 \)) and primary dendrite (\( P < 0.05 \)) (Figure 5.2 D). These morphological evidence suggest that remodeling of GABAergic induced by AR blockade might influence and rescue normal GnRH neuron activity in PNA mice as observed by others (Sullivan and Moenter, 2004).

Figure 5.2. Androgen receptor (AR) blockade reverse the altered GABAergic wiring onto GnRH neurons. Assessment of GABA inputs on GnRH-GFP neurons from control+oil (\( N = 6; 61 \) neurons), control+Flut (\( N = 7; 72 \) neurons), PNA+oil (\( N = 5; 53 \) neurons) and PNA+Flut (\( N = 7; 72 \) neurons) groups. VGAT appositions are shown for each neuronal compartment. (A) Total VGAT-ir density on GnRH neurons. (B) Total VGAT apposition density onto non-spiny GnRH neuron membrane. (C) Total VGAT-ir apposition density onto GnRH neuron spines. (D) Number of GnRH neuron spines. Histogram values
are represented by mean ± SEM with dot plots of individual values. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.01; two-way ANOVA with Tukey’s post hoc test.

A comparison was made between VGAT density in adult animals described here and the prepubertal mice (PND 25) presented in Chapter 3. Analysis revealed that PNA treatment was the major determinant for GABA-to-GnRH neuron circuit abnormalities (phenotype: PNA vs. control; F (1, 16) = 29.96; P < 0.0001; two-way ANOVA with Bonferroni’s post hoc test), but not age in relation to puberty (age: PNA vs. control; F (1, 16) = 0.38; P = 0.55) (Figure 5.3). In addition, total VGAT apposition density on GnRH neurons in Flut treated mice was reduced by 53 % to levels not different to controls (Figure 5.3). This result illustrates that the enhanced GABAergic innervation to GnRH neurons in PCOS-like mice is programed during prenatal androgen insult but remains plastic in adulthood through AR signaling blockade.

Figure 5.3. PNA mice show similar amount of GABA inputs onto GnRH neurons across pubertal development. Total VGAT appositions onto GnRH neuron were compared among prepubertal control (N = 4) and PNA (N = 5) mice, and adult oil-vehicle control (N = 6) and oil-vehicle PNA (N = 5) mice. Histogram values are represented as mean ± SEM. Different letters indicate significant statistical differences with P < 0.05; two-way
ANOVA with Bonferroni’s post hoc test. Graphical representation illustrates the effect of androgen receptor (AR) blockade attenuating GABA inputs onto GnRH neurons (percentage of decrease) in PNA+Flut mice when compared to PNA+oil group.

5.3.2 Long-term AR blockade restores estrous cyclicity in PNA mice

Estrous cycles were followed during the 20-day treatment to determine whether changes in brain wiring would be associated with an improvement of reproductive cycles. Cycle stage characterization showed that all PNA+oil mice were acyclic with a complete absence of proestrus stage vaginal smears (Figure 5.4 A). In contrast, PNA+flut group displayed recurrent proestrus stage similar to control groups (Figure 5.4 A). Examination of the percentage of days in each stage cycle showed that while PNA+oil did not show evidence of ovulation through vaginal smear examination, control groups and PNA+flut groups presented proestrus smear at a similar frequency during the 20-days of treatment (Figure 5.4 B). These data together illustrate that androgen signaling blockade was able to overcome PNA-programed disruption of fertility and restore reproductive cycles in parallel with anatomical changes in the brain.
Figure 5.4. Blockade of androgen receptor (AR) signaling restores reproductive cycles in PNA mice. AR signaling blockade was achieved with the administration of flutamide (Flut) s.c. 25 mg/kg/day during 20 days. (A) Representative estrous cycle plots over 20 days from control+oil (N = 6), control+Flut (N = 7), PNA+oil (N = 5) and PNA+Flut (N = 6) groups. P: proestrus; D: diestrus; M: metestrus; E: estrus. (B) Histogram showing mean ± SEM percentage of days spent in each stage of the estrous cycle (ND = not detected). *P < 0.05; two-way ANOVA with Tukey’s post hoc test.
5.3.3 Long-term blockade of AR signaling improves the recruitment of preovulatory follicles and their morphological features in PNA mice

Ovarian morphology was investigated to determine whether restoration of normal brain circuitry following AR blockade was also able to improve ovarian function (Figure 5.5 A). PNA mice presented a greater number of primordial follicles than controls and this was not modified by Flut treatment (effect of the phenotype: $P < 0.001$; $F_{1,21} = 23.84$; effect of treatment: $P = 0.89$; $F_{1,21} = 0.02$; two-way ANOVA with Tukey’s post hoc test) (Figure 5.5 B). PNA mice groups show significantly fewer primary follicles when compared to the control+oil group (Figure 5.5 B). Although, not restored completely to control levels, flutamide treatment in PNA mice increased the number of primary follicles to levels similar to the control+Flut group (control+oil: 39.00 ± 3.56; control+Flut: 35.71 ± 3.30; PNA+oil: 16.60 ± 0.75; PNA+Flut: 26.17 ± 3.55 primary follicles per ovary). The number of secondary follicles, early antral follicles and total atretic follicles were not different across the four groups (Figure 5.5 B). These results reaffirm that PNA mice exhibit altered folliculogenesis and suggest that pharmacological blockade of androgen-mediated actions has minimal impact on the arrest of small follicles.
Figure 5.5. Androgen receptor (AR) blockade improves the recruitment of preovulatory follicles and their features in PNA mice. Ovarian morphology of adult control and PNA mice undergoing s.c. injection with oil-vehicle or flutamide (Flut) 25 mg/kg/day from PND 40 to PND 60. (A) *First row*: 5-μm thick ovarian sections from control and PNA mice in diestrus. Corpora lutea are indicated by black asterisks; (scale bar = 500 μm). *Second row*: representative images of a preovulatory follicle wall from each group; yellow dashed lines delineate the theca cell layer (TLC) from granulosa cell layer (GCL); (scale bar = 50 μm). (B) Total number of primordial, primary, secondary, early antral and atretic follicles. (C) Total number of preovulatory follicles and corpora lutea. (D) Percentage of the ovarian follicle area made up of corpora lutea. (E) Percentage of the follicle wall area made up of GCL and TLC from the largest preovulatory follicle. Different letters indicate significant statistical differences with $P < 0.05$; two-way ANOVA followed by Tukey’s post hoc test. Histograms show mean ± SEM data from control+oil (N = 6), control+Flut (N = 7), PNA+oil (N = 5) and PNA+Flut (N = 6) groups.

Competent preovulatory follicles are able to ovulate in response to coordinated gonadotropin actions and an LH surge which occurs on the evening of the proestrus stage in mice. PNA+oil mice had significantly fewer preovulatory follicles compared to control+oil mice (PNA+oil: 7.40 ± 2.89 vs. control+oil: 26.86 ± 3.15 follicles per ovary; $P < 0.001$; **Figure 5.5 C**). Flut treatment resulted in divergent effects in PNA and control animals. Flut increased the number of preovulatory follicles of PNA mice ($P < 0.01$), whereas it attenuated the number of preovulatory follicles in diestrus control mice when compared to controls treated with oil ($P < 0.01$) (**Figure 5.5 C**). Thus, AR signaling blockade can restore the number of preovulatory follicles in a hyperandrogenic state, however direct androgen actions in the ovaries are also critical for the normal final steps of folliculogenesis.

In line with an absence of the proestrous cycle stage detected by vaginal cytology, most PNA mice showed dramatically fewer or a complete absence of corpora lutea (CL) compared to
control mice (PNA+oil: 0.60 ± 0.24 vs. control+oil: 9.57 ± 1.09 CL per ovary; \( P < 0.0001 \); Figure 5.5 C). Although not significantly different, PNA+Flut mice displayed a ~ 4-fold increase in the number of CL when compared to the PNA+oil group (PNA+oil: 0.6 ± 0.24 vs. PNA+Flut: 3.79 ± 0.8 CL per ovary; Figure 5.5 C). Quantification of the percentage of ovarian area containing corpora lutea was significantly decreased in PNA+oil group when compared with control+oil (\( P < 0.001 \)) and control+Flut groups (\( P < 0.05 \)), whereas PNA+Flut exhibit a trend of increase toward similar values of control groups (Figure 5.5 D). Together, these data suggest that long-term AR signaling blockade, associated with changes in brain wiring, is able to increase the recruitment of preovulatory follicles, and ultimately support the return of ovulation and the capacity for fertility.

The composition of the preovulatory follicle wall thickness is indicative of gonadotropin-mediated actions in the ovaries (Gilling-Smith et al., 1994; Moore, Prescott and Campbell, 2013). Hence, the thickness of the granulosa cell layer (as a mean of determining FSH actions) and the theca cell layer (as a mean of determining LH actions) were measured. The granulosa cell layer was significantly thinner in PNA+oil mice compared to control groups (\( P < 0.05 \)). Flutamide treatment restored the granulosa cell layer thickness to levels not different to controls (Figure 5.5 E). The theca cell layer was significantly thicker in PNA+oil mice compared to controls (PNA+oil: 18.60 ± 1.51 vs. control+oil: 12.21 ± 0.57 vs. control+Flut: 12.51 ± 0.82 % of percentage of the preovulatory follicle wall area; \( P < 0.05 \)). AR signaling blockade significantly reduced the thickness of the theca cell layer in PNA mice to control levels (11.06 ± 0.97 %; Figure 5.5 E), suggesting that long-term androgen receptor blockade leads to the normalization of gonadotropin-mediated actions in the ovary.
5.4 Discussion

Previous results elucidated that altered GABA-to-GnRH neuron circuit in the brain is present prior to puberty (Chapter 3) and androgen excess occurs during early adulthood in a PCOS-like condition (Chapter 4). Those findings suggested that PNA treatment promoted an abnormal and hard wired GABAergic innervation to GnRH neurons, leading to the neuroendocrine and reproductive impairment following the activation of the HPG axis. However, clinical evidence demonstrated that chronic Flut treatment is able to restore normal neuroendocrine regulation of LH secretion in PCOS women (Eagleson et al., 2000). This implied that a certain degree of plasticity within the GnRH neuronal network might be achieved by blocking androgen-mediated actions in PCOS. Therefore, the present study tested the hypothesis that this recovery of normal neuroendocrine function driven by Flut actions could be associated with the rescue of normal GABAergic wiring on GnRH neurons. The present results revealed that long-term blockade of AR signaling is sufficient to reverse abnormal GABAergic innervation to GnRH neurons. Flut treatment in PNA mice was able to reduce GABA inputs on different GnRH neuron compartments and membrane sites leading to a similar pattern of diestrus control animals. In addition, rescuing of altered brain wiring was followed by an improvement of estrous cyclicity and an increase in the recruitment of preovulatory follicles in PNA mice.

Androgens can induce synaptogenesis in the female brain and modify pre-established circuits through neuronal plasticity mechanisms (Leranth, Hajszan and MacLusky, 2004a). Elevated androgen levels negatively impact the female brain and the reproductive function as observed with anabolic steroid abuse animals models (Penatti et al., 2011) and in PCOS models (Roland and Moenter, 2014). AR is expressed in the hypothalamus of adult female rodents (Roselli, Handa and Resko, 1989; Brock, De Mees and Bakker, 2015) and humans (Fernández-Guasti et al., 2014).
2000); nevertheless, and the biological relevance of AR signaling during adulthood is better characterized in pathological states. The requirement of AR signaling for the development of PCOS was recently addressed using a postpubertal androgenized (PPA) mouse model (Caldwell et al., 2017). This study reported that a neuron-specific deletion of AR protects mice from developing the reproductive impairment of PCOS induced by PPA treatment (Caldwell et al., 2017). In contrast, granulosa-specific AR knockout, which targets androgen actions in the ovary, was not sufficient to protect mice from presenting PCOS-like traits (Caldwell et al., 2017). These data indicated that androgen actions in neurons are implicated in the full manifestation of PCOS. The present findings suggest that Flut treatment might have a direct impact on female brain wiring, which ultimately led to the improvement of reproductive cycles and ovarian function in PNA mice.

Although GnRH neurons do not express AR (Huang and Harlan, 1993; Herbison et al., 1996), central actions of androgens, which impact reproduction, are place upstream in steroid-sensitive afferent neurons. GABA neurons are considered important afferent neurons capable of integrating sex steroid-mediated feedbacks to GnRH neurons and regulate GnRH neuron activity. Specifically, DHT-induced hyperandrogenemia in adult female mice increases GABAergic transmission to GnRH neurons and disrupts progesterone-mediated feedback (Sullivan and Moenter, 2005). In addition, transgenic approaches using PNA mice have identified greater GABAergic transmission (Sullivan and Moenter, 2004) and innervation (Moore et al., 2015) to GnRH neurons that may underlie the pathophysiology of PCOS. Considering that GABA actions on GABA_{A} receptors are mainly depolarizing in GnRH neurons (DeFazio et al., 2002), enhanced GABA actions may ultimately increase GnRH/LH release. The downstream response of enhanced LH actions on theca cells in the ovaries is an elevated androgen production, which in turn, may act back upstream in androgen-sensitive GABA neurons to increase GABAergic transmission to GnRH neurons. The results presented in this chapter illustrate that chronic AR signaling blockade
might be a potential therapeutic target to cease this continuous cycle in PCOS by rescuing altered neuroendocrine brain circuits. Current work in our laboratory is investigating the expression of AR in GABA neurons in the hypothalamus in normal and PNA female mice to determine whether Flut treatment could directly signal to these neurons.

In the clinic, chronic flutamide (Flut) treatment is able to restore progesterone sensitivity to decrease abnormal LH pulses (Eagleson et al., 2000), improve menstrual regularities (Paradisi et al., 2013) and rescue normal levels of circulating testosterone (De Leo et al., 1998) in PCOS patients. In the present study Flut treatment was also able to restore estrous cyclicity and improve the recruitment of preovulatory follicles in PNA mice. These findings suggest that these improvements may be downstream from Flut-induced changes in the neuroendocrine wiring in the brain. In fact, studies using brain slices have shown that Flut treatment reduce action-potential-independent GABAergic transmission to GnRH neurons in adult PNA mice (Sullivan and Moenter, 2004), suggesting that Flut treatment is able to induce structural changes within the GnRH neuronal network. The ability of AR blockade to restore normal GABA-to-GnRH innervation and rescue downstream reproductive function indicate that circuit abnormalities remain plastic in adulthood and are dependent upon on-going androgen signaling. Future studies in our laboratory will investigate whether these changes in brain wiring can remain following the cessation of Flut treatment.

Prenatal exposure to androgens ultimately leads to ovarian dysfunction later in life as it is evident in women with congenital adrenal hyperplasia (Hague et al., 1990), PCOS daughters (Sir-Petermann et al., 2009) and PCOS animal models (Dumesic et al., 1997; Smith et al., 2009; Moore, Prescott and Campbell, 2013). While AR blockade did not change the follicular arrest of small follicles (primordial and primary), a typical trait of PCOS ovaries (Webber et al., 2003), a
marked effect was observed in recovering the number of preovulatory follicles. Long-term blockade of AR signaling also promoted the recovery of normal granulosa and theca cell layer thickness in preovulatory follicles of PNA mice. LH overstimulation is a primary driver of a condition known as hyperthecosis, which mirrors the increased androgen synthesis by ovarian theca cells (Hughesdon, 1982; Eisner et al., 2002). The present results showed that long-term AR blockade reduced the thickness of the theca cell layer to control levels, probably reflecting a reduced LH pulse frequency drive. Granulosa cells are the main target of androgen actions in preovulatory follicles (Sen et al., 2014); however, granulosa cell specific AR knockout mice does not protect mice from developing the PCOS phenotype with chronic DHT administration (Caldwell et al., 2017). This indicates that the restoration of normal follicle morphology after AR blockade is more likely to be indirect, through the normalization of gonadotropin action, which may reflect normalized regulation of GnRH neuron activity by GABAergic neurons. In addition, Flut treatment significantly decreased the number of preovulatory follicles in control mice. This might be a direct effect of Flut in the ovaries as AR signaling in granulosa cells is known to decrease the expression of pro-apoptotic markers in antral follicles and Flut might be driving the apoptosis of preovulatory follicles in normal mice (Prizant, Gleicher and Sen, 2014; Sen et al., 2014).

In summary, the present findings demonstrated that long-term AR signaling blockade rescues normal GABA-to-GnRH neuron innervation and the restoration of downstream reproductive function in a PCOS-like condition. These results also indicate that circuit abnormalities remain plastic in adulthood and are dependent upon on-going androgen excess in PCOS. Together, our observations suggest that early blockade of androgen signaling in PCOS might be able to restore a normal GnRH neuronal network and highlight AR antagonism as a therapeutic target.
Chapter Six: Functional role of ARN GABA neurons in the modulation of LH secretion in normal and PNA mice
6.1 Introduction

The central control of reproduction in mammals is highly dependent upon the proper functioning of gonadotropin-releasing hormone (GnRH) neurons. Brain circuits mediating gonadal steroid hormone feedback regulate GnRH neuron activity, which in turn, provides the pace of GnRH peptide secretion and the downstream performance of the hypothalamic-pituitary-gonadal (HPG) axis (Herbison, 2014). A series of studies and ongoing debate in the field indicates that regulatory mechanisms through upstream afferents define the pulsatile GnRH secretion defining the so-called GnRH pulse generator (Wuttke et al., 1996; Navarro et al., 2009; Goodman, Hileman, Casey C Nestor, et al., 2013). The location of the GnRH pulse generator was initially suggest in early studies using deafferentation of the medial basal hypothalamus (MBH) from the rostral hypothalamus in rats (Halász and Gorski, 1967; Taleisnik, Velasco and Astrada, 1970; Blake and Sawyer, 1974). Within the MBH, the arcuate nucleus (ARN) has been considered the central node of the pulsatile GnRH/LH secretion as electrolytic lesion of the ARN completely blocks LH pulses in ovariectomized rats (Soper and Weick, 1980).

Early studies demonstrated that anterior hypothalamic deafferentation (AHD) did not change LH pulse frequency in ovariectomized or estradiol-treated ewes (Whisnant and Goodman, 1994). In contrast, AHD ewes treated with progesterone significantly decrease the pulsatile LH secretion suggesting that progesterone-mediated negative feedback in the ARN regulate the GnRH/LH pulse frequency (Whisnant and Goodman, 1994). Additionally, placing microimplants in ewes (Goodman et al., 2011) or microinjections in rats (He et al., 2017) containing RU486, a progesterone receptor (PR) antagonist, disrupts progesterone-mediated attenuation of LH pulse frequency. Together, these data suggest that PR-expressing neurons in the ARN are required to restrain GnRH pulsatile release during times of negative feedback.
An impairment of the negative feedback and, therefore, the desensitization of the GnRH pulse generator, is present in women with polycystic ovary syndrome (PCOS) (Daniels and Berga, 1997; Pastor et al., 1998; Chhabra et al., 2005). Chronic pharmacological blockade of androgen receptor (AR) signaling can re-establish the ability of progesterone to attenuate LH pulse frequency in these patients (Eagleson et al., 2000). This indicates that AR and PR signaling may act antagonistically at the cellular level in the brain to regulate GnRH neuron activity. In fact, electrophysiological studies with mouse brain slice preparations demonstrated that AR signaling disrupts progesterone-mediated attenuation of GnRH neuron activity through GABAergic transmission (Sullivan and Moenter, 2005; Pielecka, Quaynor and Moenter, 2006). Therefore, progesterone-sensitive GABA neurons might be critical in mediating the PR signaling required to negative feedback and enhanced androgen signaling antagonizes progesterone as seen in PCOS.

Previous work from our research group employed a viral-mediated tract-tracing technique and revealed that GABA neurons in the ARN (ARN GABA) innervate GnRH neurons and this afferent input is significantly increased in PNA mice (Moore et al., 2015). In addition, PNA mice also displayed decreased PR expression in ARN GABA neurons and impaired progesterone-mediated negative feedback associated with elevated LH pulse frequency (Moore et al., 2015). These data leads to the hypothesis that ARN GABA neurons may drive the excitation of GnRH neurons and modulate GnRH/LH secretion. Furthermore, these evidence raises the question whether the activation of ARN GABA neurons evokes enhanced LH secretion or whether the requirement to activate these neurons is modified in a PCOS-like condition.

In Chapter 3 of the present thesis, my work revealed that this abnormal GABAergic innervation on GnRH neurons is established before the onset of puberty in PNA mice and these alterations are not dependent on prepubertal elevation of circulating androgen levels. Furthermore,
in Chapter 5 of this study, I presented that these circuit abnormalities remain plastic during adulthood and can be reversed by blocking androgen receptor signaling. These data, together with referenced literature, outline a potential circuit-mediated mechanism through which GABA actions are biologically meaningful to drive LH secretion. In this chapter, I aimed to specifically target and activate ARN GABA neurons by using an optogenetic strategy to express a type of channelrhodopisin-2 and follow LH secretion outcome in male, diestrus female and PNA female mice. In order to understand the dynamics and possible players modulating the LH response following optogenetic activation of ARN GABA neurons, two different frequencies of stimulation were applied: a small stimulation of 2 Hz, which may mainly drive fast-synaptic transmission via neurotransmitters such as GABA, and a ten-fold higher stimulation of 20 Hz, which may drive the release of neurotransmitters and other modulators such as neuropeptides (Liu et al., 2011).

6.2 Methods

6.2.1 Animals

Adult male, diestrus female and diestrus PNA mice were used for all experiments. Transgenic and wild-type C57BL/6 mice (8-12 weeks) were housed at the Hercus Resource Unit (University of Otago - Dunedin) under 12h: 12h light-dark cycles (lights on at 0700h) with ad libitum access to water and food. Selective targeting of ARN GABA neurons was accomplished using VGAT-Cre mouse line (Vong et al., 2011). Homozygous VGAT-Cre (VGAT-Cre^{+/+}) mice were crossed with TdTomato floxed-stoped reporter mice to generate VGAT-Cre^{+/+}-TdTomato male (N = 4) and female (N = 5) mice to evaluate efficacy of viral transfection. Optogenetic activation was performed in heterozygous VGAT-Cre (VGAT-Cre^{+/+}) male (N = 6) and female (N = 7) mice, and Cre negative C57BL/6 (Control Cre^{−/−}) mice (N = 4-5). Mouse lines were confirmed with
genotyping procedures performed by Mrs. Mell Prescott, the Assistant Research Fellow in our laboratory. PNA mice (N = 5) were generated as previously described in Chapter 2 and elsewhere (Sullivan and Moenter, 2004; Moore, Prescott and Campbell, 2013). Briefly, time-mated pregnant dams were injected with 100 µL of dihydrotestosterone (250 µg) in oil, or oil alone to serve as a control, on gestational days 16, 17 and 18. Control vehicle mice (N = 3) were grouped with non-treated female mice (N = 7) to compose the VGAT-Cre+/- diestrus female group (N = 10) as LH release following optogenetic activation was similar among these animals.

6.2.2 Viral transfection and stereotaxic intracerebral surgery

6.2.2.1 Selective targeting and expression of Adeno-Associated Virus (AAV) in ARN GABA neurons

To specifically activate ARN GABA neurons, VGAT-expressing neurons were transfected with a blue light-sensitive channelrhodopsin-2 (ChR-2), a Na+ channel that controls electrical excitability (Zhang et al., 2010). All optogenetic experiments used an engineered channelrhodopsin-2 E123T accelerated variant (ChETA). As previously characterized, ChETA is considered an optimized version of ChR-2 allowing high spike fidelity, decreased spurious depolarizations and faster recovery from post-stimulus inactivation (Gunaydin et al., 2010; Urban et al., 2012a). To express ChETA in ARN GABA neurons specifically, a Cre-Lox system was used including the Cre recombinase-dependent adeno-associated virus (AAV) with a ChETA-eYFP expression cassette under the control of an elongation factor 1α (EF1α) promoter; and the expression driver transgenic VGAT-Cre mice (Figure 6.1 A). Briefly, within the AAV, the transgene ChETA-eYFP was inserted in a double-floxed inverted orientation (DIO) flanked by loxP and lox2272 sites. The viral vector used in all experiments was the AAV2/9-EF1α-DIO-ChETA-eYFP (AAV9-ChETA; 3.15 x
10^{13}\text{ GC/mL; PennVector Core}). In the presence of Cre-recombinase enzyme, which is only present in VGAT-expressing neurons, the transgene is “flipped” to the right orientation to allow for the expression of ChETA-eYFP in GABA neurons with high spatial and temporal control. Under anesthesia, adult VGAT-Cre^{+/+} mice were bilaterally injected with AAV9-ChETA into the ARN. The expression of the transgene could be visualized 2-3 weeks following injection. Diestrus female (N = 4) and male (N = 5) Cre-negative control (Cre^{-/-}) mice were also injected with AAV9-ChETA. Additional control female VGAT-Cre^{+/+} mice (N = 2) were injected with a control virus containing the reporter tag eYFP without ChETA expression (AAV2/9-EF1α-DIO-EYFP.WPRE.hGH; 6.69 x 10^{13}\text{ GC/mL; Penn Vector Core}).
Figure 6.1. Cre-Lox recombinase technology and optogenetics to specifically activate ARN GABA neurons in vivo. Schematic design showing how the Cre-Lox system is used to express the adeno-associated virus 2/9 (AAV2/9)-ChETA-eYFP in vesicular GABA transporter (VGAT) neurons. A double-floxed inverted (DIO) ChETA-eYFP sequence cassette is flanked by loxP and lox2272 sites, under the control of the ubiquitous EF1α promoter. This genomic sequence is then packaged into the AAV2/9 vector to allow viral delivery and expression of ChETA-eYFP in the presence of Cre. When viral vector is injected into a Cre-driver mouse line, such as VGAT-cre mice, the Cre-dependent expression of ChETA-eYFP is driven exclusively in GABA neurons. ChETA is a blue light-sensitive channelrhodopsin that allows high fidelity optogenetic control of neuronal depolarization (Figure adapted from Urban et al., 2012).

6.2.2.2 Surgical procedure of stereotaxic intracerebral injections

Adult mice were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus (Stoeling®, Digital Lab Standard) with head and nose fixed. All experiments were performed with sterile instruments and aseptic conditions. Mice were given s.c. injection of carprofen (5 mg/kg body weight) after anesthesia was confirmed. A small midline incision was made in the skin to expose the skull with scalpel blade. Connective tissue was removed from the bone to allow better visualization using a dissection microscope (20X; Leica®, Wetzlar, Germany). Bregma position was used as stereotaxic reference zero and X and Y coordinates were calculated to target the transition region from tuberal (a.k.a. middle) to caudal ARN (tARN-cARN) of the hypothalamus. Coordinates according to the mouse brain atlas (Paxinos and Franklin, 2004) were -0.9 mm anterior-posterior, ±0.3 mm medial-lateral and -6.0 mm dorsal-ventral from the dura mater layer. Mice were given simultaneous bilateral 200-nL injection of AAV9-ChETA or AAV9-eYFP into the ARN at a rate of 50 nL per minute. Syringes were left in situ for 5 minutes prior to injection.
and for 10 minutes after injection. Mice were left to recover and to allow viral transfection for 3 weeks.

6.2.3 *In vivo* optogenetic activation protocol in unconscious mice

6.2.3.1 Surgical procedure

All surgical procedures for optical fiber implantation and the optogenetic activation protocol were modelled after previous reports (Campos and Herbison, 2014; Han *et al.*, 2015) and following pilot studies from the initial stage of this PhD project (see more information in Appendix IV). Briefly, following 3 weeks post AAV injection, mice were given 2% isoflurane anesthesia, and head and nose were placed and fixed in stereotaxic apparatus (Stoeling®, Digital Lab Standard). Animals were positioned on a heat-pad and internal body temperature was kept at 35°C during the whole procedure. A small midline incision was made in the skin to expose the skull with scalpel blade and craniotomy was performed with surgical drill. Cannula with a 100-μm diameter optical fiber (Thorlabs, Inc.) was implanted in the rostral preoptic area (rPOA) with the following mouse brain coordinates: +0.9 to +1.0 mm anterior-posterior, 0.0mm medial-lateral (pulling the sagittal sinus aside) and -4.2 mm dorsal-ventral (Paxinos and Franklin, 2004). The optical fiber was left *in situ* for at least 30 minutes before commencing serial blood collection as described below. Following the light activation protocol, pituitary responsiveness to GnRH was tested by administering GnRH (s.c. 200 ng/kg in 100-nL saline 0.9% solution) and collecting additional blood samples. At the end of the protocol, all mice underwent transcardial perfusion with paraformaldehyde (PFA) 4% and brain tissue was collected for post-mortem examination as described in Chapter 2 (*Figure 6.2*).
Figure 6.2. In vivo optogenetic activation protocol in unconscious mice. (A) Mice were kept under isoflurane anesthesia (2%) with head and nose fixed in a stereotaxic frame during the whole protocol. Cannula with a 100-μm diameter optical fiber was implanted into the brain parenchyma aiming for the arcuate nucleus of (ARN) of the hypothalamus in VGAT-Cre+/+ and Cre−/− control mice. Target was achieved based on coordinates identified in the mouse brain atlas (Paxinos and Franklin, 2004). Mice were kept on top of a heat-pad and the internal temperature was measured and controlled during protocol. (B) Following the implantation of the fiber, mice were kept at rest for at least 30 min before commencing blood sampling. Blood samples were taken every 6 minutes (black circles) in all times and...
every 3 minutes (red circles) during and after blue light was turned on (blue bloxes). Blood
samples were later used to measure LH pulsatile release following optogenetic activation of
ARN GABA neurons. Following light stimulation, mice were injected with GnRH (200
ng/kg) to test pituitary gland responsiveness. Animals were perfused with paraformadehyde
(PFA) 4% at the end brain tissue was collected.

6.2.3.2 Optical stimulation & serial blood sampling

The light stimulation consisted of 5-ms blue light (473 nm) pulses using a diode-pumped solid-
state (DPSS) laser (IkeCool©) controlled by Grass S88X stimulator. The power light at the tip of
the optical fiber was set at 5mW. Each mouse received two optical stimulations in a random order
and delivered at 2 or 20 Hz for 10 minutes. Serial blood samples (3 µL each) were firstly collected
at the baseline -13, -7 and -1 minutes considering time 0 as the start of optical stimulation. Blood
sample collection was performed every 3 minutes (at 2, 5, 8, 11, 14 and 17 minutes from time 0)
followed by collection every 6 minutes until 75 minutes of optogenetic protocol. The a second both
baseline and blood sample collection are performed with the second optical stimulation. Following
light stimulation, mice were injected with GnRH (200 ng/kg) to test pituitary gland responsiveness,
and blood samples were collected at –5 (baseline), 0 and 15 minutes. Whole blood samples were
diluted in 57 µL of 0.1 M phosphate buffered saline-tween (PBS-T) pH 7.4, frozen in dry ice and
stored at -20 °C for subsequent hormone measurement.

6.2.4 Hormone measurement – LH ELISA

A well-established ultrasensitive ELISA method was used to measure circulating LH in mice as
previously reported (Steyn et al., 2013; Czieselsky et al., 2016). Briefly, 96-well high affinity
binding plates (Corning©) were coated with a bovine LHβ518B7 monoclonal antibody (1:1,000
in 1X PBS; 50 µL per well). Circulating hormone levels were determined using a standard curve for LH generated with serial dilutions of a mouse LH-RP reference provided by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Standards, positive controls and samples were incubated in the plates for 2h in a humidified chamber on an orbital shaker at room temperature. A rabbit polyclonal primary antibody for LH (1:10,000; rabbit antiserum AFP240580Rb; National Hormone and Peptide Program, USA) and a polyclonal goat anti-rabbit IgG secondary antibody (1:1,000 in 50% blocking + 50% PBS-tween buffer solution; DAKO) were used for this assay. LH levels were determined using an absorbance plate reader at 490 nm and 650 nm wavelengths (more details about the protocol can be found in Chapter 2). The sensitivity of this routine LH ELISA assay was 0.002 ng/mL. The average intra-assay coefficient of variation was 3.1% and the inter-assay coefficient of variation was 10.7%.

6.2.5 Brain slice electrophysiology (Spike fidelity)

Acute coronal brain slices (250-µm thick) accommodating the ARN were prepared from either VGAT-Cre+/-TdToma or VGAT Cre +/- GnRH GFP male mice (N = 3 each, but pooled since no difference could be detected) injected with AVV9-ChETA-eYFP as reported elsewhere (de Croft et al., 2012; Han et al., 2015). These experiments were performed in collaboration with Dr Sabine Hessler, a postdoctoral fellow in our laboratory. Briefly, brain slices were kept at 32°C in 95%/O2/CO2 artificial cerebrospinal fluid with (in mM) 120 NaCl, 3 KCl, 1 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, 1.2 MgCl2, 10 HEPES, and 10 glucose. Cell-attached voltage-clamp (10-30 MΩ) recordings of ARN ChETA-expressing neurons were performed using an upright epifluorescence microscope (Olympus). Following brief illumination with green light, ARN ChETA-expressing neurons were patched using differential interference contrast optics. Blue light (473 nm) stimulation consisted of 5-ms blue light pulses using a Grass S88X stimulator controlled
by diode-pumped solid-state (DPSS) laser (IkeCool©). Light pulses were delivered at 2, 5, 10, 20, 50 and 100 Hz, and power light at the tip of the optical fiber was set at 10% of the light (~ 5mW). Action potentials were recorded (3- to 5-MΩ pipettes) were recorded using 0-mV command voltage. Data acquisition used a Multiclamp 700B amplifier (Molecular Devices) coupled with Digidata 1322A.

6.2.6 Immunohistochemistry

Perfusion PFA 4%-fixed brains were saturated in a 30% sucrose-Tris buffer saline (TBS) solution overnight and cut into three series of 30-μm thick coronal sections using a freezing stage microtome (Leica®, Wetzlar, Germany). Coronal brain sections throughout the ARN and rostral preoptic area (rPOA) of the hypothalamus were chosen according to the mouse brain atlas (Paxinos and Franklin, 2004). To determine the proportion of ARN GABA neurons transfected with AAV9-ChETA-eYFP, free-floating dual-label immunohistochemistry was performed using the primary antibodies polyclonal chicken anti-GFP (1:2,500, Aves Labs Inc.) and polyclonal rabbit anti-GnRH antiserum (1:5,000, gift from Dr Allan Herbison, University of Otago). Brain sections were incubated with the primary antibodies and 2% normal goat serum for 48 h at -4 °C. AlexaFluor488 goat anti-chicken (1:200) and AlexaFluor647 Cy5 donkey anti-rabbit (1:500, Abcam®) were used as secondary antibodies. TdTomato reporter expression was visualized by its endogenous fluorescent signal without any immunolabeling. Primary antibody omission served as a negative control of the procedure.
6.2.7 Image acquisition and analysis

Brain sections were imaged using a Nikon A1R Multi-photon confocal microscope with 488, 543, and 647 nm diode lasers and a Plan NeoFluor 40X oil objective (1.30 NA). High magnification images were acquired using a 3x or 5.2x zoom function when suitable and taking Z-stacks of 0.5 μm steps (pinhole at 1 AU). Two representative coronal sections from both the tARN and cARN were selected to determine the colocalization of TdTomato (GABA)- and eYFP (ChETA)-expressing neurons in male (N = 4) and female (N = 5) VGAT-Cre^{+/−}-TdTomato mice and imaged using Plan NeoFluor 20X objective. As observed during imaging, the detection of TdTomato is mainly restricted to the nucleus and cell bodies, while eYFP immunostaining is prevalent at the cell membrane and neuronal fibers. Therefore, to confirm double labelled ARN GABA neurons, images were acquired with Z-stacks of 1-μm steps and analysis was undertaken identifying positive cells throughout the Z-stacks. Injection site in the ARN and optical fiber position in the rPOA were checked for all mice. Confocal images were analyzed with software NIS-Elements AR 4.00.00 (Nikon® Instruments Inc.). Off-target injections or optical fiber placement outside the rPOA were excluded from data analysis.

6.2.8 Statistical analysis

Statistical analysis was performed with PRISM software 7.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS 24.0 Statistics software (IBM Corp., Armonk, NY, USA). Normal distribution was determined with D’Agostino-Pearson’s normality test. Statistical analysis of LH release values was analyzed with a one-way repeated measures ANOVA with Dunnet’s post hoc test to compare each point of blood sample collection to the baseline (average of -7 and -1 minute time points). Fold-change in LH levels was calculated by subtracting the average baseline from the
average of the three highest amplitude values of the LH release curve (14, 17 and 23 minutes) following optogenetic activation as previously reported (Han et al., 2015). To calculate exogenous GnRH-evoked LH secretion (pituitary responsiveness test) average baseline values was subtracted from LH peak release at 15 minutes after s.c. injection of GnRH. Statistical analysis of change in LH and GnRH-evoked LH release among male, female and PNA mice used a one-way ANOVA with Tukey’s post hoc test. GnRH-evoked LH release between female and male Cre– mice was determined using Student’s t test. Analysis of optogenetic-evoked spike fidelity in ARN GABA neurons used Kruskal-Wallis H test with Dunn’s post hoc test. Exogenous GnRH-evoked LH secretion between Control Cre–/male and female mice used Mann-Whitney U test. Statistical significance was accepted when \( P < 0.05 \). Graphical representation to display significant different are shown in figures as \( * P < 0.05, ** P < 0.01, *** P < 0.001 \) and **** \( P < 0.0001 \).

6.3 Results

6.3.1 Selective targeting and expression of ChETA in ARN GABA neurons

Specific targeting and expression of ChETA in ARN GABA neurons was evaluated in bilaterally injected diestrus female (N = 5) and male (N = 4) VGAT-Cre+/–-TdTomato mice. Three weeks following stereotaxic surgery, brains were analyzed, and confocal imaging revealed that expression of ChETA-eYFP was spread within the ARN (Figure 6.3 A). Viral transfection was present around the injection site (Figure 6.3 B) and also extended beyond this range throughout the ARN (Figure 6.3 C) reflecting the anterograde and retrograde properties of the AAV2/9 in neurons (Rothermel et al., 2013). Expression of ChETA-eYFP in the ARN was mainly observed in cell bodies and neuronal fibers, whereas TdTomato reporter expression was typically found around the nucleus of VGAT neurons (Figure 6.3 B and C). Interestingly, VGAT neurons, which were not transfected
with AAV9-ChETA, were observed receiving putative contact from ChETA-expressing fibers (Figure 6.3 D).

**Figure 6.3. Expression of AAV9-ChETA-eYFP in ARN GABA neurons.** Dual fluorescence images show ARN GABA neurons (TdTomato in red) expressing AAV9-ChETA (eYFP in green) in a representative section from a VGAT-cre⁺/--TdTomato male mouse (A) (12.5-µm optical thickness) (scale bar = 100 µm). The site of injection is shown with white dotted lines. Viral transfection was observed around the site of injection in the dorsal part of the ARN (B) and spread widely throughout the nucleus reaching its ventral part (C) (scale bar = 10 µm). ARN GABA neurons also shared ChETA-expressing fibers as observed in neurons which were not transfected with AAV9-ChETA-eYFP but received putative contact (D). (White arrows) (Scale bar = 100 µm).

Initial findings showed that viral injections into the rostral ARN (rARN) did not achieve significant viral transfection and did not present any effect of optogenetic activation protocol and were not included in this study. Hence, viral injections targeted the middle part, which represents the tuberal region of the ARN, and caudal part of the ARN (tARN↔cARN), which represents the
tuberal part of the arcuate nucleus (Figure 6.4 D i and D ii) and caudal aspect of the nucleus (Figure 6.4 D iii). Despite double labeled cells being strongly detected in the whole extension of tARN (Figure 6.4 D i and D ii), ChETA expression was restricted to the medial-ventral portion of the cARN with eYFP-expressing GABAergic fibers.

Figure 6.4. Characterization of the selective targeting and expression of channelrhodopsin-2 E123T accelerated (ChETA) variant in ARN GABA neurons. (A) Sagittal view of the mouse brain showing the site of injection into the ARN to drive the expression of ChETA-eYFP in VGAT-Cre-TdTomato-expressing neurons. (B) Coronal view of the mouse brain with high magnification focus showing the bilateral ventral position of
the ARN along the third ventricle (3V) of the brain. (C) Representative photomicrographs showing VGAT-Cre-TdTomato (red) and ChETA-eYFP (green) expression and overlay image in the tARN (scale bar = 100 µm) (D) Double-labelled photomicrographs displaying viral transfection in the tuberal (i, ii) to caudal (iii) extension of the ARN (tARN↔cARN). Dashed white line delineates the lateral borders of the ARN (3V = third ventricle; scale bar = 100 µm).

Immunohistochemical analysis revealed that ChETA-eYFP was expressed in the great majority of tARN GABA neurons in both diestrus female (93.3 ± 1.6%) and male (94.4 ± 1.5%) mice (Table 6.1). The proportion of GABA neurons within the cARN expressing eYFP was slightly lower than tARN yet with a satisfactory ChETA-eYFP expression of over 80 % in both sexes (Table 6.1). A very small number of neurons displayed a single eYFP signal without TdTomato co-expression (~ 1 %) in both sexes, confirming the specificity of transfection only in Cre+ cells. These results show that ChETA expression can be targeted in ARN GABA neurons using a Cre-dependent AAV to allow optogenetic control of these cells in mice.

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<th>FEMALES</th>
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<tr>
<td></td>
<td>No. VGAT neurons</td>
<td>No. ChETA neurons</td>
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<tr>
<td>tARN</td>
<td>226.3 ± 29.9</td>
<td>213.9 ± 28.1</td>
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<tr>
<td>cARN</td>
<td>260.4 ± 51.3</td>
<td>208.0 ± 33.6</td>
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Table 6.1. Expression of channelrhodopsin-2 E123T accelerated (ChETA) variant in ARN GABA neurons of the mice. The table provides mean ± SEM of the average number per section of single labelled vesicular GABA transporter (VGAT) neurons and ChETA-expressing neurons in the tARN and cARN from diestrus female (N = 5) and male (N = 4) mice. VGAT neurons were identified with the detection of TdTomato reporter gene (red fluorescent tag) present in these cells. The table also provides the mean ± SEM percentage of dual-labelled VGAT and ChETA neurons along the tARN and cARN extent.
from both sexes. Colocalization analysis considered two representative sections of each tARN and cARN regions.

6.3.2 ChETA-assisted circuit mapping: an optical strategy to activate ARN GABA neuronal fibers in the rPOA

Next, we investigated whether ChETA-expressing neurons can faithfully be activated by blue light (473 nm) pulses. In collaboration with Dr Sabine Hessler, a postdoctoral fellow and electrophysiologist in our laboratory, we aimed to target and optogenetically activate ARN GABA neurons in vitro. Dr Hessler was able to show that these neurons can optogenetically respond with high spike fidelity at 2, 5, 10, 20 and 50 Hz with 5-ms blue laser pulses (N = 6/19 GABA neurons) (Silva, Hessler, Herbison and Campbell, unpublished data). When testing a high frequency stimulation of 100 Hz, the spike fidelity was significantly decreased when compared to lower frequencies (100 Hz: 72.5 ± 10.0 % vs. 2-50 Hz: 100.0 ± 0.0 %; P < 0.001; Figure 6.5).

![Figure 6.5. Characterization of ARN GABA neurons transfected with ChETA-eYFP. Histogram shows mean ± SEM evoked spike fidelity at 2, 5, 10, 20, 50 and 100 Hz of optogenetic activation from cell-attached voltage-clamp recordings in ChETA-expressing](Image)
ARN GABA neurons in vitro from VGAT-Cre<sup>+</sup>-male mice (N = 6). Kruskal-Wallis H test with Dunn’s post hoc test; ***P < 0.001. (Silva, Hessler, Herbison and Campbell, unpublished data).

To activate ARN GABA neuronal in vivo in the vicinity of GnRH neuron soma, a ChETA-assisted circuit mapping based on previous reports (Petreanu et al., 2007) was performed. Briefly, VGAT-Cre<sup>+</sup>-mice were bilaterally injected with AAV9-ChETA into ARN at the levels of ARN GABA neuron cell bodies, and the optical fiber was positioned in the rPOA above and around GnRH neurons (Figure 6.6 A and B). Confocal imaging revealed that GnRH neurons were densely innervated by ChETA-expressing fibers in the rPOA which originated in the ARN (Figure 6.6 C) as previously described (Moore et al., 2015). Together, these data indicate that the expression of ChETA in ARN GABA neurons allow precise control of action potential firing in these neurons and that we can map and investigate the function of this specific GABAergic pathway using an optogenetic tool.
Figure 6.6. ChETA-assisted circuit mapping. (A) Coronal mouse brain template showing the position of optical fiber in the rPOA. (B) Photomicrograph show optical fiber (white dashed lines) above GnRH neurons (in blue) and ARN GABA neuronal fibers (in green), which were transfected with AAV9-ChETA-eYFP (scale bar = 100 µm). (C) Projected confocal image and 3D reconstruction of GnRH neuron (in blue) in the rPOA being innervated by ChETA-expressing fibers (magenta arrowheads point putative contact) from ARN GABA neurons (scale bar = 10 µm). High-magnification 3D reconstruction shows ChETA-expressing fiber contact onto GnRH neuron soma (i) and proximal dendrite (ii, iii) (scale bar = 1.5 µm). rPOA = rostral preoptic area; OVLT = organum vasculosum of the lamina terminalis.
6.3.3 Optogenetic activation of ARN GABA neurons in vivo elicits LH secretion and reveals a different dynamic of LH release between diestrus female and PNA mice

To determine whether ARN GABA-to-GnRH neuron circuit is functionally relevant to modulate LH secretion, ARN GABA neuronal fibers in the rPOA were activated in male (N = 6), diestrus female (N = 10) and diestrus female PNA (N = 5) VGAT-cre<sup>+/−</sup> mice using in vivo optogenetics. Previous studies have shown that mice under isoflurane anesthesia do not display LH pulsatile secretion and exhibit low circulating LH levels during the whole protocol (Campos and Herbison, 2014; Han et al., 2015). Therefore, changes in LH release during light stimulation protocol in isoflurane-anesthetized mice are triggered by the stimulation of GnRH neurons. Optogenetic activation of ARN GABA neurons at 2 Hz for 10 minutes did not alter blood LH levels; however, 20-Hz optical stimulation for the same period elicited a robust increase in LH levels as shown in representative examples in Figure 6.7 A. Increment changes were typically observed over ~ 10 minutes from the start of the optical stimulation and remained higher after the blue light was turned off in all animals. In diestrus female (N = 4) and male (N = 5) Cre<sup>−/−</sup> control mice injected with AAV9-ChETA and in VGAT-Cre<sup>−/−</sup> female mice (N = 2) injected with AAV9-eYFP, blue light stimulation had no effect on LH secretion (Figure 6.7 B).
Figure 6.7. Optogenetic activation of ARN GABA neurons in vivo generates LH release in mice. (A) Representative examples of circulating LH levels from VGAT-Cre<sup>+/−</sup> male (N = 6), diestrus female or female oil-vehicle (grouped together; see in methods 6.2.1) (N = 10), and diestrus female PNA (N = 5) mice during protocol to optogenetically activate ARN GABA neurons. Blue bars indicate 5-ms blue light stimulation delivered at 2 or 20 Hz during 10 minutes (B) Representative traces of LH levels from diestrus female (N = 4) and male (N = 5) Cre<sup>+/</sup> control mice injected with AAV9-ChETA and in VGAT-Cre<sup>+/−</sup> female mice (N = 2) injected with AAV9-eYFP. Blue bars indicate 5-ms blue light stimulation delivered at 2 or 20 Hz during 10 minutes.
To investigate the dynamics of LH release in male, diestrus females and PNA mice, each time point was compared to baseline values. All three groups did not show any significant changes when 5-ms blue light pulses were delivered at 2 Hz (Figure 6.8 A). The activation of ARN GABA neurons at 20 Hz for 10 minutes in male and diestrus female groups generated robust increase in LH release, which typically started at 8 minutes from the start of optical stimulation and LH levels remained significantly higher than baseline values ($P < 0.05$) for over 50 minutes (Figure 6.8 B). Conversely, PNA mice exhibited a delayed rise in LH release, which occurred ~11 minutes following optogenetic activation of ARN GABA neurons and, despite LH levels being elevated for 18 minutes ($P < 0.05$), hormone levels returned to baseline shortly after peak values (Figure 6.8 B).
Figure 6.8. Dynamics of LH release following the activation in vivo of ARN GABA neurons. Effect of optogenetic activation of ARN GABA neurons on LH secretion in male (N = 6; blue circles), diestrus female (N = 10; red circles), diestrus PNA (N = 5; black circles). Cre-/- control male (N = 5) and female (N = 4) mice injected with AAV9-ChETA are shown with respective gender matching groups (open black squares). Blue bars indicate 5-ms blue light stimulation delivered at 2 or 20 Hz during 10 minutes. (A) Summary graphs show mean ± SEM values of LH levels during 2-Hz optogenetic activation of ARN GABA neurons in males, diestrus females and diestrus PNA mice. (B) Summary graphs show mean ± SEM values of LH levels following optogenetic activation of ARN GABA neurons with 20-Hz stimulation in males, diestrus females and diestrus PNA mice. Triangles in yellow indicate statistically different LH levels compared with pre-stimulus baseline values (-7 and -1 min) analyzed with repeated-measures one-way ANOVA with Dunnet’s post hoc test.
To detect and compare changes in LH among the male, diestrus female and PNA mice, the magnitude of hormone secretion was calculated by analyzing the average of LH peak values during 20-Hz optogenetic stimulation (17, 23 and 29 minutes after the start of the stimulus). Although PNA mice displayed lower LH peak levels when compared with male and female mice (average LH peak levels: males = 4.94 ± 1.32; diestrus females = 5.18 ± 1.02; diestrus PNA = 2.36 ± 0.50 ng/mL), no significant statistical differences among the groups were detected ($P = 0.21$). During the performance of LH measurement experiments, great differences in baseline values within each group were observed, which ultimately might influence the magnitude of LH peak levels (Patel et al., 2004). To overcome this problem, fold-changes in LH release were determined for each group. Results showed that LH release was significantly higher in males and females when compared with PNA mice following 20-Hz optogenetic activation of ARN GABA neurons ($P < 0.05$), whereas no changes were detected when blue light was delivered at 2 Hz (Figure 6.9). These findings showed that \textit{in vivo} high-frequency stimulation (20 Hz) of ARN GABA neurons can elicit LH release in mice.
Figure 6.9. High-frequency stimulation of ARN GABA neurons induces LH release in mice. Optogenetically-evoked change in LH levels following 2-Hz and 20-Hz optical stimulation in male (N = 6), diestrus female (N = 10) and diestrus PNA (N = 5) mice. *P < 0.05; One-way ANOVA with Tukey’s post hoc test was applied at each frequency.

6.3.4 Pituitary responsiveness to exogenous GnRH

The pituitary responsiveness to exogenous GnRH was tested at the end of the optogenetic activation protocol, in which mice were given s.c. injection of GnRH (200 ng/kg). The pituitary response to s.c. GnRH is also important to evaluate the functioning of the pituitary gland after the optogenetic protocol. Although observed as a decreasing trend for pituitary responsiveness in VGAT-Cre+/− male (4.3 ± 1.2 ng/mL; N = 6) to female (3.4 ± 0.6 ng/mL; N = 9) to PNA (2.3 ± 0.6 ng/mL; N = 5) mice, no statistical differences were detected among the groups (F2, 16 = 1.8; P = 0.19; Figure 6.10 A). On the other hand, GnRH-evoked changes in LH release of control Cre−/− diestrus female mice (N = 4) was significantly lower than control Cre−/− male mice (N = 5) (3.1 ±
0.3 vs. 5.9 ± 0.7 ng/mL; \( P = 0.02 \) (Figure 6.10 B). These data suggest that sex differences in LH release may rise from a differential responsiveness from the pituitary gland as observed in control mice, although pre-exposure to optogenetically-evoked high LH levels might change the pituitary response to exogenous GnRH.

**Figure 6.10.** Exogenous GnRH-evoked change in LH release following optogenetic activation protocol. Graphs show data analysis of s.c. GnRH (200 ng/kg)-evoked increase in LH release (mean ± SEM) at the end of optogenetic activation protocol. (A) Histogram shows comparison of s.c. GnRH-evoked change in LH secretion among VGAT-Cre\(^{+/−}\) male (\( N = 6 \)), diestrus female (\( N = 9 \)) and PNA (\( N = 5 \)) mice. One-way ANOVA with Tukey’s post hoc test. (B) Histogram presents pituitary responsiveness dictating change in LH secretion in control Cre\(^{−/−}\) male (\( N = 5 \)) and female (\( N = 4 \)) mice. \(* P < 0.05; \) Mann-Whitney \( U \) test.
Chapter Six

6.4 Discussion

6.4.1 Summary of findings

To dissect the functional role of ARN GABA neurons, ChETA expression was selectively targeted in these neurons using Cre-Lox recombinase technology. This strategy yielded effective transfection with ChETA of ~ 90% and 80% of VGAT neurons in the tARN and cARN, respectively. Electrophysiological studies in our laboratory have demonstrated that ChETA display high spike fidelity with laser pulses delivered up to 50 Hz and effectively activate ARN GABA neurons. Taking advantage of the AAV9 anterograde/retrograde properties, transgene expression was widely spread within the ARN and elsewhere in the mouse brain. ChETA expression was present in ARN GABA neuronal fibers reaching the rPOA over 1000 µm from the injection site. The present study revealed that an in vivo 20-Hz optogenetic stimulation of ARN GABA neurons for 10 minutes elicits robust LH release for over an hour in male and diestrus female mice similarly, whereas a lower frequency of stimulation (2 Hz) failed to induce changes in LH levels. Despite diestrus female and male mice exhibiting similar optogenetically-evoked increase in LH secretion, PNA female mice presented significantly lower magnitude of LH release when light pulses were delivered at 20 Hz. To determine the nature of these differences, the pituitary responsiveness to exogenous GnRH was tested in all groups. Sex differences in GnRH-evoked LH release were found in control Cre−/− mice with males exhibiting greater LH release when compared with females. Although no significant differences were identified among VGAT-cre+/− male, diestrus female and PNA mice, a smaller response in PNA mice was observed during data analysis.
6.4.2 Using optogenetics to decipher the functional role of ARN GABA neurons modulates GnRH/LH secretion

To map and probe the functional role of ARN GABA neurons modulating GnRH/LH secretion, a ChETA-assisted circuit mapping was performed targeting ARN GABA neuron fibers in the rPOA, which contact GnRH neurons. This approach, commonly used to study other circuits in the brain (Tye and Deisseroth, 2012), may be an effective tool to interrogate the biological relevance of different afferent inputs within the GnRH neuronal network and their role in fertility. Our study confirmed that ChETA exhibits high spike fidelity and reports elsewhere have indicated that this Ch-2 variant shows fast on/off kinetics, fast post-spiking recovery (Gunaydin et al., 2010) and efficient during acute activation in vivo (Jego et al., 2013). Although ChETA has been used to activate fast spiking GABA neurons such as parvalbumin interneurons (Urban et al., 2012b; Assaf and Schiller, 2016), the present study is novel in the sense of applying this powerful strategy to interrogate the role of a specific GABAergic input within the GnRH neuronal network.

The investigation of GABA actions in GnRH neurons has empirical challenges leading to controversial findings across different reports. Early studies demonstrated that injection of GABA into the third ventricle (3V) of the brain promotes LH secretion in a dose dependent manner in male (Ondo, 1974) and female rats (Vijayan and McCann, 1978). Conversely, the majority of pharmacological paradigms reported inhibitory actions of GABA blocking the LH surge by infusing GABA in the medial preoptic area (Herbison and Dyer, 1991) or intraperitoneal (i.p.) administration of GABA A R agonist muscimol in rats (Adler and Crowley, 1986). These divergent findings were likely due to different experimental manipulations, which ultimately obscured the functional relevance of specific GABAergic afferents regulating GnRH neuron activity (Herbison and Moenter, 2011). This PhD thesis advanced our existing knowledge by applying optogenetics
to investigate GABAergic transmission from ARN GABA neurons, which may modulate GnRH neuron activity to promote GnRH/LH secretion. Ongoing studies in our laboratory using brain slice electrophysiology in VGAT-Cre\(^{+/-}\)-GnRH-GFP male and diestrus female mice, which were injected with AAV9-ChETA into the ARN, will determine the direct effect of the activation of ARN GABA neuron on GnRH neuron firing activity. To date, Dr Sabine Hessler has performed on-cell recordings from a GnRH-GFP neuron while optogenetically activating ARN GABA neuron fibers in rPOA brain slices from VGAT-Cre\(^{+/-}\) male mice. Preliminary results show that about 30% of GnRH neurons show immediate activation to 10- and 20-Hz blue light stimulation and around 20% of the cells showed a delayed activation (Figure 6.1). In addition, most GnRH neurons did not respond to 2-Hz stimulation (Silva, Hessler, Herbison and Campbell, unpublished data). Further experiments are being addressed to uncover whether these responses in GnRH neurons are mediated by GABA\(_{A}\)R activation or neuropeptidergic signaling.
Figure 6.11. **Optogenetic activation of arcuate (ARN) GABA neuron fibers directly depolarize GnRH-GFP neurons in vitro.** Representative on-cell voltage-clamped recordings of GnRH-GFP neuron following the activation of ChETA-expressing neuronal fibers from ARN GABA neurons in the rPOA. Coronal brain slices were prepared from VGAT-cre+/− male mice injected with AVV9-ChETA-eYFP into the ARN. Optogenetic protocol was performed with 5-ms blue light pulses (indicated by blue traces) delivered at 10 and 20 Hz over 5 minutes.

Excitatory and inhibitory actions of GABA on GnRH neurons were also investigated in isoflurane-anesthetized GnRH-GFP mice by Constantin and colleagues (Constantin, Iremonger and Herbison, 2013). Researchers showed that GnRH neurons located in the anterior hypothalamic area (AHA) exhibit excitation or inhibition in response to muscimol administration in on-cell recordings. Interestingly, the same group demonstrated early on that the net effect of GABA actions is likely to be determined by a differential expression of GABAAR and GABABR along the GnRH
neuron extent (Constantin et al., 2012). This study showed that GABA$_{\alpha}$R-mediated net excitation was predominantly observed in rPOA brain slices, which preserves the GnRH neuron soma and proximal dendrites, whereas an increase in GABA$_{\beta}$R-mediated net inhibition was seen using parahorizontal brain slices that maintain AHA GnRH neurons and distal segments of other GnRH neurons (Constantin et al., 2012). In addition, ARN GABA neurons are known to robustly innervate the proximal dendrites and cell bodies of GnRH neurons located in the rPOA/OVLT region (Moore et al., 2015). Based on these studies, I performed pilot studies at the beginning of the PhD project (Appendix IV) and discovered that performing viral transfection with ChR-2 at the level of the ARN and optically stimulating ARN GABA fibers in the rPOA led to a robust LH release in mice as opposed to aiming the activation of these neurons directly in the ARN. Different ARN GABA neurons might project to different parts of the GnRH neuron extent; therefore, using this optogenetic strategy might enable us to investigate GABA actions that are known to have a more net excitation in GnRH neurons and be involved in the pathophysiology of PCOS.

6.4.3 Dynamics of LH release following the activation of ARN GABA neurons

Optogenetic activation of ARN GABA neurons using low frequency stimulation of 2 Hz was not effective to induce changes in LH secretion whereas 20-Hz stimulation generated a fourfold LH release in male and diestrus female mice and likely to be through the upregulation of GnRH neuron activity. The activation of GABA neurons did not promote LH pulse-like release as seen, for instance, following the activation of kisspeptin neurons (Han et al., 2015) or GnRH neurons (Campos and Herbison, 2014). Instead, the LH secretion observed after optogenetic activation of ARN GABA neurons lasted longer than those observed in previous reports, promoting LH release above baseline levels for over 60 minutes in male and diestrus female mice groups.
Previous studies showed that low frequencies of stimulation (< 1 Hz) of anteroventral periventricular (AVPV) nucleus monosynaptic inputs to GnRH neurons generate predominantly GABAAR postsynaptic currents (PSCs), whereas higher frequencies promote delayed activation by the neuropeptide kisspeptin in mice (Liu et al., 2011). The present findings, along with the literature may indicate two main possibilities. Firstly, different requirements in vitro versus in vivo to activate GnRH neurons that are sufficient to promote LH secretion might explain these differences. Secondly, the long-lasting LH release at high-frequency stimulation is also suggestive of neuropeptidergic transmission along with GABA (Liu et al., 2011). ARN GABA neurons are known to express a wide range of neuropeptides and other neuromodulators (Zuure et al., 2013; Marshall et al., 2016; Campbell et al., 2017; Chaclaki et al., 2017), which present direct influence in GnRH neurons and for the control of fertility. For instance, neuropeptide Y (NPY), which represents around one third of the ARN GABA neuronal population (Marshall et al., 2016), is able to evoke direct depolarizing and hyperpolarizing responses in GnRH neurons (Roa and Herbison, 2012). Agouti-related peptide (AgRP), which is also colocalized in NPY neurons, exhibits different types of responses in GnRH neurons (Roa and Herbison, 2012). Although ARN kisspeptin neurons are known to elicit strong LH release following optogenetic activation (Han et al., 2015), our laboratory showed that in fact a small population of VGAT neurons in the ARN express kisspeptin (Marshall et al., 2016), which seems unlikely to drive such long-lasting LH secretion as seen in our results. A dissection of possible candidates that might drive or be release with GABA from ARN GABA neurons is beyond the scope of the present study; however, future studies in our laboratory will further investigate this issue.

Although significant increase in LH levels were observed at 8 minutes following the start of optical stimulation, 20-Hz-induced LH peak levels occurred around 23 minutes after the beginning of the light stimulation (0 min) in all groups. The present study was limited to determine
the mechanism of this delayed rise in LH release. Studies using electrophysiological recordings in brain slices from GnRH-GFP male mice and dynamic current clamp showed that excitatory GABA induces a delayed temporal shift for excitatory actions of glutamate via AMPA receptors in GnRH neurons (Roberts, Hemond and Suter, 2008). Therefore, a pre-exposure to excitatory GABA actions might “charge” the membrane capacitance of GnRH neurons in order to increase their response to following additional excitatory inputs (Roberts, Hemond and Suter, 2008), which could explain the delayed yet long-lasting LH release following optogenetic activation of ARN GABA neurons.

6.4.4 Activation of ARN GABA neuron promotes altered LH secretion in PNA mice

Enhanced GABAergic transmission in GnRH neuron activity in PNA mice (Sullivan and Moenter, 2004) may be culprit for the elevated LH pulse frequency secretion in adulthood (Moore et al., 2015). Based on these evidence, we firstly postulated that the activation of ARN GABA neurons would promote a greater LH release in PNA mice than in normal females. Contrary to this initial hypothesis, 20-Hz optogenetic activation of ARN GABA neurons induced smaller magnitude and changes in LH release of PNA mice when compared with male and diestrus female mice. Of note, early studies had shown that small changes in the amplitude of LH secretion are a direct readout of a high GnRH pulse frequency. Specifically, high frequency secretion of GnRH generates lower LH release amplitude as a reflection of the pituitary LH releasable pool stores in ewes (Clarke and Cummins, 1985). Evidence from studies in non-human primates (Wildt et al., 1981) and rodents (Lerrant et al., 1995) demonstrated that high-frequency GnRH stimulation decreases LH synthesis and secretion. Therefore, we might attribute the small magnitude release of LH in PNA mice due to the abnormally elevated GnRH/LH pulsatile secretion in a PCOS-like condition.
The pituitary sensitivity following optogenetic protocol was similar among PNA, male and diestrus female mice, which indicates that altered LH release might be a primary outcome of altered GABAergic wiring but not pituitary responsiveness. Sexually dimorphic differences in the pituitary sensitivity could be detected in control Cre^{-/-} mice as previously reported in that males display greater GnRH-evoked LH release than female mice (Han et al., 2015). Accordingly, serial pulse bleeding studies showed that the LH pulse frequency of male mice is lower than female mice (Steyn et al., 2013; Czieselsky et al., 2016), implying that the GnRH/LH frequency determines the LH releasable pool in the pituitary gland. However, following robust optogenetically-evoked LH secretion in VGAT-cre^{+/-} female and male showed similar exogenous GnRH-evoked LH release. This might indicate that a GnRH self-priming effect following optogenetic activation protocol might alter the pituitary outcome (Pickering and Fink, 1976, 1979). Future experiments are required to determine the pituitary responsiveness in PNA and normal female mice without optogenetic activation of ARN GABA neurons.

6.4.5 Conclusion

The results in these chapter outline a potential circuit-mediated mechanism by which ARN GABA neurons may drive GnRH/LH secretion and be biologically meaningful for the control of fertility. Furthermore, I was able to characterize an optogenetic approach, which can be applied to specifically target and activate discrete GABAergic populations within the hypothalamus. These data show critical support for the hypothesis that ARN GABA neurons are a functional component of the GnRH neuronal network and that abnormalities in this circuit generates altered LH release such as seen in PCOS.
Chapter Seven: Final Discussion
7.1 Physiological and clinical relevance of the study

PCOS is a major hyperandrogenic endocrine disorder leading to female infertility and affecting approximately one in ten women around the world (Dumesic et al., 2015). Women with PCOS present distressing effects of circulating androgen excess such as hirsutism, alopecia and acne problems (Azziz et al., 2009). PCOS is also strongly associated with metabolic comorbidities, for instance obesity and diabetes mellitus type 2 (Ladrón de Guevara et al., 2014), and psychosocial disorders such as depression (Milsom et al., 2013). Although PCOS is a prevalent and complex disorder, the etiological mechanisms causing the disease remain unclear. Both clinical and basic research supports the idea that prenatal androgen excess may change the architecture of the female brain and contribute to the development of PCOS during adult life. Specifically, disordered GABAergic innervation and activity transmission to GnRH neurons occurs in a PCOS (Sullivan and Moenter, 2004; Moore et al., 2015) and women with PCOS display high levels of cerebrospinal fluid GABA (Kawwass et al., 2017). Other conditions which leads to high androgen levels, such anabolic steroid abuse, impact GABA actions in GnRH neurons (Nieschlag and Vorona, 2015). In addition, women under treatment for epilepsy, which ultimately increases the GABAergic tone, commonly develop PCOS-like reproductive impairments (Bilo and Meo, 2008). Therefore, this triad of androgen excess, enhanced GABAergic innervation to GnRH neurons and reproductive dysfunction may underlie the pathophysiology of PCOS.
7.2 Background

The present PhD thesis aimed to investigate the ontogeny, plasticity and functional role of GABAergic innervation on GnRH neurons in PCOS. The examination of altered wiring in the developing female brain has been highlighted in clinical and pre-clinical studies as one possible culprit for the origin of PCOS (Daniels and Berga, 1997; Pastor et al., 1998; Sullivan and Moenter, 2004). Despite the speculated heritable etiology of PCOS, large genetic studies have failed to detect defects in single genes that could explain the complexity and prevalence of this broad disorder. On the other hand, prenatal exposure to elevated androgens is strongly associated with the developmental origin of the neuroendocrine derangements in PCOS and the postpubertal manifestation of the disease (Hague et al., 1990; Sir-Petermann et al., 2002; Padmanabhan and Veiga-Lopez, 2013b).

Prenatal androgen excess is known to induce increased GABAergic transmission (Sullivan and Moenter, 2004) and innervation (Moore et al., 2015), which is associated with impairment of reproductive function in PNA mice. Elevated GABA actions within the GnRH neuronal network may lead to an overexcitation of GnRH neurons that ultimately increase GnRH/LH secretion. In addition, clinical evidence identified that altered LH secretion in PCOS can be restored through the chronic blockade of androgen-mediated actions (Eagleson et al., 2000) indicating a possible rescue of normal brain wiring. Based on these important findings, this PhD project was aimed to answer critical questions which remained elusive about the development and functional role of altered GABA actions in the brain leading to the manifestation of PCOS.

The initial studies presented in this thesis aimed to determine the ontogeny of abnormal GABA-to-GnRH neuron innervation in relation to the onset of puberty and time of androgen
excess. To address this specific object, immuistochemical studies were performed to identify GABA inputs on GnRH neurons in prepubertal PNA mice and circulating testosterone levels were assessed (Chapter 3). Further studies aimed to determine the developmental timing of androgen excess from puberty to adulthood in PNA mice, and whether the removal of hyperandrogenic ovaries in these animals would promote similar LH pulse secretion as detected in normal females (Chapter 4). Exploring the requirement of androgen receptor (AR) signaling in PCOS, this thesis investigated the effect of long-term AR blockade on GABAergic contact onto GnRH neurons in PNA mice (Chapter 5). Emphasizing the role of altered brain wiring for the manifestation of PCOS, this PhD work also interrogated whether rescuing normal GABA-to-GnRH neuron circuit would promote improve reproductive cycles and ovarian morphology in PNA mice (Chapter 5). Finally, the functional role of ARN GABA neurons in the regulation of LH secretion was investigated by applying an optogenetic strategy, which specifically target and activate this GABAergic population in normal and PNA mice (Chapter 6).

This final discussion will summarize and integrate the main findings of this PhD work and highlight the contributions and limitations of these studies. The results of this PhD project will be critically outlined and discussed to envision their implications for the understanding of PCOS pathophysiology. Lastly, this final discussion will feature some possible avenues for future research based on the present findings.
7.3 Summary of findings

The initial findings of this PhD work revealed that enhanced GABAergic wiring onto GnRH neurons is established early (PND 25), prior to the development of post-pubertal circulating androgen excess (Chapter 3). GABAergic innervation was visualized with immunolabeling of VGAT contact onto GnRH-GFP neurons. VGAT appositions were significantly greater on GnRH neuron soma and both primary and secondary dendrites in PNA females. Very recently, another research group has published evidence that GABAergic postsynaptic currents (PSC) are elevated in 3-week old PNA mice (Berg, Silveira and Moenter, 2018). This increase GABAergic drive might contribute to abnormal GnRH neuron activity in these animals in the same prepubertal stage (Dulka and Moenter, 2017). These complimentary findings suggest that prenatal androgen exposure drives a biological program that disrupts normal GABA-to-GnRH neuron circuit wiring before puberty and may drive the neuroendocrine impairment of PCOS in adulthood.

As outlined in the results of Chapter 3, the presence of brain wiring abnormalities might be dependent upon early androgen excess in PNA mice and not prepubertal androgen elevation. Therefore, the developmental timing of androgen excess in PNA mice was addressed in this study. Androgen excess was found to be increased in PNA mice during early adulthood at PND 50 and PND 60 (Chapter 4). Interestingly, during pubertal transition at PND 40, control and PNA mice exhibited highly variable testosterone levels, which significantly decrease in normal females toward adulthood. In contrast, increasing increments of testosterone levels were observed in PNA mice leading to elevated circulating androgen levels in adulthood (Chapter 4). All hyperandrogenic PNA mice displayed disrupted estrous cyclicity, showing longer metestrous stages and lacking the detection of proestrous, which indicate anovulatory cycles (Chapter 4). The same cohort of mice were followed to address the question whether hyperandrogenic ovaries were
the only culprit in the neuroendocrine impairment of a PCOS-like condition. From this study, findings revealed that while post-castration LH pulse frequency and basal LH levels were similar between control and PNA mice, PNA mice had a higher LH pulse amplitude and a higher magnitude of LH release compared to controls (Chapter 4). These results suggest that even in the absence of elevated circulating androgens, the regulation of pituitary gonadotropin secretion is different to healthy controls. This suggests that wiring changes in the brain that drive increased LH secretion might still be present maintained in a short-term absence of testosterone.

Androgen excess might be involved in both the initial developmental programming of the PCOS-like phenotype and the progression of the disease during adulthood. Pharmacological treatment with flutamide (Flut), over the defined time when androgen excess develops (from PND 40 to PND 60), tested the hypothesis that blockade of AR signaling would restore reproductive cycles by rescuing normal GABA-to-GnRH neuron innervation in PNA mice. I discovered that long-term blockade of AR signaling is sufficient to reverse abnormal GABAergic innervation to GnRH neurons, improve estrous cyclicity and an increase in the recruitment of preovulatory follicles in PNA mice (Chapter 5). Although, I could not detect a significant improvement of corpora lutea (CL) in PNA+Flut mice, this group exhibited a ~ 4-fold increase in the number of CL when compared to PNA+oil group, suggesting a progressing recovery of the ovulatory rate. In addition, AR blockade rescued the normal morphological features of both preovulatory follicle granulosa and theca cell layers suggesting an improvement in gonadotropin-mediated actions in the ovary (Chapter 5). These compelling findings indicate that improvement of reproductive function in PNA mice might result from upstream restoration of normal GABA-to-GnRH neuron wiring. It also indicates that, although prenatal androgen programs altered GABAergic innervation, this circuit remains plastic by blocking androgen actions in the adult female brain.
The neuroanatomical evidence of robust innervation from ARN GABA neurons to GnRH neurons (Moore et al., 2015) and the current findings implied that this GABA neuronal population could have a functional role for the regulation of GnRH/LH secretion. The last chapter of this thesis proposed to investigate this idea by applying an optogenetic strategy to activate ARN GABA neuronal fibers, which putatively innervate GnRH neurons, located in the rPOA/OVLT. Using viral tracing and Cre-LoxP technology, a selective expression of ChETA in ~ 80% of ARN GABA neurons was achieved and in vitro studies showed that activation of ChETA-expressing ARN GABA neurons could trigger action potentials with high fidelity (Chapter 6). Novel results demonstrated that high-frequency optogenetic activation (20 Hz/10 minutes) of ARN GABA neurons in vivo can evoke robust LH release in both female and male mice (Chapter 6). These findings presented that ARN GABA-to-GnRH neuron circuit is functionally relevant to modulate GnRH/LH secretion in both sexes. The activation of ARN GABA neurons also stimulated LH release over a similar timeframe and in response to the same frequency in PNA mice; however, these animals presented a significantly lower magnitude of LH release. The responsiveness of gonadotropes to exogenous GnRH was also decreased in PNA mice, although not statistically different from both male and diestrus female mice (Chapter 6). Evidence from an early study suggested that high GnRH pulse frequency generates lower LH release amplitude in ewes (Clarke and Cummins, 1985). Therefore, this inverted relationship between elevated GnRH pulse frequency in a PCOS-like condition and a lower LH releasable pool in the pituitary might drive the altered LH pulse amplitude in PNA mice. Future studies may investigate the LH releasable pool in non-optogenetically activated PNA and normal female mice to dissect out the nature of the current findings.
7.4 Contributions and limitations of the study

7.4.1 Understanding the ontogeny of altered brain wiring in PCOS

The results of this thesis have shown that prepubertal GnRH neurons receive pronounced GABAergic innervation and that this is significantly increased in PNA mice when compared to normal females. This enhanced GABAergic contact on GnRH neurons was present before the elevation of circulating androgen levels in PNA mice (PND 25), implying that brain abnormalities were programmed by androgen excess in utero, not result of the precocious manifestation of hyperandrogenism. These data unveil an important aspect of the ontogeny of the PCOS-like condition, suggesting that a prenatal biological program promotes altered GABAergic synaptogenesis within the GnRH neuronal network.

PNA treatment occurs during late pregnancy in mice from gestational day (GD) 16 to 18. Before this time, around GD 14 to 15, the vast majority of GnRH neurons have already migrated from the olfactory nasal placode into the preoptic area and caudal diencephalon in the mouse brain (Wray, Grant and Gainer, 1989). A subset of GnRH neurons can produce GABA during early embryonic development and the neurotransmitter synthesis peaks around embryonic day (ED) 15 and decreases significantly toward a virtual absence in GnRH neurons during perinatal period in rodents (Tobet et al., 1996). The restraint of GABA synthesis by embryonic GnRH neurons is essential for the normal positioning of these neurons in the forebrain and normal reproductive function (Heger et al., 2003). To date, no study has emphasized differences in the number of GnRH neurons along the frontal to caudal extent of the hypothalamus in PNA mice, indicating that PNA treatment may not impair fertility by changing the organization or migration of hypophysiotropic GnRH neurons (Prescott and Campbell, unpublished data). Therefore, it seems unlikely that PNA treatment disrupts GnRH neuron migration to the forebrain and changes in GABA-to-GnRH
neuron circuit might happen due to altered organization of presynaptic GABAergic innervation that projects to GnRH neurons.

Recently published findings have also shed some light on the temporal resolution of altered GABAergic innervation of GnRH neurons during prepubertal development. GnRH neurons are reported to receive GABAergic transmission as early as at one week of age in mice (Berg, Silveira and Moenter, 2018). Interestingly, in PNA female mice, GABAergic transmission becomes robustly elevated at 3-weeks of age, returns to low values similar to controls at 4 weeks and it is abnormally high during adulthood (Berg, Silveira and Moenter, 2018). GnRH neurons are surprisingly active during prepubertal stages and, specifically, elevated neuronal activity is observed around 3-weeks of age in normal female mice (Dulka and Moenter, 2017). In contrast, PNA female mice exhibit a decrease of GnRH neuron activity during the same prepubertal stage (Dulka and Moenter, 2017). Interestingly, around 4 weeks of age, PNA mice shows GnRH neuron firing (Dulka and Moenter, 2017) and GABA PSCs in GnRH neurons (Berg, Silveira and Moenter, 2018) similar to normal females; however, these are lost over pubertal development and become abnormal during adulthood. Together, these findings with the results in this thesis suggest that altered GABA inputs and transmission to GnRH neurons manifests around PND 25 but remains under some form of homeostatic control that blunts the postsynaptic response to GABA until activation of the HPG axis. During pubertal development, this control is lost and an enhanced response to GABAergic transmission is initiated and drives inappropriate GnRH/LH secretion. This elevated GABAergic innervation actions is likely to convey an altered feedback signal to GnRH neurons (Moore et al., 2015), allowing their overexcitation. The establishment of hyperandrogenemia may contribute to permit continuous impairment and desensitization of the GnRH pulse generator during adulthood (Figure 7.1).
Figure 7.1. Development of altered GABAergic innervation to GnRH neurons induced by prenatal androgenization. Prenatal androgenized (PNA) mice are generated by the induction of androgen excess during late pregnancy in mice from gestational day (GD) 16 to GD 18. During prenatal development, overactivation of androgen signaling in steroid-sensitive GABAergic afferents program altered brain wiring through unknown mechanisms (e.g.: via epigenetic remodeling). On PND 25, altered GABAergic innervation to GnRH neurons can be detected in the hypothalamus, which may promote the increase of GABAergic transmission to GnRH neurons detected at this time (Berg, Silveira and Moenter, 2018) in PNA mice. During pubertal development and maturation of the reproductive system, homeostatic control that maintains reduced GnRH neuron activity through prepubertal development (Dulka paper here) might be lost in PNA mice, which allows the manifestation of the neuroendocrine impairment of PCOS such as elevated LH pulse frequency secretion.
There is increasing evidence indicating that prenatal androgen excess imprints hypothalamic circuits, potentially leading to the development of PCOS features (Moore and Campbell, 2017). The mechanisms by which PNA treatment induces altered GABAergic wiring to GnRH neurons remains to be determined. To date, it is unclear whether AR signaling acts directly on steroid-sensitive GABA neurons. AR is expressed in the developing female brain (Brock, De Mees and Bakker, 2015), therefore, AR signaling could directly drive the increased innervation. It also remains to be investigated whether increased GABAergic signaling has any excito-cytotoxic effect in GnRH neurons, which could affect synaptogenesis as observed in androgen-treated prepubertal hippocampal neurons (Nuñez, Alt and McCarthy, 2003; Nuñez et al., 2005).

This study investigated only one prepubertal developmental time point. The specific time course of neuronal plasticity remains to be determined. It would be of interest to determine whether greater GABAergic innervation is already present before birth and whether is functionally required to induce prepubertal alterations within the GnRH neuronal network.

7.4.2 Understanding the developmental timing of androgen excess and the post-pubertal manifestation of PCOS

Although the origin of PCOS may happen during prenatal life, clinical features commonly manifest later during the peripubertal period (D.H. Abbott et al., 1998; Crisosto et al., 2007). There is long standing evidence that mild elevation of androgen levels during adolescence might be required for the establishment of hyperandrogenemia during adulthood and for the development of PCOS (Azziz et al., 2009; McGee et al., 2012). However, it is unknown whether androgen excess occurs prior to the neuroendocrine impairment of PCOS or whether it could be the downstream result of altered development of the HPG axis. This thesis showed that androgen excess takes places during young adulthood and it is associated with the development of anovulatory cycles in PNA mice.
The identification of time course of androgen excess in PNA mice contributes with the evidence that prepubertal altered brain wiring is not dependent upon precocious elevated testosterone levels. It also strengthens the idea that abnormal GABAergic wiring on GnRH neurons might be primary to the ovarian dysfunction, reassuring the brain a primary site for PCOS pathogenesis.

Various studies assert the conclusion that unbalanced levels of circulating androgens in adult females culminate in derangements of neuroendocrine function and infertility (Lizneva, Gavrilova-Jordan, et al., 2016). Clinical evidence indicates that chronic blockade of the classical AR with Flut can improve menstrual irregularities (Paradisi et al., 2013) and attenuate circulating androgen levels (De Leo et al., 1998). Notably, Flut treatment also rescues the ability of progesterone to decrease high LH pulse frequency in PCOS women (Eagleson et al., 2000), suggesting that AR blockade may restore normal wiring within the GnRH neuronal network. Initial electrophysiological studies revealed that Flut treatment decrease GABAergic transmission to GnRH neurons in PNA mice to normal levels (Sullivan and Moenter, 2004). However, it was unclear whether Flut treatment was able to change an abnormal brain wiring, which was thought to be hardwired before pubertal onset (PND 25) in PNA mice. Despite this evidence supporting early organization of wiring defects, long-term AR blockade significantly rescued normal GABAergic innervation on GnRH neurons and effectively improved reproductive function in PNA mice. The observed changes in the brain were more dramatic when compared to changes in the ovarian morphology, which the latest seemed to be gradually recovered. These findings may either indicate that the female brain is more sensitive to AR signaling blockade than the ovaries or that changes in brain wiring are necessary for the downstream improvement of ovarian function. The improvements in granulosa and theca cell layer features suggest that the restoration of normal follicle morphology after AR blockade is more likely to be indirect, through the normalization of gonadotropin action, which could reflect normalized GnRH/LH secretion. In addition to the current
findings, it would be interesting to test in future studies whether Flut treatment improves LH pulse frequency and future work is needed to clarify this subject. The present findings advance our knowledge of the pathophysiology of PCOS and highlight AR antagonism as a therapeutic target (Figure 7.2).

**Figure 7.2. Ontogeny and plasticity of GABA-to-GnRH neuron circuit abnormalities in a preclinical model of PCOS.** Typically, female androgen levels are relatively low throughout pubertal development and into adulthood, with the exception of a small peripubertal rise (blue line). Elevated perinatal androgens can drive a hyperandrogenic PCOS condition in adulthood (red line). GABA to GnRH neuron circuit abnormalities are present before the onset of puberty in prenatally androgenized PCOS-like mice, suggesting early programming of this aberrant wiring. Adult PCOS-like mice remain with enhanced GABAergic inputs onto GnRH neurons associated with an impairment of steroid hormone-mediated negative feedback,
disruption of reproductive cycles and ovarian dysfunction. Remarkably, GABAergic wiring to GnRH neurons is still plastic through androgen receptor signaling blockade, which also positively impacts reproductive cycles, and improves the recruitment and features of preovulatory follicles. (Red dots indicate GABAergic innervation on GnRH neurons).

Flut treatment can both inhibit androgen signaling pathway in androgen-dependent manner (Ilagan et al., 2005) and decrease the expression of AR in male and female mice (Kontula et al., 1985). Studies have mapped the expression of AR throughout the female hypothalamus and showed that the number of AR-expressing cells in the AVPV is ~ 30% greater in PNA mice when compared to diestrus controls (Moore et al., 2015). This raises the question of whether chronic Flut treatment is able to decrease the AR expression in the AVPV or other regions in the hypothalamus involved in the regulation of feedback mechanisms such as the ARN. Ongoing work in our laboratory is addressing this specific issue by mapping the expression of AR in the hypothalamus of diestrus female and PNA mice after AR signaling blockade.

Neuronal circuits are dynamic and the androgen milieu influences synaptogenesis and plasticity (Leranth, Hajszan and MacLusky, 2004b; MacLusky et al., 2006). The observed changes in GABA-to-GnRH neuron wiring in response to Flut poses the possibility that long-term AR blockade might act directly on AR-sensitive neurons to either induce synaptic plasticity or desensitize androgen-dependent synaptogenesis (Pettorossi et al., 2013). Considering that AR expression exhibits a relatively fast turnover (Kontula et al., 1985), it remains to be investigated whether the normal GABA-to-GnRH neuron circuit is maintained following cessation of Flut treatment or whether this improvement would be lost. It also remains to be determined whether the functionally restored ovarian morphology in PNA mice following Flut treatment would achieve
readily fertilizable oocytes. The present study was limited to assess these issues due to a restrain of time for the completion of further experiments and future work in our laboratory will address these questions.

The developmental window of AR blockade might also influence the impact on brain circuit plasticity. Clinical reports suggest that treatments for PCOS to prevent the progression of the disorder may be more effective when applied in adolescence rather than later in life (Ibáñez and de Zegher, 2004; Ibáñez et al., 2011; Ornstein, Copperman and Jacobson, 2011). The confirmation of this evidence is challenging as long-term investigations are less feasible and commonly have ethical burdens. The use of PCOS animal models, such as PNA mice, promotes the opportunity to investigate the effectiveness of different interventional paradigms and prospective outcomes to be further applied in the clinics (Franks, 2015). The present PhD study revealed that Flut treatment during the therapeutic window targeting the early rise of circulating testosterone levels (from PND 40 to PND 60) was effective to rescue normal brain wiring and estrous cyclicity in PNA mice. However, the effectiveness of other prospective therapeutic windows, for instance, before onset of puberty starting at PND 25, or during adulthood after PND 60 remains to be investigated.

7.4.3 Understanding the functional role of ARN GABA neurons in the GnRH neuronal network

Previous work suggests that increased GABA innervation to GnRH neurons arises largely from the ARN. ARN GABA neurons densely contact GnRH neurons and this innervation is $\sim 30\%$ greater in PNA mice compared to diestrous controls (Moore et al., 2015). In addition, ARN GABA neurons express progesterone receptor (PR) and this PR expression is dramatically decreased in PNA mice, asserting that a loss of progesterone sensitivity in these neurons might underlie the neuroendocrine
impairments in PNA mice (Moore et al., 2015). Based on these neuroanatomical observations, the present study aimed to address the biological relevance ARN GABA neurons in the modulation of GnRH/LH secretion in both normal and PCOS-like condition. The results presented in Chapter 6 provide compelling evidence that ARN GABA neurons are functionally important within the GnRH neuronal network and that activation of ARN GABA neuron fibers around the vicinity of GnRH neurons (rPOA/OVLT region) elicits LH release in vivo.

As discussed in Chapter 6, different attempts have been made to identify the role of specific GABAergic inputs to GnRH neurons mostly using electrophysiological preparations (Glanowska and Moenter, 2011; Liu et al., 2011; Constantin et al., 2012). The distance between the ARN and the rPOA/OVLT regions (over 1000 µm) poses a technical impediment to study this specific pathway as typical coronal brain slice preparations are only 200-300 µm thick. Of note, reported GABA actions in GnRH neurons vary from in vitro studies to in vivo reports (Herbison and Moenter, 2011; Constantin, Iremonger and Herbison, 2013), suggesting that brain slice preparations may damage GABAergic inputs to GnRH neurons. This PhD study utilised in vivo optogenetics to study this neurocircuitry and interrogate the function of ARN GABA neurons in HPG axis regulation. Optogenetic experimental designs with channelrhodopsin-2 (ChR-2) are widely employed to map and probe circuit function and address specific biological questions in the mammalian brain (Yizhar et al., 2011; Tye and Deisseroth, 2012). This PhD study applied ChETA-assisted circuit mapping to investigate the role of ARN GABA neurons. This approach rendered the target of ChETA expression in a substantial proportion of ARN GABA neurons (≥ 80 %) using Cre-Lox recombinase technology and revealed that high-frequency optogenetic activation (20 Hz) was able to elicit LH release in vivo.
Optogenetically-evoked LH release did not display a pulse-like profile as reported following the optogenetic activation of other GnRH neuronal afferents such as ARN kisspeptin neurons (Han et al., 2015). It remains unclear whether other optogenetic strategies using alternative light stimulation protocols may influence the magnitude of LH release and promote LH pulse-like secretion through the activation of ARN GABA neurons. Additional experiments are addressing some of these questions and, to date, ongoing examination indicates that 10-Hz optogenetic activation of ARN GABA neurons over 10 minutes does not induce significant change in LH release (Figure 7.3). To evaluate whether the extent of light pulses could influence LH secretion rather than the pace of stimulation (frequency), ongoing experiments have tested optogenetic activation using 10-Hz stimulation for 20 minutes. To date, the increment changes in LH secretion following this paradigm of optogenetic activation of ARN GABA neurons promoted smaller responses than 20-Hz stimulation for 10 minutes (Figure 7.3). Therefore, these preliminary data suggest that the pace of neuronal firing rather than the number of delivered spikes might be important to evoke sufficient changes in GnRH neuron activity and elicit LH secretion.
Figure 7.3. Effect of optogenetic activation of ARN GABA neurons using different stimulation patterns. Example of VGAT-cre+/-female mice injected with AVV9-ChETA-eYFP into the arcuate nucleus (ARN) and optical fiber implanted into the rostral preoptic area (rPOA). The 20-Hz optogenetic activation (10 min) could elicit LH secretion, while 10-Hz stimulation (10 min) did not change the increment in LH release. The same number of light pulses of a 20-Hz (10 min) stimulation were delivered over 20 minutes at a frequency of 10 Hz. At the end of the protocol, pituitary response was tested with s.c. injection of GnRH (200 ng/kg).

Having the evidence ARN GABA neurons express sex steroid hormone receptors (Moore et al., 2015) and may contribute to negative feedback mechanisms, we might speculate that the activity of ARN GABA neurons is tightly controlled by gonadal hormones to avoid overexcitation of GnRH neurons and elevated GnRH/LH release. Decreased PR expression in these cells might be linked to a blunted negative feedback and result in unrestrained activity that leads to high GnRH/LH secretion (Figure 7.4). The molecular and cellular mechanisms by which ARN GABA neurons specifically control GnRH neuron activity in males and females, and throughout the female reproductive cycles remain to be investigated. The ARN GABA neuronal population is diverse and has been involved in the control of reproduction, metabolism, and underlying some of the pathological features of polycystic ovary syndrome (PCOS) (Moore and Campbell, 2016). Using transcriptomic technology with Drop-seq generation of single-cell sequencing, researchers discovered that around 19 distinct GABA neuron phenotypes reside in the ARN (Campbell et al., 2017). These ARN GABA neurons co-express important neuromodulators which are also involved in the regulation of reproductive function such as somatostatin, NPY, thyrotropin releasing hormone (TRH) and dopamine (Campbell et al., 2017). Considering this vast heterogeneity within the ARN GABA neuronal population, the optogenetic activation of these neurons is likely to drive changes in other physiological systems. Future electrophysiological experiments are needed to
address the nature of specific neurotransmitter/neuropeptides that GnRH neurons are responding to following the activation of ARN GABA neurons. Therefore, the results introduced in this thesis may open the opportunity for further dissection of the biological role of different ARN GABA neuronal populations for the modulation of GnRH/LH secretion and fertility.

Figure 7.4. The ARN GABA-to-GnRH neuron circuit in females. ARN GABA neuron project to GnRH neurons and are a functional component within the GnRH neuronal network for the regulation of GnRH/LH secretion. Evidence from previous studies suggest that ovarian steroid mediated-negative feedback rely upon the proper functioning of steroid-sensitive ARN GABA neurons (green neurons with light blue nucleus) (Moore et al., 2015). ARN GABA also communicate among themselves (dashed green lines) and project to other areas in the brain as observed in the present study and in previous reports (Kong et al., 2012; Zhang and van den Pol, 2015). The activation of ARN GABA fibers around the vicinity of GnRH neurons can promote LH release in a frequency-depend manner and alterations within this circuit might be involved in the pathophysiology of PCOS.
As noted in Chapter 6, 20-Hz optogenetic activation of ARN GABA neurons induced smaller magnitude and increment change of LH release in PNA mice when compared with male and normal female mice. The amplitude of LH secretion is a direct readout of GnRH pulse frequency and pituitary responsiveness to pulsatile GnRH actions (Thompson and Kaiser, 2014). Specifically, it has been demonstrated in the ewe that high frequency GnRH secretion results in a lower amplitude of LH release reflecting a smaller LH releasable pool in the pituitary (Clarke and Cummins, 1985). The small amplitude LH release in PNA mice points toward an on-going higher GnRH pulse frequency that has decreased pituitary stores of LH leading small magnitude release (Figure 7.5). These data suggest that long-term exposure to high GnRH pulse frequency may remodel the pituitary responsiveness to GnRH in the presence of ovarian hormones. In fact, this thesis also showed that castrated PNA mice exhibit an abnormal increase of LH amplitude, suggesting that in the absence of negative feedback onto the pituitary gland and in the brain, the LH releasable pool stores might be increased in response to high GnRH pulse frequency (Chapter 4). It is unlikely that postpubertal androgen excess may be directly altering LH synthesis in the pituitary as studies showed that conditional AR knockout in the gland mice present normal mRNA and content of LH (Wu et al., 2015). It remains to be investigated the pituitary response of control versus PNA mice with exogenous GnRH, which future experiments in our laboratory will be addressing.
Figure 7.5. PNA mice shows decreased amplitude of LH secretion following optogenetic activation of ARN GABA neurons. The scheme shows a model of possible neuroendocrine mechanism by which PNA mice display smaller increment changes in LH release following the optogenetic activation of ARN GABA neurons. Enhanced GABAergic innervation may drive a hyperactive state of GnRH neurons, which in turn present a high GnRH/LH pulse frequency. Elevated GnRH actions in the pituitary gland might induce high LH pulse frequency and smaller LH releasable pool stores. Optogenetic activation of ARN GABA neurons promotes a robust LH release, which reveals the altered gonadotropin secretion reflecting the high GnRH/LH pulse frequency in a PCOS-like condition.

Although this thesis provides compelling evidence that ARN GABA neurons have a meaningful role in the modulation of GnRH/LH secretion, the functional requirement of this circuit remains to be defined. During the PhD project, many attempts were made to address the hypothesis that ARN GABA neurons are required for normal pulsatile LH secretion and fertility. To test this
hypothesis, I aimed to ablate ARN GABA neurons and follow LH pulse release in freely moving mice. Firstly, to permanently ablate ARN GABA neurons, stereotaxic injections using rAAV-flex-taCasp3-TEVp (UNC Vector Core) into the ARN of VGAT-cre<sup>+/−</sup>-TdTomato were performed in male and female mice. This approach triggers a Cre-dependent cell-autonomous apoptosis through the activation of an exogenous Caspase-3 in neurons as observed in previous reports (Yang et al., 2013). To date, viral-mediated ablation of ARN GABA neurons could not be achieved using smaller volumes of injections (50-100 nL/side). In contrast, a complete ablation of ARN GABA neurons was accomplished with a larger volume of injection (1-2 µL) (Figure 7.6). The ablation was widespread in the hypothalamus reaching other GABA neurons which project to the ARN such as the dorsomedial hypothalamic (DMH) GABA neurons (Garfield et al., 2016) (Figure 7.6). Although neuronal ablation was achieved in the latest experiments, mice presented an abrupt weight loss and recurrent seizures after 2 weeks of viral transfection, which were not ethically acceptable, and trials were discontinued. Future studies may attempt different titers and volumes to achieve a safe range of neuronal ablation and evaluate the impact of arcuate-specific GABA neuron loss on reproductive function.
Figure 7.6. Permanent ablation of ARN GABA neurons with Cre-dependent expression of Caspase-3 in mice. (A) VGAT-cre\textsuperscript{+/−}-TdTomato were injected with saline vehicle showing the expression of TdTomato (red) in GABAergic neurons in the arcuate nucleus (ARN) and dorsomedial hypothalamus (DMH). (B) Higher magnification showing VGAT-TdTomato-expressing neurons in the ARN. (C) VGAT-cre\textsuperscript{+/−}-TdTomato were injected with rAAV-flex-taCasp3-TEVp, which drives the expression of exogenous caspase-3 in neurons. TdTomato (red) signal was absent from ARN and other regions such as DMH. (D) Higher magnification showing the absence of VGAT-TdTomato-expressing neurons in the ARN following viral transfection. (Scale bar = 300 µm).
7.5 Future directions

7.5.1 Investigating prepubertal markers and mechanisms of altered brain wiring in PCOS

Various studies have attempted to find genetic (polymorphisms) and epigenetic markers for PCOS, which are associated with the manifestation of the disorder. The majority of these studies have include women of reproductive age (Cui et al., 2013; Mutharasan et al., 2013; Day et al., 2015) as the identification of prepubertal girls who will later develop PCOS remains unclear. Animal models represent an important tool to investigate developmental changes in PCOS and to identify possible markers before the pubertal manifestation of the disease. One example that future investigations could take advantage of is the recent discovery showing the relationship between epidermal growth factor receptor (EGFR) family members such as ERBB4 and ERBB3 and the pathogenesis of PCOS (Day et al., 2015). EGFRs are known to participate in the regulation of ovarian LH-mediated steroidogenesis (Park et al., 2004) and have a strong influence on brain circuit development (Namba et al., 2017). Polymorphisms of EGFR family members could be identified through skin samples as they are expressed in the human and in the mouse epidermis (Hoesl et al., 2018) and serve as a potential marker for the development of PCOS. Therefore, it could be possible that PNA treatment may promote epigenetic changes that affect EGFR signaling in GABAergic inputs within the GnRH neuronal network. These changes could be detected early in life and contribute to the development of PCOS-like features in PNA mice.

Early detection and treatment of PCOS could be ideal as the disorder does not only affect reproductive function but is strongly associated with diabetes mellitus, obesity and an increased risk of cardiovascular disease (Apter, 1998). Bearing the evidence that neuroanatomical alterations occur within the GnRH neuronal network, it would be reasonable to address the question whether prenatal androgen excess can program altered GABAergic innervation in brain circuits involved
in the regulation of metabolism and cardiovascular function. For instance, hypothalamic circuits involved in the control of reproduction and metabolism as the ARN agouti related peptide (AgRP)/NPY/GABA neurons (Padilla et al., 2016) and TH/GABA neurons (Brown et al., 2016) could also be affected in prepubertal PNA mice. Interestingly, AgRP/NPY/GABA neurons can modulate arterial pressure and heart rate and influence obesity-related cardiovascular dysfunction (Shi, Madden and Brooks, 2017). Future work may define whether prenatal androgen excess may also affect these different brain circuits and contribute for the complex and heterogeneous manifestation of PCOS.

It is known whether AR signaling is required in GABA neurons to drive the development of PCOS-like features in PNA mice. To address this specific question a Cre-loxP approach might be effective to genetically knockout AR expression from GABA neurons in the brain. Future experiments in our laboratory will address this issue by crossing AR-floxed transgenic mice (De Gendt and Verhoeven, 2012) with VGAT-cre+/+ mice to investigate whether PNA treatment is able or not to recapitulate brain abnormalities and neuroendocrine dysfunction in the absence of AR signaling in GABA neurons.

Future studies may also define whether prenatal androgen exposure alters GABAergic wiring specifically through the AR. Androgens such as DHT can be converted into 5α-androstane-3β-17β-diol (3β-diol), which have high affinity for estrogen receptor β (ERβ) (Lund, Hinds and Handa, 2006). GnRH neurons express ERβ (Abrahám et al., 2003) and could be indirectly affect by PNA treatment with DHT. Future studies could address this subject by generating PNA mice which are also transgenic knockout animals for ERβ in order to evaluate whether PNA treatment remains able to induce the PCOS-like phenotype.
7.5.2 Investigating the mechanisms of AR signaling blockade which contribute for the reversal of brain abnormalities in PNA mice

The present study demonstrated that long-term blockade of AR signaling from PND 40 to PND 60 can reverse altered GABAergic wiring on GnRH neurons, restore estrous cyclicity and improve the recruitment of preovulatory follicles in PNA mice. It is possible that different windows of treatment can be more efficient or that a loss in efficiency when testing AR blockade in different developmental stages may give new insights about the pathophysiology of PCOS. Future studies could determine the impact of Flut treatment either at the time of prenatal androgen exposure or postnatally before altered GABAergic wiring can be identified.

Flut is a non-steroidal anti-androgen, which may present some adverse effects as early clinical report indicated the possibility of liver failure due to hepatotoxicity during treatment for hirsutism (Andrade et al., 1999). Despite this initial finding, the risk of Flut side-effects was assessed in clinical (Ibáñez et al., 2005; Dikensoy et al., 2009) and meta-analysis studies (Domecq et al., 2013) and the majority of the findings suggest a minimal or total absence of hepatotoxicity of Flut doses, which are used for the treatment of PCOS symptoms. Future studies may address the impact of other AR blockers on rescuing normal sensitization of the GnRH pulse generator and restoring fertility in PCOS. For instance, bicalutamide, branded as Casodex, has been recently found to improve hirsutism in PCOS patients (Moretti et al., 2017) and remains to be investigated whether this anti-androgen could also improve normal neuroendocrine regulation of LH secretion. In addition, a third-generation of AR blocker currently applied for the treatment of prostate cancer named tetraaryl cyclobutane, or CB (Pollock et al., 2016). The CB compounds may be more efficient than other AR antagonists (e.g.: flutamide and enzalutamide) as it acts by impeding the translocation of AR into the nucleus of the cell and may present less side-effects (Pollock et al.,
Therefore, future pre-clinical and clinical work could investigate the potential therapeutic application of this drug for the treatment of PCOS.

7.5.3 Investigating the biological function and requirement of ARN GABA neurons in the control of GnRH/LH secretion and female fertility

This thesis provided evidence that ARN GABA neurons might be involved in the control of GnRH/LH secretion as optogenetic activation of these neurons evoked robust LH release in female and male mice. Interestingly, optogenetic activation of ARN GABA neurons in PNA mice also evoked LH secretion but with a significant smaller amplitude of LH release. As discussed previously (Chapter 6), this altered magnitude of LH secretion might be the result of an elevated GnRH/LH pulse frequency in PNA mice. If abnormal pituitary responsiveness is driven by altered GnRH pulse frequency, future studies may combine Flut treatment and optogenetic activation of ARN GABA neurons in order to test whether rescuing the abnormal GABAergic wiring from this specific GnRH neuronal afferent may promote robust LH release similar to normal female mice.

Increased GnRH/LH secretion following optogenetic activation of ARN GABA neurons may rise from other neurotransmitters/neuropeptides which are also expressed in ARN GABA neurons and have an impact in reproduction (Marshall et al., 2016; Campbell et al., 2017). Future electrophysiological studies may determine the nature of signals which are being released from ARN GABA neuron terminals during optogenetic stimulation. Transgenic and Cre-LoxP technologies have provided important tools to investigate the physiological function of distinctive neuronal phenotypes involved in the control of reproduction using in vivo optogenetics (Qiu et al., 2016; Clarkson et al., 2017). Different Cre-driver transgenic lines of interest are available to examine the role of specific ARN GABAergic inputs to GnRH neurons such as TH-cre mice (Runegaard et al., 2017), dopamine transporter (DAT)-cre mice (Yip et al., 2017), AgRP-cre mice...
(Aponte, Atasoy and Sternson, 2011), among others. Future work may also attempt to decipher whether PNA treatment affects ARN GABA neurons in general or whether prenatal androgen insult is phenotype-specific.

As mentioned above, during this PhD project, experiments aiming to test the requirement of ARN GABA neurons for GnRH/LH pulsatility and fertility were firstly performed driving the expression of exogenous caspase-3 in VGAT-expressing neurons. The detection of wide spread cell ablation generated real concerns about the severity of side-effects. As an alternative for this objective, future studies will address the same question using a technique that leads to neuronal silencing of ARN GABA neurons. In this approach, VGAT-cre<sup>+/−</sup> mice will receive bilateral injection of Cre-dependent AAV-DJ-TeNT (AAV-TetTox) virus into the ARN. AAV-TetTox drives the expression of tetanus toxin light chain in neurons, which inhibit neurotransmitter release and altered postsynaptic sites by decreasing the density of dendritic spines in the receptor cell (Heimer-McGinn et al., 2013). This approach has been successfully used by others when investigating the requirement of AgRP/NPY neurons in adaptive behaviors such as food seeking (Padilla et al., 2016).
7.6 Concluding remarks

The work presented in this PhD thesis supports the hypothesis that prenatal androgen excess is an important factor for the development of PCOS and that brain circuit abnormalities, which may underlie the pathogenesis of the disorder, are present well before the onset of puberty. Additionally, this altered GABAergic innervation on GnRH neurons in the prepubertal brain may not be dependent upon early rise of circulating androgen levels in PNA mice; however, the molecular and cellular mechanisms triggering these brain abnormalities remain elusive. Major changes in circulating testosterone levels occur after pubertal development during early adulthood in PNA mice, which may be the result of an abnormal maturation of the HPG axis in a PCOS-like condition. Targeting this rise of circulating androgen levels in PNA with long-term AR blockade, resulted in the rescue of normal GABAergic wiring to GnRH neurons followed by an improvement of reproductive function. ARN GABA neurons are considered to cause the increased GABAergic innervation on GnRH neurons in PNA mice and were suggest to regulate LH secretion. Functional studies addressed this subject and showed that in vivo optogenetic activation of ARN GABA neurons result in robust and sustained LH secretion. Conversely, activation of ARN GABA neurons in PNA mice led to small LH release amplitude, which may reflect an altered regulation of the LH releasable pool in the pituitary by a high GnRH pulse secretion in these animals. The results presented in this PhD thesis advance our existing knowledge in the fields of PCOS and androgen excess in the female brain, providing new insights about the pathophysiology of PCOS and prospective therapeutic interventions for future use in the clinics.


References


References


References

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Goodman, R. L. et al. (2011) ‘Evidence that the arcuate nucleus is an important site of progesterone negative feedback in the ewe’, *Endocrinology*, 152(9), pp. 3451–60.


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Appendices
### 9.1 Appendix I: List of chemical reagents

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagents</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Phosphate-buffered saline (PBS)</td>
<td>0.1 M sodium phosphate monobasic</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>0.1 M sodium phosphate dibasic</td>
<td></td>
</tr>
<tr>
<td>4% Paraformaldehyde</td>
<td>4% (w/v) paraformaldehyde</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>0.1 M phosphate buffer</td>
<td></td>
</tr>
<tr>
<td>1X Tris-buffered saline (TBS)</td>
<td>0.5 M Tris-hydrochloride</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>0.5 M Tris-base</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15 M sodium chloride</td>
<td></td>
</tr>
<tr>
<td>30% Sucrose (cryoprotectant sucrose)</td>
<td>30g of sucrose in 100 mL of 1X TBS</td>
<td>7.4</td>
</tr>
<tr>
<td>Cryoprotectant (to store brain sections at -20°C)</td>
<td>1.0 M phosphate buffer</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>0.9% (w/v) NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30% (w/v) sucrose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1% (w/v) polyvinylpyrrolidone (PVP-40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30% (w/v) ethylene glycol</td>
<td></td>
</tr>
<tr>
<td>Citrate buffer</td>
<td>1.03% (w/v) citric acid [5.15g]</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>1.816% (w/v) sodium phosphate [9.08g] [values for 500 mL of solution]</td>
<td></td>
</tr>
<tr>
<td>PBS-Tween 20</td>
<td>0.1 M PBS</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>0.05% Tween-20</td>
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<tr>
<td>Incubation solution</td>
<td>1X TBS</td>
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<tr>
<td></td>
<td>0.25% (w/v) Triton-X-100</td>
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<tr>
<td></td>
<td>0.3% (w/v) bovine serum albumin (BSA)</td>
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</tr>
<tr>
<td></td>
<td>2% (w/v) normal goat serum</td>
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</tr>
<tr>
<td>Flutamide (Sigma-Aldrich, St Louis, MO, USA)</td>
<td>▪ 0.10 g of Flutamide + 1 mL of absolute ethanol to make up a 10X stock solution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>▪ Flutamide ethanol solution is diluted in nine parts of sesame oil to create a 1x solution for use</td>
<td></td>
</tr>
</tbody>
</table>

Table 9.1. List of chemical reagents for solutions used in all experiments.
9.2 Appendix II: Characterization of primary antibodies for immunohistochemistry

List of primary antibodies:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Manufacturer</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green fluorescent protein (GFP)</strong></td>
<td>Polyclonal Chicken</td>
<td>Aves Labs # GFP - 1020</td>
<td>Specificity was verified by western blot (Aves Labs, data sheet) and by the absence of immunoreactivity in wild-type mice that lacked endogenous GFP expression (Roberts et al., 2014)</td>
</tr>
<tr>
<td><strong>Vesicular GABA transporter (VGAT)</strong></td>
<td>Polyclonal Rabbit</td>
<td>Synaptic Systems # 131 003</td>
<td>Specificity verified in knockout studies (Synaptic systems, data sheet) and by western blot (Bragina et al., 2010)</td>
</tr>
<tr>
<td><strong>Gonadotropin-releasing hormone (GnRH)</strong></td>
<td>Polyclonal Guinea-pig</td>
<td>Gift by Dr Greg Anderson (University of Otago, New Zealand)</td>
<td>Specificity verified by peptide pre-absorption (Rizwan et al., 2012)</td>
</tr>
</tbody>
</table>

Table 9.2. Validity of primary antibodies.
9.3 Appendix III: Verification of anti-androgenic effects of Flutamide dose

Background and objectives:

Pharmacological therapies aiming to ameliorate the impact of hyperandrogenemia (HA) in PCOS use antiandrogens such as Flutamide (Flut). Flut is a nonsteroidal anti-androgen acting as a selective antagonist of the androgen receptor (AR), competing with other androgens such as testosterone and dihydrotestosterone (Schulz et al., 1988). Previous studies reported that a 10mg/kg/day dose of Flut s.c. was able to improve reproductive cycles in PNA mice and restore normal GABAergic transmission to GnRH neurons (Sullivan and Moenter, 2004). Early pilot studies in our laboratory tested this dose and could not find any impact or improvement of estrous cyclicity in PNA mice. Initially, a pilot study aimed to confirm anti-androgenic effect of a higher dose of s.c. Flut 25 mg/kg/day as used in other previous reports (Shouqi Luo et al., 1997; Kim et al., 2002).

Methods:

Male (N = 4) and female (N = 4) C57BL6 mice were injected daily with 100μL of Flut at a dose of 25mg/kg/day for 15 days. Control vehicle animals received 100μL of sesame oil during the same period. Male mice were used as biological control to assess the effectiveness of Flut treatment according to studies elsewhere (Poyet and Labrie, 1985; Shouqi Luo et al., 1997). Animals were euthanized using cervical dislocation at the end of treatment and tissues were collected. Seminal vesicles and adrenal gland weights were measured from treated intact male mice, and ovaries and adrenal gland weights were measured from treated female mice. Statistical analysis was performed
with PRISM software 7.0 (GraphPad Software, La Jolla, CA, USA). Control and Flut-treated mice were compared using Mann-Whitney U test.

**Results and conclusion:**

In males, s.c. Flut 25 mg/kg/day for 15 days significantly decreased seminal vesicle weight ($P < 0.05$) and adrenal gland weight ($P < 0.05$) compared to oil-treated controls, confirming the antiandrogenic effect of this dose (Figure 9.1). In healthy females, the same treatment protocol had no effect on ovarian or adrenal gland weight. The results demonstrated that s.c. Flut 25 mg/kg/day has pronounced antiandrogenic effects in androgen-responsive tissues of male mice, and benchmarked this dose to be used in further experiments of Chapter 5. The absence of changes in either adrenal or ovarian weight were expected and confirm that antiandrogenic treatment effects are better visualized in male mice.
Figure 9.1. Effects of flutamide (Flut: 25mg/kg/day) administration in male and female mice. Flut was administered by subcutaneous (s.c.) injections daily for 15 days. Antiandrogenic effects at the dose of 25mg/kg/day were determined by measuring the weight of (A) seminal vesicles and adrenal glands from male mice (N = 4), our positive biological control, and the weight of (B) ovaries and adrenal glands from female mice (N = 4). Data are expressed as means ± SEM. *P < 0.05; Mann-Whitney U test.
9.4 Appendix IV: Trials for optogenetic activation of ARN GABA neurons

Background and objectives:

Optogenetics have opened opportunities to investigate the physiological role of discrete neuronal circuits in different systems (Tye and Deisseroth, 2012). During the initial stage of this PhD project, a series of pilot studies aimed to determine the best optogenetic strategy to activate ARN GABA neurons, which could promote LH secretion in mice. Following the establishment of the technique in our laboratory, pilot studies which firstly aimed the activation of either ARN GABA neuron cell bodies or neuronal processes in the rPOA. Trials undertook different protocols, which mainly varied the time and frequency of light stimulation. This appendix resumes the results found during the performance of the optogenetic pilot studies.

Methods:

Animals: Male and female mice from either GnRH-Cre+/– mice or VGAT-Cre+/– transgenic lines were housed at the Hercus Resource Unit (University of Otago - Dunedin) under 12h: 12h light-dark cycles (lights on at 0700h) with ad libitum access to water and food.

AAVs:

- AAV9-ChR2-mCherry = AAV2/9-EF1-dflox-hChR2-(H134R)-mCherry-WPRE-hGH; 4.35x10^{13}GC/mL (PennVector Core).
- AAV9-ChR2-eYFP = EF1a-DIO-hChR2-(H134R)-eYFP-WPRE-hGH; 6.12x10^{13}GC/mL (PennVector Core).
- AAV9-ChETA-eYFP = AAV2/9-EF1α-DIO-ChETA(E123T)-eYFP; 3.15 x 10^{13} GC/mL (PennVector Core)
**General protocol for optogenetic stimulation:** Three weeks after AAV injection, mice were given 2% isoflurane anesthesia and same surgical procedures as reported in Chapter 6. Cannula with a 100-μm diameter optical fiber (Thorlabs, Inc.) was implanted either into the rPOA or into the ARN. The light stimulation consisted of 5-ms blue light (473 nm) pulses using a Grass S88X stimulator controlled by diode-pumped solid-state (DPSS) laser (IkeCool©). The power light at the tip of the optical fiber was set at 5mW. Serial blood samples and LH measurements were performed as described above (Chapters 2 and 6).

**Results and conclusion:**

Table 9.1 presents an overview of the results from all optogenetic pilot studies, which predated the findings showed in Chapter 6. Initial pilot study (Pilot 1) was performed in order to establish optogenetics in our laboratory based on previous reports (Campos and Herbison, 2014). As expected, optogenetic activation of GnRH neuron cell bodies in the rPOA at 5, 10 and 20 Hz promoted robust LH secretion (**Figure 9.2 A**). Pilot study 2 aimed to target the expression of ChR-2 in the ARN and stimulate ARN GABA neurons cell bodies using the same light stimulation protocol as used in Pilot 1. In contrast, results showed that this optogenetic activation protocol did not promote changes in LH release (**Figure 9.2 B**). A similar outcome with no changes in LH levels was observed during the execution of pilot study 3, which tested 2 Hz (10 and 20 min) and 10 Hz (10min) light stimulation (**Figure 9.2 C**). Taking advantage of the retrograde properties of AAV9-ChR2-mCherry, Pilot 4 attempted to backfill ARN GABA neurons which project to the rPOA. Viral transfection was not efficiently present in the ARN and optogenetic activation of ARN
GABA neurons did not elicit LH secretion (Figure 9.2 D). In pilot 5, viral transfection with AAV9-ChR-2 was aimed at the level of ARN GABA neuron cell bodies and optical fiber was positioned in the rPOA, around the vicinity of GnRH neurons. Results showed that 2 mice (1 male/1 female) out of 7 showed sufficient transfection and LH secretion following 20-Hz stimulation for 10 minutes (Figure 9.2 E). Pilot 6 applied the same transfection and light stimulation strategy, but with AAV9-ChETA and all mice with sufficient transfection exhibited robust LH secretion following 20-Hz stimulation for 10 minutes (Figure 9.2 F). Therefore, the same optogenetic strategy from pilot study 6, named ChETA-assisted circuit mapping, was applied to investigate the functional role of ARN GABA neurons in mice.
<table>
<thead>
<tr>
<th>Pilot</th>
<th>Mice</th>
<th>Sex</th>
<th>AAV</th>
<th>Local of viral transfection</th>
<th>Local of optogenetic activation</th>
<th>Light stimulation protocol</th>
<th>Outcome</th>
<th>Figure</th>
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<td>1</td>
<td>GnRH-Cre&lt;sup&gt;+&lt;/sup&gt; -</td>
<td>Female (N = 7)</td>
<td>AV9-ChR2-mCherry</td>
<td>ME</td>
<td>rPOA</td>
<td>2, 5, 10 and 20 Hz (5 min each)</td>
<td>LH release was confirmed as reported previously (Campos and Herbison, 2014)</td>
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<td>2</td>
<td>VGAT-Cre&lt;sup&gt;+&lt;/sup&gt;-</td>
<td>Female (N = 8); Male (N = 5)</td>
<td>AV9-ChR2-mCherry</td>
<td>ARN</td>
<td>ARN</td>
<td>2, 5, 10 and 20 Hz (5 min each)</td>
<td>No LH</td>
<td>9.1 B</td>
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<tr>
<td>3</td>
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<td>Female (N = 5)</td>
<td>AV9-ChR2-mCherry</td>
<td>ARN</td>
<td>ARN</td>
<td>2 Hz (10 / 20 min); 10 Hz (10 min)</td>
<td>No LH</td>
<td>9.1 C</td>
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<tr>
<td>4</td>
<td>VGAT-Cre&lt;sup&gt;+&lt;/sup&gt;-</td>
<td>Female (N = 6)</td>
<td>AV9-ChR2-mCherry</td>
<td>rPOA</td>
<td>ARN</td>
<td>2 and 20 Hz (10 min each)</td>
<td>No LH</td>
<td>9.1 D</td>
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<tr>
<td>5</td>
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<td>Female (N = 5); Male (N = 2)</td>
<td>AV9-ChR2-eYFP</td>
<td>ARN</td>
<td>rPOA</td>
<td>2 and 20 Hz (10 min each)</td>
<td>LH release with 20 Hz during 10 min in 2 mice</td>
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<td>6</td>
<td>VGAT-Cre&lt;sup&gt;+&lt;/sup&gt;-</td>
<td>Female (N = 3); Male (N = 5)</td>
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<td>rPOA</td>
<td>2 and 20 Hz (10 min each)</td>
<td>LH release with 20 Hz during 10 min in all mice</td>
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**Table 9.3. Results from optogenetic pilot studies.**
Figure 9.1. Optogenetic pilot studies. Graphical examples from pilot studies 1 (A), 2 (B), 3 (C), 4 (D), 5 (E) and 6 (F) to investigate the functional role of ARN GABA neurons in the modulation of LH secretion.