EFFECTS OF PRENATAL TESTOSTERONE ON KNDY- GNRH CIRCUITRY IN A SHEEP MODEL OF PCOS

Maria Cernea

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EFFECTS OF PRENATAL TESTOSTERONE ON KNDY-GNRH CIRCUITRY IN A SHEEP MODEL OF PCOS

(Short title: Effects of prenatal testosterone on the KNDy cells)

(Thesis format: Integrated-Article)

by

Maria Cernea

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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The thesis by

Maria Cernea

entitled:

Effects of Prenatal Testosterone on KNDy-GnRH Circuitry in a Sheep Model of PCOS

is accepted in partial fulfillment of the requirements for the degree of Master of Science

Date ____________________________

Chair of the Thesis Examination Board
Women with polycystic ovarian syndrome (PCOS) and prenatal testosterone (T)-treated ewes show similar reproductive neuroendocrine defects, including reduced responsiveness to feedback control of GnRH secretion, suggesting alterations in the hypothalamic circuitry mediating control of reproduction. KNDy (kisspeptin/neurokinin B/dynorphin) neurons of the arcuate nucleus (ARC) play a critical role in relaying the influence of steroid hormones to GnRH neurons. We tested the hypothesis that prenatal T exposure alters the synaptic connections of KNDy cells and their connections to GnRH neurons by examining inputs to kisspeptin and GnRH neurons in control and prenatal T-treated adult ewes. Prenatal T-treated animals showed a significant reduction in the total numbers of synaptic inputs onto ARC kisspeptin (KNDy) neurons, kisspeptin cells in the preoptic area (POA) and GnRH neurons in the medial basal hypothalamus (MBH) and POA. We also examined the effects of prenatal T treatment on the size of KNDy cell bodies and found that prenatal T ewes showed a significant increase in the somal size of KNDy cells compared to control ewes. Overall, prenatal T treatment results in morphological changes both in the synaptology of the KNDy population and its connections to GnRH, as well as its cell size. These changes may contribute to defects in the ability of the KNDy population to convey steroid feedback signals to GnRH neurons.

Keywords: Kisspeptin, KNDy, GnRH, Prenatal testosterone (T), Pulse generator, Hypertrophy, Synaptic inputs, GnRH surge, estrogen positive feedback, immunohistochemistry, hypothalamus, confocal microscopy.
Acknowledgements

This thesis could not have been completed without the encouragement and support from Dr. Michael Lehman, who was not only my supervisor but my mentor throughout my academic program. His constant optimism, patience and guidance allowed me to grow and think in ways that I never thought I could. His tremendous experience and passion for science truly inspires me in my research. I would also like to acknowledge the immense contributions of Dr. Lique Coolen. Her dedication to research, her passion for quality and her inspiring work ethic is truly amazing. Both Dr. Coolen and Dr. Lehman, in their individual ways, have been great mentors during my program, allowing me to think freely while always guiding me in the right direction. I hope to one day be as invaluable to the science world as they are.

I would like to thank all of my fellow colleagues and lab technicians, who were always helpful when I needed direction, teaching me different techniques or just offering advice when I needed it. I am so proud to have worked with so many amazing individuals. Specifically, I would like to acknowledge Christina Merkley, whose hard work and endless dedication to neuroendocrine research has provided the groundwork for my project.

I would like to acknowledge our collaborators Dr. Vasantha Padmanabhan and Dr. Teresa Lee who spearheaded the prenatal T sheep projects and whose contributions are invaluable to our research field. Also, I would like to thank the countless numbers of individuals who were involved in the care of animals and tissue collections. Having experienced this work myself, I can honestly say that there is an enormous amount of effort that goes into the development of these animals and I am grateful to all those involved. Finally, a very special thank you to the entire Lehman/Coolen Lab, who have made this experience one of the most memorable and enjoyable of my life.
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<tr>
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<td>AgRP</td>
<td>agouti-related peptide expressing cells</td>
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<td>ARC</td>
<td>Arcuate nucleus</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<td>AVPV</td>
<td>Anteroventral periventricular nucleus</td>
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<td>E$_2$</td>
<td>Estradiol</td>
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<td>Estrogen Receptor Alpha</td>
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<td>FSH</td>
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<td>GABA</td>
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<td>Gonadotropin-releasing Hormone</td>
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<td>GPR54</td>
<td>G Protein coupled Receptor 54</td>
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<td>Kiss</td>
<td>Kisspeptin</td>
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<td>KNDy</td>
<td>Kisspeptin/Neurokinin B/Dynorphin neurons</td>
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<td>LH</td>
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<td>MBH</td>
<td>Medial basal hypothalamus</td>
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<td>ME</td>
<td>Median eminence</td>
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<td>NKB</td>
<td>Neurokinin B</td>
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<td>NK3R</td>
<td>Neurokinin B receptor</td>
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<td>OVX</td>
<td>Ovariectomized</td>
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<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
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<tr>
<td>POA</td>
<td>Preoptic area</td>
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<td>POMC</td>
<td>pro-opiomelanocortin expressing cells</td>
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<td>Progesterone receptor</td>
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<td>T</td>
<td>Testosterone</td>
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<td>vGlut2</td>
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Chapter 1: Literature Review and Introduction

Prenatal programming of reproduction and the origins of polycystic ovarian syndrome

Exposure to steroid hormones during the critical period of prenatal development permanently changes the organization and function of the brain [1]. In females, the prenatal programming of the brain and its neural pathways is essential for reproductive success during adulthood. The gonadal steroid hormones, testosterone (T) and estrogen (E2) are important for organizing the brain during early development as well as regulating growth, maintenance and function of various reproductive tissues. However, abnormal exposure to excess T during a critical period of prenatal development leads to phenotypic, behavioural and reproductive abnormalities in humans [2, 3] as well as in other mammalian species such as monkey[4, 5], sheep[6-9], rat[10], mouse[11], and guinea pig[12].

Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders, affecting approximately 6-8 percent of reproductively aged women [13]. Although the specific origin of PCOS is still under study, genetic and environmental factors, such as exposure to excess T during a critical stage of prenatal development, are thought to play an important role in the manifestation of PCOS [8, 14, 15]. PCOS is characterised by a constellation of reproductive and metabolic deficits, including hyperandrogenemia, polycystic ovaries, disrupted menstrual cycles and fertility complications, hypersecretion of gonadotropin releasing hormone (GnRH) and luteinising
hormone (LH), reduced sensitivity to sex steroid feedback, insulin resistance, and hyperinsulinemia [16-20]. The Rotterdam conference of reproductive medicine in this field in 2003 characterized PCOS as having two of the following three features: oligo-ovulation, clinical or biochemical evidence of androgen excess and the presence of multicystic ovaries [21].

Animal experiments have strongly suggested that PCOS is a result of prenatal exposure to excess testosterone (T), which interferes with the development of the hypothalamus-pituitary-gonadal (HPG) axis and contributes to reproductive dysfunction in adults [6, 15, 22, 23]. The sheep model of PCOS provides a reliable means of studying the programming effects of prenatal T on the developing brain [6-9]. My research has focused on analyzing the specific effects of prenatal T on the reproductive control center of the brain: the hypothalamus. Sheep are excellent models for studying developmental programming of adult disorders and have long been used to study foetal development [24]. With a gestation length of 147 days, and time of puberty in females at 28 weeks, the sheep is ideally suited for integrative studies that investigate progression of reproductive/metabolic disruption from the initial developmental insult to manifestation of adult consequences. In addition, sheep can be studied in natural social settings which reduce the level of stress. From a reproductive perspective, ovarian differentiation in sheep is similar to humans with full follicular differentiation occurring by birth [25]. Neuroendocrine aspects of reproductive cyclicity are also similar to human [26, 27]. In addition, sheep are reliable subjects for assaying hormone levels because large quantities of blood can be sampled with ease and minimal stress to the animal. By monitoring the
patterns of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) throughout postnatal development and adult life, changes in reproductive stages can be easily monitored.

**Prenatal Testosterone Sheep Model**

In our model (Fig. 1), pregnant Suffolk ewes were given twice weekly intramuscular (i.m.) injections of testosterone propionate (100 mg/injection) and control females received an equal volume of vehicle (2 ml cottonseed oil) in the same regimen as testosterone [8]. The lambs were born in March and April and after weaning, were maintained outdoors under natural photoperiods with a daily maintenance feeding and
free access to water at the Sheep Research Facility of the University of Michigan (Ann Arbor, MI) until the age of 2 yr.

At this time, in order to normalize the endogenous hormonal milieu, control and prenatal T-treated ewes were ovariectomized and received estradiol implants (OVX+E) designed to mimic follicular phase levels of E2, and were perfused 19-22 hours later at the time of the predicted GnRH/LH surge.

**Control of the estrous cycle in normal females, and its disruption by prenatal T exposure**

Adult reproductive success is determined by a complex series of events that occur during the early stages of brain development. During this critical time, gonadal steroids act to permanently organize the structure of the reproductive center of the brain, the hypothalamus, which includes GnRH neurons. Prenatal changes in the organization of GnRH neurons and their connections determine the control of GnRH secretion, the key mediator of the release of pituitary gonadotropins (LH and FSH) and thus, ovulation [28, 29]. In fact, prenatal T decreases (masculinizes) synaptic input to GnRH neurons to about half the number seen in normal ewes[30], leading to the assumption that a change in synaptic inputs to KNDy and GnRH neurons will occur in our prenatal T treated sheep.

In the female, the pattern of GnRH and gonadotropin release varies during different reproductive stages. There are two modes of GnRH secretion: pulsatile and surge secretion, which are regulated independently [31]. The frequency and amplitude of the
GnRH pulse are crucial for normal gonadotropin secretion [32]. Pulsatile secretion of GnRH occurs in both males and females and is under the negative feedback control of estradiol, while surge secretion of GnRH occurs only in females and is under positive feedback influence of rising levels of estradiol during the follicular phase of the estrous cycle. This preovulatory GnRH surge causes the release of gonadotropins from the anterior pituitary and subsequently, there is a surge in luteinizing hormone (LH) which triggers ovulation [33, 34].

In female sheep, prenatal T exposure from days 30-90 of gestation results in a set of adult reproductive disorders [6-8, 35-39], the major of which include: a deterioration of estrous cycles [8, 35, 40], development of multifollicular ovaries [7, 41] and eventually, infertility [35, 40]. Irregularities in the mode of gonadotropin secretion appear as hypersecretion of LH but not FSH, along with a reduction in sensitivity to gonadal steroid feedback [6, 23, 36]. This hypersecretion of LH associated with reproductive irregularity is also evident following prenatal T exposure in female rhesus monkeys [42], mice[43], and rats [44], and of course, in women with PCOS[45, 46]. Exposure to excess prenatal T also causes malformation of the ovaries in sheep[7], manifested as large multifollicular ovaries, similar to those seen in women with PCOS. Therefore, ewes exposed to prenatal T provide an ideal model for studying PCOS as they display disruptions at a peripheral as well as neuroendocrine level, similar to women with PCOS [17, 47].
Prenatal testosterone alters the feedback sensitivity of the GnRH system to estradiol and progesterone

As mentioned, estradiol (E2) and progesterone (P) are the main regulators of GnRH pulsatility throughout the estrous cycle. Estradiol is important for providing a negative and positive feedback signal to GnRH throughout the cycle. During the luteal phase, estradiol exerts an inhibitory influence on GnRH/LH pulse amplitude, and during the follicular phase, increasing E2 levels exert a stimulatory influence, ultimately triggering the GnRH/LH surge and ovulation. Progesterone, on the other hand, acts to inhibit the frequency of GnRH/LH pulses through a negative feedback loop during the luteal phase of the estrous cycle [48]. Following regression of the corpora luteum (the major source of progesterone), GnRH secretion is released from inhibition, and GnRH/LH pulse frequency increases. In fact, if P levels are artificially maintained in the follicular phase, the GnRH/LH surge is blocked in sheep[49]. For normal estrous cyclity, both stimulatory and inhibitory feedback mechanisms must work effectively.

Exposure to prenatal T reduces hypothalamic sensitivity to the feedback systems involved in the control of cyclic variations of GnRH/gonadotropin secretion: E2 negative and positive feedback [6, 23, 36, 37] and P negative feedback [38]. E2 negative feedback is the main type of feedback operating before puberty and responsible for the inhibition of GnRH/LH pulses in the prepubertal animal [50]. When sensitivity to E2 negative feedback is reduced at puberty, GnRH/LH secretion escapes from inhibition affecting the timing of puberty [50, 51]. Sarma et al. (2005) found ovary-intact ewes to be hypergonadotropic and show reduced sensitivity to E2 negative feedback when exposed
to T from days 30–90 of gestation [23], and as a consequence these animals showed an early onset of puberty. Support for alterations of steroid negative feedback also comes from studies in adult mice [43] and monkeys [5], where an increase in LH pulsatility is seen following prenatal T treatment.

In addition to disrupting E2 negative feedback, prenatal T exposure disrupts E2 positive feedback [6, 36, 37]. In the ovariectomized E2-replaced sheep model, disruption of the E2 positive feedback system is seen as the absence of GnRH/LH surges [36, 52]. However, the type of disruption varies depending on the time of prenatal T exposure; with T60–90 females displaying a delayed LH surge and the T30–90 females showing a delayed LH surge as well as a severe dampening of its amplitude [6]. These studies show that both the timing and duration of T exposure during prenatal life is important and can influence the features of neuroendocrine disruption.

Progesterone feedback acts to inhibit the pulsatile release of GnRH during the luteal phase [48]. The progesterone feedback system is also disrupted by exposure to prenatal T. Specifically, Robinson et al. (1999) showed that control ewes were more responsive to progesterone negative feedback when compared to either males or prenatal T-treated females [38]. Importantly, a reduction in responsiveness to progesterone negative feedback has been shown to be a key characteristic of the neuroendocrine system in women with PCOS [17].
To summarize, disruption of E2 positive feedback may lead to a dampened or delayed LH surge, while disruption of E2 or progesterone negative feedback may lead to an incorrectly timed increase in pulsatile LH secretion during puberty, or a failure to inhibit LH pulses during the estrous cycle. Thus, T acts on the developing central nervous system to permanently alter the feedback responses of the steroid-sensitive stimulatory and inhibitory neural populations regulating GnRH neurons and their secretion.

**Kisspeptin and the HPG axis**

Over the past decade, kisspeptin has received much attention as a key player in the control of reproduction. The discovery in 2003 that mutations and deletions of the G protein-coupled receptor for kisspeptin (GPR54) in humans and mice resulted in hypogonadotropic hypogonadism provided a link between kisspeptin and reproduction [53, 54]. Since then, kisspeptin has become the focus of research centering on the feedback control of GnRH secretion by gonadal steroids [55-57]. A long standing question in reproductive neuroendocrinology has been the identity of sex steroid-responsive afferents that can relay feedback information to GnRH, as the latter neurons do not express estrogen receptor alpha (ERα) [58, 59] or progesterone receptor (PR) [60], the gonadal steroid receptors mediating feedback control. Moreover, in terms of their numbers and distribution, GnRH neurons are not sexually dimorphic [61]. This suggests that sex differences in the control of the reproductive axis are caused by differences in afferent neural circuits and other mechanisms that regulate GnRH. The kisspeptin neurons are ideally located to convey information between the gonadal steroids and the
GnRH neurons. By further investigating the kisspeptin population, we can begin to understand how control of GnRH and gonadotropin release is organized and differs between males and females.

**How does kisspeptin affect GnRH secretion?**

Kisspeptin is a powerful stimulator of GnRH release [62, 63] as well as gonadotropin secretion [64, 65] in different species including rodents, primates and sheep [66]. Kisspeptin fibres have also been shown to project directly to GnRH neurons [67, 68], and GnRH neurons express GPR54 mRNA [62, 63]. Moreover, kisspeptin neurons are distributed throughout regions of the brain known to play important roles in sex steroid feedback [64, 69]. Finally, kisspeptin neurons express estrogen and progesterone receptors (ERα and PR), thus sex steroids are able to affect kisspeptin mRNA and peptide expression and both negative and positive feedback signals can be conveyed to GnRH [70, 71].

In sheep, kisspeptin neurons are located in the hypothalamus, with two distinct populations, one in the preoptic area (POA) and the other in the arcuate nucleus (ARC) [69, 71]. In the POA, approximately half of the kisspeptin neurons co-express ERα [70]. However, it is not clear how these cells are directly affected by E2 and if this immediately affects GnRH secretion. In the rodent AVPV, POA kisspeptin neurons participate in the E2-induced preovulatory LH surge [53, 54, 68]. In the sheep however, E2 acts in the mediobasal hypothalamus (specifically the ARC), not the POA, to induce
the preovulatory LH surge [72, 73]. This may indicate that the ovine POA kisspeptin population is activated indirectly by the positive feedback action of E2 acting upon kisspeptin neurons in the ARC. Almost 100% of kisspeptin cells in the ARC colocalize ERα. In addition, E2 has both stimulatory and inhibitory effects on the expression of Kiss1 in the ARC, consistent with both positive and negative feedback control being mediated through this population at least in the sheep [69, 71].

The KNDy cells

Figure 2. Schematic diagram of the HPG axis showing the potential connections between the POA and ARC kisspeptin/KNDy populations and their interactions with GnRH neurons.
The ARC kisspeptin population co-expresses two other important peptides: the opioid peptide, dynorphin and the tachykinin peptide, neurokinin B (NKB). The colocalization of the three peptides in the ARC is nearly 100%, and this subpopulation has been collectively termed “KNDy” (kisspeptin, neurokinin B, dynorphin) cells [74]. GnRH neurons have been shown to receive inputs specifically from KNDy cells [74]. Interestingly the KNDy population is reciprocally interconnected, suggesting that it may be responsible for synchronous activity recorded from the ARC associated with the pulsatile release of GnRH. Based on this, and other features, the KNDy population has been proposed to be a critical component of the “GnRH pulse generator” with the three peptides serving roles as the start signal (NKB), stop signal (dynorphin) and output (kisspeptin) responsible for each pulse [74-79].

Dynorphin and NKB are important players in the control of GnRH release and work in conjunction with kisspeptin to relay steroid information to GnRH during different stages of the estrous cycle. In sheep, there is strong evidence that dynorphin, an inhibitory peptide, is responsible for progesterone-negative feedback on GnRH neurons during the luteal phase of the estrous cycle [80-82]. The role of NKB is still unclear; however recent studies show that loss-of-function mutations of genes encoding either NKB or its receptor resulted in hypogonadotropic hypogonadism, revealing a critical role for NKB in the central control of reproduction [83, 84]. Moreover, NKB expression appears to be under the control of estradiol, with findings in rats [85] and monkeys [86] showing an upregulation of the preprotachykinin B gene (which encodes neurokinin B) after ovariectomy. Findings in the sheep suggest that NKB is stimulatory specifically during the follicular phase of the estrous cycle. The activation of NK3R with an agonist
(senktide) stimulates LH secretion, suggesting a role in control of the GnRH/LH surge [87]. To summarize, KNDy neurons express ERα and PR, allowing for the regulation of neuropeptide expression in a manner appropriate for both stimulatory and inhibitory signals. Collectively, the KNDy neuropeptides work together to regulate the two modes of GnRH release because they each play distinct roles in conveying the feedback signals of estrogen and progesterone onto GnRH neurons.

While the KNDy cell population is a target for the organizational effects of prenatal T [88], the specific mechanisms by which this programming occurs are still under investigation. Recent work in sheep by Cheng et al. (2009) has found that prenatal T treatment reduces the number of dynorphin, neurokinin B, and progesterone receptor-positive cells in the female ARC, however the number of kisspeptin cells in the ARC remains high and at levels similar to control females [89]. These results suggest that there is an imbalance between inhibitory (dynorphin) and stimulatory (kisspeptin) neuropeptides in this population, and that this imbalance may play a role in the disrupted steroid feedback that is typical of the prenatal T model. Specifically, the imbalance between inhibitory and stimulatory neuropeptides in this subpopulation provides a potential explanation for the decreased ability of progesterone to inhibit GnRH pulses in prenatal T-treated sheep. However, in addition to the defect in pulsatile GnRH secretion, the GnRH/LH surge is dampened or absent in prenatal T treated sheep. How can the GnRH/LH surge be disrupted if kisspeptin, the potent stimulator of GnRH and its preovulatory surge, is not seemingly affected by prenatal T treatment? Are there
disruptions at other levels of this system? What other components of the circuitry are affected and might contribute to these functional changes?

**Glutamate**

Glutamate is the major excitatory neurotransmitter in the brain [90] and has been implicated in the direct and indirect regulation of GnRH/LH secretion. Glutamate receptors (NMDA receptor subtypes and kainite receptors) are present in GnRH cells in the rat [91, 92] and glutamatergic terminals directly contact GnRH cells [93-95]. In the monkey, glutamatergic neurons in the hypothalamus express ER alpha[96], and the effects of glutamate and its agonists and antagonists on GnRH secretion appear to be E2-dependent. In fact, treatment with E2 increases glutamate receptor levels in the POA of ovariectomized rats [97], and levels of glutamate in the POA are increased during the E2-induced LH surge and at the time of puberty in female rats and monkeys [98-101]. Thus, glutamate appears well positioned to mediate the positive feedback ability of E2 to induce a GnRH/LH surge. Furthermore, intravenous infusion of NMDA increases GnRH mRNA levels in the POA of rats [92, 102].

The vesicular glutamate transporter vGlut2 has been used as a means of visualizing glutamatergic terminals. Research in rats [103] and sheep [104] reveals that KNDy cells and their terminals co-localize vGlut2, indicating that these neurons are glutamatergic and well as peptidergic. Importantly, this co-localization has also been noted within the reciprocal connections of the arcuate KNDy population in sheep, suggesting a major
stimulatory role for glutamate in this system. Given evidence that KNDy cells may participate in surge as well as pulsatile modes of GnRH secretion (refs), changes in glutamate across the estrous cycle within KNDy cells may contribute to the positive feedback ability of estradiol (E2) to induce a GnRH surge[75, 76].

**Linking the KNDy cells to ovarian failure and menopause**

There is a selective hypertrophy in neurons of the infundibular nucleus (analogous to the arcuate nucleus in sheep) of the hypothalamus in postmenopausal women[105]. Evidence from Naomi Rance and her colleagues has shown that the majority of these neurons contain kisspeptin[106], NKB [107] and dynorphin [108] as well as ER-alpha [109] mRNA and thus, these neurons likely represent the KNDy cells of the human hypothalamus. Several studies in women show that this hypertrophy is a secondary response to ovarian failure [106, 109], and associated with declining levels of circulating E2 concentrations [110]. Like women with PCOS and prenatal T-treated ewes, postmenopausal women show an erosion of LH-FSH pulse regularity, with increased LH pulsatility and dampened LH surge [111]. Thus, we can propose that at least in some respects, these three share similar disruptive mechanisms. More examples of this are seen in the female rat, where the sensitivity to E2 positive feedback declines with age, resulting in an attenuated and delayed LH surge [112, 113]. Moreover, E2 activation of the AVPV neurons and GnRH neurons decline simultaneously in middle-aged rats [114], implying that E2 responsiveness is age-related in the rat AVPV and may play a role in the delayed and attenuated GnRH/LH surge. Another example is the ovariectomized or
menopausal rhesus monkey, which displays an increase in GnRH pulsatility and gonadotropin concentration, much like the prenatal T treated ewe[115-117]. Dominique et al. [118] also found that only long term ovariectomy (~ 4 yr) significantly increased kiss-1 and NKB expression, and this was reversed by E2 administration, further linking these changes to menopausal ovarian failure and relating the disruption of the HPG axis with the neuroendocrine defects of prenatal T treatment. The previous studies point to alterations in the systems that modulate GnRH as the main elements of reproductive decline. The inability of prenatal T treated ewes to generate the necessary GnRH/LH surge needed for ovulation suggests that these females may be also be experiencing a form of ovarian failure and that their KNDy cells might also show a marked hypertrophy.

To summarize, stimulatory and inhibitory signals influence GnRH secretion by converging on the KNDy cells of the ARC. In women with PCOS and prenatal T treated sheep however, this pathway is differentially altered during early development. In the sheep, the KNDy neurons become less sensitive to steroid negative and positive feedback, which interferes with the reproductive success of the animal. Because the KNDy cells are a critical component of the GnRH pulse generator, it is essential that we investigate this population in a framework that allows us to compare a reproductively malfunctioning model with a normal one. It is important to note that all evidence to date of neural changes associated with prenatal T treatment are based on immunocytochemical detection of peptides and transmitter enzymes which themselves are under steroidal regulation. Thus it is difficult to distinguish those long lasting changes which are due to true morphological rearrangements from those that reflect changes in peptide/protein
expression. Thus, the goal of this study is to explore the potential neuroplasticity and morphology of the KNDy population and its connections to GnRH cells using markers that are independent of changes in peptide/protein levels, and analyze which alterations may be responsible for the altered sensitivity to steroid feedback in prenatal T treated ewes. In turn, exploring the KNDy network and the organizational programming by prenatal T leading to irregularities in steroid feedback (as seen in women with PCOS) can helps us understand the normal pathways involved in the control of reproduction.

Our hypothesis is that prenatal T females will exhibit altered synaptic connections in the circuitry that interconnects KNDy neurons with each other and with GnRH cells, as well as an altered morphology of KNDy cell bodies consistent with that seen in postmenopausal women. We speculate that these changes lead to decreased synchronicity of the KNDy network and a reduction in the stimulatory drive to GnRH neurons resulting in an impaired ability to generate a GnRH/LH surge.
Chapter 1 References


Chapter 2: Manuscript

Effects of Prenatal Testosterone on KNDy-GnRH Circuitry in a Sheep Model of PCOS

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ABSTRACT

Polycystic ovarian syndrome (PCOS) is a reproductive disorder that includes altered sensitivity to steroid feedback control of GnRH secretion. Like women with PCOS, prenatal testosterone (T)-treated ewes show various reproductive neuroendocrine defects, including reduced responsiveness to feedback control of GnRH secretion, suggesting alterations in the hypothalamic circuitry mediating neuroendocrine control of reproduction. KNDy (kisspeptin/neurokinin B/dynorphin) neurons of the arcuate nucleus (ARC) comprise an interconnected subpopulation of neurons that have been shown to play a critical role in relaying the influence of steroid hormones to GnRH neurons. We tested the hypothesis that prenatal T exposure alters the synaptic connections of KNDy cells, both with each other and in their connections to GnRH cells. Using the synaptic vesicle marker, synaptophysin, we examined inputs to kisspeptin and GnRH neurons in control (n=5) and prenatal T-treated (n=4) adult ewes, who were ovariectomized and received estradiol implants (OVX+E). Prenatal T-treated animals showed a significant reduction (P<0.05) in the total numbers of synaptic inputs onto ARC kisspeptin (KNDy) neurons, kisspeptin cells in the preoptic area (POA) and GnRH neurons in the medial basal hypothalamus (MBH) and POA. Similarly GnRH neurons in the medial basal hypothalamus (MBH) and POA showed a reduction (P<0.05) in the number of dual kisspeptin/vGlut2 inputs due to prenatal T treatment. In addition to this evidence for morphological changes in synaptic inputs, we also examined the possible effects of prenatal T treatment on the size of KNDy cell bodies, based on evidence that the soma size of kisspeptin cells in the human hypothalamus increases in postmenopausal women.
Prenatal T sheep showed a significant increase (P<0.05) in the somal size of KNDy cells compared to control ewes, while kisspeptin cells in preoptic area, GnRH neurons, and other cells in the ARC (AgRP) were each unchanged in size. Overall, prenatal T treatment results in morphological changes both in the synaptology of the KNDy population and its connections to GnRH, as well as its cell size. We speculate that these changes may contribute to defects in the ability of the KNDy population to convey steroid feedback signals to GnRH neurons, specifically the ability of estradiol to induce a GnRH/LH surge.

Keywords: Kisspeptin, KNDy, GnRH, Prenatal testosterone (T), Pulse generator, Hypertrophy, Synaptic inputs, GnRH surge, estrogen positive feedback, immunohistochemistry, hypothalamus, confocal microscopy.
INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a common heterogeneous endocrine disorder, the reproductive deficits of which are characterized by hyperandrogenism, polycystic ovaries and anovulatory infertility [119-123]. These characteristics can be further broken down to include disturbances at the hypothalamic-pituitary-gonadal (HPG) axis, with decreased sensitivity to steroid feedback and control of GnRH secretion [6, 23, 46, 124, 125] and abnormally rapid LH pulsatility [126, 127]. Although the reproductive and clinical symptoms of PCOS manifest during adolescence, the neuroendocrine programming of PCOS originates in prenatal life. Prenatal testosterone (T)-treated female sheep (treated from days 30 to 90 of the 150-day gestation period) show a constellation of symptoms almost identical to that of women with PCOS [6-8, 35, 40, 41, 128], including deterioration of estrous cycles [8, 35, 40], development of multifollicular ovaries [7, 41] and, ultimately, infertility [35, 40]. Associated with these abnormalities is an alteration in gonadotropin secretion characterized as a hypersecretion of LH but not FSH [6, 23].

Extensive studies using the sheep animal model have shown that prenatal (T) treatment interferes with estradiol (E2) negative and positive feedback [6, 23, 36-38]. For example, ewes exposed to excess T in utero during days 30-90 show a delayed and attenuated LH surge [6], highlighting the importance timing and duration of T exposure in the level of disruption. This altered sensitivity to steroid feedback and reproductive abnormalities have been shown in female rhesus monkeys [42], rats [44] and mice [43] exposed to prenatal testosterone, and are also seen in women with PCOS [45, 46].
The reproductive outcomes of prenatal (T) exposure are well described in the ewe, but the hypothalamic mechanisms responsible for these detrimental effects are not yet understood. Recent work has focused on the role of the KNDy cells, a subset of neurons in the arcuate nucleus (ARC) of the sheep that co-express the neuropeptides, kisspeptin, neurokinin B, and dynorphin (referred to as KNDy cells) [74, 75, 89, 129]. These cells play a critical role in mediating steroid feedback regulation of GnRH and represent a final common pathway of signal convergence before the activation of GnRH [75, 76]. Because of their reciprocal connections with each other, and the presence of synchronous activity in this portion of the ARC corresponding to GnRH/LH pulses, the KNDy cells have been hypothesized to be the "GnRH pulse generator" driving pulsatile secretion of GnRH [78, 79]. In addition, in the sheep, there is evidence that KNDy cells may also be involved in the generation of the preovulatory GnRH/LH surge (see below). KNDy neurons are found in both males and females and they express estrogen, progesterone and androgen receptors [53, 54, 70, 71, 80, 130], consistent with the view that these neurons are direct targets for the positive and negative feedback actions of sex steroids and prenatal organization by these steroids. Supporting this is evidence that prenatal T-treated ewes show a reduction in the neuropeptide expression of DYN and NKB in KNDy cells, while the expression of kisspeptin in this population remains similar to control females [89], a peptide imbalance which may underlie the defect in progesterone negative feedback seen in this model and women with PCOS [40, 82, 125].

Recent work in rats [103], mice [131] and sheep [104] has indicated that, in addition to the colocalization of peptides, KNDy cells contain the classical excitatory
neurotransmitter, glutamate, based on colocalization of the vesicular glutamate transporter-2 (vGlut2). The presence of glutamate in KNDy cells is consistent with other evidence that estrogenic regulation of GnRH neurons is controlled by glutamatergic E2-responsive cells in the ARC and/or POA [94], and that changes in glutamate expression across the estrous cycle within these cells may play a role the positive feedback ability of E2 to induce a GnRH surge (Merkley, In Prep.). As noted above, KNDy cells form a reciprocally interconnected network [74], a feature that may play a critical role in the generation and synchronization of GnRH pulses. In addition, there is also evidence that KNDy cells may be involved in the preovulatory GnRH/LH surge. KNDy cells, along with kisspeptin cells in the sheep POA, are activated during the follicular phase of the estrous cycle after the onset of the GnRH/LH surge [75, 104, 132, 133], and there is an increase in kisspeptin mRNA expression in KNDy cells at that time [132, 134]. In addition, in the sheep (unlike the rodent), the MBH is the primary site of action of E2 in inducing the GnRH surge [73]. Thus, alterations in KNDy cells could potentially affect both major modes of GnRH secretion, pulses and the surge, and their control by steroid hormones.

To date, the changes in KNDy cells seen in prenatal T animals have been based on immunocytochemical detection of peptide or protein [89]. Thus it is unclear whether such changes in the appearance of cells and fibres reflect genuine morphological changes or the presence/absence of immunodetectable peptide/protein. Thus, we used markers of synapses, and measurements of cell size, to determine whether prenatal T exposure results in morphological changes in the KNDy-GnRH network, either at the level of
synaptic connections onto KNDy neurons, their projections to GnRH cells, or the soma size of KNDy neurons. We decided to analyze the latter (soma size) based on evidence that KNDy cells in the human hypothalamus show hypertrophy in the brains of postmenopausal women [85, 107, 109, 135]. We speculate that changes in the morphology and synaptic connections of the KNDy population and its connections to GnRH neurons may in part be responsible for the altered sensitivity to steroid feedback in prenatal (T)-treated ewes specifically underlying defects in the ability of E2 to induce a GnRH surge.
METHODS

Animals: To generate prenatal T-treated ewes, pregnant Suffolk ewes (mothers) were administered twice weekly i.m. injections of testosterone propionate (100 mg/injection, catalog item T1875; Sigma- Aldrich, St. Louis, MO) suspended in cottonseed oil (catalog item C7767; Sigma-Aldrich) in the hind leg from 30–90 d of pregnancy (term = 147 d). This dose of testosterone propionate administered results in levels of T in the female fetus comparable to those seen in fetal males [136]. Control ewes received an equal volume of vehicle (2 ml cottonseed oil) using the same regimen as T. Lambs were born in March and April. After weaning, they were maintained outdoors under natural photoperiods with a daily maintenance feeding and free access to water at the Sheep Research Facility of the University of Michigan (Ann Arbor, MI) until the age of 2 yr. Four prenatal T-treated ewes and five control, prenatal T-treated ewes were used in this experiment. As part of studies to characterize the estrous cycles of these animals [137], ovarian cycles of all ewes were synchronized by two i.m. injections of 10 mg prostaglandin F2 (Luteolyse; Pharmacia & Upjohn, Kalamazoo, MI) given 11 d apart. On d 11 from the second injection (mid-luteal phase of the estrous cycle), blood samples were collected every 20 min for 8 h by jugular venipuncture. After estrous cycle characterization studies, animals were ovariectomized, and for 2 months before use and adult, control and prenatal T-treated ewes were ovariectomized and received estradiol implants (OVX+E) designed to normalize the hormonal milieu of the animals. These implants mimic follicular phase levels of E2, and animals were perfused 19-22 hours later at the time of the predicted
GnRH surge. All experimental procedures were approved by the University Animal Care and Use Committee at the University of Michigan (Ann Arbor, MI).

Tissue collection and preparation

Animals received two i.v. injections of heparin (25,000 U given 10 min apart; catalog item 402588B; Abraxiz Pharmaceutical Products, Schumberry, IL) and then deeply anesthetised with sodium pentobarbital (2–3 g, iv, catalog item P3761; Sigma-Aldrich) and were immediately decapitated. The heads were perfused via both internal carotids with 6 liters 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) containing 0.1% sodium nitrate and 10 U/ml heparin. The brains were removed after perfusion, and a block of tissue containing the preoptic area and hypothalamus was dissected out. Tissues were placed in 4% paraformaldehyde in 0.1 M PB overnight for post fixation at 4°C and then transferred into 30% sucrose in 0.1 M PB for cryoprotection until the filtration was completed. Frozen coronal sections (45 μm) were cut using a freezing microtome (Microm HM400R, Walldorf, Germany) and stored at -20°C in a cryopreservative solution [138] until being processed using immunohistochemistry or immunofluorescent staining for kisspeptin, GnRH, vGlut2 (vesicular glutamate transporter-2), synaptophysin (marker of all synaptic terminals), and DAPI (nuclear marker). Within each experiment, tissue sections from all experimental groups were processed simultaneously as described below.
Immunohistochemistry: General Methods

Sections were processed free floating with gentle agitation and all steps were performed at room temperature. Antibodies were dissolved in incubation solution consisting of 0.1M PBS, 0.4% Triton X-100 (catalog item BP151-500; Sigma-Aldrich) containing 4% normal goat serum (NGS) (catalog item 005-000-121; Jackson Immuno Research Laboratories, West Grove, PA). Unless otherwise specified, tissue sections were washed extensively with 0.1 M PBS (pH 7.35) between steps. Prior to incubation with first primary antibody sections were incubated with 1% hydrogen peroxide (10 min, H$_2$O$_2$; catalog item H325; Fisher Scientific, Pittsburgh, PA) and incubation solution (1hr) to prevent non-specific background labelling. Negative controls were performed by omission of primary antibody which eliminated the signal corresponding to that antigen.

Quadruple-label detection of Kisspeptin, vGlut2, Synaptophysin, and DAPI

In order to investigate synaptic inputs on Kiss cells in POA and Arc, one series of every sixth section containing POA or MBH from each animal were processed for kisspeptin, vGlut2, synaptophysin, and DAPI using immunostaining protocol as previously described [75, 76, 104]. First, tissue sections were incubated with rabbit anti-kisspeptin (1:300,000 dilution, 17 h; kp10, lot 564; gift from Dr. Alain Caraty, Nouzilly, France), biotinylated goat anti-rabbit IgG (1:500, 1 hr; catalog item BA-9200; Vector Laboratories, Burlingame, CA), avidin and biotinylated horseradish peroxidase complex (ABC; 1:500,
Burlingame, CA), avidin and biotinylated horseradish peroxidase complex (ABC; 1:500, 1 hr, catalog item PK-6100; Vector Laboratories), biotinylated tyramine (BT, 1:250; diluted PerkinElmer Life Sciences, Cat # NEL700A), adding 1 μl of 3% H₂O₂ per ml of total volume (10 mins.), and Alexa 488-streptavidin (1:100, 30 mins.; green; Invitrogen/Molecular Probe, S-32354). Tissue was protected from light from this step forward. Next, sections were co-incubated with rabbit anti-vGlut2 (1:5000, 17 h; Synaptic Systems, Lot # 135402) and mouse anti-synaptophysin (1:200, 17h; Sigma, S-5768). After overnight incubation, sections were washed and incubated with goat anti-rabbit Alexa 555 (1:100, 30 mins.; red; Invitrogen/Molecular Probe, Cat # A-21428), then donkey anti-mouse Cy5 (1:100, 30 mins.; blue; Jackson ImmunoResearch 715-175-151, Lot # 75139). Finally, sections triple labelled for kisspeptin, vGlut2 and synaptophysin were incubated in DAPI (10 mg/ml, 10 mins.; Invitrogen, Cat # D-3571) to visualize the nuclear area of the cells. Tissue sections were mounted onto slides, air dried, coverslipped with Gelvatol mounting medium, and stored at 4°C until analyzed.

**Triple-label detection of GnRH, kisspeptin, and vGlut2**

In order to examine synaptic inputs on GnRH cells in POA and MBH, one series of every sixth section containing POA or MBH from each animal were processed for GnRH, kisspeptin, and vGlut2 [75, 76, 104]. As previously described, sections were co-incubated with rabbit anti-kisspeptin and mouse anti-GnRH (1:400, 17h; Sternberger Monoclonals, Inc., catalogue #SMI-41R, Lot #3). Next, sections were washed and incubated with goat anti-mouse Alexa 488 (1:100, 30 min.; green; Molecular probe, Lot # 41139A). Tissue
was protected from light from this step forward and sections were incubated with biotinylated goat anti-mouse IgG (1:500, 1 h; catalog item BA-9200; Vector Laboratories, Burlingame, CA), followed by ABC reagent, BT (as previously described) and Cy5- Streptavidin (1:100, 30 mins.; blue; Jxn Lot# 55606). Sections were incubated with the primary antibody rabbit anti-vGlut2 (17h), then with goat anti-rabbit Alexa 555 (1:100, 30 mins., red; Invitrogen/Molecular Probe, Cat # A-21428). Sections were mounted onto slides, cover-slipped with Gelvatol mounting medium, covered, and stored at 4°C until analyzed.

**Image capture and analysis: synaptic inputs**

Images of immunostained sections were captured at ×60 magnification using LSM 510 META/ConfoCor2 Confocal Microscope at the Robarts Research Institute at the University of Western Ontario. Sections through the ARC and POA (experiment 1) and the MBH and POA (experiment 2) in each animal were examined for kisspeptin- or GnRH positive cells, respectively, and their synaptic inputs. For quantitative analysis, 15 or more kisspeptin-positive cells were analyzed per animal from the ARC and the POA and 10 or more GnRH positive cells per animal were analyzed from the MBH and POA. The number of triple-labelled (kiss/vGlut2/syn), double labelled (vGlut2/syn; Kiss/syn) and single-labelled synaptophysin inputs were counted for each kisspeptin positive cell body (soma) in the ARC (KNDy) and POA and numbers were compared between control (OVX+E) and prenatal (T)-treated females (OVX+E). A synaptic input was defined as an immunolabelled terminal 1) positive for synaptophysin, 2) in close apposition to a cell
body and 3) without any intervening pixels. Orthogonal views confirmed contacts so
markers in all planes were accepted as inputs. Markers were placed to ensure inputs were
not counted twice (see figure 5). The numbers of triple-, double-, or single-labelled inputs
were recorded at each section pertaining to a cell (see image 1 and 3). The somal
perimeter of each cell was measured for each section of the cell where the nucleus was
seen (see image 4) and the total soma membrane analyzed calculated for each cell. The
number of inputs per KNDy cell soma was normalized per 10 μm of soma perimeter to
control for any change in soma perimeter between the two groups. Similarly, the number
of double-labelled (kiss/vGlut2) and single-labelled (kiss and vGlut2) inputs were
counted for each GnRH soma in the MBH and POA and numbers were compared
between control and prenatal (T)-treated females. The number of inputs was calculated
per 10μm of GnRH soma perimeter. All analysis was performed blind to the animal
number or group.

**Image capture and analysis: soma size analysis**

Images of immunostained sections were captured at ×60 magnification using LSM 510
META/ConfoCor2 Confocal Microscope or the LSM 510 META Confocal Microscope
(laser diode 405nm to visualize the DAPI staining) at the Robarts Research Institute at
the University of Western Ontario. To determine if prenatal T treatment induced
morphological changes in the KNDy, GnRH cells, or other neuronal populations of the
ARC, the soma perimeters of kisspeptin cells in the ARC (KNDy) and POA, GnRH cells
(MBH and POA), AgRP and POMC cells (ARC) were measured. Using the same animals
(Control n=5, Prenatal T n=4) as for the synaptic input analysis, the somal and nuclear perimeter (DAPI) of approximately 15-20 kisspeptin (see figure 10) in the ARC and POA and 10 GnRH positive-labelled cells in the MBH and POA was measured and averaged over several stacks per cell using the Zeiss Image Browser (Version 4.2.0.121). Confocal images of AgRP and POMC cells in Control and Prenatal T treated sheep were obtained from Rebecca Phillips (Phillips, unpublished) and approximately 15-20 cells were measure per animal.

Statistical analysis

Microsoft Excel 2000 and Sigma Stat 3.0 were used for all the statistical analysis in this study. Comparisons of somal perimeter and synaptic inputs between control and prenatal T-treated ewes were analyzed using t test. $P < 0.05$ was considered statistically significant for all analyses.
RESULTS

EXPERIMENT 1a) KNDy-KNDy connections

To analyze the effects of prenatal T treatment on the KNDy reciprocal circuitry, the number of triple-labelled (kiss/vGlut2/syn), double-labelled (vGlut2/syn and kiss/syn), single-labelled synaptophysin, and total number of inputs per KNDy neuron were compared between control and prenatal T treated females. Prenatal T treated ewes had a significantly lower number of triple-labelled kiss/vGlut2/syn inputs (Control: 0.69 ± 0.08, Prenatal T: 0.26 ± 0.16, p=0.003), double-labelled vGlut2/syn (Control: 0.60 ± 0.04, Prenatal T: 0.43 ± 0.12, p=0.005) and single-labelled synaptophysin inputs (Control: 0.95 ± 0.07, Prenatal T: 0.63 ± 0.04, p=0.006) per 10 μm soma perimeter than control ewes (Figure 1a). Moreover, when the total number of synaptophysin-positive inputs (single-, double-, and triple-labelled) was analyzed, KNDy cells in prenatal T treated ewes received significantly fewer contacts than in control ewes (Control: 2.25 ± 0.16 Prenatal T: 1.34 ± 0.07, p=0.002) (Figure 1b). There was no difference in the number of double-labelled kisspeptin/synaptophysin inputs (Control: 0.013 ± 0.001, Prenatal T: 0.011 ± 0.001, p=0.15) between the two groups. The number of inputs was also calculated per average cell soma perimeter and the same differences in inputs were seen.
Figure 3. Effects of Prenatal T treatment on the A) mean number of triple-labelled (kiss/vGLU2/syn), dual-labelled (vGlut2/syn and kiss/syn), and single-labelled synaptophysin inputs and B) mean number of total (triple-labelled, dual-labelled and single-labelled) inputs (±SEM) per 10 μm of KNDy soma perimeter in Control (n=5) vs. Prenatal T treated (n=4) females.
Figure 4. An example of a 0.38 μm fluorescent layer captured with the Confocal microscope. A) DAPI, B) Kisspeptin, C) vGlut2, D) Synaptophysin, and E) Overlay.

Figure 5. An example of triple-, double- and single-labelled inputs onto a kisspeptin neuron in the ARC. Magenta arrow: vGlut2+synaptophysin (single vGlut2 input), Blue arrow: synaptophysin (single other input), Black arrow: synaptophysin+kisspeptin (single kiss input), White arrow: synaptophysin+kisspeptin+vGlut2 (KNDy input), Blue circle marks a synaptophysin input (circle remains through entire stack).
EXPERIMENT 1b) Kisspeptin cells in the POA

To analyze the effects of prenatal T treatment on the number inputs onto POA kisspeptin cells, triple-labelled (kiss/vGlut2/syn), double-labelled (kiss/syn and vGlut2/syn), and single-labelled (synaptophysin) inputs were quantified. There was no significant difference in the number of triple-, double-, or single-labelled inputs between control and prenatal T-treated females (Figure 6a). However, there was a trend towards decreased number of vGlut2 single inputs and synaptophysin single inputs in prenatal T sheep (vGlut2: Prenatal T: 0.57 ± 0.05, Control: 0.76 ± 0.08, p=0.068; synaptophysin: Prenatal T: 0.74 ± 0.05, Control: 0.88 ± 0.09, p=0.27).

A  Inputs onto Kisspeptin neurons (POA) per 10μm of soma perimeter
**B Total inputs onto POA Kisspeptin cells**

![Bar graph showing total inputs onto POA Kisspeptin cells](image)

Figure 6. Effects of Prenatal T treatment on A) the mean number of triple-labelled (kiss/vGLU2/syn), dual-labelled (vGlut2/syn and kiss/syn) and single-labelled synaptophysin inputs (±SEM) and B) total number of inputs per 10 µm of POA kisspeptin soma perimeter in Control (n=5) vs. Prenatal T treated (n=4) females.

**EXPERIMENT 2) KNDy inputs to GnRH**

To analyze the effects of prenatal T treatment on KNDy projections to GnRH neurons, the number of double-labelled (kiss/vGlut2) and single-labelled (kisspeptin and vGlut2) inputs were quantified onto GnRH somas in the MBH and POA. vGlut-2 was used as a marker for kisspeptin cells of the ARC (KNDy) since preliminary observations indicate that kisspeptin cells of the POA do not colocalize vGlut-2 in the sheep [139]. Because of the inclusion of GnRH as a third immunocytochemical label in this experiment, we were not able to confirm the synaptic nature of the close contacts observed by using synaptophysin as a marker, as in experiments 1a and 1b. Nonetheless, in other experiments we have shown that kisspeptin terminals in direct contact with GnRH somas,
observed in confocal 1 μm optical sections, always colocalize synaptophysin suggesting they are bona fide synaptic inputs (Merkley, unpublished).

Prenatal T animals showed a significant decrease in the number of kisspeptin/vGlut2 dual inputs per 10 μm soma perimeter for GnRH cells in the MBH (Control: 0.28 ± 0.02, Prenatal T: 0.15 ± 0.02, p=0.0009) and POA (Control: 0.23 ± 0.02, Prenatal T: 0.14 ± 0.01, p=0.003). In addition, the total number of inputs (here defined as kiss/vGlut2 dual, kisspeptin single and vGlut2 single) were fewer for GnRH cells in the MBH (Control: 0.70 ± 0.05, Prenatal T: 0.49 ± 0.05, p=0.017) and POA (Control: 0.73 ± 0.05, Prenatal T: 0.59 ± 0.05, p<0.036) in prenatal T females than controls. No significant difference was found in the number of kisspeptin or vGlut2 single inputs onto GnRH neurons (Figure 7a and b).
Figure 7. Effects of Prenatal T treatment on the A) mean number of dual-labelled kisspeptin/vGLU2, single-labelled kisspeptin and single-labelled vGlut2 inputs and B) mean number of total (dual and single-labelled) inputs (±SEM) per 10 μm of GnRH soma perimeter in Control (n=5) vs. Prenatal T treated (n=4) females.

Figure 8. An example of a fluorescent image captured with the Confocal microscope. A) GnRH, B) Kisspeptin, C) vGlut2, D) Overlay.
EXPERIMENT 3) Somal Perimeter

To determine if prenatal T treatment induced morphological changes in the KNDy, GnRH cells, or other neuronal populations of the ARC, the soma perimeters of kisspeptin cells in the ARC (KNDy) and POA, GnRH cells (MBH and POA), AgRP and POMC cells (ARC) were measured (see figure 10 and 11). KNDy cells in prenatal T treated females had a significantly larger somal perimeter (Control: 43.46 ± 0.64, Prenatal T: 51.23 ± 0.90, p=0.009) compared to control females (Figure 9a and b). In the same animals, there were no changes in the somal perimeter of kisspeptin cells in the POA, nor of GnRH cells in either the MBH or POA (Figure 9c). The somal perimeter of two other cell populations in the ARC was measured: proopiomelanocortin expressing neurons (POMC) and agouti-related protein expressing neurons (AgRP), both appetite regulating neurons, responsible for suppression and stimulation of food intake, respectively. AgRP neurons, like KNDy neurons, have recently shown to be altered by prenatal T exposure in sheep however, whereas prenatal T decreases dynorphin and NKB in KNDy cells, it results in an increase in the number of AgRP neurons [85]. Despite this effect, the somal size of AgRP cells did not differ between prenatal T and control animals; similarly there was no change observed in somal size for POMC neurons (Figure 9c).
Figure 9. Effects of Prenatal T treatment on the A) soma and nuclear perimeter of KNDy neurons in the ARC, B) soma perimeter of dual-labelled (kisspeptin and dynorphin) KNDy neurons in the ARC and C) Soma perimeter of AgRP and POMC neurons in the ARC and kisspeptin and GnRH neurons in the POA (±SEM) in Control (n=5) vs. Prenatal T treated (n=4) females.
Figure 10. An example of a confocal image showing A) DAPI nuclear staining, B) kisspeptin staining, C) synaptophysin staining, and D) an overlay of all three. *Red circles* outline the perimeter of the nucleus (A) and soma (D).

Figure 11. ARC KNDy cell showing C) co-localization of A) kisspeptin and B) dynorphin.
DISCUSSION

The experiments described here allowed us to analyze morphological changes in the ARC KNDy cells on two different levels as a result of prenatal T treatment: 1) the number of KNDy reciprocal connections and inputs to GnRH neurons and 2) the size of KNDy cell bodies (Fig. 12). Prenatal T treated ewes had decreased KNDy reciprocal connections, with a lower number of dual (kisspeptin/vGlut2) inputs and single (vGlut2 and synaptophysin) inputs, as well as a lower number of total inputs. In addition to changes in KNDy reciprocal connections, prenatal T treatment also altered KNDy inputs to GnRH, resulting in a reduction in the number of dual (kiss/vGlut2) inputs and total inputs (Kiss and vGlut2 dual and single) onto GnRH neurons in the POA and MBH. Finally, prenatal T also resulted in an increased somal size which was specific to KNDy cells, and not seen in other kisspeptin cells (POA), in GnRH cells, or in other adjacent but separate cell populations of the ARC (AgRP, POMC).

What is the functional impact of these changes?

We observed a decrease in KNDy reciprocal (triple-labelled kiss/vGlut2/syn) inputs as well as other inputs (synaptophysin) synapsing onto the KNDy cells in prenatal T animals suggests an imbalance between the stimulatory and inhibitory signals within the ARC population. It is conceivable that the decreased number of reciprocal connections among KNDy cells, and their decreased inputs to GnRH cells, might results in reduced coordination of synchronous activity among KNDy cells and its output in the control of GnRH pulses. However, there is no evidence that the GnRH pulse generator is compromised in prenatal T animals; rather, the responsiveness of GnRH neurons to
steroid negative feedback control is impaired resulting in increased GnRH/LH pulse frequency in prenatal T animals as in PCOS patients. Instead, we speculate that the morphological alterations seen in this study may contribute to the defects seen in the GnRH surge in prenatal T sheep. Previous work by Sharma et al. 2002 shows that prenatal T exposure (T30–90) results in a delayed and severe dampened LH surge [6]. Moreover, recent findings in our lab have revealed that in normal, ovary-intact sheep, there is an increase in the number of synaptic inputs onto ARC kisspeptin neurons (KNDy) at the time of the preovulatory GnRH surge [77]. Specifically, the total number of synaptophysin-positive inputs onto KNDy cells is significantly greater in animals perfused during the follicular phase of the estrous cycle than during the luteal phase (ref).

In addition, GnRH cells in both the POA and MBH receive a greater number of inputs during the follicular compared to luteal phase in normal animals [77]. Given evidence that KNDy cells may, in part, mediate the positive feedback ability of E2 to induce a GnRH surge (see Introduction), we hypothesize that the reduced amount of input to KNDy cells, and their connections to GnRH cells, in prenatal T sheep maintains the KNDy-GnRH circuit in “luteal phase” state even though the animals have been treated with E2 implants mimicking the follicular phase, i.e., sufficient to induce a GnRH surge. In fact, if one compares the percentage of reduction in total amount of synaptic input, using follicular phase animals as controls, then prenatal T treated females show a 25% decrease in total inputs, similar to the 35% decrease seen in normal, luteal phase animals (Fig. 14). Thus, the decreased synaptic input to KNDy and GnRH cells seen in prenatal T sheep, parallels defects in the positive feedback influence of E2, and suggests that the KNDy-GnRH circuitry is impaired in its ability to respond (morphologically) to follicular
phase levels of E2. Interesting, recent work in our lab has shown that there are no
differences between prenatal T and control ewes in the expression of ER-alpha in KNDy
cells, suggesting that the defect is either due to impaired signalling downstream of ER-
alpha or changes in upstream signals that control the responsiveness of KNDy cells to E2.

Which neuropeptides/transmitters in KNDy cells might be functionally involved in
control of the surge, and in the defects seen in prenatal T animals? Kisspeptin has been
implicated as a key transmitter in the role of the GnRH surge [67, 69, 133, 140-144] and
mutations of the kisspeptin receptor (GPR54) have been shown to cause a failure to enter
puberty in humans and mice [145-147]. However, as mentioned previously, prenatal T
treatment in sheep does not alter kisspeptin peptide in KNDy cells or in the POA
kisspeptin population. On the other hand, prenatal T reduces NKB in KNDy cells [89] as
well as decreasing NK3R, the high affinity receptor for NKB, in the ARC [148].

Although NKB was initially believed to be inhibitory to GnRH/LH secretion [85, 86],
recent work in sheep, primates, and rodents have shown this tachykinin to be stimulatory
to GnRH/LH release [79, 149, 150]. In the sheep, NKB causes a surge-like secretion of
LH when administered intraventricularly during the follicular, but not luteal, phase of the
estrous cycle [150]. In human, mutations of NKB or its receptor (termed TAC3 and
TAC3R, respectively) leads to hypothalamic hypogonadism [83, 84] consistent with the
loss of a stimulatory influence on GnRH secretion.

Another KNDy transmitter that may contribute to the role of KNDy cells in the surge is
 glutamate. Levels of glutamate in the POA are increased during the E2-induced LH
surge and at the time of puberty in female rats and monkeys [98, 100, 101, 151] and intravenous infusion of NMDA increases GnRH mRNA levels in the POA of rats [92, 102]. Moreover, immunofluorescence studies in sheep found an increase in the number of vGlut2 positive terminals apposing KNDy cells and GnRH neurons at the time of the GnRH/LH surge [77]. Because changes in glutamate across the estrous cycle within the KNDy cells and their connections may act as a permissive signal, allowing the positive feedback actions of $E_2$ to generate a GnRH surge [75, 76], it is possible that a decrease in KNDy-KNDy connections (containing glutamate as well as NKB) would diminish the ability of $E_2$ to elicit a GnRH/LH surge. Thus a loss of NKB and/or glutamate signalling with the KNDy circuitry may be amplified by the reduced number of synaptic connections among these cells, and there projections to GnRH neurons, both decreases in peptide/transmitter and morphological changes contributing to defects in the generation of the surge.

KNDy inputs to GnRH cells: effects of prenatal T compared to sexual differentiation

The decrease in KNDy (double-labelled kiss/vGlut2) inputs onto GnRH neurons suggests a reduced stimulatory drive of GnRH secretion in prenatal T animals. Interestingly, in the rodent, GnRH cell bodies receive roughly twice as many inputs in females compared to males [152]. In the sheep, prenatal T treatment masculinizes synaptic input to GnRH neurons [30] and thus the changes observed in this study are consistent and in the same direction of the effects of prenatal T during normal sexual differentiation. However, a difference between the prenatal T model and normal sexual differentiation is that not all
sex differences in the KNDy cell population are mimicked by prenatal T. Specifically, although sex differences are seen in dynorphin and NKB in KNDy cells, they are also seen in kisspeptin expression in both KNDy cells and preoptic kisspeptin neurons in the sheep, with females having greater numbers of cells than males. By contrast the number of kisspeptin cells does not differ between control and prenatal T sheep [2], in either the ARC (KNDy cells) or POA, suggesting that the critical periods for sexual differentiation of the three peptide co-expressed in the same cells are distinct, with dynorphin and NKB being dependent on androgen exposure during days 30-90 of gestation, and kisspeptin being dependent on either prolonged or later steroid exposure.

Changes in KNDy soma size

Prenatal T treated ewes exhibited a significantly increased KNDy somal size compared to control females. It is unclear what causes this hypertrophy, however similar changes have been reported by Rance and her colleagues in postmenopausal women, where a population of neurons in the infundibular (arcuate) nucleus expressing KiSS-1, neurokinin B (NKB), substance P, dynorphin and estrogen receptor a (ERα) mRNA displayed an increase in somal size [107, 109, 135]. In women, this hypertrophy is believed to be a secondary response to ovarian failure [106, 109], and associated with declining levels of circulating E$_2$ concentrations [110, 113]. Interestingly, Birch et al. (2003) [8] found that while prenatal T treated ewes cycled during the first breeding season, reproductive cycles were absent in the second breeding season (T60 females), suggesting that prenatal T treatment leads to a rapid and premature ovarian failure. Eghlidi et al. [118] found that only long term ovariectomy (~ 4 yr) significantly increased
KISS-1 and NKB expression, and this was reversed by E2 administration, further linking these changes to menopausal ovarian failure and relating the disruption of the HPG axis with the neuroendocrine defects of prenatal T treatment. As seen in postmenopausal women, a decline in E2 concentrations (or perhaps a decreased sensitivity to E2), causes an increase in KISS-1 and NKB expression [85, 107, 118]. However, the number of kisspeptin-positive cells do not change in prenatal T treated sheep and thus, an increase in protein production unlikely to fully explain the increase in cell size. Analysis of the ARC POMC and AgRP soma size provides additional confirmation that the KNDy cell hypertrophy seen in prenatal T treated ewes is a change occurring exclusively in the KNDy population. There was no effect of prenatal T treatment on the soma size of AgRP or POMC neurons even though previous work by Sheppard et al. (2011) [153] clearly shows that prenatal T treated ewes had significantly more AgRP-immunoreactive neurons suggesting an increase in peptide expression. Thus, the changes in somal size we report here for prenatal T sheep do not appear to be a direct consequence of changes in intracellular peptide levels. Future work must be done to determine the functional significance of the KNDy cell hypertrophy.
Summary and future directions

Previous studies point to alterations in the systems that modulate GnRH as being the main elements responsible for reproductive defects in the prenatal T model. Bringing this back to the KNDy circuitry, one possibility is that a reduction in the number of reciprocal KNDy-KNDy connections and their inputs to GnRH cells may be the initial step of a cascade of events contributing to the decreased responsiveness of the KNDy cells to $E_2$ and thus, defects in the timing or amplitude of the GnRH/LH surge.

Specifically, we hypothesize that the decreased number of kisspeptin/glutamate terminals, together with the reduced expression of NKB, creates a weakened network, incapable of coordinating a large enough stimulatory drive to GnRH cells, and thus, incapable of generating the robust GnRH surge needed for ovulation. This in turn, leads to ovarian failure and hypertrophy of the KNDy neurons. One prediction of this working hypothesis would be that glutamate and NKB agonists may be able to reverse the functional defect in the GnRH/LH surge observed in prenatal T animals; if this reversal is sufficient to produce ovarian recovery we would also expect to see a normalization of the hypertrophy of KNDy neurons.

A major limitation of the results reported here is that they focus on morphological changes seen at the level of KNDy cell somas and not dendrites; this is because immunodetectable kisspeptin only fills the proximal dendrites of KNDy cells in the sheep. Other approaches could be developed and exploited to allow visualization of the complete KNDy dendritic arbor: these include the possibility of transfection of KNDy
cells by viral vectors incorporating KNDy-specific promoters to drive reported genes (e.g., EGFP) [154], or the use of diolistics to label KNDy cells with carbocyanine dyes and produce a “Golgi-like” appearance [155].

In summary, we have found evidence of morphological changes in the synaptic connections and size of KNDy cells that may underlie some of the reproductive neuroendocrine defects seen in female sheep as a consequence of prenatal T exposure. Given the close correspondence of the defects seen in this animal model to the multifaceted symptoms of PCOS in women, it may be that this reproductive disease involves long-term neuroplastic changes in brain circuitry similar to those underlying a number of neuropsychiatric and neurologic disorders such as anxiety and depression. However, whether these morphological changes are causal or not for the defects seen remains to be determined. Overall, these findings are single puzzle pieces, but when put together in a big picture context, we hope to understand the complex mechanisms that normally organize the reproductive neuroendocrine system, and what goes awry in the development of reproductive disease.
Table 1. Linking the reproductive and metabolic attributes of prenatal T-treated ewes with alterations seen at the neuroendocrine level and the expected results of effective treatment. Adapted from Lehman, M.N. EAC Meeting 2011.

<table>
<thead>
<tr>
<th>Prenatal T attribute</th>
<th>Neural correlate</th>
<th>Result of effective prevention/treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced P negative feedback</td>
<td>↓ DYN [89]</td>
<td>↑ DYN</td>
</tr>
<tr>
<td>Reduced E2 positive feedback</td>
<td>↓ NKB, NK3R[89, 148]</td>
<td>↑ NKB, NK3R (senktide?)</td>
</tr>
<tr>
<td></td>
<td>↓ Synaptic inputs to KNDy and GnRH cells</td>
<td>↑ Synaptic inputs to KNDy and GnRH cells (Glutamate agonist?)</td>
</tr>
<tr>
<td>Metabolic dysfunction/obesity</td>
<td>↑ AgRP[153]</td>
<td>↓ AgRP</td>
</tr>
<tr>
<td></td>
<td>↓ Insulin receptor (IR) in AgRP cells (Phillips, unpublished)</td>
<td>↑ Insulin receptor (IR) in AgRP cells</td>
</tr>
</tbody>
</table>

Figure 12. Total number of contacts per KNDy neuron in ovary-intact follicular and luteal phase females vs. control and prenatal T treated females sacrificed during the late follicular phase. (Ovary-intact animal data from Merkley, C.M.[56])
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Chapter 3: Conclusions and Discussions

Overview and Main Findings

The previous experiments allowed us to analyze morphological changes to the KNDy-GnRH circuit on two different levels – the number of inputs/reciprocal connections among the KNDy cells and the size of the KNDy cells. The results indicate changes in both as a response to prenatal T: (1) a decrease in the number of KNDy-KNDy connections, and in the number of their connections to GnRH cell bodies; and (2) an increase in the soma size of KNDy cells but not that of GnRH or other kisspeptin cells (Figure 12). With respect to synaptic changes, we specifically found a reduction in the number of kiss/vGlut2/syn triple-labelled inputs, as well as a lower number of total inputs, to KNDy cells as a result of prenatal T treatment. Given evidence that vGlut2 is a marker of KNDy cells but not kisspeptin cells in the preoptic area [139], the decrease in Kiss/vGlut2 inputs reflects a decrease in KNDy-KNDy connections; whether they are truly reciprocal (a two-neuron, bidirectional chain) remains to be determined. Since there is no evidence that kisspeptin levels are altered in prenatal T sheep [89], we interpret the decrease in Kiss/vGlut2 inputs as being a morphological change. The fact that we saw a parallel decrease in total synaptophysin-positive inputs provides further evidence of this morphological rearrangement.

We also saw a decrease in the number of KNDy inputs to GnRH, identified by kisspeptin and vGlut2 colocalization, as well as a decrease in total inputs onto GnRH neurons in the POA and MBH. The nature of the species of origin for the antibodies we used precluded
us from adding synaptophysin as a fourth marker in our analyses of inputs to GnRH neurons. Nonetheless, the changes in total number of kisspeptin and vGlut2 inputs to GnRH cells parallels that seen for KNDy-KNDy connections, suggesting that morphological rearrangements occur at the level of KNDy projections to GnRH cells as well as to each other.

Finally, we found that prenatal T affected the somal size of KNDy neurons, with prenatal T females displaying increased soma size compared to control females. This change was specific to KNDy cells, and not seen in GnRH neurons, in kisspeptin cells localized in the preoptic area, or in adjacent AgRP or POMC neurons in the arcuate nucleus.

**KNDy reciprocal connections**

The decrease in KNDy-KNDy (triple-labelled kiss/vGlut2/syn) inputs suggests a decreased degree of communication within this ARC subpopulation. Since prenatal T animals still show pulsatile GnRH/LH (albeit reduced steroid feedback control of pulsatile secretion), it is unlikely that this change has a significant impact on operation of the “GnRH pulse generator”. However, prenatal T animals do show a decrease in the expression of dynorphin within KNDy cells [89]. If dynorphin serves as the stop signal for GnRH pulses, then its decrease may account for the increased pulse frequency seen in prenatal T sheep, and in particular, the reduced sensitivity to the negative feedback effects of progesterone [8, 49, 82]. A decreased total number of KNDy-KNDy connections may augment this change, leaving the network overall less responsive to the inhibitory influence of progesterone.
On the other hand, as mentioned above, there is evidence that KNDy cells in sheep may play a functional role in the generation of the GnRH/LH surge, as well as pulsatile secretion [73, 75, 79, 87]. Previous work by Sharma et al. 2002 shows that prenatal T exposure (T30–90) results in a delayed and severe dampened LH surge [6]. Moreover, recent evidence shows that there is an increase in the number of synaptic inputs onto ARC kisspeptin neurons (KNDy) at the time of the preovulatory GnRH surge, further highlighting the possible role of the KNDy cells as a key component of the E2 positive feedback mechanism [77].

Glutamate has been shown to play a critical role in the GnRH surge. In fact, levels of glutamate in the POA are increased during the E-induced LH surge and at the time of puberty in female rats and monkeys [98, 100, 101, 151] and intravenous infusion of NMDA increases GnRH mRNA levels in the POA of rats [92, 102]. Moreover, as previously mentioned, immunofluorescence studies in sheep found an increase in the number of vGlut2 positive-inputs to KNDy cells at the time of the LH surge [77]. Changes in glutamate inputs across the estrous cycle may act as a permissive signal, allowing the positive feedback actions of E2 to generate a GnRH surge [75, 76]. In this view, a decrease in KNDy-KNDy (kiss/vGlut2) connections would decrease excitatory coupling among KNDy cells, and alter the ability of E2 to elicit a synchronized GnRH/LH surge. Thus, prenatal T treatment, in addition to altering the balance of neuropeptides within individual KNDy cells, causes a reduction in KNDy-KNDy
glutamatergic inputs impairing the ability of this population to help generate and coordinate a robust preovulatory GnRH surge.

**KNDy inputs to GnRH**

The decrease in KNDy (dual-labelled kiss/vGlut2) inputs onto GnRH neurons suggests a reduced stimulatory drive of GnRH secretion, perhaps contributing to defects in E2 positive feedback influence seen in prenatal T animals [6, 37, 156, 157]. Thus, changes at the level of both KNDy-KNDy connections, and KNDy inputs to GnRH cells are consistent with a functional impairment of the surge mechanism. Interestingly, in the rodent, GnRH cell bodies receive roughly twice as many inputs in females compared to males [152]. In the sheep, prenatal T treatment masculinizes synaptic input to GnRH neurons[30] and produces an absent LH surge [136, 156, 158]. Therefore, we can propose that the inability of prenatal T treated ewes to generate a GnRH/LH surge partially stems from a reduction in the number of KNDy inputs to GnRH neurons. It is notable that women with PCOS also show defects in the inability to respond to E2 with an LH surge [17, 45, 46].

**KNDy inputs to POA kisspeptin cells**

There were no significant changes in the number of triple-labelled (kiss/vGlut2/syn) and double/labelled (kisspeptin/syn or vGlut2/syn) inputs onto POA kisspeptin cells between our groups, although there was a trend toward decreased inputs. On the other hand, we did see a significant decrease in total inputs onto the POA kiss population. It may be that
with greater numbers of animals, the differences in triple- or dual-labelled inputs would also become significant. Since the colocalization of kisspeptin and vGlut2 is a marker for KNDy cell axons, the presence of these triple-labelled inputs to POA kisspeptin cells suggest that the two kisspeptin populations (KNDy and POA) are connected, at least in one direction (KNDy to POA). The significant change in overall input to POA kisspeptin cells in prenatal T sheep suggest that this population, like the KNDy cells, may also participate in the generation of the GnRH surge. In fact, studies in rodents have deemed the POA kisspeptin population as critical players in the GnRH/LH surge. About 50% of kisspeptin neurons in the sheep POA express ERα (compared to 93% in the ARC) [70] and at the time of the LH surge, the majority of Kiss neurons in the preoptic region of the rat [159] and sheep [104] co-express the immediate early gene Fos. Thus, POA kisspeptin neurons play an active and complementary role in the E2-induced preovulatory LH surge.

KNDy soma size

KNDy cells in prenatal T treated ewes displayed a specific and dramatic increase in somal size compared to the same cells in control females. It is unclear what causes this hypertrophy, however a similar findings have been reported in postmenopausal women by Rance and colleagues, where a population of neurons in the infundibular (arcuate) nucleus colocalizing KiSS-1, neurokinin B (NKB), dynorphin and estrogen receptor a (ERα) mRNA displayed an increase in somal size [107, 109, 135]. In women, this hypertrophy is believed to be a secondary response to ovarian failure [106, 109], and associated with declining levels of circulating E2 concentrations [110, 113]. Like women with PCOS and prenatal T treated ewes, postmenopausal women also show an erosion of
LH-FSH pulse regularity, with increased LH pulsatility and dampened LH surge [111], suggesting a loss of synchrony control within the HPG axis and decreased GnRH responsiveness with aging [160]. When compared to young rats during proestrous, middle-aged rats showed a 50% decrease in the number of GnRH neurons expressing c-fos during a steroid-induced LH surge [114], further linking the reduction of stimulatory inputs onto GnRH neurons in the prenatal T model with aging of the reproductive neuroendocrine system. Thus, the increased somal size of KNDy neurons seen in this study may reflect accelerated aging of the sheep reproductive neuroendocrine system.

Interestingly, Birch et al. (2003) [8] found that while prenatal T treated ewes cycled during the first breeding season, reproductive cycles were absent in the second breeding season (T60 females), suggesting that prenatal T treatment leads to a rapid and premature reproductive failure. This most likely stems from alterations at the neuroendocrine level (decreased sensitivity to estrogen) and not from a decline in estrogen from the ovaries, as our prenatal T sheep have elevated E2 levels [23] and the groups of sheep in this study each received the same E2 implant to mimic late follicular phase levels.

Another possible explanation for the increased somal size of KNDy neurons in prenatal T sheep is that it reflects an increase in peptide/protein production. In postmenopausal women, there is an increase in KISS-1 and NKB expression [118] and this is thought, in part, to account for the hypertrophy of KNDy neurons observed. However, nearby AgRP neurons in the arcuate nucleus showed no change in their size, despite the fact that prenatal T treatment more than doubles the number of immunodetectable AgRP neurons [153]. Thus the increased soma size may not reflect
increased protein synthesis per se, but may instead be a response to other changes, perhaps in responsiveness to E2, as suggested above, or to progesterone. Support for the latter possibility comes from evidence that progesterone receptor expression is decreased in the ARC of prenatal T sheep [89], while ER-alpha expression in KNDy cells is unchanged [161]. Thus, the KNDy cell hypertrophy seen in prenatal T treated sheep may be a reflection of decreased activation of these cells by progesterone or even E2 if downstream elements of this receptor are altered during prenatal T programming.
Final Thoughts

The current findings point to alterations in the systems that modulate GnRH as the main neuroendocrine elements responsible for reproductive defects due to prenatal T exposure. Prenatal T treatment resulted in neuroplastic changes in the ARC KNDy population on two different levels: the number reciprocal connections among the KNDy cells and their inputs to GnRH cells, and their somal size. We propose that a reduction in reciprocal KNDy connections and their inputs to GnRH results in the decreased stimulatory that disrupts its ability to convey $E_2$ positive feedback to GnRH neurons. In turn, these morphological alterations induced by prenatal T result in defects in the timing or amplitude of the GnRH/LH surge. The functional impact of the changes in KNDy cell size are less clear, but may reflect accelerated aging of the system and/or a response to diminished steroid hormone feedback efficacy. Because the reproductive and metabolic phenotype of prenatal T-treated sheep parallels the features of women with PCOS, we speculate that the same morphological changes may be present in the hypothalamic networks of PCOS patients.

Studying the hypothalamic reproductive networks and specifically, those driving GnRH secretion, and how they are organized by prenatal T, will help us understand the events leading to reproductive dysfunction. These findings are single puzzle pieces, and when put together, will hopefully assist us to understand the complex mechanisms that organize the reproductive axis and govern female reproduction, and the defects in those mechanisms that underlie some types of reproductive disease.
Figure 13. Schematic diagram showing the differences in synaptic connections (among the KNDy cells and to GnRH neurons) and soma size between control and prenatal T treated females.
Future Directions

The changes in synaptic connections seen in prenatal T treated females provide us with exciting path for future work. To determine if the dampened and delayed GnRH/LH surge is a result of the changes in KNDy-KNDy connections (and perhaps a decrease in NKB or glutamate), future experiments can confirm this with the use of agonist and/or antagonists. Infusing of a NK3R (senktide) or glutamate agonist into the MBH of a prenatal T treated female during the late follicular phase should restore the GnRH/LH surge. In reverse, infusing a NK3R or glutamate antagonist into the MBH of a normal female during the preovulatory period should block the GnRH/LH surge and ovulation.

It is important to think of the KNDy-GnRH alterations as acting collectively with other changes of the metabolic and behavioural neuronal systems as well as the postnatal environment. Recent work has shown that KNDy cells receive GABAergic inputs (Merkley et al., unpublished) and dopaminergic inputs [162]. Analyzing these types of inputs in a prenatal T model will provide us with a wider scope on the various systems that regulate KNDy and GnRH activation and their specific role in reproduction. It would also be interesting to examine AgRP and POMC connections to KNDy cells and GnRH neurons. These appetite stimulating and suppressing neurons (respectively) are prenatally programmed by testosterone [153] and play a critical role in the disrupted metabolic (and probably reproductive) pathways seen prenatal T treated sheep. Recent work suggests that leptin does not modulate reproduction by direct action on KNDy or GnRH neurons, but most likely communicates with the reproductive axis via populations of LepRb (leptin receptor) neurons that are afferent to KNDY or GnRH neurons[163]. AgRP and POMC
cells express leptin receptors and are leptin responsive[164]. Therefore, it would be interesting to understand how prenatal T treatment affects AgRP and POMC inputs to KNDy and GnRH neurons and determine how this fits in with leptin's role in reproduction.

Finally, in terms of KNDy-GnRH circuitry, although the focus in this thesis has been upon the influence of KNDy cells in stimulating GnRH neurons, recent evidence suggests that the relationship may be bidirectional, with GnRH neurons providing a feedback signal back to the KNDy population. As seen below (Fig. 13) in our analysis of dual-labelled kisspeptin/GnRH material, we noticed a few examples of GnRH terminals in direct contact with KNDy cell bodies in the ARC. Ramaswamy et al. (2008) also found GnRH contacts on kisspeptin cell body and dendrites in the gonadectomized male rhesus monkey [165]. There is also evidence in rodents and sheep that GnRH may act in a short loop autoregulatory fashion to inhibit its own secretion [166, 167], although the site of that influence (the ARC?) has yet to be determined. Thus, prenatal T treatment may not only alter KNDy projections to GnRH neurons, but also the circuitry by which GnRH feeds back upon KNDy cells; further research is needed to examine this possibility.
The increase in KNDy cell size we uncovered in prenatal T treated females was a very exciting finding and one worth investigating further. Finding a link between the causes of KNDy hypertrophy in postmenopausal women and prenatal T treated sheep will be a critical step in determining the functional significance of this neuronal response.

It would also be interesting to explore the differences in synaptic inputs and somal morphology in T30 ewes (treated with testosterone from gestational days 60-90). Unlike the T60 females who do not cycle at all during the second breeding season (complete reproductive failure), 71% of T30 females continue to cycle in the second breeding season. Will the T30 females show similar changes in KNDy-GnRH synaptic
connections? How about somal size? Answering these questions would bring us a step closer to understanding how the timing and duration affect the KNDy-GnRH network.

One important limitation to our studies is that due to the nature of labelling we could only analyze cell bodies and not dendrites. Diolistic labelling would help us visualize the cell body and dendritic tree in its entirety. More specifically, performing immunofluorescence for kisspeptin and dynorphin/NKB in combination with diolistic labelling would provide us with an additional marker of soma area and would allow us to visualize the differences between control and prenatal T treated females in terms of dendritic fibres.

Finally, the role of kisspeptin POA population in the sheep is still unclear. Correlating Fos expression in POA kisspeptin cells with the presence/absence of the GnRH surge would be helpful in determining whether POA kisspeptin cells are activated in prenatal T treated females at the time of the surge (which can be gathered from blood sample data of each female in our experiments).

Additional experiments comparing GPR54 receptor levels in GnRH neurons, AR and insulin receptor levels in KNDy neurons would help us understand the changes leading to reproductive dysfunction and the complex mechanisms that organize the reproductive axis in prenatal T treated females.
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