

**Metabolic regulation of fertility: an emerging role for AMP-activated protein kinase
in the control of gonadotropin-releasing hormone neuron function**

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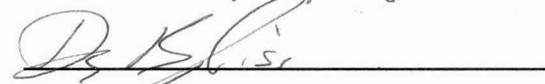
A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Pharmacology

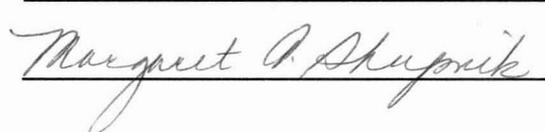
University of Virginia
September, 2010











Abstract

Gonadotropin-releasing hormone (GnRH) neurons integrate steroidal and metabolic cues to regulate fertility centrally. Abnormalities in GnRH release play a role in polycystic ovary syndrome (PCOS), the leading cause of infertility in women. PCOS is characterized by elevated androgen and luteinizing hormone (LH) levels, oligo- or anovulation, and/or ovarian cysts. Increased central stimulation of the reproductive axis due to increased frequency and amplitude of GnRH secretion is thought to play an important pathophysiological role in this disorder. Metabolic abnormalities, including insulin resistance, are also extremely common in PCOS.

Because glucose homeostasis is perturbed in PCOS, we examined metabolic characteristics in female mice prenatally androgenized (PNA) with dihydrotestosterone. PNA mice exhibit increased central drive to the reproductive axis and irregular reproductive cycles, similar to PCOS. PNA mice had impaired glucose tolerance, but did not exhibit peripheral insulin resistance and hyperinsulinemia, suggesting that excess prenatal androgen receptor activation may be insufficient to cause the full spectrum of metabolic abnormalities seen in PCOS.

Evidence indicates that glucose is critical in regulating reproduction centrally, but the cellular mechanisms are poorly understood. We examined if changes in extracellular glucose could alter the activity of GnRH neurons, and if glucosensing was affected by dihydrotestosterone and/or estradiol. GnRH neurons were sensitive to physiological reductions in extracellular glucose; androgens inhibited glucosensing. Mechanistically, AMPK proved to be important mediator of glucosensing, and AMPK activation inhibited GnRH neurons.

The inhibitory effect of AMPK activation led us to hypothesize that metformin, an AMPK activator that restores reproductive function in PCOS, might blunt excessive GnRH release through direct central actions. We tested this hypothesis in PNA mice. Metformin restored estrous cyclicity to PNA mice and reduced LH and GnRH neuronal activity. GnRH neurons from metformin-treated mice were stimulated by an AMPK antagonist and relatively insensitive to low glucose, suggesting AMPK was activated centrally by metformin.

Together, these studies have provided novel insight into the regulation of GnRH neuronal activity by metabolic and steroidal cues, shown how interactions between these cues might contribute to the PCOS phenotype, and elucidated the mechanisms by which metformin improves reproductive function in women with PCOS.

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Acknowledgements

I would like to express my gratitude to the many people who helped me surmount one of my biggest challenges.

To Sue, for being accessible, taking the time to teach me hands-on, conveying your knowledge in a way that was understandable, encouraging me to do more, and making this a fun experience.

To my lab mates, for always making me laugh, even when none of my experiments were working. Thank you for all of your help with experiments, equipment, surgeries, etc. – this really was a team effort!

To my committee members, for all of your time and helpful suggestions with regard to experiments, despite my habitual failure to provide donuts.

To my husband Joe, my family, and my friends, for supporting me and reminding me of what is truly important.

Dedication

To my parents, I dedicate this work: the first science project I've ever completed without your help!

Chapter 1
Introduction

Fertility in vertebrates is governed centrally by a small population of around 1000 gonadotropin-releasing hormone (GnRH) neurons scattered throughout the preoptic area and mediobasal hypothalamus (1). The primary function of these neurons is the synthesis and secretion of the decapeptide hormone GnRH, which is delivered to the hypophyseal portal vessel system via nerve terminals located in the median eminence. GnRH travels to the anterior pituitary via portal vessels, then binds to its receptors, stimulating the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH stimulate gonadal maturation, gametogenesis, and steroid secretion.

GnRH release and subsequent LH/FSH release occur in distinct episodes of secretion termed pulses. Pulsatile release of GnRH is critical to prevent downregulation of the pituitary response (2). The response of the pituitary is encoded by the frequency of GnRH release: low frequency favors FSH, whereas high frequency favors LH secretion (3). Circulating gonadal steroids feed back to the hypothalamus to regulate central GnRH secretion. Castration experiments have demonstrated that removal of steroids increases GnRH release, indicating a classical endocrine negative feedback mechanism whereby GnRH via LH and FSH stimulates steroid secretion, and steroids in turn inhibit GnRH release to maintain a constant pulse frequency in the male (see Fig 1-1). Steroid feedback can be mediated by direct effects on the GnRH neuron and also through afferent neurons. In males, central negative feedback by testosterone is mediated mainly by estradiol (4), the product of testosterone aromatization.

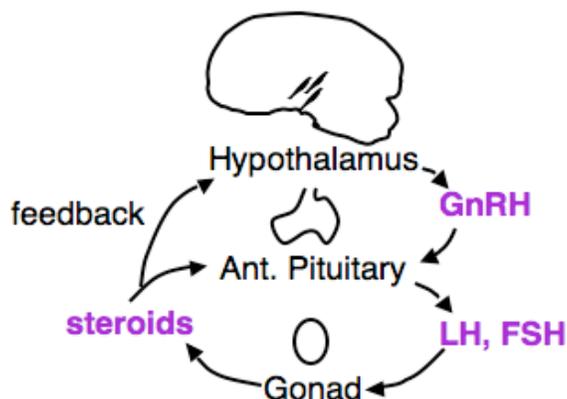


Fig 1-1. The hypothalamic-pituitary-gonadal axis.

In the female, regulation of this system is more complex than in the male given the requirements for ovulation and subsequent pregnancy. Cyclical changes in activity of GnRH neurons (and thereby GnRH release) are critical to produce the changes in levels of LH and FSH required for follicle maturation and ovulation (5). In the early follicular phase of the cycle, GnRH pulses are infrequent, leading to a rise in FSH that stimulates ovarian follicle development. The follicle releases estradiol, which rises throughout the follicular phase. Although most of the time central estradiol feedback is negative, mid-cycle rising estradiol exerts positive central feedback by mechanisms still being elucidated. The resultant increasing GnRH pulse frequency culminates in a surge of GnRH that induces a surge of LH release from the pituitary. The LH surge is the stimulus for ovulation. Following ovulation, the remnants of the follicle become the corpus luteum (CL). In rodents, the CL rapidly degenerates, and there is no true luteal phase. In primates and most other mammals, the CL secretes high levels of progesterone and continued estradiol throughout the luteal phase. Progesterone, and at this time estradiol, exert a negative feedback effect in the hypothalamus to reduce GnRH pulse frequency. In the absence of fertilization, the follicle will degenerate, and the cycle repeats.

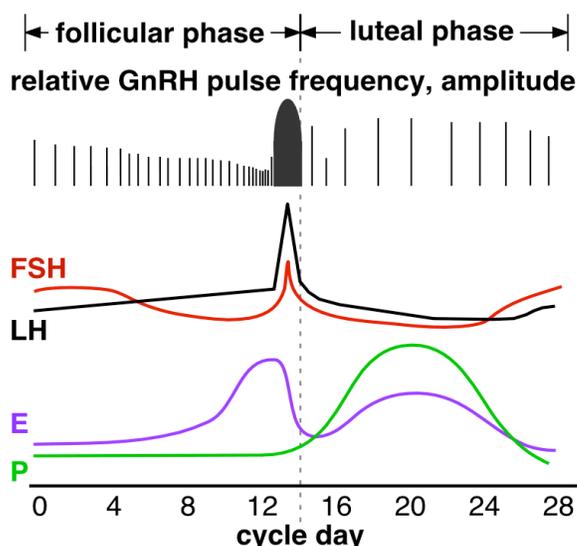


Figure 1-2. Relative hormone levels throughout the human menstrual cycle. E, estradiol. P, progesterone.

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy among women of childbearing age, occurring in 6-8% of women. PCOS is defined by the presence of 2 out of 3 of the following: oligo- or anovulation, clinical or biochemical signs of hyperandrogenemia, and polycystic ovaries diagnosed by ultrasound (6). In this disorder, the exquisite control of the female reproductive cycle is disrupted by an interplay of steroidal and metabolic factors. LH pulses become more frequent, due to increased GnRH pulse frequency (7), and increased in amplitude, due to an exaggerated pituitary response to GnRH (8). Resultant high LH levels increase androgen production by the ovary (9). In women, androgen receptor antagonists have been used to show that elevated androgens interfere with the ability of progesterone to slow GnRH pulses during the luteal phase (10); thus the low frequency of release necessary to stimulate FSH and subsequent follicular development during the early follicular phase is rarely achieved.

In animal models, the combination of dihydrotestosterone (DHT), a non-aromatizable androgen, and estradiol interferes with progesterone's ability to reduce GnRH neuron activity (11). This combination also activates GnRH neurons (11). Since women with PCOS do not ovulate frequently, they seldom have elevated progesterone, whereas androgens are nearly always elevated. Work investigating the neurobiological mechanisms of androgen and progesterone action suggests that they act in part by altering GABAergic transmission to GnRH neurons (12). Unlike most mature neurons, which extrude chloride, GnRH neurons express a chloride accumulating transporter. High intracellular chloride changes the electrochemical driving force so that opening GABA_A receptor-operated chloride channels causes chloride to flow out. This outward movement of negatively charged chloride ions depolarizes the membrane, causing GABA to have an excitatory effect (13, 14). In accordance with an excitatory effect of GABA, progesterone reduces and DHT increases GABA transmission to GnRH neurons (12), consistent with their respective inhibitory and excitatory effects on GnRH neuron activity. In addition, progesterone inhibits whereas DHT potentiates voltage-gated calcium currents in GnRH neurons, suggesting effects of these steroids on the intrinsic properties of GnRH neurons that may be direct or induced by changes in neuromodulation (15).

Another important aspect of PCOS is the prevalence of metabolic abnormalities (16). Thirty-five to forty percent of women with PCOS are insulin resistant and half are obese, with a tendency to accumulate fat centrally. This visceral obesity contributes to insulin resistance due to changes in the secretion of adipocyte-derived hormones and cytokines that impair insulin sensitivity in muscle. Some women with PCOS also exhibit marked insulin resistance despite a normal body weight, suggesting that insulin resistance in

PCOS can be independent of adiposity. Recent work examining intrinsic abnormalities in insulin-resistant women with PCOS demonstrated increased serine phosphorylation of the insulin receptor that interferes with tyrosine autophosphorylation, a critical step in insulin signaling (16). This abnormality may be unique to PCOS. Elevated insulin levels in PCOS further increase serum androgens by activating 17 α -hydroxylase, the androgen synthesizing enzyme in the ovary. Insulin also suppresses levels of sex hormone binding globulin (SHBG), leading to higher levels of unbound testosterone. Insulin may have additional effects in the brain or pituitary; for example, insulin is thought to be one factor that increases the pituitary's LH response to GnRH (17). Women with PCOS have a four-fold increased risk of developing type 2 diabetes mellitus compared to women in the general population. Metformin, an anti-hyperglycemic drug with insulin-sensitizing properties, is a commonly used therapeutic in this disorder. In addition to its metabolic effects, metformin can improve menstrual cyclicity, reduce hyperandrogenemia, and increase the rate of ovulation (18-22). It is thought that insulin sensitization, and a subsequent reduction in insulin levels, is the primary mechanism by which metformin exerts these reproductive system effects. A reduction in insulin levels by metformin can potentially lower both total and free androgen levels, attenuating reproductive abnormalities driven by hyperandrogenemia (16, 18, 22, 23). However, recent studies have shown that metformin can improve PCOS symptoms in normoinsulinemic women (24-27), suggesting it may have reproductive effects that are independent of insulin sensitization. This led us to assess other possible mechanisms of metformin action, presented in Chapter 4 of this dissertation.

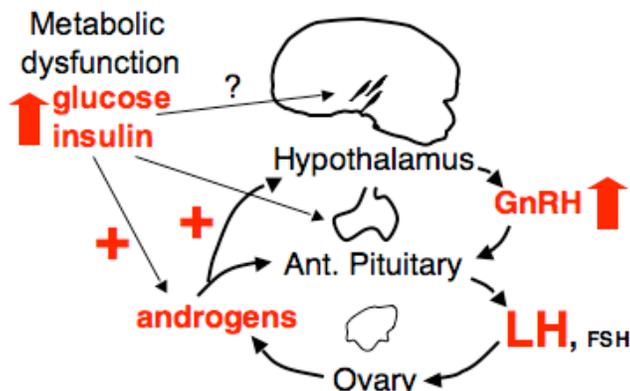


Fig 1-3. Disruption of the hypothalamic-pituitary gonadal axis in PCOS.

Prenatal androgen programming: a potential etiology for PCOS?

A single etiology for PCOS has not been identified, and considering the heterogeneity of clinical presentation, it may not exist. Major areas of investigation include searching for genetic mutations common to large subsets of PCOS patients, and exploring prenatal programming induced by maternal androgen excess. PCOS clusters in families, suggesting a genetic basis for the syndrome. In terms of identifying PCOS genes, research has focused on calpain-10, insulin receptor substrate proteins, sex hormone binding globulin, and fibrilin, among others (28); although found to be PCOS-associated, mutations in each of these genes likely play a role in only a small number of cases. Another possibility is that epigenetic changes caused by fetal programming play a role in the etiology of PCOS.

The first suggestion of a prenatal androgen origin for PCOS came from studies of females with congenital adrenal hyperplasia. These women are deficient in 21-hydroxylase, the cortisol-synthesizing enzyme in the adrenal gland, resulting in shunting of the cortisol precursor (17-hydroxy-progesterone) to androgen synthesis (29). Elevated

androgens are thus present from the time the hypothalamic-pituitary-adrenal axis is activated *in utero*. Despite resolution of adrenal androgen excess by exogenous cortisol administration after birth, some of these women develop elevated basal and GnRH-stimulated LH levels, ovarian hyperandrogenism, polycystic ovaries, reduced fertility, and insulin resistance (30), suggesting that organizational effects of androgens during gestation caused these symptoms. Pregnant women with PCOS have elevated androgens during mid- to late gestation (31). Thus, androgen exposure during a specific period in gestation has been linked to PCOS phenotypes in offspring, and a mechanism for exposure exists in the elevated maternal androgens in women with PCOS. The familial nature of PCOS might therefore be explained by prenatal programming in addition to genetic mechanisms.

Animal studies have explored this hypothesis further. This began with studies in rhesus monkeys examining the behavioral consequences of fetal testosterone excess. Prenatally androgenized female monkeys were found to have many features mimicking human PCOS, and subsequent animal models in sheep and rats were developed based on these observations. Female rhesus monkeys exposed to testosterone propionate *in utero* exhibited a high frequency of anovulation in adulthood, accompanied by large, multifollicular (polycystic) ovaries (32). LH levels tended to be high and FSH low in these monkeys, even in those that were normally cycling. Although body mass index (BMI) and fasting insulin were normal in the treatment group on average, body weight-dependent anovulation and hyperinsulinemia were evident in some prenatally androgenized monkeys, but not in any controls, indicating that weight gain had negative effects on fertility and metabolism only in the androgenized group. The authors postulated that this finding was due to increased visceral adiposity. Likewise, prenatal exposure of ewes to

testosterone propionate resulted in abnormal ovarian morphology, LH and androgen excess, anovulation, and impaired insulin sensitivity. High LH was shown to be at least partly a consequence of increased pituitary responsiveness to GnRH (33). Abnormal metabolic function in androgenized female sheep was exacerbated by weight gain, similar to that in androgenized monkeys. Rat models exhibited similar phenotypes. Prenatal treatment of female rats with either testosterone or DHT prevented the ability of estradiol to induce LH surges, and increased the LH pulse frequency in DHT-exposed rats (34). Testosterone-exposed rats also had increased visceral adiposity and insulin resistance (35).

Our lab developed a prenatal androgenization (PNA) model in mice (36). A mouse model provides several advantages: large numbers of animals can be produced in a short timeframe; lifespan is compressed allowing observation of phenotype changes over time; and genetic manipulability allows for creation of transgenics, such as our green fluorescent protein (GFP)-GnRH mouse, which expresses GFP under the control of the GnRH promoter. This mouse permits GnRH neurons to be directly targeted for electrophysiologic recordings, which is unique to our animal model. Mouse models can be useful for the study of human reproductive disorders, as the rodent estrous cycle is similar to the human menstrual cycle. The main difference is the time course: the cycle in the mouse is shorter to maximize reproductive potential in the short rodent lifespan. The sequence of hormonal changes is the same, and progesterone, estradiol, and testosterone provide feedback to modulate the activity of the GnRH system in parallel ways. However, no animal model can perfectly mimic human physiology, and species differences must be kept in mind when interpreting data from these models.

PNA mice exhibited reproductive neuroendocrine abnormalities similar to PCOS. Estrous cyclicity was disrupted, LH levels were elevated (consistent with high GnRH release or increased responsiveness to this hormone), and serum testosterone was increased. PNA mice had increased afferent GABAergic drive to GnRH neurons, similar to mice with experimentally-elevated DHT as adults. Electrophysiological as well as estrous cycle observations in PNA mice were completely reversed by treating them with flutamide, an androgen receptor antagonist, suggesting that the observed results were due to activational effects of elevated androgens. PNA thus induced organizational effects resulting in elevated androgens, which caused activational changes in the function of the hypothalamic-pituitary-gonadal axis. The original characterization of this model did not examine metabolic phenotypes. My research began with an examination of metabolic characteristics in PNA mice, and the results of these studies are discussed in Chapter 2 of this dissertation. If PNA mice exhibited both reproductive neuroendocrine and metabolic aspects of PCOS, this model would enable us to study the interaction of these factors at the level of the GnRH neuron. As there are vast interactions between metabolic and reproductive systems, these are likely to be of importance to PCOS. The following section will review what is known about metabolic control of fertility at the central level.

Metabolic cues in the central regulation of fertility

A large body of work has examined the role of metabolic cues in reproductive neuroendocrine regulation. Particularly well studied is the central inhibition of reproduction by negative energy balance. When energy stores are low, non-essential processes -- including growth and reproduction -- are shut down to preserve energy for basic survival functions. Numerous studies indicate that low energy availability is sensed

centrally and relayed to GnRH neurons, which ultimately determine reproductive status. Food deprivation suppresses pulsatile LH secretion in rats (37, 38), sheep (39, 40), monkeys (41), and humans (42), consistent with inhibition of pulsatile GnRH release. Multiunit activity recordings of electrical discharges in the mediobasal hypothalamus, where GnRH neurons reside, demonstrated a reduction in the frequency of these discharges during fasting in goats (43, 44). Although progress has been made in elucidating the mechanisms underlying the central suppression of reproduction by energy insufficiency, much remains to be learned about the specific signals that mediate this response and how they are transmitted to GnRH neurons. As with other systems that govern reproduction, multiple redundant pathways and cues likely mediate the interplay between metabolism and GnRH neuron function. These cues include nutrients, hormones, neuropeptides, and neurotransmitters. The current knowledge regarding each class of signal is discussed below.

Nutrients

Changes in levels of circulating nutrients are the most rapid and thus sensitive signal of a change in bodily energy availability. As circulating glucose and fatty acids become depleted, specialized neurons in the brain engage responses aimed at restoring homeostasis, by stimulating gluco-counterregulatory responses and food intake and decreasing energy expenditure. Glucose and fatty acids have also been shown to regulate the reproductive axis by acting centrally to alter GnRH/LH release. Most existing evidence for glucose regulation of GnRH release is derived from studies of experimental glucoprivation induced by fasting or intracerebroventricular (i.c.v.) infusion of insulin or glucose antimetabolites (2-deoxyglucose or 2-thioglucoase). A reduction in central glucose availability suppresses LH levels and pulse frequency (45-47). Multi-unit

activity recordings in the preoptic area of goats treated with 2-deoxyglucose or insulin showed a reduction in the pulses of electrical activity thought to correspond with GnRH release (48). Using steroid-primed ovariectomized rats as a model of the GnRH/LH surge, caudal hindbrain infusion of 2-thioglucose both attenuated the LH surge and reduced c-fos expression in GnRH neurons (49), suggesting an inhibition of GnRH neuronal activity. Restoration of glucose, but not other metabolic substrates, restored LH pulsatility in insulin-induced hypoglycemic rats, suggesting that low glucose rather than high insulin mediated the suppression of LH (50). In addition to the negative effect of reduced glucose, increased glucose can positively influence GnRH/LH secretion. Goats provided with dietary supplementation showed parallel increases in serum glucose levels and LH pulse frequency; pulses declined as food availability was reduced (51). In studies that utilized glucoprivic agents, these agents were infused into either the lateral or 4th ventricle, which are devoid of GnRH neurons, suggesting other neuronal populations relay these signals to GnRH neurons synaptically. Only one study has suggested direct sensing of glucose by GnRH neurons (52). GnRH neuron firing rate was reduced by a switch from high to low glucose in supraphysiological K^+ and high Mg^{2+} /low Ca^{2+} solution; this combination of divalent cations inhibits presynaptic vesicle release. Some evidence also suggests that fatty acids are sensed centrally and regulate reproductive function. Acute lipoprivation by fourth ventricle injection of mercaptoacetate, an inhibitor of fatty acid oxidation, inhibits LH pulse frequency and amplitude in female rats (53).

Peripherally-secreted hormones

The metabolic hormone that has received the most attention for its influence on fertility is leptin. Leptin, the product of the ob gene, is an adipocyte-derived satiety hormone that

circulates at concentrations proportional to body adiposity. Leptin's profound influence on body weight regulation is illustrated by the leptin-deficient ob/ob mouse, which develops severe obesity (54). These mice are also infertile. Along with body weight, fertility can be restored in ob/ob mice by the administration of exogenous leptin. Ob/ob mice that are pair-fed with wildtype controls do not maintain fertility, suggesting it is the absence of leptin rather than excess adiposity that causes infertility. Leptin has been postulated to be a trigger for the onset of puberty (55). Individuals with leptin mutations do not progress through puberty and are hypogonadic and infertile (56). Food restriction delays puberty, but peripheral or central leptin administration at least partially reverses this delay. Leptin administration can also accelerate puberty onset in normal mice (57). In adult animals, leptin is reduced by fasting (58), and its administration reverses the inhibition of estrous cyclicity (59) and LH pulsatility (60) that occurs during fasting. Leptin stimulates GnRH release in hypothalamic explants from rats (61). Although leptin receptors have not been detected on GnRH neurons (60, 62), they are expressed in the arcuate and ventromedial nuclei of the hypothalamus, which extend axons to brain areas containing GnRH neurons (63). Studies in our lab have demonstrated effects of leptin on the activity of GnRH neurons via both pre- and postsynaptic mechanisms. Leptin reverses the decrement in GABA transmission to GnRH neurons induced by fasting, and increases the postsynaptic response of GnRH neurons to GABA (64).

Other metabolic hormones involved in the central regulation of reproduction include insulin, adiponectin, and ghrelin, but their roles are less well characterized. Neuronal insulin receptor knockout (NIRKO) mice exhibit subfertility attributable to reduced central stimulation of LH secretion (65). Body weight is normal in NIRKO males on chow diet (65), suggesting that the loss of neuronal insulin receptors rather than a change in

adiposity is responsible for hypothalamic infertility. A more recent study showed that GnRH neuron-specific deletion of the insulin receptor did not affect fertility; this suggests that insulin may be more important for synaptic regulation of GnRH neurons (66). Insulin withdrawal in diabetic lambs results in a decrease in LH pulse frequency, whereas insulin supplementation increases pulse frequency in an estradiol-potentiated manner (67), consistent with a stimulatory effect of insulin on GnRH pulsatility. Preliminary unpublished data from our lab have failed to find effects of acute insulin application on the activity of GnRH neurons; however, the effect of insulin may be state-dependent and observable only in particular steroid treatment conditions or glucose concentrations, or dependent on synaptic connections abolished by the slice preparation used. Adiponectin is an adipocyte-derived hormone of particular relevance to PCOS as its levels are reduced in women with this disorder, independently of body mass index (68). In contrast to leptin, adiponectin concentrations in blood are typically inversely related to adiposity (69); thus, high levels may indicate insufficient food intake. In accordance with this, female mice with 3-fold overexpression of adiponectin are infertile (70), while fertility is preserved in adiponectin knockout mice (71). Adiponectin reduces GnRH secretion from GT1-7 immortalized GnRH neurons (72), consistent with a possible function as a “starvation” signal to the reproductive system. Ghrelin is a peptide hormone secreted by the gut and elevated during food deprivation. Recent work demonstrated that a five-hour ghrelin infusion decreases LH pulse frequency, but not amplitude, in rhesus monkeys, indicating an effect of ghrelin on GnRH pulse activity (73). Intraperitoneal ghrelin in male rats increased GnRH interpulse interval examined in hypothalamic explants (74). Interestingly, leptin, insulin, adiponectin, and ghrelin have all been shown to have effects on AMP-activated protein kinase, a metabolic regulating enzyme that emerged as an important player in the studies presented in this dissertation (75-77).

Neuropeptides

Many of the neuropeptides involved in the central regulation of food intake and energy expenditure have also been shown to play a role in regulating reproduction. These include anorexigenic peptides such as pro-opiomelanocortin (POMC) and its derivatives, as well as orexigenic peptides such as neuropeptide Y (NPY). Neurons that secrete these peptides are located in the arcuate, ventromedial, and lateral hypothalamus, which project to brain regions containing GnRH neurons (63). These peptidergic neurons are also sensitive to metabolic hormones and nutrient cues described above (78), and may constitute a pathway by which these cues are relayed to GnRH neurons.

The role of NPY in the central regulation of reproduction is well characterized, both morphologically and functionally. Studies using retrograde tracers and electron microscopy indicate that GnRH neurons receive synaptic contacts from NPY axons originating in the arcuate nucleus and brainstem (79, 80). There exist 5 NPY receptor subtypes, Y1-Y5; of these, Y5 colocalizes with GnRH neurons (81), while Y1 appears to be expressed on presynaptic terminals in close proximity to GnRH neurons (82). Numerous studies indicate an inhibitory effect of NPY on GnRH/LH release, consistent with its general role as a starvation signal. NPY hyperpolarizes GnRH neurons via Y5 receptors (83), as well as decreases the frequency of GABAergic PSCs to GnRH neurons (84). NPY appears to have a restraining effect on puberty; puberty can be triggered prematurely by administration of a Y1 antagonist (85), and NPY knockouts proceed through puberty even when nutritionally deprived (86). In some studies, infusion of NPY has been shown to increase rather than decrease GnRH release (87, 88); the direction of its effect appears to be dependent on estrous cycle stage and steroid milieu (89), and possibly location of its application (90). Consistent with potential stimulatory

effects on GnRH release, NPY has been implicated in the generation of the GnRH/LH surge. NPY knockout mice have attenuated LH surges (91). Thus, NPY may serve dual roles in reproductive neuroendocrine regulation, as both a metabolic intermediary and as a regulator of the pre-ovulatory GnRH/LH surge. NPY neurons are inhibited by increased glucose and also responsive to leptin, insulin, and ghrelin (78).

Agouti-related peptide (AgRP), the endogenous antagonist of melanocortin receptors and an orexigenic signal, is co-expressed with NPY and is a marker for NPY-positive axons arising from the arcuate nucleus (80). About half of NPY contacts to GnRH neurons express AgRP (80). EM analysis indicates that symmetric, presumably inhibitory synapses exist between AgRP fibers and GnRH cell bodies (80). Physiologically, central AgRP infusion suppresses pulsatile LH in ovariectomized rhesus monkeys (92), and AgRP increases the interval between GnRH pulses in hypothalamic explants from male rats (74), consistent with an inhibitory role of AgRP on GnRH release. In contrast, other work showed that AgRP increases plasma LH when infused i.c.v., and stimulates GnRH release from hypothalamic explants from male rats (93). The reason for the discrepancy between these studies is unclear. No role has been shown thus far for AgRP in the GnRH surge.

Kisspeptin is a peptide expressed in brain as well as peripheral tissues, and was recently discovered to be critical for maturation and function of the reproductive axis. Humans with mutations in the receptor for kisspeptin fail to progress through puberty and manifest hypogonadotropic hypogonadism. Kisspeptin is one of the strongest stimulators of GnRH release discovered to date. As kisspeptin is indispensable for fertility and is expressed in the arcuate nucleus, it has been postulated as conduit for the transmission of metabolic information to GnRH neurons (94). Fasting reduces the

expression of kiss1, the gene for kisspeptin, in prepubertal and adult rats (95-97) and also reduces expression of its cognate receptor GPR54 (96). Interestingly, the reduction in kisspeptin expression precedes the reduction in hypothalamic GnRH expression induced by fasting. Ob/ob mice, which are infertile, were shown to have reduced kiss-1 expression in the arcuate nucleus; this was restored by leptin administration, suggesting an interaction between leptin and kisspeptin (98). Similarly, NPY knockout mice have reduced kisspeptin expression, and NPY stimulates kisspeptin expression in hypothalamic cell cultures (96). While these findings are of interest, the majority are studies of gene expression, which are limited without correlative functional and physiological observations. As such, the available data have not yet supported a critical role for kisspeptin in the metabolic control of GnRH/LH secretion.

Orexins, also called hypocretins, are involved in the maintenance of wakefulness as well as the arousal that accompanies energy restriction. Orexin neurons in the lateral hypothalamus project to and directly contact GnRH neurons, and orexin receptors are expressed on GnRH neurons (99, 100). One study showed that third ventricle infusion of orexins A and B suppressed pulsatile LH secretion in ovariectomized rats (101). Another study demonstrated site-specific effects; orexin A injected into the rostral preoptic area at the level of organum vasculosum of the lamina terminalis stimulated LH release in estradiol-primed ovariectomized rats, whereas it inhibited the estradiol+progesterone-induced surge when injected into the medial preoptic area or arcuate/median eminence (102). Orexin A stimulated GnRH release in hypothalamic explants from male rats and from females on proestrus (the time of the preovulatory GnRH/LH surge), with no effect during other cycle stages. Infusion of anti-orexin A antibodies abolished the surge in steroid-primed ovx rats (103). In orexin-deficient narcoleptic men, pulsatile LH release

was diminished while pituitary responsiveness to GnRH was maintained, suggesting orexin tone may be necessary to maintain proper GnRH release (104). Like NPY neurons discussed above, orexin neurons are glucose-inhibited.

Similar to orexin, melanin-concentrating hormone (MCH) is an orexigenic peptide expressed in the lateral hypothalamus and upregulated in response to food restriction. MCH fibers were identified to be in close apposition with 90% of GnRH neurons (105, 106), and about half of GnRH neurons were found to express MCHR1 (105). MCH increased GnRH release from isolated median eminence from rats, but only on proestrus (107). MCH and GnRH content of the median eminence showed a corresponding increase on the day of proestrus (108). Together these studies suggest involvement of MCH in regulating preovulatory GnRH and gonadotropin secretion. MCH injected into the preoptic area stimulated LH release in estrogen-primed ovariectomized rats, and evoked GnRH release from hypothalamic explants (109). The stimulatory effect of MCH was mediated by either MCH1-R or MCH5-R, depending on location. A recent study found that MCH inhibits the subpopulation of septal vesicular glutamate transporter 2-expressing GnRH neurons that is sensitive to kisspeptin, and blocked the excitatory effect of kisspeptin on these cells (110). Thus, like other neuropeptides that modulate GnRH neuronal activity, the direction of MCH's effect is controversial, and may be dependent on the steroid milieu.

Cocaine and amphetamine related transcript (CART) is a widely expressed neuropeptide with anorexigenic properties and additional roles in reward, stress, and nociceptive brain pathways. CART neurons arising from the arcuate nucleus, dorsomedial nucleus of the hypothalamus, and ventral premammillary nucleus project to areas with GnRH neurons and to the anteroventral periventricular nucleus (111), which is involved in regulation of

GnRH release (112). Close appositions occur between CART and GnRH neurons (113). In hypothalamic explants from female rats, incubation with anti-CART antiserum reduced GnRH pulse amplitude and prevented the increase in GnRH pulse amplitude caused by leptin (114). In a similar hypothalamic explant model from prepubertal rats, CART independently accelerated GnRH pulse frequency, and anti-CART antiserum prevented the stimulatory effects of leptin, similar to the above study (115). These studies indicate a stimulatory role of CART on GnRH release, consistent with its role as a “fed” signal. Further, the expression of CART in the AVPV suggests it may be important for regulation of the GnRH/LH surge. However, any role in this regard is not likely to be critical, as genetic CART deletion has no apparent impact on fertility (116).

Pro-opiomelanocortin (POMC) is a precursor polypeptide that is cleaved to form many additional biologically active peptides. POMC is coexpressed with CART in the arcuate nucleus. Knockout mice have demonstrated that POMC is critical for body weight regulation, and neurons that express POMC are sensitive to glucose, leptin, and insulin (78, 117). In the hypothalamus, cleavage products include alpha-MSH and beta-endorphin. Alpha-MSH binds to melanocortin receptors, whereas beta-endorphin is a ligand for opioid receptors. Axons immunoreactive for products of the POMC gene originate in the arcuate nucleus and form a network in the preoptic area (118), forming close appositions with GnRH neurons (106). Synaptic connections have been demonstrated immunohistochemically between POMC neurons and GnRH neurons in the preoptic area (63). Interestingly, GnRH fibers contact POMC perikarya in the arcuate nucleus (119), suggesting a potential reciprocal interaction between these systems.

Alpha-MSH has steroid-dependent effects on the reproductive axis. Intravenous administration of alpha-MSH stimulates gonadotropin release in men, and in women

during the luteal phase. It also stimulates gonadotropin release in women with PCOS, suggesting that either androgen or progesterone can convey sensitivity to alpha-MSH (120). A more recent study showed that the stimulatory effect of melanocortins during the luteal phase may be dependent on activation of kisspeptin and/or orexin neurons (121). Third-ventricle injection of alpha-MSH reduced plasma LH in ovariectomized rats, but only in the absence of estradiol (122).

GnRH neurons express mu-opioid receptor mRNA (123), and EM analysis demonstrated symmetrical (i.e., inhibitory) synapses between opioid-positive axons and GnRH somata and axons (124). Interestingly, a sexual dimorphism in beta-endorphin innervation of GnRH neurons has been observed (125). Beta-endorphin, in addition to other endogenous opiates, exerts an inhibitory effect on GnRH release (126). It may also mediate the suppressive effects of other metabolic cues, including ghrelin (127) and orexin (128). Met-enkephalin, a nonspecific opiate agonist, inhibited GABAergic postsynaptic currents to GnRH neurons. Mu-opioid receptor antagonism attenuated the suppression of LH by hindbrain 2-deoxyglucose infusion (49, 129). In contrast, naloxone failed to reverse the suppression of LH by insulin-induced hypoglycemia in female macaques (130). This could reflect the site of glucoprivation. Opioids may be specific for hindbrain effects, whereas systemic hypoglycemia might affect a larger area of the brain.

Corticotropin-releasing factor (CRF) is the central regulator of the stress axis. Food insufficiency is a strong physiological stressor that stimulates the central release of CRF, which inhibits reproduction. Preliminary work by our lab has shown that CRF inhibits GnRH neuron activity. CRF may be important for glucoprivic suppression of GnRH/LH pulses. Insulin-induced hypoglycemia inhibits LH while concomitantly activating CRF-expressing neurons (131). In female rats, a CRF antagonist attenuated glucoprivic

suppression of LH secretion, but only in the presence of estrogen, suggesting that there are both estrogen-dependent and -independent mechanisms of glucoprivic LH suppression (132). In monkeys it was shown that CRF, but not endogenous opiates, are required for the suppression of LH by insulin-induced hypoglycemia (133).

The divergent response to many of the described neuropeptides during different estrous cycle stages, with a switch to stimulatory on proestrus at the time of the GnRH surge, suggests that these neuropeptides do more than just signal undernutrition. It is interesting that their effects switch from negative to positive, similar to the effect of estradiol, to possibly assist in surge generation. Thus the relevance of these metabolic cues may extend beyond nutritional infertility, as they appear to govern the central control of ovulation under non-pathological (fed) conditions. This may be of importance to PCOS, given that anovulation is a principal characteristic of this disorder.

Neurotransmitters

GABA is thought to be particularly important for the central regulation of fertility, as GnRH neurons receive significant GABAergic neurotransmission, indicated by histological studies and electrophysiological recordings of GABA postsynaptic currents. GABA is also thought to integrate and deliver metabolic cues to GnRH neurons, including cues from NPY, opiates, and leptin (84). Hindbrain glucoprivation increases GABA release in areas proximal to GnRH neurons (134), and the GABA_A receptor antagonist bicuculline prevents blockade of the LH surge by glucoprivation (135). There is also some evidence that serotonin may play a role in the integration of metabolism and fertility. Administration of fluoxetine, which acts throughout the brain to increase serotonin levels in the synaptic cleft, restored estrous cyclicity to fasted mice (59).

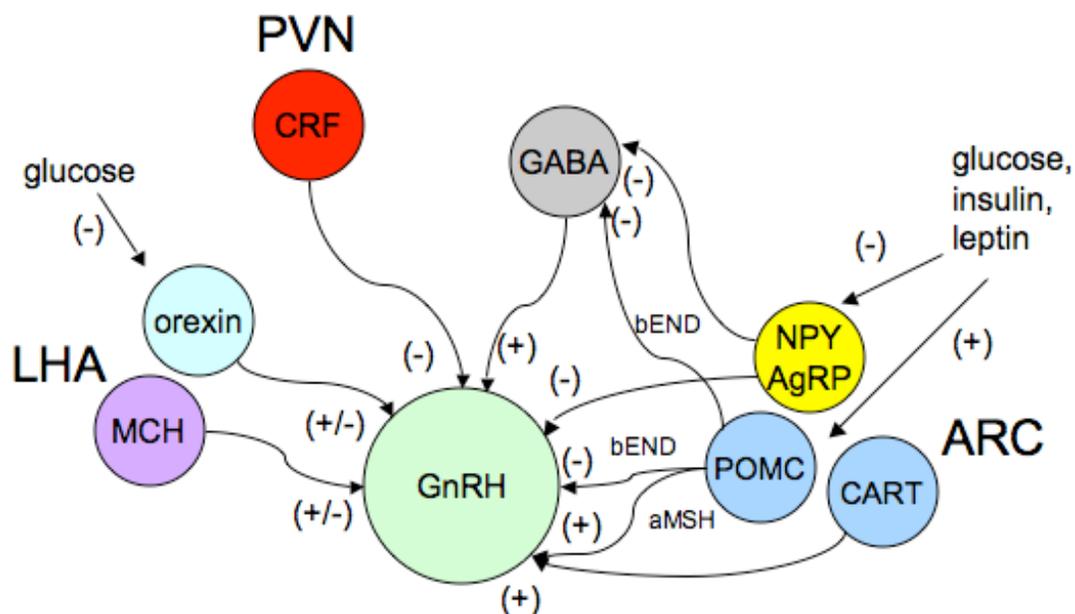


Figure 1-4. Metabolic inputs to GnRH neurons. LHA, lateral hypothalamic area. PVN, paraventricular nucleus. ARC, arcuate nucleus.

Among the multitude of metabolic cues likely to be altered in PCOS, glucose is of particular interest as many previous studies have suggested its involvement in reproductive function, and extensive anatomical connections have been demonstrated between glucosensing areas of the brain and GnRH neurons. Elevated blood glucose in PCOS may stimulate GnRH neurons, contributing to excessive GnRH release in this disorder. Although existing studies have provided valuable evidence for the regulation of reproductive neuroendocrine function by glucose, only one examined the activity of GnRH neurons directly, and none assessed the underlying mechanisms associated with activity changes, or the effect of steroids. We addressed these questions by directly recording the activity of GFP-GnRH neurons in response to changing glucose concentrations in defined animal models treated with physiological levels of steroids. The results of these studies are presented in Chapter 4. In order to provide context to

these studies, the following section will address known cellular mechanisms of glucosensing.

Neuronal glucosensing

The high metabolic requirements of the brain require a constant supply of glucose. Glucose enters the brain via facilitated transport through GLUT1 transporters in the blood brain barrier (BBB), reaching an extracellular concentration of approximately 10-30 percent of the blood level. Specialized neurons in the brain respond to changes in ambient glucose concentration by changing their action potential firing rate, modulating glucose counterregulatory responses, energy expenditure, and feeding behavior. These neurons are subdivided into two classes: glucose-inhibited, such as orexin neurons, which hyperpolarize when the extracellular glucose concentration is increased, and glucose-excited, such as POMC neurons, which depolarize when glucose is increased. Recent studies have suggested that one mechanism of glucose action in glucose-inhibited orexin neurons is to enhance the activity of tandem pore potassium channels (136). However, these findings were disputed by the observation that glucose inhibition of excitability was intact in mice with genetic deletion of various two-pore K⁺ channels (TASK, TREK, and TRAAK) (137). Alternatively, glucose-inhibited neurons of the VMH have been shown to sense low glucose by activation of AMPK, which phosphorylates cystic fibrosis transmembrane regulator (CFTR) chloride channels, reducing chloride current (138). This process was shown to be amplified by AMPK-dependent generation of nitric oxide (NO). NO activates soluble guanylyl cyclase, which increases phosphorylation and thus activation of AMPK.

Glucose-excited neurons sense glucose via ATP-sensitive potassium (K_{ATP}) channels, at least in part. This was demonstrated in mice with deletion of the pore-forming subunit of

the channel, which resulted in an absence of functional glucose-excited neurons in the VMH and impaired counterregulatory and feeding responses to glucoprivation (139). Originally established as a glucosensor in the pancreatic beta cell, the K_{ATP} channel is comprised of four Kir6.2 pore-forming subunits and four sulfonylurea (SUR1) subunits. The latter are sensitive to sulfonylureas such as tolbutamide, which close K_{ATP} channels. In both beta cells and neurons, rising extracellular glucose enters the cells through GLUT transporters and generates ATP through glycolysis. The rate-limiting step in this process is hexokinase conversion of glucose to glucose-6-phosphate; beta cells and some neurons express a form of this enzyme called glucokinase. ATP generated through glycolysis blocks membrane K_{ATP} channels, resulting in decreased potassium efflux and membrane depolarization.

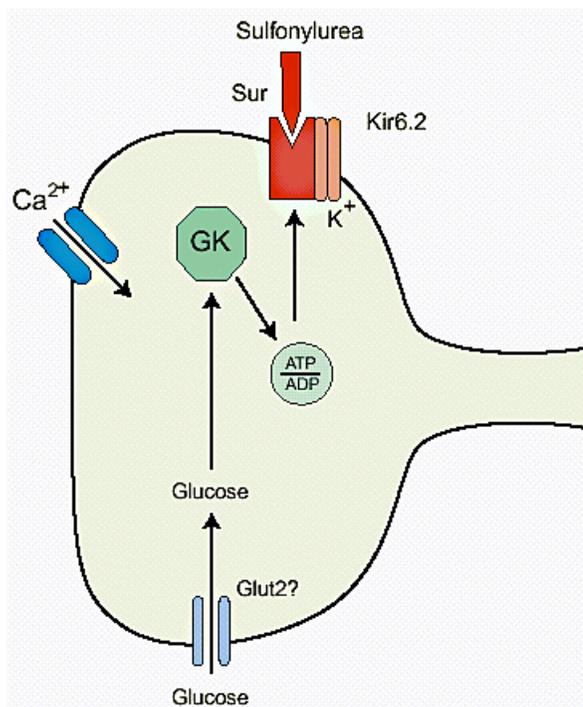


Fig 1-5. Proposed model of glucosensing by glucose-excited neurons. GK, glucokinase. Adapted from reference (139).

GnRH neurons express K_{ATP} channels that appear to be estradiol-modulated, indicating that they have at least one of the requirements for direct glucosensing. A recent study of mice heterozygous for an ablated glucokinase gene, in which neuronal glucosensing

was impaired despite functional pancreatic glucosensing, found reduced fertility in these mice. This suggests that glucokinase-dependent glucosensing in the brain is required for maintenance of reproduction; as the central component of the reproductive system, GnRH neurons are likely to be involved, either directly or via alterations in their afferents.

One possible limitation to the hypothesis that GnRH neurons are overstimulated by the high glucose of diabetes and PCOS is that some studies have suggested CSF glucose transport is reduced in chronic hyperglycemia, although other studies have found no change or an increase in brain glucose. Despite this possibility, GnRH neurons and their terminals are located close to or within circumventricular organs. Both the arcuate and ventromedial nuclei, from which GnRH neurons receive synaptic contacts, are adjacent to the median eminence. The blood-brain barrier is deficient in this area, where highly permeable fenestrated capillaries permit glucose entry at higher concentrations than in CSF. The third ventricle is also lined by ependymal glia and tanycyte cells that are thought to play an accessory role in transmitting glucose signals. Thus, regardless of the level of BBB glucose transport, GnRH neurons either directly or through synaptic connections are likely exposed to high glucose approaching blood levels in hyperglycemic disorders. Furthermore, hyperglycemia in PCOS is not necessarily chronic; some women have abnormally elevated glucose levels in response to a glucose challenge, but normal fasting levels. The effects of repeated transient exposures to hyperglycemia on glucose transport in the brain do not appear to have been investigated.

It is important to note that other mechanisms for glucose-responsiveness have been identified in addition to the classical K_{ATP} -dependent model. One mechanism is electrogenic glucose entry, whereby glucose transport into cells is coupled to ion

movement. An example is the sodium-glucose cotransporter, which concurrently transports sodium and glucose into cells, generating depolarizing inward currents. A second alternative is glucose receptors on the surface of cells that do not require glucose entry for excitation. An example is the sodium-glucose transporter SGLT3. SGLT3 activation by glucose leads to sodium-dependent membrane depolarization. Lastly, as alluded to above, glial cells, which perform trophic functions for neurons, have also been implicated in glucosensing. Glycolysis in astrocytes increases when extracellular glucose rises and may be conveyed to neurons by the release of lactate, which can be converted to ATP intracellularly or may directly close K_{ATP} channels. Interestingly, there is some evidence for glial modulation of GnRH neuronal function.

Interaction between steroids and metabolism

We also examined a possible interaction between androgens and glucosensing, as androgens are elevated in women with PCOS. One possibility was that androgens might enhance the response to glucose, exacerbating the effect of an elevation. A second possibility was that androgens might impair glucosensing, as several lines of evidence support this hypothesis. Male and female reproductive systems respond differently to metabolic stimuli; female fertility is particularly susceptible to metabolic fluctuations. Caloric restriction leads to a cessation of reproductive cycles in the female, whereas males remain fertile even under severe (40%) food deprivation (140). Similarly, female mice and rats on high fat diets have irregular estrous cycles and reduced fecundity, while males maintain their ability to sire pups despite similar diet-induced metabolic changes (140). These studies indicate that both nutritional deprivation and excess have sexually differentiated effects on reproduction, suggesting that GnRH neuron responses to metabolic cues may be enhanced by female-predominant steroids (estrogens) or

downregulated by male-predominant steroids (androgens). Although glucoprivic suppression of LH pulses is potentiated by gonadal steroids in both sexes, the effect of testosterone in males may be mediated by estradiol. Overall, the reproductive axis of female rats is more sensitive to decreased glucose availability than that of males (47).

The following chapters present work in three major areas to address metabolic and steroid interactions in the control of GnRH neurons. Chapter 2 focuses on metabolic characterization of the PNA model. Chapter 3 examines glucosensing in GnRH neurons, revealing a possible role for AMPK. Chapter 4 integrates the findings of the two preceding chapters by assessing neuronal AMPK activation in PNA mice treated with metformin, a commonly administered therapeutic for PCOS. Finally, Chapter 5 will discuss these findings and their implications and propose ideas for future work.

Chapter 2

Prenatal androgen exposure programs metabolic dysfunction in female mice

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Published in the *Journal of Endocrinology*
August 2010

Abstract

Polycystic ovary syndrome (PCOS) is a common fertility disorder with metabolic sequelae. Our lab previously characterized reproductive phenotypes in a prenatally androgenized (PNA) mouse model for PCOS. PNA mice exhibited elevated testosterone and luteinising hormone (LH) levels, irregular oestrous cycles, and neuroendocrine abnormalities suggesting increased central drive to the reproductive system. In this study we examined metabolic characteristics of female PNA mice. PNA mice exhibited increased fasting glucose and impaired glucose tolerance (IGT) that were independent of age and were not associated with changes in body composition or peripheral insulin sensitivity. IGT was associated with defects in pancreatic islet function leading to an impaired response to high glucose, consistent with impaired insulin secretion. Exposure of isolated pancreatic islets to androgen *in vitro* demonstrated an impaired response to glucose stimulation similar to that in PNA mice, suggesting androgens may have activational in addition to organizational effects on pancreatic islet function. PNA mice also exhibited increased size of visceral adipocytes, suggesting androgens programmed differences in adipocyte differentiation and/or function. These studies demonstrate that in addition to causing reproductive axis abnormalities, *in utero* androgen exposure can induce long-term metabolic alterations in female mice.

Introduction

Developmental programming by steroid hormones is important to establish sex differences in the reproductive tract and in other physiological systems. Androgen levels surge during gestation and postnatally in the male (141, 142), while in females androgens typically remain low during embryonic development, except in rare instances of pathologic exposure from intrinsic (fetal) or extrinsic (maternal or environmental) sources. Recent work on endocrine disruptors has demonstrated the existence of environmental substances with androgenic actions, such as 17- β -trenbolone and triclocarban, which are a potential cause of abnormal fetal androgenisation (143-145). Because hormonal perturbations during this critical time may have adverse effects that persist into adulthood, it is important to study the consequences of androgen exposure *in utero*.

Polycystic ovary syndrome (PCOS), the most common cause of infertility in women, is one disorder that may originate in prenatal androgen excess. PCOS is characterized by hyperandrogenemia, elevated central drive to the reproductive system, and irregular or absent menstrual cycles due to oligo- or anovulation (146). PCOS also predisposes women to metabolic dysfunction characterized by impaired glucose homeostasis and abdominal adiposity (147). Animal models have exhibited reproductive and metabolic abnormalities similar to PCOS following prenatal androgenisation (32, 35, 148). The finding that prenatal androgen can lead to defects in both reproduction and metabolism suggests it may play a major role in the etiology of at least some cases of PCOS. Consistent with this idea, women with PCOS have elevated circulating androgens during late gestation (31), potentially exposing their offspring, who are at increased risk for

PCOS (149). Alternatively, it has been proposed that the fetal ovary itself is the source of androgen due to abnormalities in genes controlling steroidogenesis (150).

To mimic the gestational androgen excess associated with PCOS and other inappropriate androgen exposures, our lab developed a mouse model treated with dihydrotestosterone (DHT) late in gestation. Previously, we described reproductive neuroendocrine abnormalities in this model (36), including elevated androgen and LH levels, irregular oestrous cycles, and increased excitatory neurotransmission to GnRH neurons. Here we investigate metabolic phenotypes in PNA mice.

Materials and Methods

Generation of PNA mice

Adult (2-4 mo) female GnRH-GFP (descended from CBB6/F1 founder, currently ~75% C57Bl/6J by speed congenics) transgenic mice were used to generate PNA mice. Mice were housed under a 14 h light:10 h dark cycle with chow (2916, Harlan, Indianapolis, IN) and water available *ad libitum*. Females were paired with males and checked for copulatory plugs. The date of plug was considered day 1 of gestation. Pregnant mice were injected daily subcutaneously with 50 μ l sesame oil containing 250 μ g of dihydrotestosterone (DHT) on day 16-18 of gestation. DHT was used to eliminate the possibility of aromatisation to oestradiol, thereby permitting the study of primarily androgen receptor-mediated effects. Of note, DHT can be metabolized to 5 α -androstane-3 β ,17 β -diol, which can bind ER β (151); however, the levels of this metabolite attained in the fetal compartment are unknown. Female offspring were subjected to glucose tolerance tests beginning at one month of age; all other studies were performed at 3-6 months. Control mice (CON) were offspring of either oil-injected

dams or untreated mice; no differences were observed between these groups, and they were combined for analysis. Three rounds of PNA mice were generated for use in this study: 9 CON and 7 PNA mice for repeated glucose tolerance testing; 22 CON and 21 PNA mice for DEXA, adipocyte studies, islet studies, and hormone measurements; and 10 CON and 10 PNA mice for insulin tolerance testing. For *in vitro* examination of steroid effects on pancreatic islets, C57BL/6J female mice 8-12 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). These mice were ovariectomized (OVX) three days before islet isolation under isoflurane anesthesia (Burns Veterinary Supply, Westbury, NY). Long-acting postoperative local analgesia was provided by 0.25% bupivacaine (Abbott Laboratories, North Chicago, IL). All procedures were approved by the University of Virginia Animal Care and Use Committee and conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Glucose tolerance tests (GTT)

Mice were singly housed on Sani-Chip bedding (Harlan, Indianapolis, IN) and fasted overnight for sixteen hours (1600h to 0800h) prior to the test. The tail was anesthetized with the skin refrigerant ethyl chloride (Gebauer, Cleveland, OH) and the tip removed with a sterile scalpel blade. Tail blood (~1 μ l/ sample) was collected for glucose measurement with a OneTouch Ultra glucometer (Lifescan, Milpitas, CA). Following a fasting glucose measurement, mice were injected intraperitoneally with a bolus of 1g/kg glucose in 0.9% NaCl. Blood glucose was assessed at 10, 20, 30, 45, 60, 75, 90, and 120 minutes post injection. Glucose tolerance tests were performed monthly from 1 to 6 months.

Insulin tolerance tests (ITT)

Studies were performed 10 hours after lights-on in singly-housed fed mice. Although mice had free access to food prior to testing, their active (feeding) period normally ends at the time of lights-on. Following an initial glucose measurement, mice were injected intraperitoneally with a bolus of 0.75 U/kg of insulin in sterile 0.9% NaCl. Blood glucose was determined at 10, 20, 30, 45, 60, and 75 minutes post injection as described above.

Fasting insulin measurements

Fasting insulin was measured following an overnight fast (1600h to 0800h). Five- to 6-month-old mice were restrained, and ~50 μ l blood was collected from the tail vein using a heparinized capillary tube. Plasma insulin was determined by radioimmunoassay (Millipore, cat# SRI-13K). All samples were determined in a single assay with a sensitivity of 0.1 ng/mL and intra-assay coefficient of variation (CV) of <10%.

Dual Energy X-ray Absorptiometry (DEXA)

Mice were anesthetized by intraperitoneal injection of 100-150 μ l of a ketamine/xylazine mix in saline (ketamine at 20 mg/ml and xylazine at 2 mg/ml). Anesthetized mice were introduced into the DEXA machine (GE Lunar Piximus II) and subjected to total body imaging. Lean body mass and fat mass were determined using the Lunar Piximus II software and percent fat mass was calculated. Abdominal fat percentage was calculated by selecting a region of interest (ROI) in the scanned image. Mice were scanned at age 3, 4, and 5 months.

Measurement of adipocyte size

Mice were euthanized with CO₂. The left parametrial fat pad and adjacent uterus were fixed in 10% formalin for 48 hours and embedded in paraffin. Sections (5 μ m) were cut

and stained with hematoxylin-eosin. Pictures of stained sections were taken at 10x magnification with a Zeiss Axioplan Universal Microscope (Thornwood, NY); the uterus was used for photographic orientation and the adipocytes sized were in the same frame as the uterus to minimize bias due to regional differences. Cell areas (in μm^2) of 50 adipocytes were determined for each mouse using Scion Image Corporation software (Frederick, MD). Only 40 cells were used for one animal in which tissue damage precluded analysis of 50 cells.

Glucose uptake assays in adipocytes

The right parametrial adipose tissues were dissected, and adipocytes were isolated and subjected to glucose uptake assays as previously described in detail (152). In brief, isolated adipocytes were pre-incubated without (basal) or with different concentrations of insulin (0-10 nM) for 30 min. [U-14C] D-glucose was then added. Thirty min after the addition of radiolabeled glucose, the cell suspension was harvested and adipocytes separated from the medium. Cell-associated radioactivity was determined and glucose uptake expressed in amol/min/cell. Cell numbers were determined by measuring lipid content of aliquots of cell suspension and measuring sizes of the adipocytes in aliquots of the cell suspension as described above. For each condition, the measurements were done in quadruplicate.

Islet isolation

Islets were harvested from 5-month-old PNA and CON mice on dioestrus (determined by vaginal lavage). Although non-cycling animals likely have a very different steroid milieu despite similar vaginal cytology, this is a consequence of the considerable reproductive disruption of the model, and dioestrous vs. long-term dioestrous animals is the most practical comparison to make. Mice were euthanized with CO_2 and cardiac puncture was

performed for blood collection. The pancreas was dissected and islets were isolated by collagenase digestion and Histopaque centrifugation using previously detailed methods (153). Isolated islets were transferred to a Petri dish containing standard RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and penicillin/streptomycin. Islets were incubated in this medium overnight to allow recovery from digestion prior to experiments.

Intracellular calcium imaging of islets

The day following isolation, imaging recordings of intracellular calcium ($[Ca^{2+}]_i$) were made from groups of 10-20 islets from a single mouse approximately 18-26 hours post-isolation; no differences were noted based on time of recording. $[Ca^{2+}]_i$ was measured using the ratiometric dye fura-2 AM and previously described methods (154). Briefly, islets were incubated with 1 μ M fura-2 AM for 30 minutes in a modified KRB solution containing 3 mM glucose, washed, then transferred to a small volume chamber (Warner Instruments, Hamden, CT) mounted on the stage of an upright Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan). Islets were perfused using a peristaltic pump (Minipuls 2, Gilson, France) and maintained at 35 C with an in-line heater (model SF-28 with automatic temperature controller model TC-324B, Warner Instruments, Hamden, CT). A Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan) was used to take sequential images during 340 and 380 nm excitation, and the ratio of emitted light at 510 nm used to determine the $[Ca^{2+}]_i$. Excitation from a xenon burner was accomplished using a light pipe and filter wheel (Sutter Instrument Company, Novato, CA). Paired images were recorded every 5 seconds for 15 minutes. After 5 minutes in 3 mM glucose, 11 mM glucose was applied for 10 minutes, during which fluorescence levels were recorded continuously.

Insulin release in vitro

A subset of islets isolated from PNA and CON mice were used for studies of *in vitro* insulin release. Following the overnight culture, islets were incubated at 37 C and 5% CO₂ for one hour in standard KRB solution. Islets were then washed and treated for one hour in KRB supplemented with 3 mM glucose, followed by one hour in KRB containing 11 mM glucose. Supernatants were collected after each treatment. Insulin concentration in the supernatant was measured by an ELISA insulin assay kit (Merckodia Inc., Winston Salem, NC) according to manufacturer instructions. This assay differed from that used for mouse serum because insulin levels in media differ from those *in vivo*. Intra-assay variation was <10% and inter-assay variation was <5%. *In vitro* insulin release was also assessed in islets isolated from 3-month-old female C57BL/6J mice three days post ovariectomy. Islets were harvested and cultured as described above. During the overnight culture, 50 islets per mouse were incubated in DHT, DHT+oestradiol (DHT+OE), or ethanol vehicle (0.0001%). All steroids were used at a final concentration of 10 nM. The following day, islets from each steroid treatment group were incubated in standard KRB solution with steroids omitted to preclude acute effects. Insulin release in 3 and 11 mM glucose was quantified by ELISA as above.

Endocrine measures

Testosterone was measured in serum using a radioimmunoassay kit according to the manufacturer's instructions (Siemens Medical Solutions, cat# TKTT2, Los Angeles, CA). Sensitivity averaged 10 ng/dl, and the intra- and inter-assay CVs were 4.4 and 8.1%, respectively. An adipokine panel was used to assess insulin, leptin, IL-6, TNF- α , PAI-1, and resistin (mouse serum adipokine kit, Millipore, cat# MADPK-71K-07). Sensitivity was 12 pg/mL for insulin, leptin, PAI-1, and resistin, 2 pg/mL for TNF- α , and 5 pg/mL for IL-6.

Intra- and inter-assay CV were <10% for all analytes. Adiponectin was measured via radioimmunoassay (Millipore, cat# MADP-60K); assay sensitivity was 1.3 ng/ml and inter- and intra-assay CVs were <9% and <5%, respectively.

Oestrous cycle monitoring

Oestrous cycles were monitored by vaginal lavage. Cycle stage was classified as oestrus (primarily cornified cells), dioestrus (primarily leukocytes), or prooestrus (primarily nucleated cells).

Analyses and statistics

GraphPad Prism software (La Jolla, CA) was used for all analyses unless otherwise indicated. For glucose and insulin tolerance tests, glucose values from PNA and CON mice were compared at each time point using a two-tailed Student's t-test. Area under the curve (AUC) for the GTT was calculated using Igor Pro software (Wavemetrics, Lake Oswego, OR); AUC across age was compared in PNA vs CON mice using a repeated-measures analysis of variance (ANOVA) and Fisher's protected LSD post-hoc test. Two-tailed Student's t-test or Mann-Whitney test was used to compare body mass and fat pad mass, fat percentages, fat cell sizes, adipokines, HOMA indices, and glucose uptake in adipocytes. For islet calcium imaging studies, calcium measurements from 10-20 islets per mouse were averaged, and AUC was calculated using Igor Pro. Insulin secretion in 3 mM and 11 mM glucose from control, DHT, and DHT+OE groups were compared by two-way ANOVA. For all statistical tests, significance was set at $p < 0.05$. Parametric or non-parametric comparisons were used as dictated by data distribution.

Results

PNA mice do not have altered body composition but do have enlarged visceral adipocytes

Because adiposity contributes to metabolic disease, we assessed body mass and composition in PNA (n=8) and CON (n=9) mice. Body mass at age 3, 4, and 5 months did not differ between groups (all comparisons $p>0.8$); representative data from age 5 months are shown (Fig 1A). Body fat percentages as measured by DEXA also showed no difference in total body fat or abdominal fat at these ages ($p>0.4$, Fig 1B). Since DEXA cannot differentiate visceral and subcutaneous fat compartments, we could not exclude the possibility that PNA mice have changes in fat distribution. To better assess visceral adiposity, parametrial fat pads were weighed in a subsequent group of PNA mice and further analyzed for adipocyte size. Fat pad weights were similar (n=8 CON, n=11 PNA, $p>0.5$, Fig 2A), indicating no increase in visceral adiposity in PNA mice, consistent with the DEXA measurements. However, PNA mice had larger visceral adipocytes ($2592\pm150\ \mu\text{m}^2$ CON, n=10, $3230\pm211\ \mu\text{m}^2$ PNA, n=12, $p<0.05$, Fig 2B-D), indicating that despite similar total amounts of parametrial fat in these mice, prenatal androgenisation induced changes in adipocyte differentiation and/or function leading to larger fat cells. Adipokine assays showed no differences between PNA and CON mice in the fed or fasted state, except for a strong trend (two-tailed $p=0.08$) for reduced adiponectin in fed PNA mice (Table 1). Fasting decreased insulin, leptin, PAI-1, IL-6, and resistin in CON mice ($p<0.05$). In PNA mice, insulin was reduced and IL-6 and resistin showed a tendency ($p<0.06$) to be lower in fasted animals. Of note, however, leptin was not reduced by fasting in PNA mice. Glucose uptake assays in isolated adipocytes showed no change in insulin sensitivity or maximal insulin-stimulated glucose uptake,

but higher basal glucose uptake in PNA adipocytes (basal: 4.0 ± 0.5 amol/glc/min/cell CON, 6.4 ± 1.0 PNA, $n=5$ each, $p < 0.05$; at 0.1 nM insulin: 17.4 ± 2.4 CON, 20.6 ± 3.3 PNA, $n=5$ each, $p > 0.4$; at 1 nM insulin: 27.1 ± 3.3 CON, $n=5$, 26.9 ± 5.7 PNA, $n=4$, $p > 0.9$; Fig 2E).

Analyte	CON fed	PNA fed	CON fasted	PNA fasted
insulin (pg/ml)	1071 ± 178^a	1331 ± 306^a	146 ± 22.5	107 ± 16.5
leptin (pg/ml)	2476 ± 350^a	1849 ± 435	926 ± 192	1468 ± 366
TNF- α (pg/ml)	4.7 ± 0.4	5.3 ± 0.5	6.4 ± 2.0	4.1 ± 0.5
PAI-1 (pg/ml)	8361 ± 1049^a	6858 ± 1377	3635 ± 635	4246 ± 664
IL-6 (pg/ml)	17.7 ± 2.2^a	24.6 ± 5.4^b	9.2 ± 1.7	5.3 ± 0.8
resistin (pg/ml)	1758 ± 160^a	1497 ± 144^b	891 ± 95	1073 ± 159
adiponectin (ng/ml)	16.2 ± 0.7^c	14.0 ± 1.0	--	--

Table 1. Adipokine levels in plasma from control (CON) and prenatally androgenized (PNA) mice under fed and fasted conditions ($n=10-16$ per group). ^a $p < 0.05$ vs fasted. ^b $p < 0.06$ vs fasted. ^c $p = 0.08$ vs PNA.

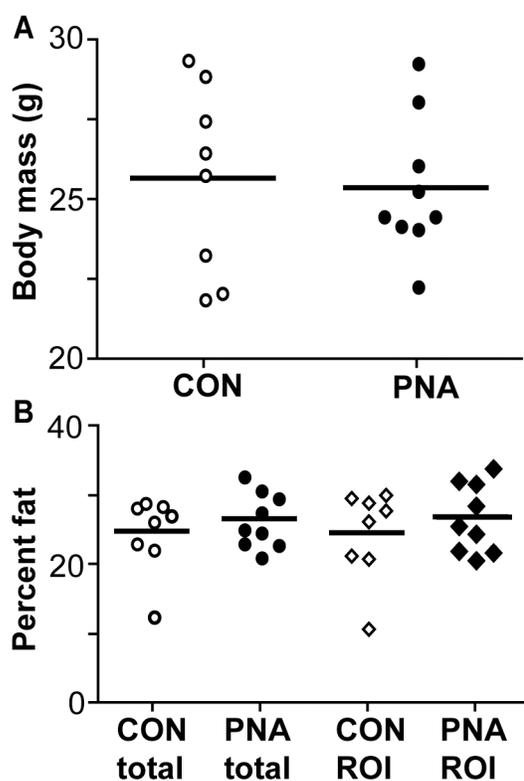


Figure 1. PNA does not alter body mass or composition in PNA mice at 5 months of age. A. Body mass in CON (open circles, $n=8$) and PNA mice (closed circles, $n=9$) ($p > 0.8$). B. Total body fat or abdominal fat (region of interest, ROI, subcutaneous and visceral fat combined) in CON and PNA ($p > 0.4$).

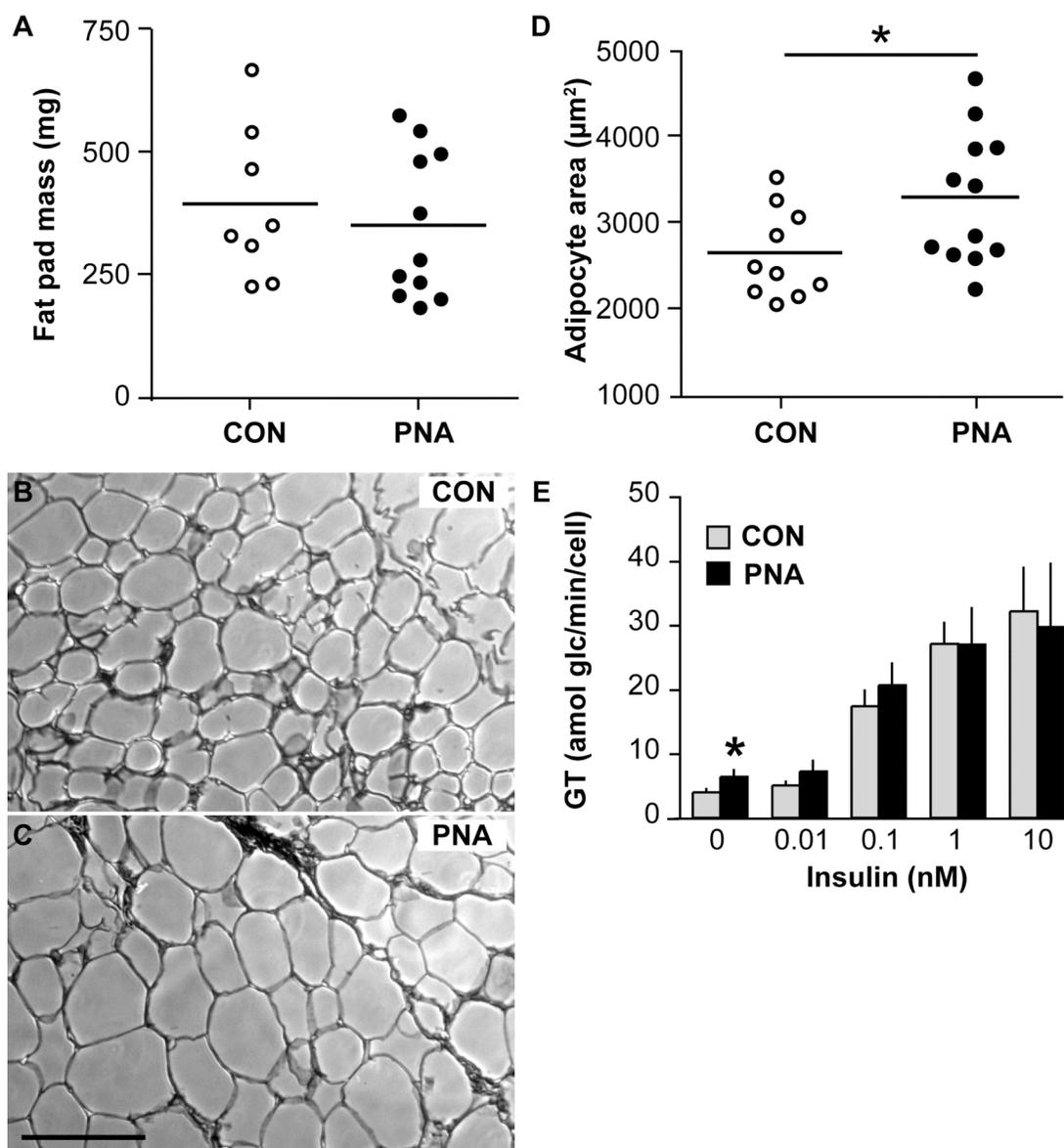


Figure 2. PNA does not alter fat pad mass but increases adipocyte size. A. Fat pad mass in CON (open circles) and PNA mice (closed circles) ($n=8$ CON, $n=11$ PNA, $p>0.5$). B, C. Representative photomicrographs of adipose tissue from control (B) and PNA (C) mice. Scale bar represents $200\ \mu\text{M}$. D. Mean adipocyte size ($n=10$ CON, $n=12$ PNA). E. Mean \pm SEM glucose uptake (GU) into CON (gray bars) and PNA (black bars) adipocytes in response to varying concentrations of insulin ($n=2$ to 5 assays per insulin concentration). Basal uptake was higher in PNA adipocytes, but insulin sensitivity was similar. * $p<0.05$.

PNA mice exhibit impaired glucose tolerance

To test if prenatal androgenisation alters glucose disposal in PNA mice, glucose tolerance tests were performed. PNA mice exhibited impaired glucose tolerance at all ages studied, with the exception of 2 months, when glucose tolerance transiently worsened in controls (n=9 CON, n=7 PNA, $p<0.05$ at age 1, 3-6 months at 45 -120 min time points). Figure 3A shows representative average glucose curves at age 5 months; Figure 3B shows the average area under the glucose curve at each age studied (repeated measures ANOVA, $p<0.05$). Fasting glucose was significantly higher in PNA mice (glucose: CON 68.7 ± 4.2 mg/dL, n=9, PNA 86.7 ± 4.9 mg/dL, n=7, $p<0.02$).

PNA mice exhibit normal peripheral insulin sensitivity

Glucose intolerance occurs due to the failure of insulin target tissues to adequately dispose of circulating glucose. This can be a consequence of impaired insulin secretion and/or impaired insulin action. To assess the latter, we performed an insulin tolerance test in a group of three-month-old mice. No differences were found between PNA and CON mice (n=10 per group, all comparisons $p>0.2$, Fig 3C). Fed basal glucose levels were also not different (CON 164.8 ± 11.8 mg/dl, PNA 162.7 ± 10.1 mg/dl, n=10 per group, $p>0.8$). Additionally, fasting insulin and glucose values were used to determine homeostasis model assessment (HOMA) as a surrogate measure of insulin resistance. This index was developed using human data but subsequently has been validated in rodents (155, 156). No difference was found between groups (0.78 ± 0.10 CON vs 0.86 ± 0.18 PNA, $p>0.6$).

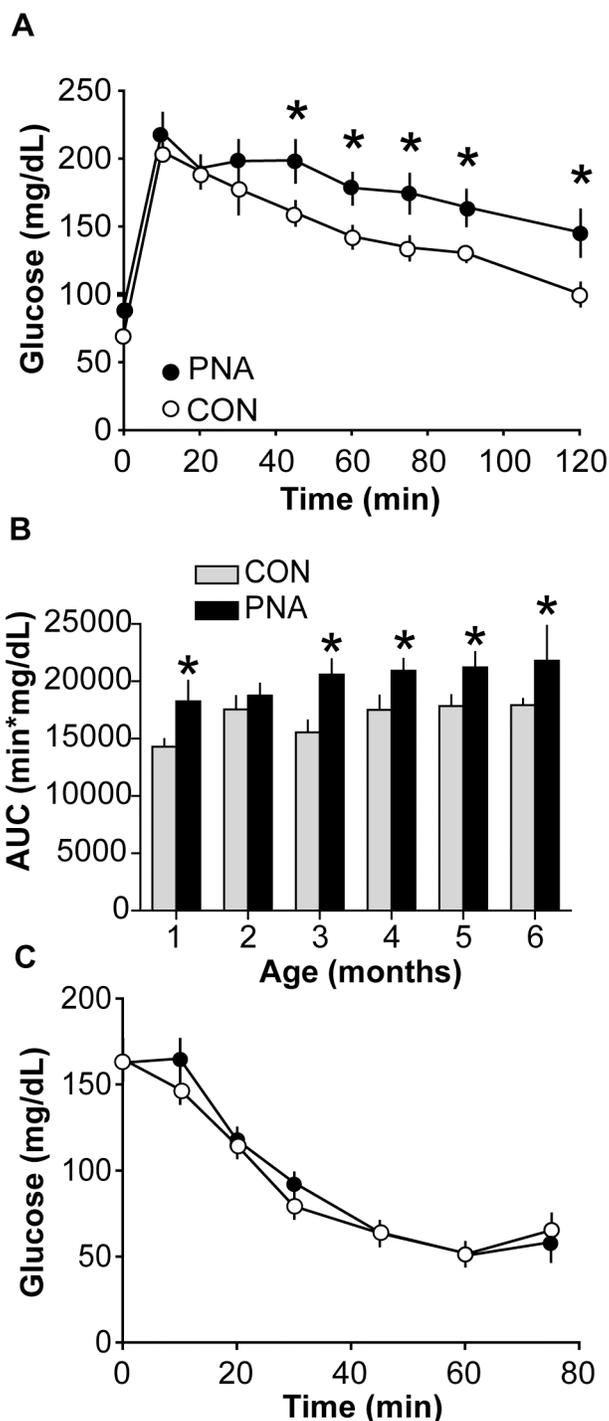


Figure 3. PNA mice exhibit impaired glucose tolerance but not insulin resistance. A. Glucose tolerance was impaired in 5 month-old PNA (closed circles) compared to CON (open circles) mice. B. Area under the curve (AUC) in CON (gray bars) and PNA (black bars) mice examined at different ages illustrates that glucose intolerance develops by 1 month of age. C. PNA mice are not insulin-resistant based on insulin tolerance testing (n=10 per group, $p>0.2$). * $p<0.05$.

PNA mice have an early form of islet dysfunction

The presence of apparently normal insulin sensitivity in PNA mice suggested glucose intolerance may have originated in an insulin secretion defect at the level of the

pancreatic beta cell. To assess pancreatic islet function, we used the ratiometric fluorescent probe fura-2 AM to measure glucose-stimulated calcium (GSCa) in islets from PNA and CON mice. GSCa is a measure of islet glucose sensitivity that allows high frequency sampling, which captures the dynamics of the biphasic response (154, 157) and approximates that of glucose-stimulated insulin secretion (158). $[Ca^{2+}]_i$ was monitored in islets during perfusion with 3 mM glucose and following a switch to 11 mM glucose. Islets from PNA (n=6) and CON (n=8) mice had similar calcium levels in 3 mM glucose ($p>0.4$), but the rise in intracellular calcium following a switch to 11 mM glucose was blunted in islets from PNA mice ($p<0.05$, Fig 4).

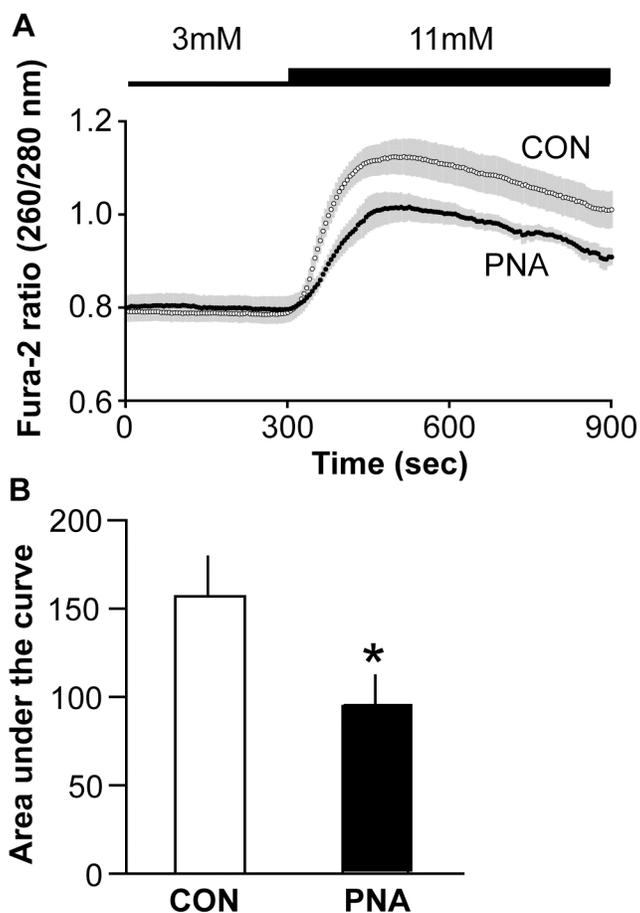


Figure 4. PNA mice have impaired pancreatic response to elevated glucose. A. Mean \pm SEM ratios (n=8 CON, n=6 PNA) of fura-2 fluorescence at 260 and 280 nm excitation, demonstrating the impaired response of islets from PNA mice upon an increase in glucose concentration from 3 mM to 11 mM. Shaded area indicates SEM. B. Area under the curve was lower in PNA mice. * $p<0.05$.

The limited number of islets precluded performing parallel insulin secretion studies in islets from all mice. Insulin secretion was measured in islets from 2 mice per group. While this sample size was insufficient to perform statistical comparisons, there was good agreement between calcium responses and insulin release in high glucose, consistent with observations in the literature (159, 160) (3 mM: CON 2.1 ± 0.06 pg/mL, PNA 5.7 ± 1.5 pg/ml; 11 mM: CON 33.0 ± 2.1 pg/mL, PNA 20.8 ± 4.2 pg/ml).

Insulin release from isolated islets

Steroids can exert organizational effects, which are mediated by developmental programming and persist in the absence of hormone, as well as activational effects, which require the immediate presence of hormone (161). To probe the activational role of androgens in adult islet function, we performed an *in vitro* study of islet insulin secretion after incubation with different steroids. Pancreatic islets express steroid receptors, including receptors for androgen (162, 163), but few studies have examined the direct roles of androgens in the islet. Islets were harvested from ovariectomized mice three days post surgery. Ovariectomized mice were used to control for effects of intrinsic steroids and oestrous cycles. Isolated islets were incubated overnight in 10 nM DHT, DHT+OE, or ethanol vehicle. Insulin secretion was measured in 3 mM and 11 mM glucose. Neither DHT nor the combination of DHT+OE had an effect on insulin secretion in 3 mM glucose ($p > 0.05$, $n = 12$ per group, Fig 5). However, DHT and the combination of DHT+OE significantly inhibited insulin secretion in 11 mM glucose ($p < 0.01$ and $p < 0.05$, respectively). This blunting of the islet response to high glucose was similar to that observed in PNA mice.

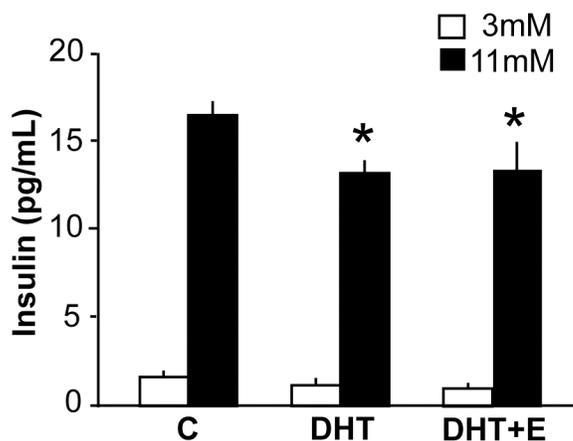


Figure 5. Steroids can act directly at the islet to alter insulin release in response to a glucose challenge. DHT or DHT in combination with oestradiol (DHT+OE) impair the release of insulin in response to a high glucose challenge (n=12 mice) *p<0.05 versus control (C, ethanol vehicle) in 11 mM glucose.

Reproductive measures

The lack of a difference in glucose tolerance at 2 months was due to an increase in glucose levels in control mice (p<0.05 by paired t-test), rather than an improvement in glucose tolerance in PNA mice. Interestingly, two months is around the age of final sexual maturation in mice (164). Given that puberty is a period of relative insulin resistance (165), we speculated that the increase in glucose levels in CON mice may be due to pubertal changes, and PNA mice may have experienced puberty earlier or later than control mice. To address this question, vaginal opening was monitored in a subsequent group of mice and found to occur earlier in PNA mice (CON 34.4±1.3 d, n=18, PNA 29.5±1.7 d, n=16, p<0.05). Body mass at the time of vaginal opening was significantly lower in PNA mice (CON 14.9±0.3 g, PNA 12.7±0.5 g, p<0.001), suggesting that increased body mass was not the cause of early vaginal opening.

Serum testosterone was assayed in blood samples taken at euthanasia on the day of islet harvest. In contrast to our previous report (36), PNA mice in this study did not exhibit elevated testosterone levels at 5 months of age (CON 10.5±3.6 ng/dL, n=9, PNA 11.9±3.0 ng/dL, n=8, p>0.7). However, testosterone levels measured in a different group of mice from this cohort of PNA animals at 8 months of age revealed significantly higher

levels than CON mice (CON 17.7 ± 2.7 ng/dL, $n=7$, PNA 28.9 ± 1.9 ng/dL, $n=5$, $p < 0.01$). Thus, as the mice continue to age, differences in androgen levels may become apparent. In this report, metabolic studies were performed up to age 6 months.

Despite the absence of elevated testosterone, the primary reproductive phenotype of disrupted oestrous cycles was apparent in PNA compared to CON mice. Cycle duration, defined as the oestrus-to-oestrus interval, was significantly lengthened (6.6 ± 0.4 d CON, 15.7 ± 2.6 d PNA, $n=10$ each, $p=0.002$) with the percent of time in oestrus significantly decreased ($15.2 \pm 1.6\%$ CON, $4.8 \pm 1.6\%$ PNA, $p=0.0002$). PNA mice exhibited prolonged periods in which leukocytes were the primary cell in the vaginal lavage. This finding differs somewhat from the original report of this model, in which similar disruptions were observed in cyclicity but prolonged periods of cornified cells were observed. We believe the difference may be attributed to a switch from phytoestrogen-containing to reduced-phytoestrogen chow. The observations of cyclicity for the three rounds of prenatal androgenization used for this study, as well as ongoing studies in the lab, are consistent with prolonged dioestrus.

PNA mice exhibit normal birth weight

Some of the metabolic effects observed in this model coincide with those induced by intrauterine growth restriction, which has been demonstrated in sheep prenatally androgenized with testosterone (166). Birth weight was assessed in subsequent litters of PNA mice but was not altered by prenatal treatment (CON 1.4 ± 0.03 g, $n=33$, PNA 1.4 ± 0.03 g, $n=29$, $p > 0.7$).

Discussion

Prenatal androgenisation of female mammals has profound lasting effects on reproductive function in adulthood, and may underlie the fertility and metabolic disorder polycystic ovary syndrome. In this study we assessed whether the same prenatal DHT treatment that caused reproductive abnormalities in female mice (36) could also induce metabolic dysfunction. PNA mice exhibited glucose intolerance that was present before puberty and persisted into adulthood. Impaired glucose tolerance was not associated with increased adiposity or peripheral insulin resistance; however, pancreatic islet function was altered in PNA mice and may be a causative factor in glucose intolerance.

Glucose tolerance was studied across postnatal development since increasing adiposity with age, or changes in circulating hormones following puberty, might influence the phenotype. Of interest, PNA mice exhibited glucose intolerance at the earliest age studied, four weeks, and the difference relative to controls remained stable throughout the study, except at the two-month time point. A difference in the timing of puberty may account for the disparity at two months. PNA mice underwent vaginal opening earlier than control mice; thus, we may have missed the window of pubertal insulin resistance in this group. Alternatively, the already impaired glucose handling in PNA mice may have masked the effects of pubertal insulin resistance. The finding of earlier puberty in PNA mice is supported by work in sheep showing pubertal advancement following prenatal androgen exposure in females (167, 168), and recent studies implicating androgens in the timing of puberty (169). Further, the observation of impaired glucose tolerance in PNA mice at only one month of age corresponds with the appearance of some aspects of PCOS in adolescents (170).

PNA mice exhibited elevated fasting glucose levels in the presence of normal fasting insulin. Impaired fasting glucose is associated with hepatic insulin resistance (171), which is characterized by a failure of insulin to suppress gluconeogenesis under fasting conditions. Typically insulin would also be elevated in this situation, but this assumes normal pancreatic beta cell compensation. Another possibility is that PNA mice have a higher stress response to fasting and handling, leading to acutely elevated glucose. However, studies of steroid programming of the hypothalamic-pituitary-adrenal axis indicate that adult stress responses are blunted by developmental androgen exposure (172). DHT administered close to parturition as in our study could potentially change maternal nurturing behaviour, which could also affect offspring stress responses (173).

Isolated islet studies were performed because the limited blood volume of mice makes sequential measurements of insulin secretion *in vivo* difficult to perform. $[Ca^{2+}]_i$ is closely coupled to insulin release in the beta cell, permitting $[Ca^{2+}]_i$ changes to be monitored as a surrogate for insulin secretion amid changing glucose concentrations (154). Islets are a heterogeneous tissue comprised of alpha, beta, delta, PP, and epsilon cells (174), but beta cells comprise 65-80% of the islet mass; thus glucose-stimulated $[Ca^{2+}]_i$ variations primarily reflect changes in this cell type. Impaired glucose tolerance in PNA mice did not progress to frank diabetes. Nevertheless, the observed defects in islet function are similar to the early islet dysfunction in type 2 diabetes mellitus (154). Hallmarks of pending islet failure include elevated basal calcium, loss of oscillatory activity, and failure to generate an appropriate rise in calcium (and thus insulin secretion) upon high glucose stimulation (154, 159). Type 2 diabetes occurs in the context of peripheral insulin resistance, when pancreatic compensation to increase insulin production is no longer adequate (175). Thus, impaired pancreatic islet function in PNA mice may predispose

them to develop type 2 diabetes in the presence of other risk factors, such as obesity. Similarly, women with PCOS have impaired beta cell function and are at increased risk for diabetes (176, 177).

An additional tissue-specific abnormality was identified, with PNA mice exhibiting increased visceral adipocyte size. Although enlarged adipocytes are often observed with increased fat mass, total fat pad mass was unchanged in PNA mice, suggesting that adipocyte number may be reduced. This idea is speculative; however, reports in the literature suggest that androgens can indeed alter adipocyte differentiation and size. DHT reduces omental adipocyte differentiation in tissue culture, and both DHT and testosterone inhibit differentiation of pluripotent cells into the adipogenic lineage (178, 179). Androgen receptor knockout mice have smaller adipocytes than wildtype controls, suggesting androgen receptor activation increases adipocyte size (180). It must be emphasized that the change in adipocyte size observed here was small and did not result in associated changes in adipocyte insulin sensitivity (181) or changes in circulating levels of the majority of adipokines measured (182, 183). In PNA mice, there was a strong trend for decreased adiponectin in the fed state, which is in agreement with reduced adiponectin levels in women with PCOS (184). Further, leptin levels were not reduced by fasting in PNA mice, suggesting altered regulation of this hormone, which is permissive for fertility (185). Differences in other adipokines, or in adipocyte insulin sensitivity, could appear under conditions of metabolic stress, such as diet-induced obesity. This is an interesting area for future studies.

Activational effects of androgens in islets were assessed because PNA mice previously exhibited elevated androgens (36), a cardinal feature of PCOS. The androgen receptor (186) and synthesis enzyme cytochrome P450(17 α) (187) are expressed in beta

cells, but their roles in this tissue are unclear. One study showed that micromolar concentrations of testosterone increase insulin transcription and secretion in the rat *in vivo* and in islets *in vitro* (188), but the possibility that effects were mediated by aromatisation to oestradiol or by non-genomic pathways were not examined. We found that overnight treatment with nanomolar concentrations of DHT impaired high-glucose-stimulated insulin release in islets from ovariectomized mice. This effect was most likely genomic, as steroids were not present when insulin release was measured. Oestradiol had no added effect. The reduction in glucose-stimulated insulin secretion following islet exposure to DHT *in vitro* was similar to the decrement in glucose-stimulated calcium flux in islets from PNA mice. Since we did not observe elevated circulating androgens at five months of age when islets were isolated, islet dysfunction could not be attributed to activational effects of androgens. However, the effects of elevated androgen in adult life may be additive to those induced by prenatal androgen. PCOS is thought to involve elevated androgen prenatally and in adulthood, potentially generating a double insult on pancreatic function.

The absence of peripheral insulin resistance in PNA mice differs from previously published studies of prenatally androgenized monkeys, sheep, and rats (35, 189, 190). In these models, insulin resistance was accompanied by an increase in total and/or visceral adiposity; the association between these conditions is well recognized (191). PNA mice did not exhibit changes in body mass or composition, which may correlate with the absence of insulin resistance. An important distinction that may account for these differences is the androgen used. This study employed DHT to examine primarily androgen receptor-mediated effects, whereas other studies used testosterone, which can be aromatised to oestradiol. This suggests a possible role of excess prenatal

oestrogen, or oestrogen in combination with androgen, in programming obesity and peripheral insulin resistance. Prenatal exposure to oestrogenic substances such as bisphenol A and diethylstilbestrol increases postnatal weight and adiposity (192, 193). Oestrogens may also potentiate the effects of androgens by upregulating the androgen receptor in specific tissues, including adipocytes and brain (194, 195). Further, intrauterine growth restriction and its metabolic sequelae have been shown to be an effect of prenatal testosterone, but not DHT exposure (166, 196). In addition to the type of steroid treatment, other differences that may account for phenotypic discrepancies include the difference in species and variations in the timing of androgen administration. Sexual differentiation occurs during gestation in primates and sheep, whereas in rodents, it is incomplete at birth (197, 198). Thus, androgen exposure late in gestation in mice may be comparable to an early treatment in other species. Nevertheless, the developmental timeline in rats is similar to that in mice, and the treatment period in the prenatally androgenized rat (d16-19) overlaps ours. Hence the steroid may be most important in establishing differences among models.

In this study, we have shown that prenatal androgens program long-term alterations in metabolic function in female mice. These findings have implications for gestational androgen exposure that originates from endogenous or exogenous sources. The absence of changes in circulating glucose, insulin, or the majority of adipokines assessed under fed conditions suggests that the previously observed reproductive dysfunction in this model was likely caused by androgen programming of the reproductive axis, and was not secondary to metabolic changes. Further, as the previously reported effects of prenatal androgenisation to induce adiposity and insulin resistance were absent from PNA mice, it appears that metabolic programming by

testosterone may be dependent on aromatisation to oestradiol in addition to androgen receptor-mediated effects. The impairments observed in glucose tolerance and pancreatic islet function, as well as increased adipocyte size, may predispose PNA mice to develop diabetes in the presence of aggravating factors. This suggests a two-hit hypothesis, in which prenatal androgen programming sets the stage for metabolic dysfunction, and weight gain and insulin resistance secondary to prenatal oestrogens or postnatal weight management drive the progression to diabetes.

Chapter 3

Glucosensing by gonadotropin-releasing hormone neurons: inhibition by androgens and involvement of AMP-activated protein kinase

Alison V. Roland and Suzanne M. Moenter

Abstract

Gonadotropin-releasing hormone (GnRH) neurons integrate steroidal and metabolic cues to regulate fertility centrally. Central glucoprivation reduces luteinizing hormone secretion, which is governed by GnRH release, suggesting GnRH neuron activity is modulated by glucose availability. Here we tested if GnRH neurons can sense physiological glucose changes, and if glucosensing is altered by the steroids dihydrotestosterone (DHT) and/or estradiol (E). Extracellular recordings were made from GnRH neurons in brain slices from ovariectomized (OVX) mice \pm DHT and/or E implants. Firing rate was reduced by a switch from 4.5 to 0.2 mM glucose in cells from OVX, OVX+E, and OVX+DHT+E mice, but not OVX+DHT mice. This suggests androgens reduce the sensitivity of GnRH neurons to changes in extracellular glucose, but E mitigates this effect. Next we investigated potential mechanisms. In the presence of the K_{ATP} channel antagonist tolbutamide, glucosensing persisted. In contrast, glucosensing was attenuated in the presence of compound C, an antagonist of AMP-activated protein kinase (AMPK), suggesting a role for AMPK in glucosensing. The AMPK activator AICAR mimicked the effect of low glucose and was less effective in cells from DHT-treated mice. The effect of DHT to diminish responses to low glucose and AICAR was abolished by blocking fast synaptic transmission. Both AICAR and low glucose activated a current with a reversal potential near -40 mV, suggesting a nonspecific cation current. These studies indicate glucosensing is one mechanism by which GnRH neurons sense fuel availability, and point to a novel role for AMPK in the central regulation of fertility.

Introduction

GnRH neurons form the final common pathway in the central regulation of fertility. GnRH is secreted in a pulsatile manner and elicits corresponding pulses of luteinizing hormone secretion from the pituitary (199). The frequency and amplitude of GnRH secretion changes in response to steroids across the female reproductive cycle, leading to a surge of GnRH and luteinizing hormone (LH) mid-cycle that is required for ovulation (200). GnRH neurons are further regulated by metabolic cues, and interactions among steroidal and metabolic signals may be important in the regulation of reproduction under both normal and pathophysiological states (201, 202).

Numerous studies have explored the link between metabolism and fertility, but the specific metabolic signals involved and mechanisms by which they are conveyed to the reproductive system have not been fully elucidated. The majority of existing evidence for glucose regulation of GnRH release is derived from studies of experimental glucoprivation induced by fasting or intracerebroventricular infusion of insulin or glucose antimetabolites (2-deoxyglucose or 2-thioglucose). A reduction in central glucose availability causes a suppression of LH levels and LH pulse frequency (47), as well as a reduction in pulses of electrical activity in the preoptic area thought to correspond with GnRH release (48). In insulin-induced hypoglycemic rats, restoration of glucose, but not other metabolic substrates, restores LH pulsatility (50). Using steroid-primed ovariectomized rats as a model of the GnRH/LH surge, caudal hindbrain infusion of 2-thioglucose both prevents the LH surge and reduces c-fos expression in GnRH neurons (49), suggesting an inhibition of GnRH neuronal activity. Importantly, these studies implicate a central site in the sensing of low glucose availability by the GnRH-LH axis.

The infusion of glucoprivic agents into brain regions devoid of GnRH neurons suggests other neuronal populations might relay these signals synaptically. One study suggests direct sensing of glucose by GnRH neurons (52). GnRH neuron firing rate was reduced by a switch from high to low glucose in high K⁺ and high Mg²⁺/low Ca²⁺ solution; the latter inhibits presynaptic vesicle release. However, the mechanisms and steroid-dependence of the response were not investigated.

Here we examine glucosensing in GnRH neurons, its dependence on steroids and fast synaptic transmission, and possible underlying mechanisms. We sought to determine whether GnRH neuron firing activity could be altered by changes in extracellular glucose within the physiological range. These experiments were performed in defined animal models treated with estradiol and/or the nonaromatizable androgen, dihydrotestosterone (DHT). The interaction between steroids and glucosensing is relevant to normal reproduction, as steroid levels change during the female cycle. It is also of importance to reproductive disorders such as polycystic ovary syndrome, the leading cause of infertility in women, which is characterized by both elevated androgens and poor blood glucose control (203).

Materials and Methods

Animals

Studies were performed in 2-4 month old female GnRH-GFP mice (204). Mice were housed on a 14-h light, 10-h dark cycle, with lights off at 1630 h, and were maintained on Harlan 2916 rodent chow (Harlan, Bartonville, IL) and water *ad libitum*. All procedures were approved by the Animal Care and Use Committee of the University of Virginia and

were conducted within the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals. Mice were ovariectomized (OVX) under isoflurane (Abbott Laboratories, North Chicago, IL) anesthesia. Postoperative analgesia was provided by a long-acting local anesthetic (0.25% bupivacaine; 7.5 μ l/site; Abbott Laboratories) directly to the surgical site. At the time of surgery, some mice received sc SILASTIC (Dow Corning, Midland, MI) capsules containing 400 μ g dihydrotestosterone (OVX+DHT) or 0.625 μ g estradiol (E) (OVX+E) in sesame oil. OVX+E+DHT mice received both capsules. Recordings were performed 8-12 days post surgery.

Brain slice preparation and recordings

All chemicals were from Sigma Chemical Co. unless noted. Brain slices were prepared using modifications of a previously described method (205). Solutions were bubbled with a 95% O₂-5% CO₂ mixture throughout the experiments and for at least 15 min before exposure to the tissue. The brain was rapidly removed and placed in ice-cold, high-sucrose saline solution containing (in mM) 250 sucrose, 3.5 KCl, 26 NaHCO₃, 10 glucose, 1.25 Na₂HPO₄, 1.2 MgSO₄, and 2.5 MgCl₂. Coronal 300- μ m brain slices were cut with a Vibratome 3000 (Technical Products International, Inc., St. Louis, MO). Slices were incubated for 30 min at 30–32 °C in a solution of 50% high-sucrose saline and 50% artificial cerebrospinal fluid (ACSF) containing (in mM) 135 NaCl, 26 NaHCO₃, 3.5 KCl, 10 glucose, 1.3 Na₂HPO₄, 1.2 MgSO₄, and 2.5 CaCl₂ (pH 7.4). Slices were then transferred to a solution of 100% ACSF at room temperature and kept at least 30 min and no more than 6 h before recording.

For recording, individual brain slices were placed in a recording chamber continuously superfused with oxygenated ACSF solution with glucose adjusted to 4.5 mM and

maintained at 28–30 °C using an in-line heater (Warner Instruments). Slices were incubated in this solution for 15-20 minutes prior to the start of recording. Cells were visualized with an upright fluorescent microscope with infrared differential interference contrast (Olympus). GnRH neurons were identified by brief illumination at 470 nm to visualize the GFP signal. Recording pipettes were pulled from borosilicate glass capillaries (1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Inc.) using a Flaming/Brown P-97 (Sutter Instrument) and had resistances from 1.5 to 2.5 M Ω when filled with HEPES. Pipettes were placed in contact with a GnRH neuron using an MP-285 micromanipulator (Sutter Instruments). Current and voltage traces were obtained using an EPC-10 amplifier controlled by PatchMaster (HEKA).

Experimental design

To study the effects of alterations in extracellular glucose on firing activity of GnRH neurons, targeted extracellular recordings were used. This type of recording is minimally invasive and does not alter the intracellular milieu; thus glucose and ion concentrations as well as the cell response to synaptic inputs that remain within the brain slice are maintained. Recording pipettes were filled with HEPES-buffered solution containing (in mM) 150 NaCl, 10 HEPES, 10 glucose, 2.5 CaCl₂, 1.3 MgCl₂, and 3.5 KCl. Initial resistances ranged from 6 to 30 M Ω and either remained stable or increased during recording up to as high as 50 M Ω . Recordings were made in voltage-clamp mode with a pipette holding potential of 0 mV; at low seal resistance, the amplifier potential does not influence the cell. In this type of recording, action currents are detected, which reflect changes in the action potential firing rate. The phrases “firing rate” and/or “firing activity” will be used to refer to these events.

After 15-20 min acclimation to 4.5 mM glucose and establishment of a stable recording, firing rate was monitored for 10 minutes in 4.5 mM glucose, for ten minutes following a switch to 0.2 mM glucose, and for ten minutes following a return to 4.5 mM glucose. If cells failed to fire within 10 minutes following restoration of 4.5 mM glucose, recording was continued until the cell fired, or KCl (15 mM) was applied to verify cell viability. In a subset of recordings, tolbutamide (200 μ M), compound C (20 or 40 μ M), or the combination of (2R)-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and bicuculline (each at 20 μ M) were used. Drugs were present in both the 4.5 mM and 0.2 mM glucose solutions. Cells that were quiescent or failed to achieve a stable firing pattern during the ten-minute control period were not used for extracellular experiments. Osmolarity changes were not compensated, as they were negligible (4.3 mOsm or \sim 1.4%).

To examine the effect of pharmacological activation of AMPK, N1-(b-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide (AICAR, 2 mM) was bath applied to cells for 30 minutes following a 15-20 minute control recording period. For analysis, firing rate during the last ten minutes of AICAR exposure was quantified. The AMPK activator metformin was also tested. Following a control recording period, 2mM metformin was applied to cells for 20 minutes.

Whole-cell recordings

To examine the effects of AICAR on intrinsic properties of GnRH neurons, whole-cell recordings were used. Pipettes were filled with a solution containing (in mM) the following: 125 K gluconate, 20 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, 1.0

CaCl₂, pH 7.2, 300 mOsm. The extracellular solution contained ACSF with 4.5 mM glucose and APV, CNQX, bicuculline, and in some instances 0.5 μ M tetrodotoxin (TTX) to block persistent and fast sodium currents. Input resistance (R_{in}), series resistance (R_s), and membrane capacitance (C_m) were continually measured, and only recordings with stable $R_{in} > 500 \text{ M}\Omega$, $R_s < 20 \text{ M}\Omega$, and stable C_m were used for analysis. Cells were held at -60 mV between step protocols. After attaining the whole-cell configuration, cells were allowed to stabilize for at least 5 minutes. A step protocol (shown in Fig 6B) was run under control conditions. AICAR (2 mM) was then bath applied for 15 minutes. The protocol was run intermittently during this time to monitor current changes. The data obtained at 15 minutes were used to determine the AICAR-induced current, as a clear suppression of firing was observed by this time point in extracellular recordings in the presence of fast synaptic blockers. Membrane potentials were not corrected for the calculated liquid junction potential error, estimated to be -13 mV (206).

To assess the effect of low glucose on whole-cell current, the same protocol as above was used. After cell stabilization (≥ 5 min), the protocol was run 2-4 times in 4.5 mM glucose, and then following 10 minutes in 0.2 mM glucose. If the cell remained stable, the protocol was repeated following restoration of 4.5 mM glucose for 10-20 minutes. Two traces in each condition were averaged for analysis.

Analysis and statistics

For each recording, mean firing rate (in Hz) was determined for each five-minute interval of recording time. The five minutes immediately prior to low glucose exposure was used as the control period for analysis. The last five minutes of low glucose perfusion and the first five minutes of the washout period were designated time 1 (t_1) and time 2 (t_2) post

low glucose, respectively. One-way repeated measures ANOVA with Dunnett's Multiple Comparison test was used to compare treatment versus control frequencies. To compare the effect of low glucose among groups, a two-way ANOVA with Bonferroni's post-hoc test was used. Two-tailed paired t-test was used to compare firing frequencies pre- and post-AICAR. Fisher's exact test was used to compare percent responders among groups. For all tests, significance was set at $p < 0.05$ and data are reported as mean \pm SEM.

Results

GnRH neurons respond to changes in glucose concentration within the physiological range

We first tested if GnRH neurons in brain slices from OVX mice could respond to a reduction in extracellular glucose from 4.5 mM to 0.2 mM. These represent brain extracellular glucose concentrations following a large meal and fasting, respectively (207). Acute exposure to low glucose resulted in a decrease in firing that was not immediately reversed upon restoration of 4.5 mM glucose ($n=11$, $p < 0.05$, Fig 1A). Firing rate was suppressed by greater than half in 82% (9/11) of cells. Although GnRH neurons spontaneously change their firing rate and undergo periods of quiescence (11), in cells in which the glucose concentration was maintained at 4.5 mM for a period of 30 minutes, the firing rate on average did not change ($n=6$, $p=0.98$, Fig 1B). This indicates that the decrease in firing rate in treated cells was due to low glucose exposure.

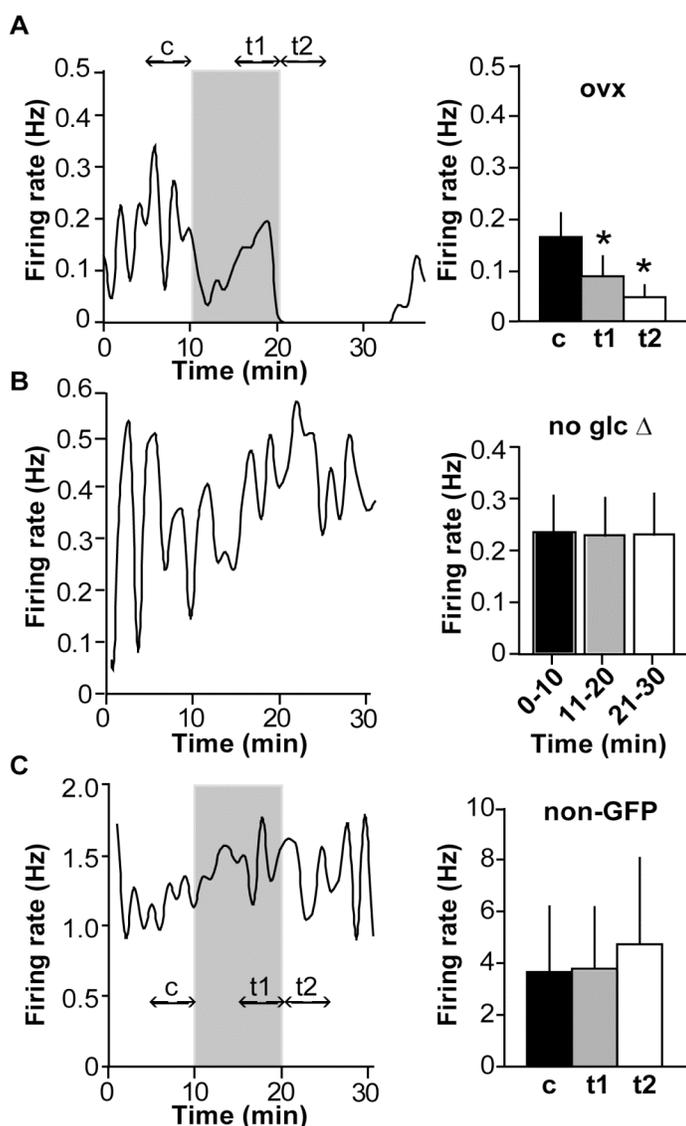


Fig 1. GnRH neurons are sensitive to a physiological reduction in extracellular glucose concentration. A, Left, Representative plot of firing rate over time in a GnRH neuron from an OVX mouse (left) and summary (n=11 cells, right). Events are binned in 60-s intervals. Glucose concentration was switched from 4.5 to 0.2 mM; shaded region indicates period of 0.2 mM treatment. Double-headed arrows indicate time intervals in which frequency was averaged for analysis, designated c for control period, and t1 and t2 for post low glucose. Mean \pm SEM firing rate for c (black bars), t1 (grey bars), and t2 (white bars) are shown in the bar graph. B, Representative plot of firing rate over time in a cell from an OVX mouse in which the glucose concentration was held constant at 4.5 mM (left), and summary (n=10 cells, right). There was no change in average firing rate from the first ten minutes to the second ten minutes of recording, in contrast to cells in which the glucose concentration was reduced. C, Representative plot of firing rate over time in a non-GFP-expressing neuron in the preoptic area (left), and summary (n=4, right). Time of 0.2 mM glucose treatment is indicated by shading. Low glucose failed to affect firing in 4/4 non-GFP (non-GnRH) neurons. *, $p < 0.05$.

In most cells, the firing rate continued to decline upon restoration of 4.5 mM glucose. To determine if this was a delayed effect of low glucose, or caused by the acute increase in glucose, some cells were incubated in low glucose for fifteen rather than ten minutes (n=3, data not shown). The firing rate continued to decline after the initial ten minutes (by an average of $80.9 \pm 13.6\%$) and did not decline when 4.5 mM glucose was restored (mean change of $-5.5 \pm 9.6\%$). This suggests that the low firing rate in the early part of the glucose restoration period is a continuation of the response to low glucose.

Activity suppression by low glucose is at least partly specific to GnRH neurons

It has been postulated that neurons throughout the brain are glucose sensitive, and some studies suggest that all neurons are silenced by glucose deprivation (208). To determine whether the observed response is ubiquitous or specific to a neuronal subpopulation that includes GnRH neurons, non-GFP-expressing neurons in the preoptic area were recorded under the same treatment protocol. 84-94% of immunofluorescently-identified GnRH neurons express GFP in this transgenic model (204). Further, some non-GFP cells studied exhibited markedly high mean firing frequencies uncharacteristic of GnRH neurons ($>15\text{Hz}$), indicating that they were likely not GnRH neurons. None of the non-GFP neurons recorded were inhibited by low glucose (Fig 1C, n=5, p=0.3). This indicates that the observed response to low glucose is not a ubiquitous neuroprotective phenomenon, and is at least partly specific to GnRH neurons.

DHT inhibits the response to low glucose but is counteracted by estradiol

To test whether steroids alter the response to a decrease in extracellular glucose, the

low glucose challenge was repeated in GnRH neurons in brain slices from OVX+DHT, OVX+E, and OVX+E+DHT mice. Hormone levels achieved with these treatments have been previously published (12, 209, 210). The E replacement is a physiological level of steroid; the DHT replacement elevates circulating androgens in these female mice to a level that is below what is required to restore seminal vesicle weight in a castrated male mouse. This “submale” level mimics the elevation in androgen seen in women with polycystic ovary syndrome (PCOS). Similar to GnRH neurons from OVX, GnRH neurons from OVX+E (n=13) and OVX+E+DHT (n=10) mice responded to 0.2 mM glucose with a decrease in firing rate ($p < 0.05$, Fig 2B,C). This effect was not observed, however, in the majority of cells from mice treated with DHT alone, and on average low glucose had no effect in this group (n=12, $p > 0.05$, Fig 2A). Percent responders (defined as $>50\%$ inhibition of firing rate) in OVX+E, OVX+E+DHT, and OVX+DHT were 64% (10/13), 70% (7/10), and 25% (3/12), respectively. Basal firing rate among cells was higher in OVX+E compared to OVX and OVX+DHT (one-way ANOVA, $p < 0.05$), which may reflect the fact that recordings were made in the afternoon, when estradiol can have a positive feedback effect (210). However, as a large proportion of cells in all steroid treatment groups were completely quiescent and thus not used in the study, basal firing rate here reflects only the average among active cells, as opposed to overall averages as previously studied. There was a negative correlation between basal firing rate and percent change in firing rate in response to low glucose (Pearson $r = -0.3$, $R^2 = 0.09$, $p < 0.05$). Cells with basal firing rate < 0.1 Hz were less likely to be inhibited by low glucose.

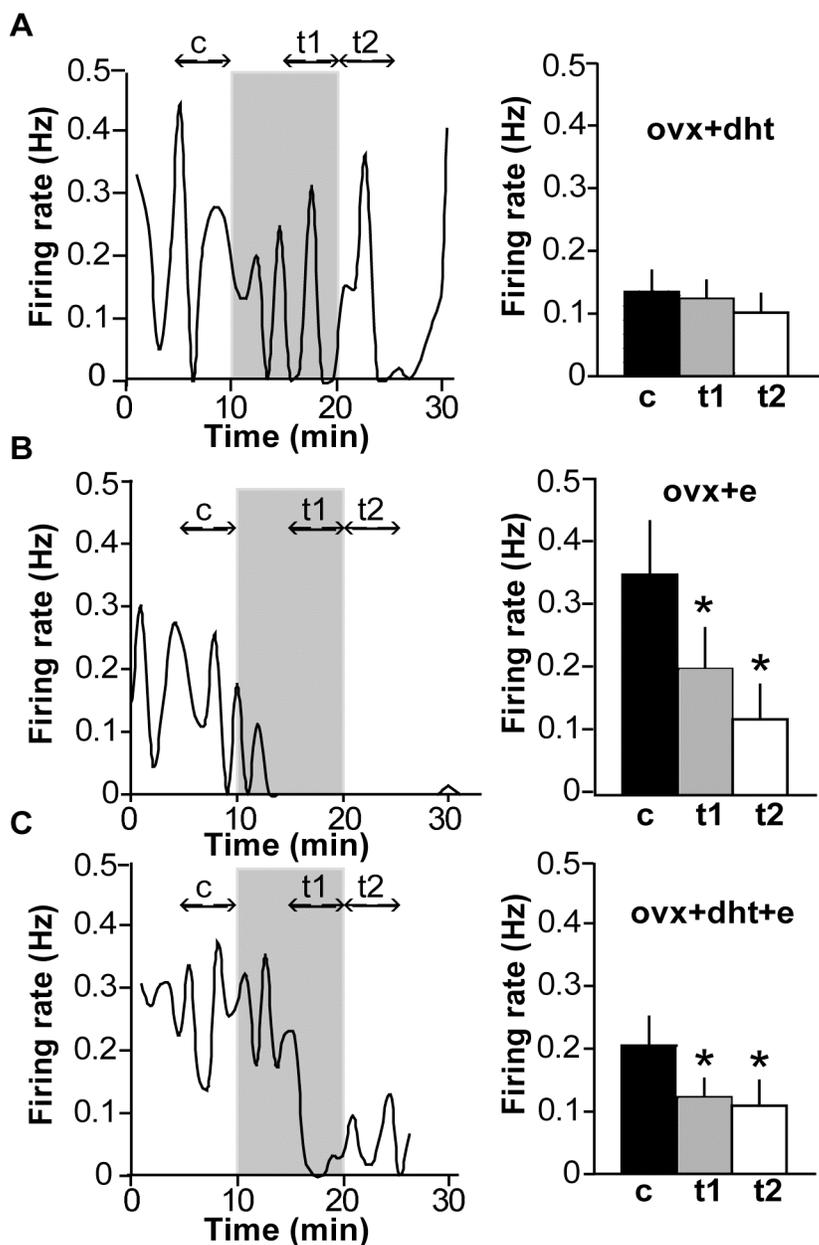


Fig 2. DHT inhibits the response to low glucose but is counteracted by estradiol. A, Representative plot of firing rate over time in a GnRH neuron from an OVX+DHT mouse and summary (n=12 cells, right). See figure 1 legend for details. Low glucose failed to inhibit firing in cells from mice treated with DHT. B, Representative plot of firing rate over time in a GnRH neuron from an OVX+E mouse and summary (n=14 cells, right). Low glucose inhibited firing in GnRH neurons from mice treated with estradiol. C, Representative plot of firing rate over time in a GnRH neuron from an OVX+DHT+E mouse and summary (n= 10 cells, right). In the presence of both DHT and E, GnRH neuron firing activity was inhibited by low glucose. *, p<0.05.

GnRH neurons respond to low glucose when fast synaptic transmission is blocked

Numerous studies suggest a role for afferent neurons in conveying glucose signals to GnRH neurons. GABA neurons, which form a major input to GnRH neurons (211-213), have been shown to relay metabolic signals to GnRH neurons (64, 84). To test if fast synaptic transmission mediated via ionotropic GABA and/or glutamatergic receptors mediates the response to low glucose, the low glucose challenge was repeated in the presence of antagonists of GABA_A, NMDA, and AMPA/KA receptors. This treatment not only blocked receptors directly on GnRH neurons, but also throughout the neuronal network maintained in the brain slice. In the presence of these receptor blockers, 83% (10/12) of GnRH neurons were inhibited by low glucose (n=12, p<0.05, Fig 3A), similar to the percentage in the absence of blockers (82%, Figure 1); additionally, the inhibitory effect of low glucose was more pronounced (2-way ANOVA OVX vs. OVX+blockers, p=0.005 for interaction). Blockers increased the basal firing rate among recorded (active) cells (p<0.0005 OVX, p<0.005 OVX+DHT). An excitatory effect of blockers has been observed previously (214) and may reflect neuromodulatory influences including increased excitatory and/or reduced inhibitory neuromodulatory input via the network, although it could also reflect a potential effect of bicuculline to block small-conductance calcium-activated potassium channels (215). This experiment indicates that fast GABA and glutamatergic transmission are not required for the response of GnRH neurons to low glucose. Overall these data suggest that low glucose is either being sensed by GnRH neurons directly or is conveyed via neuromodulatory inputs.

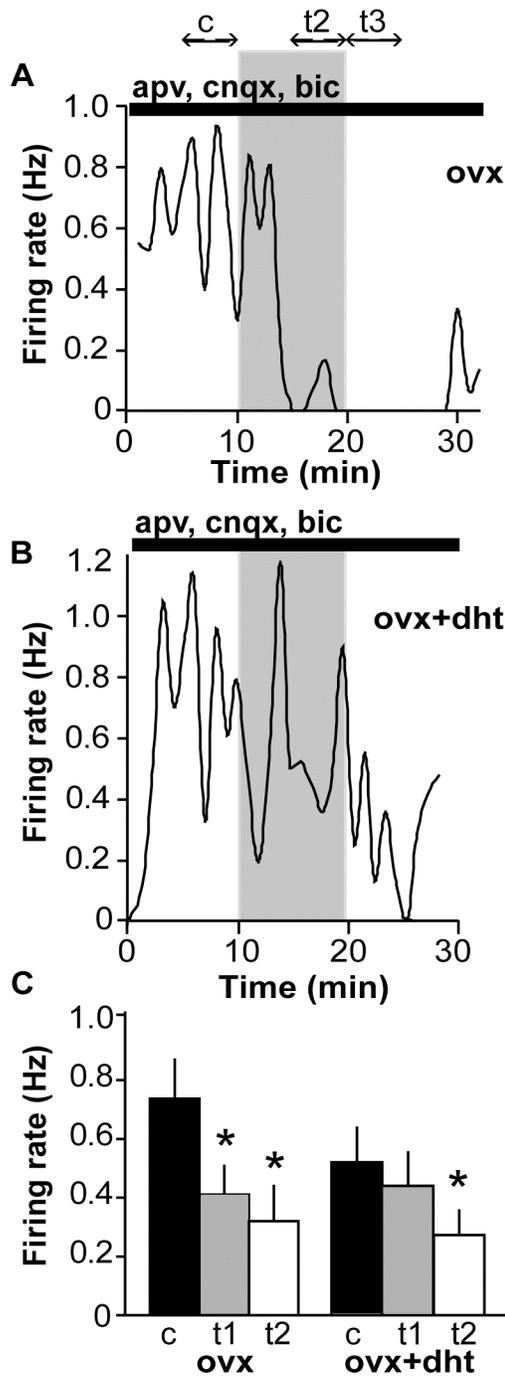


Fig 3. Glucosensing persists in OVX and is unmasked in OVX+DHT cells when fast synaptic transmission is blocked. A, Representative plot of firing rate over time in a GnRH neuron from an OVX mouse in APV, CNQX, and bicuculline (bic). See Figure 1 legend for details. B, Representative plot of firing rate over time in a cell from an OVX+DHT mouse in the presence of synaptic blockers. C, Summary of effects of low glucose on GnRH neurons from OVX and OVX+DHT mice in APV, CNQX, and bic (n=13 per group). Low glucose had an inhibitory effect in OVX cells at both times analyzed. The effect of low glucose was significant in OVX+DHT cells only at the latter time point. *, $p < 0.05$.

Blocking fast synaptic transmission unmasks glucosensing in a subpopulation of cells from DHT-treated mice

Because DHT increases GABAergic transmission to GnRH neurons (12), and androgen receptors have not been detected on GnRH neurons (216), we postulated that an intrinsic response might be masked by altered fast synaptic transmission in DHT-treated mice. We therefore repeated the low glucose challenge in cells from OVX+DHT mice in the presence of APV, CNQX, and bicuculline. With fast synaptic transmission blocked, there was a tendency for a greater proportion of cells from DHT-treated mice to be inhibited by low glucose (6/11 or 55% compared to 25% in the absence of blockers, $p=0.1$ using Fisher's exact test), and on average there was a decrease in firing during the second post-low glucose analysis interval ($n=11$, $p<0.05$, Fig 3B). The observation that the change was only evident in this latter time period suggests differences in the latency of the response between OVX and OVX+DHT cells. However, there was no statistical difference in overall response between OVX and OVX+DHT in the presence of blockers (2-way ANOVA, $p=0.27$ for interaction).

K_{ATP} channels are not required for glucosensing

K_{ATP} channels, which play an important role in glucosensing in the pancreas and in brain regions that regulate feeding behavior and peripheral glucose homeostasis (217, 218), have been demonstrated to influence GnRH neuronal activity in an estrogen-sensitive manner (52, 219). We hypothesized that K_{ATP} channels mediate the response to low glucose in GnRH neurons. To test their involvement, exposure to low glucose was repeated in the presence of the sulfonylurea tolbutamide at 200 μM , a concentration that blocks $\geq 95\%$ K_{ATP} current (220). In contrast to our hypothesis, GnRH neurons maintained their sensitivity to low glucose when K_{ATP} channels were blocked ($n=11$,

$p < 0.05$, Fig 4A), indicating that K_{ATP} channels are not required for glucosensing. In 100% of cells tested the firing rate was suppressed by $> 50\%$. The basal firing rate was not different between cells in 4.5 mM glucose in the presence or absence of tolbutamide, although it tended to be higher in tolbutamide-treated cells ($n=11$ in each condition, $p=0.1$). In a separate experiment, only 3 of 8 cells acutely treated with tolbutamide responded with an increase in firing rate ($p > 0.2$, $n=8$, Figure 4B). This suggests that in cells from OVX mice, only a fraction have functional and/or open K_{ATP} channels under these recording conditions. Because firing in 2/8 cells actually decreased ($>30\%$) after tolbutamide, another possibility is that closure of K_{ATP} channels depolarizes afferent neurons that inhibit GnRH neurons. Regardless, K_{ATP} channel activation does not appear to be required for the inhibitory response of GnRH neurons to low glucose, indicating the involvement of one or more other mechanisms. The right portion of Fig 4B shows a cell that responds to acute application of tolbutamide, and is subsequently treated with 0.2 mM glucose (also containing tolbutamide). This cell is still clearly inhibited by low glucose, indicating that even in cells that are regulated by K_{ATP} channels, another mechanism mediates the response to low glucose.

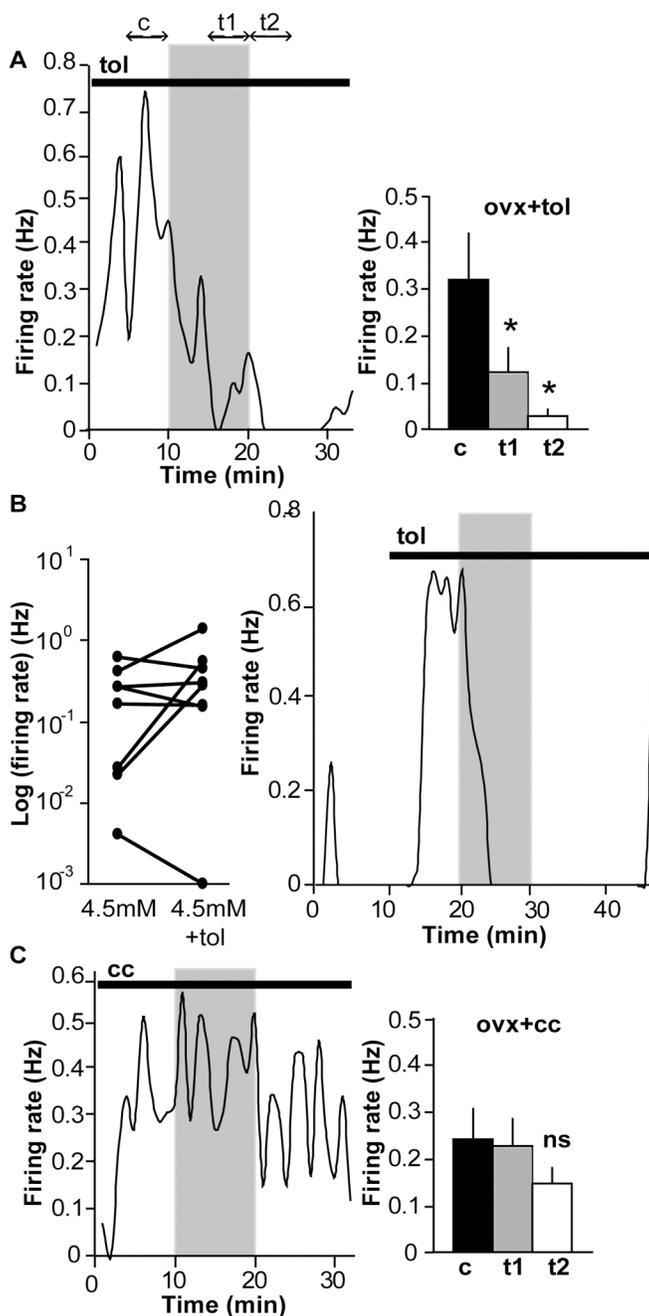


Fig 4. AMPK antagonism, but not K_{ATP} channel antagonism, attenuates glucosensing in GnRH neurons. A, Representative plot of firing rate over time in a GnRH neuron from an OVX mouse in the presence of 200 μ M tolbutamide and summary ($n=11$ cells, right). See Figure 1 legend for details. Low glucose inhibited GnRH neuron firing despite blockade of K_{ATP} channels by tolbutamide. B, Plot of firing rate over time in an OVX cell that is acutely stimulated by tolbutamide, and subsequently inhibited by low glucose application. Black bar indicates time of tolbutamide application. C, Representative plot of firing rate over time in a GnRH neuron from an OVX mouse in the presence of 20-40 μ M compound C (CC) and summary ($n=15$, right). Low glucose failed to reduce firing activity when AMPK was blocked by CC. *, $p<0.05$.

The response to low glucose is attenuated when AMPK is blocked

We next tested the hypothesis that the response to glucose was mediated by AMPK, a cellular energy sensor that is activated by increases in the intracellular AMP/ATP ratio (221). To test this, AMPK was blocked using the antagonist CC (222). Either 20 or 40 μM CC was used, but no difference in response was observed between cells treated with these concentrations, and the data have been combined for analysis. In the presence of CC, the majority of GnRH neurons (8/14 or 57%) failed to respond to low glucose ($n=14$, $p>0.05$, Fig 5C). On average a small decrement in firing rate occurred at the onset of washout, but this decrease was not statistically significant and not as robust as that in GnRH neurons in the absence of CC. Thus, AMPK antagonism attenuated the response to low glucose, indicating a potential role for AMPK in the response.

GnRH neurons are inhibited by an AMPK activator

To test the hypothesis that low glucose inhibits GnRH neurons via AMPK activation, we pharmacologically activated AMPK using AICAR. AICAR inhibited firing in GnRH neurons from OVX mice ($n=7$, $p<0.05$, Fig 5A,C). The latency of the response to AICAR was longer than that of low glucose, likely reflecting the fact that AICAR must be converted to 5-amino-4-imidazolecarboxamide ribotide (ZMP) inside the cell; it is ZMP that subsequently activates AMPK (223). The response to AICAR was maintained in the presence of blockers of fast synaptic transmission (APV, CNQX, bic; $n=4$, $p<0.05$, Fig 5D,F). Similar to AICAR, 2mM metformin also inhibited firing activity of GnRH neurons ($n=6$, control 0.51 ± 0.18 Hz, metformin 0.31 ± 0.14 Hz, $p<0.05$, data not shown.)

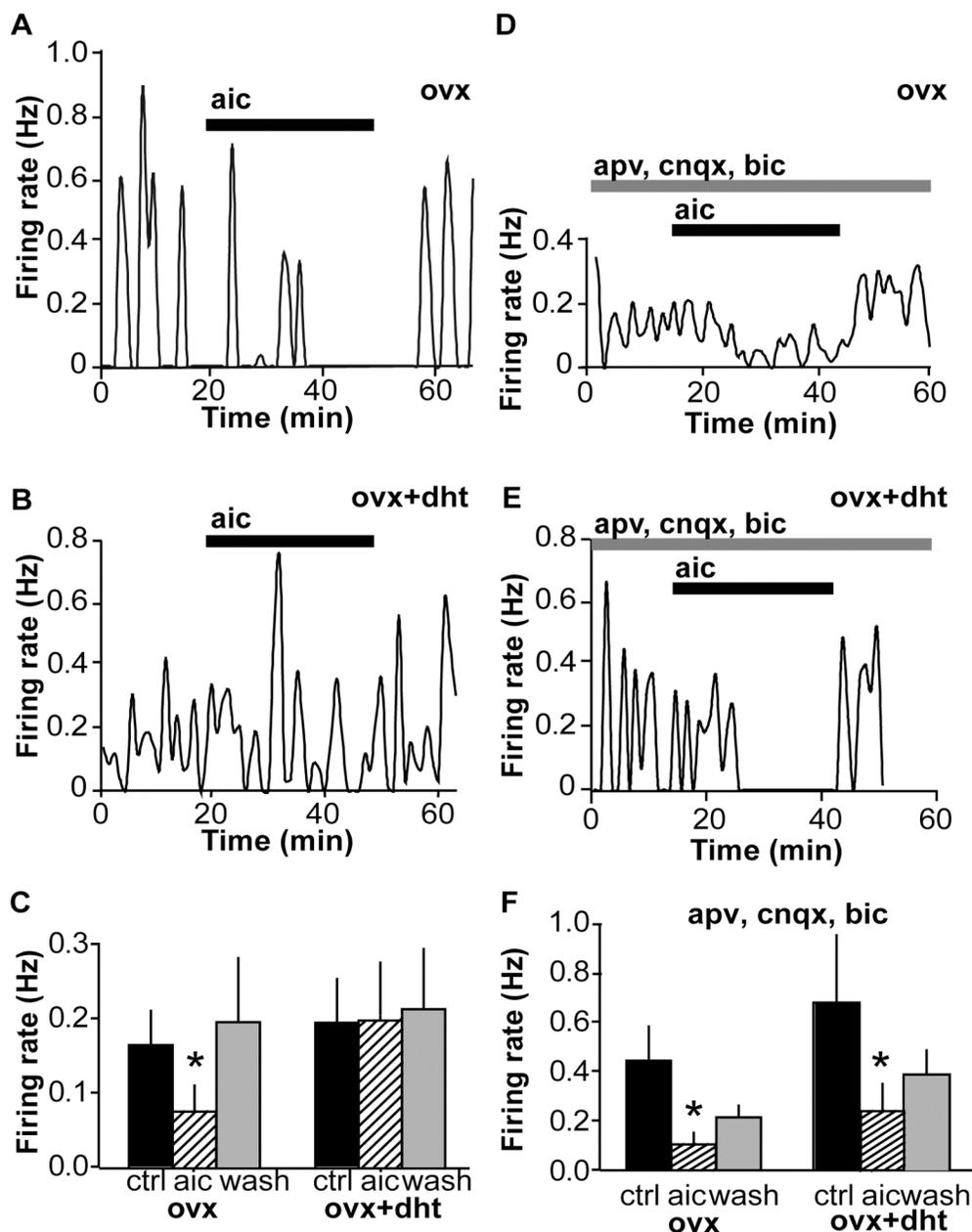


Fig 5. Effects of AICAR on firing activity of GnRH neurons from OVX and OVX+DHT mice. A, Representative plot of firing rate over time in a GnRH neuron from an OVX mouse. Events are binned in 60-s intervals. Black bar indicates time of 2 mM AICAR application. B, Representative plot of firing rate over time in a GnRH neuron from an OVX+DHT mouse. C, Summary of effect of acute AICAR application. Mean + SEM firing rate is shown for control period (black bars), AICAR (striped bars), and washout (grey bars). AICAR reversibly decreased firing rate in cells from OVX ($n=7$) but not OVX+DHT ($n=9$, $p=0.98$) mice. D,E, Firing rate over time in cells from OVX (D) and OVX+DHT (E) mice in the presence of APV, CNQX, and bic. Acute AICAR (black bar) reversibly inhibited firing in both groups in the presence of blockers of receptors for fast synaptic transmission. F, Summary of effect of acute AICAR application in the presence of blockers of fast synaptic transmission. *, $p<0.05$.

AICAR fails to inhibit GnRH neurons from DHT-treated mice

Since DHT rendered GnRH neurons less sensitive to low glucose, and the above data suggest AMPK is a major mediator of the response to low glucose, we hypothesized that GnRH neurons from DHT-treated mice would be less sensitive to the inhibitory effects of AICAR. In fact, the majority of GnRH neurons tested (7/9) were not inhibited by AICAR, and overall AICAR had no statistically significant effect on the firing rate in cells from this group ($p=0.98$, Fig 5B,C). However, similar to low glucose, AICAR inhibited firing in cells from DHT mice in the presence of blockers of fast synaptic transmission ($n=8$, $p<0.05$, Fig. 5E,F). Together these findings suggest the insensitivity of GnRH neurons from DHT-treated mice to low glucose is due to alterations in the presynaptic network.

AICAR and low glucose activate a similar current in GnRH neurons

Whole-cell recordings were employed to identify the membrane changes induced by AICAR or low glucose to generate neuronal inhibition. In voltage-clamp at a holding potential of -60mV , AICAR ($n=19$) activated a net inward current of $6.9\pm 1.5\text{ pA}$ ($p<0.001$, Fig 6A) and reduced input resistance from 0.77 ± 0.08 to $0.66\pm 0.06\text{ G}\Omega$ ($p<0.05$, Fig 6B), indicating an increase in one or more conductances. Similarly, low glucose ($n=8$) activated a net inward current of $-8.5\pm 2.0\text{ pA}$ ($p<0.01$, Fig 6A) and reduced input resistance from 0.84 ± 0.07 to 0.64 ± 0.07 ($p<0.05$, Fig 6B). These effects were at least partially reversible upon washout, assessed in a subset of cells ($p<0.05$ AICAR vs washout; $p<0.01$ low glucose vs glucose restoration). Steady-state current-voltage relationships revealed that both AICAR and low glucose activated a current that reversed around -40 mV (Fig 6C-G); the current was similar in the presence and absence of TTX (Fig F,G). The reversal potential of -40 mV indicated either a mixed

cation or chloride conductance. To assess the latter, in a subset of cells we used an internal solution with a chloride concentration of 140 mM (compared to 20 mM in standard internal). However, changing $[Cl^-]_{in}$ failed to alter the magnitude or reversal potential of the AICAR-activated current (140 mM Cl^- $n=3$, estimated $V_{rev}=-46\pm 5.7$ mV, current at -80 mV= -12 ± 3.2 pA; 20 mM Cl^- $n=19$, estimated $V_{rev}=-41.9\pm 2.3$ mV, current at -80 mV= -10.3 ± 2.1 pA), suggesting AICAR does not activate a chloride conductance. Another possibility was a change in operation of the Na^+/Ca^{2+} exchanger, which has a reversal potential of -40 mV (224). Blocking the Na/Ca exchanger with $10\ \mu M$ SN-6 failed to alter the current ($n=6$, estimated $V_{rev}=-44.6\pm 4.8$ mV, current at -80 mV= -10.8 ± 3.7 pA). $30\ \mu M$ ZD7288, an antagonist of the hyperpolarization-activated cation current I_h , also had no effect ($n=2$, estimated $V_{rev}=-37.5\pm 2.5$ mV, current at -80 mV= -11.8 ± 2.4 pA).

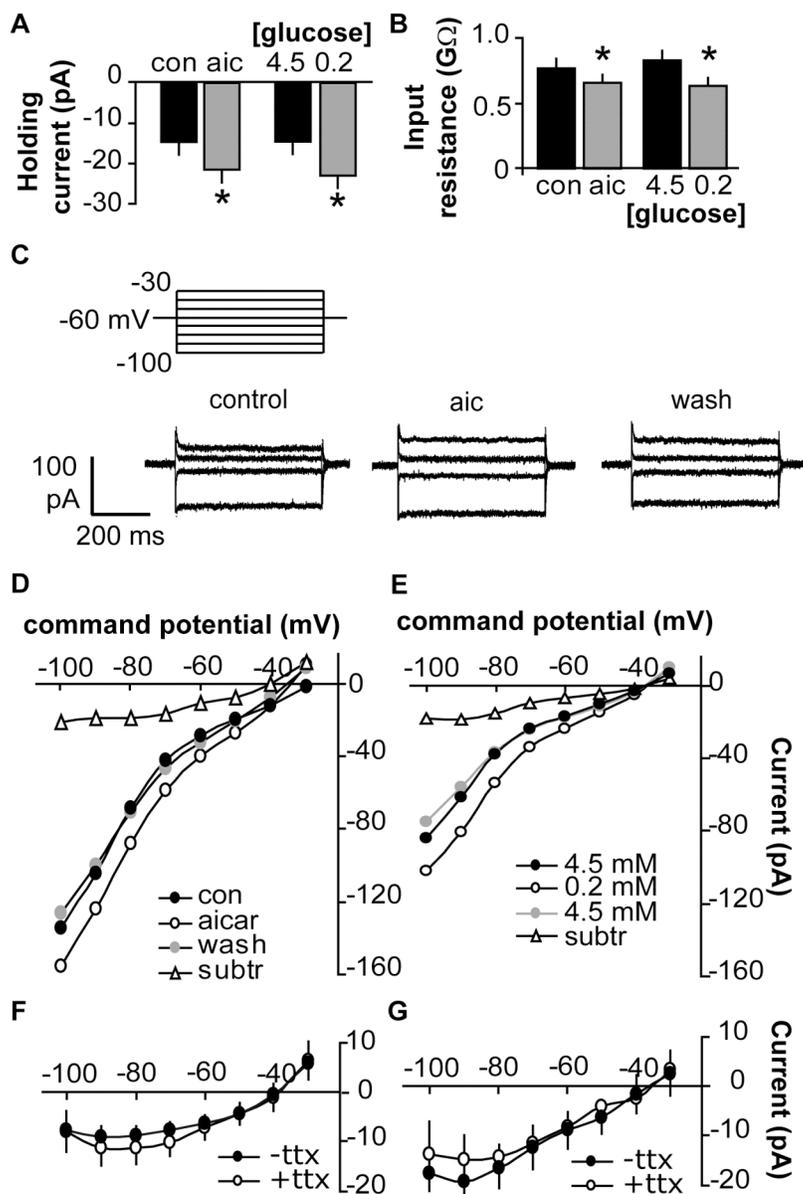


Fig 6. Both AICAR and low glucose activate a nonspecific cation current in GnRH neurons. A. AICAR and low glucose increased holding current at -60mV. *, $p < 0.05$ by paired t test. B, AICAR and low glucose decreased input resistance. *, $p < 0.05$ by paired t test. C, top, step protocol used to generate IV data. Bottom, representative traces before (con) during AICAR (aic), and after ten minutes washout (wash) (every other step shown for clarity). AICAR generated a current with a reversal potential of -40 mV that was at least partially reversible upon washout. D, E, Representative steady-state current-voltage relationships before (closed circles) and after (open circles) AICAR (D) or low glucose (E) and subtracted current (subtr, open triangles). Washout IV is shown in grey. G, Mean \pm SEM AICAR-induced (subtracted) current in cells with ($n=11$) and without ($n=8$) TTX. F, Mean \pm SEM low-glucose-induced (subtracted) current in cells with ($n=4$) and without ($n=4$) TTX. The current was similar in the presence and absence of TTX. *, $p < 0.05$.

Since the whole-cell configuration could potentially alter intracellular ion concentrations or dialyze out critical cell components, we verified that inhibitory effects of AICAR or low glucose persisted in the whole-cell configuration utilizing current-clamp recordings. DC current (0-30 pA) was injected to stimulate tonic firing. AICAR reduced mean firing rate from 0.77 ± 0.07 to 0.16 ± 0.07 Hz ($n=4$, $p<0.05$). Low glucose also reduced mean firing rate from 0.98 ± 0.4 to 0.18 ± 0.14 Hz ($n=5$, $p<0.05$).

Discussion

Numerous studies have indicated a role for glucose in the central regulation of fertility, but only one study to our knowledge has directly examined glucose regulation of GnRH neuron activity. Here we show that GnRH neurons are sensitive to changes in extracellular glucose within the physiological range. Moreover, we have demonstrated a role for AMPK in glucosensing, establishing AMPK as a novel regulator of GnRH neuron activity in brain slices. Androgen-induced changes in fast synaptic transmission appear to render GnRH neurons less sensitive to low glucose. This finding may have implications for fertility disorders such as polycystic ovary syndrome (PCOS) in which elevated circulating androgen and glucose levels are often present (225).

Since they are inhibited by low glucose (and hence fire more in high glucose), GnRH neurons could be classified as glucose-excited (208). However, they appear to employ different mechanisms than the classical hypothalamic glucose-excited neurons involved in control of food intake and energy expenditure, which utilize, at least in part, K_{ATP} channels and glucokinase (208). Although GnRH neurons express functional K_{ATP} channels and glucokinase (52), glucosensing persisted in 100% of tested neurons when

K_{ATP} channels were blocked. This suggests K_{ATP} channels are not the primary mediators of glucosensing in GnRH neurons, and these channels may have other functions in this cell type. This is supported by recent observations that the suppression of LH secretion by fasting is not reversed by tolbutamide or by genetic deletion of the sulfonylurea-1 subunit of the K_{ATP} channel (226). The existence of a distinct mechanism in GnRH neurons is fitting from a physiological standpoint, as GnRH neurons do not require sensitivity to meal-to-meal fluctuations in circulating glucose as do neurons that regulate feeding behavior.

In contrast to K_{ATP} blockade, antagonism of AMPK markedly attenuated inhibition by low glucose in GnRH neurons, suggesting it is an important mediator of glucosensing in this cell type. Accordingly, pharmacological activation of AMPK inhibited GnRH neurons and activated a similar current as low glucose. AMPK has previously been implicated in glucosensing in other types of neurons. In AgRP and POMC neurons, selective genetic deletion of AMPK $\alpha 2$ abolished glucosensing (227). Hypothalamic AMPK plays a role in the counterregulatory response to hypoglycemia (228, 229). In glucose-inhibited neurons of the ventromedial hypothalamus, activation of AMPK by low glucose is excitatory due to closure of cystic fibrosis transmembrane regulator chloride channels (138). Due to high intracellular chloride maintained by GnRH neurons (13), closure of chloride channels near the resting potential in these cells would be inhibitory. However, a 7-fold increase in intracellular Cl^- failed to shift V_{rev} of the AICAR-induced current in GnRH neurons, indicating it is not carried by chloride. In GT1-7 immortalized GnRH neurons, AICAR was shown to block the hyperpolarization-activated cation current, I_h (72). However, I_h is unlikely to be the identity of the current in this study as it is only found in about 40% of GnRH neurons, and ZD7288 had no effect on the AICAR-sensitive current.

AICAR or glucose induced a current in nearly all cells tested, including those not exhibiting I_h , which would be activated by the step protocol used in this study.

A large body of work suggests distal brain regions relay information about glucose availability to GnRH neurons. While these brain areas likely affect the response of GnRH neurons *in vivo*, their influence in this study was minimized by the use of coronal brain sections. Hindbrain neurons are thought to be particularly important for monitoring glucose availability and conveying this information to GnRH neurons (49, 230); however, connections between this brain area and GnRH neurons are severed in coronal slices. Further, GABA_A receptor activation is an important component of hindbrain-mediated glucoprivic suppression of the GnRH-LH axis (135), but the response here was enhanced rather than blocked when fast synaptic transmission was blocked. Also disrupted were synaptic connections to areas controlling feeding (ventromedial, lateral hypothalamus) and corticotropin-releasing factor neurons that mediate stress responses (paraventricular nucleus), the latter of which have been implicated in nutritional suppression of fertility *in vivo* (231). Synaptic terminals that remain intact in the brain slice may continue to influence GnRH neuron activity. These terminals can generate postsynaptic currents in isolated neurons (232), and terminal-level regulation of distal inputs could influence cellular responses. The AICAR-induced current was similar in the presence and absence of TTX, suggesting that action potential-evoked neurotransmitter release by presynaptic neurons does not contribute to this current, but AICAR may alter spontaneous presynaptic vesicle release. Another possibility is that non-neuronal cells, such as glia, contribute to the observed responses. AMPK is expressed in astrocytes where it induces morphological and functional changes in response to energy depletion (233, 234). However, the existing model of glial-dependent glucosensing involves

closure of neuronal K_{ATP} channels by glial-generated lactate (235), which is unlikely since the effect in GnRH neurons is K_{ATP} -independent. While our data do not definitively indicate AMPK signaling within the GnRH neuron, they strongly suggest this possibility.

Detection of metabolic cues by the reproductive system is critical to ensure that energy is sufficient for reproduction. This is particularly true for females, for whom reproduction and subsequent lactation are exceptionally energetically expensive. In this regard, reproductive effects of negative energy balance are sexually dimorphic, with males being less sensitive to suppressive effects of caloric restriction on fertility (140), and less sensitive to glucoprivic suppression of LH (47). These observations are consistent with the effect of androgen to prevent the suppression of GnRH neuron activity by low glucose. The present findings are also relevant to PCOS, in which women have high androgens and, due to impaired glucose disposal, elevated blood glucose levels. Increased glucose alone may enhance activity of GnRH neurons, contributing to hyperactivity of the GnRH-LH axis in this disorder. Hyperandrogenemia in PCOS may compound this effect by reducing AMPK-mediated inhibition. However, the effect of androgen may be mitigated by estradiol, also present in women with PCOS. Local changes in steroid levels due to central synthesis could also affect this response (236).

Androgen appears to inhibit glucosensing in part through an effect on presynaptic neurotransmission. Although blockade of fast synaptic transmission restored glucosensing to cells from OVX+DHT mice, the latency of the response differed in comparison to cells from OVX mice. Responses to AICAR were similar in cells from OVX and OVX+DHT mice during fast synaptic blockade. Combined, these data suggest possible differences in kinetics of glucose metabolism upstream of AMPK in cells from

DHT-treated mice. The mean basal firing rate in DHT cells is similar to the post low-glucose firing rate in the other groups, suggesting that the failure of low glucose to inhibit GnRH neurons from DHT-treated mice may be due, in part, to a “basement effect.” GnRH neuron activity in cells from DHT-treated mice may be suppressed in the range of 10 to 4.5 mM glucose, whereas sensitivity in other steroid treatment groups may be predominantly between 4.5 and 0.2 mM. Subpopulations of glucose-excited neurons in the ventromedial hypothalamus (VMH) exhibit different ranges of glucose sensitivity (237); those responding to glucose concentration changes in higher ranges are termed “high-glucose excited,” and the disparity has been attributed to differences in underlying mechanisms (237). Insulin has been shown to blunt the response of glucose-excited neurons in the VMH to low glucose (238), raising the possibility that androgen itself or androgen-induced changes in circulating factors may similarly alter the response in GnRH neurons. In this regard, leptin and melanocortin have been shown to suppress neuronal AMPK activation (75), whereas adiponectin (76) and ghrelin (77) enhance it.

The finding that low glucose or AICAR activated a nonspecific cation current was surprising, given that activation of this type of current is typically excitatory. Several possibilities may explain this observation. First, there is increased outward current at potentials positive to -40 mV. Another possibility is shunting inhibition, since the reversal potential is relatively close to the resting potential. The reduction in membrane resistance by AICAR or low glucose would require increased current to generate an action potential. Further studies are needed to clarify the mechanisms by which the observed current generates neuronal inhibition.

Overall, these studies have provided novel insight into the longstanding question of how the reproductive system senses low fuel availability, and support the idea that AMPK is

critical for neuronal glucosensing. The regulation of GnRH neuron activity by AMPK has implications for the effects of other metabolic cues that are known to interact with this kinase, opening up new areas for future research.

Chapter 4

Prenatal androgenization of female mice causes an increase in firing activity of gonadotropin-releasing hormone (GnRH) neurons that is reversed by metformin

Alison V. Roland and Suzanne M. Moenter

Abstract

Prenatal androgenization (PNA) of female mice with dihydrotestosterone recapitulates aspects of the fertility disorder polycystic ovary syndrome (PCOS). Similar to women with PCOS, PNA mice exhibit elevated luteinizing hormone levels and irregular reproductive cycles. Here we evaluated activity of GnRH neurons from PNA mice and the effects of in vivo treatment with metformin, which can activate adenosine monophosphate-activated kinase (AMPK) and can improve menstrual cyclicity in PCOS. Estrous cycles were monitored in PNA and control (CON) mice before and after metformin administration. Pre-metformin, estrous cycles were longer in PNA mice, and percent in estrus lower; metformin normalized cycles in PNA mice. Extracellular recordings were used to monitor GnRH neuron firing activity in brain slices from diestrous mice. Firing rate was higher and quiescence lower in GnRH neurons from PNA mice, consistent with increased GnRH neuron activity. In metformin-treated PNA mice, firing activity and LH levels were restored to CON levels. To assess if activation of AMPK contributes to this reduction in GnRH neuron activity, the AMPK antagonist compound C (CC) was acutely applied to cells. CC stimulated cells from metformin-treated, but not untreated mice, suggesting AMPK is activated in GnRH neurons, or afferent neurons, in metformin-treated mice. GnRH neurons from metformin-treated mice also showed less of an inhibitory response to low glucose. These studies indicate PNA causes enhanced firing activity of GnRH neurons and elevated LH that are reversible by metformin, and central AMPK activation by metformin may play a role in its restoration of reproductive cycles in PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive disorder in women defined by the presence of two of three of the following characteristics: oligo- or anovulation, clinical or biochemical evidence of hyperandrogenemia, and polycystic ovaries (6). Research into the underlying pathophysiologic mechanisms of this disorder has suggested increased central drive to the reproductive system demonstrated by increased LH pulse frequency and amplitude (7, 239-241), as well as impaired central steroid negative feedback (241-243). Metabolic abnormalities are remarkably common in PCOS and may participate in the underlying pathology in some cases, but are not prerequisite for the syndrome (6).

Our lab and others have generated animal models that recapitulate aspects of PCOS by exposing female animals to excess androgens *in utero* (32, 34, 244, 245). Prenatal androgenization of female mice with DHT causes hyperandrogenemia and irregular estrous cycles (36, 246). PNA mice also have elevated LH levels, similar to women with PCOS (247), and increased GABAergic neurotransmission to GnRH neurons (36), both of which are consistent with increased central reproductive drive (13). This is also consistent with studies in rats and sheep showing that androgen exposure in utero masculinizes activity of the GnRH pulse generator, generating a chronically high GnRH/LH pulse frequency (34, 248, 249). In terms of metabolism, PNA mice exhibit impairments in glucose tolerance and pancreatic islet function, but do not exhibit obesity or peripheral insulin resistance, suggesting that additional factors may be necessary to drive the full development of PCOS-related metabolic phenotypes (246). Disrupted reproductive cyclicity in PNA mice is therefore likely driven in large part by androgen programming of the central GnRH pulse generator.

Metformin is a biguanide anti-hyperglycemic agent frequently employed to treat women with PCOS, who often exhibit insulin resistance (146, 250, 251). In addition to its metabolic effects, metformin can improve menstrual cyclicity, reduce hyperandrogenemia, and increase the rate of ovulation in PCOS (18-22). The predominant line of thinking is that insulin sensitization, and a subsequent reduction in insulin levels, is the primary mechanism by which metformin exerts these reproductive system effects. Because insulin stimulates ovarian androgen production (23, 252, 253) and suppresses sex hormone binding globulin (254, 255), a reduction in insulin levels by metformin can potentially lower both total and free androgen levels, attenuating reproductive abnormalities driven by hyperandrogenemia (16, 18, 22, 23). However, recent studies have shown that metformin can improve PCOS symptoms in normoinsulinemic women (24-27), suggesting it may have reproductive effects that are independent of insulin sensitization. Recently, one mechanism of action of metformin was identified to be activation of AMP-activated protein kinase (AMPK) (222). AMPK is widely expressed and exhibits functional roles in metabolic tissues (muscle, liver, pancreas, adipocytes) (221), as well as the ovary (256-259), pituitary (260), and hypothalamus (75, 77, 261-264), suggesting that diverse mechanisms may account for the reproductive effects of metformin. Because PNA mice are not insulin resistant or hyperinsulinemic (246), they serve as a unique model to assess the reproductive effects of metformin that are independent of its insulin-lowering effects. Specifically, we can study the effects of metformin at the hypothalamic level by directly recording the activity of green fluorescent protein-identified GnRH neurons (204).

While it is not possible to assess the effects of metformin on hypothalamus directly in humans, studies have shown that LH pulse amplitude and LH levels are reduced by

metformin (265-267), which may be a consequence of reduced hypothalamic GnRH release. Recent work by our lab (268) and others (269) has demonstrated an inhibitory role of AMPK in GnRH neuron regulation. AMPK activation inhibits GnRH release from immortalized GnRH neurons (269) and inhibits firing of GnRH neurons in brain slices from mice (268), suggesting possible effects of metformin on GnRH neurons. It is unknown if metformin crosses the blood brain barrier; however, multiple studies have demonstrated AMPK phosphorylation in the brain following peripheral metformin administration (263, 270). Moreover, GnRH neurons and their terminals reside near the organum vasculosum of the lamina terminalis and external layer of the median eminence, which lack a blood brain barrier (271, 272). This suggests that GnRH neurons, their terminals, or their afferents may be exposed to metformin irrespective of its blood brain barrier penetrability. Thus metformin reduction of GnRH neuronal activity may suppress the increased central reproductive drive that occurs in PNA mice and PCOS, contributing to a normalization of reproductive cycles.

This study had three aims. First, we tested if metformin could restore estrous cyclicity to PNA mice. Second, we assessed GnRH neuron activity in PNA mice, which has not been previously characterized, and whether it was altered by metformin in a manner consistent with changes in estrous cycles. Third, we tested if GnRH neurons showed changes consistent with AMPK activation following systemic metformin administration.

Materials and Methods

Generation of PNA mice

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Adult (2-4 mo) female GnRH-GFP transgenic mice were used to generate PNA mice. Mice were housed under a 14 h light:10 h dark cycle with chow (2916, Harlan, Indianapolis, IN) and water available ad libitum. Females were paired with males and checked for copulatory plugs. The date of plug was considered day 1 of gestation. Pregnant mice were injected daily subcutaneously with 50 μ l sesame oil containing 250 μ g of dihydrotestosterone (DHT) on days 16, 17, and 18 of gestation. Control mice (CON) were offspring of oil-injected dams or untreated mice; no differences were observed between these, and they were combined for analysis. All procedures were approved by the University of Virginia Animal Care and Use Committee and conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Estrous cycle monitoring

Beginning at eight weeks of age, estrous cycles were monitored by vaginal cytology for 16 d prior to metformin administration, for the first 16d of administration, and during the 8th-10th week of administration. Mean cycle length was the average estrus-to-estrus interval; for mice that did not enter estrus, 16 days was used as cycle length, although actual length may have been longer.

Metformin administration

A schematic indicating the time of metformin administration relative to physiological assessments is shown in Figure 1. Mice from each litter were randomized to receive

either metformin or regular water. Metformin (1,1-dimethylbiguanide hydrochloride) was administered in drinking water at a dose of 2.5 mg/mL. Based on measured water consumption of ~4 mL/d by a 25 g mouse, this equates to approximately 400 mg/kg/d. This is approximately ten times the maximum human dose (273); however, similar high doses have been used in mice and may be necessary to induce metabolic changes in this species (274-276). We previously used this dose to reverse the effects of a high-fat diet on the onset of puberty in mice, without affecting growth or food and water intake (277). No differences in water consumption were measured between metformin-treated and untreated mice in this study (data not shown).

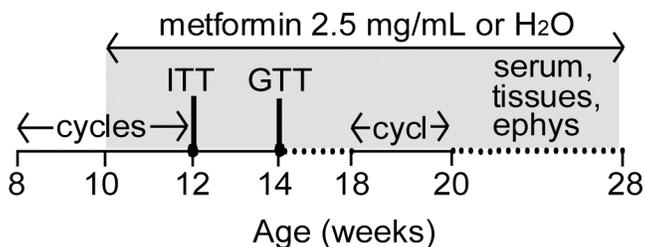


Figure 1. Timeline of metformin administration and physiological assessments.

Insulin tolerance tests (ITT)

ITTs were performed two weeks after the start of metformin administration. Studies were performed 10 hours after lights-on in singly-housed fed mice. Although mice had free access to food prior to testing, their active (feeding) period normally ends at the time of lights-on. Following an initial glucose measurement, mice were injected intraperitoneally with a bolus of 0.75 U/kg of insulin (Novolin R, Novo Nordisk, Denmark) in sterile 0.9% NaCl. Blood glucose was determined at 10, 20, 30, 45, 60, and 75 minutes post injection, and percent change from baseline calculated for each time point.

Glucose tolerance tests (GTT)

GTTs were performed after a two-week recovery period from ITTs (four weeks after initiation of metformin). Mice were singly housed on Sani-Chips (Harlan, Indianapolis, IN) and fasted overnight for sixteen hours (1600h to 0800h) prior to the test. The tail was anesthetized with the skin refrigerant ethyl chloride (Gebauer, Cleveland, OH) and the tip removed with a sterile scalpel blade. Tail blood (~1 μ l/ sample) was collected for glucose measurement with a OneTouch Ultra glucometer (Lifescan, Milpitas, CA). Following a fasting glucose measurement, mice were injected intraperitoneally with a bolus of 1g/kg glucose in 0.9% NaCl. Blood glucose was assessed at 10, 20, 30, 45, 60, 75, 90, and 120 minutes thereafter.

Brain slice preparation

Mice were euthanized on diestrus between 0900h and 1100h (5-7 hours after the time of lights-on). Blood glucose, body mass, parametrial fat mass, and uterine mass were recorded for all mice. Brain slices were prepared with slight modifications of previously described methods (205, 278). Briefly, the brain was rapidly removed and placed in ice-cold high-sucrose saline solution containing (in mM) 250 sucrose, 3.5 KCl, 26 NaHCO₃, 10 D-glucose, 1.3 Na₂HPO₄, 1.2 MgSO₄, and 3.8 MgCl₂. Coronal (300 μ m) slices were cut with a Vibratome 3000 (Ted Pella, Inc., Redding, CA). Slices were incubated for 30 min at 30–32 C in 50% high-sucrose saline and 50% artificial cerebrospinal fluid (ACSF) containing (in mM) 135 NaCl, 3.5 KCl, 26 NaHCO₃, 0 D-glucose, 1.25 Na₂HPO₄, 1.2 MgSO₄, 2.5 CaCl₂ (pH 7.4). Slices were then transferred to 100% ACSF solution (glucose adjusted to 5 mM) at room temperature (~21–23 C) for 0.5–5h.

Electrophysiological recordings

Targeted single-unit extracellular recordings (loose patch) were used in this study because this configuration minimally affects the intrinsic properties of the cell, including glucose metabolism, during long-term recordings (279). Recording pipettes (1–3 M Ω) were filled with normal HEPES-buffered solution, and low-resistance (<50 M Ω) seals were formed between the pipette and the GnRH neuron. Recordings were made in voltage-clamp mode with the pipette holding potential at 0 mV and signals were filtered at 10 kHz. Experiments were performed using an EPC 10 amplifier and Patchmaster software (HEKA Electronics, Lambrecht/Pfalz, Germany). Cells targeted for recording were located in preoptic and septal areas of hypothalamus. In some cells, the AMPK antagonist compound C (40 μ M) was acutely applied for ten minutes following a 45-60 minute baseline recording. In another subset of cells, the effect of a 10-min application of 0.2 mM glucose was tested following a 10-min control recording period.

Hormone measurements

All hormones were measured in serum from random-fed mice exhibiting diestrous vaginal cytology on the day of euthanasia. LH was measured using a two-site sandwich immunoassay with a sensitivity of 0.07 ng/ml (280-282). Testosterone was measured in serum using a radioimmunoassay kit according to the manufacturer's instructions (Siemens Medical Solutions, cat# TKTT2, Los Angeles, CA). Sensitivity was 7.5 ng/dl, and the intra- and inter-assay CVs were 4.4 and 8.1%, respectively. An adipokine panel was used to assess insulin, leptin, IL-6, TNF- α , PAI-1, and resistin (Millipore mouse serum adipokine kit, cat# MADPK-71K-07). Sensitivity was 12.2 pg/mL for resistin, 195 pg/mL for insulin, 48.8 pg/mL for leptin and PAI-1, 2 pg/mL for TNF- α , and 5 pg/mL for IL-6. Intra- and inter-assay CV were <10% for all analytes. Adiponectin was measured

via radioimmunoassay (Millipore, cat# MADP-60K); assay sensitivity was 1.3 ng/ml and inter- and intra-assay CVs were <9% and <5%, respectively.

Analysis and statistics

PNA and CON mice with body mass >2 standard deviations from the mean (greater than 29 g) were excluded from analysis of electrophysiology data to avoid possible confounding effects of excess adiposity on the function of GnRH neurons. (Mean firing rate of GnRH neurons in control mice <29 g: 0.11 ± 0.04 Hz, n=17; in control mice >29g: 0.22 ± 0.09 Hz, n=9; $p < 0.08$). The percent change in response to compound C was calculated and compared among groups using two-way ANOVA. For effects of low glucose, one-way repeated measures ANOVA with Dunnett's Multiple Comparison test was used to compare treatment versus control frequencies. ANOVA with Bonferroni or Duncan's post-hoc testing was used for all other comparisons. Data are reported as mean \pm SEM. Significance was set at $p < 0.05$.

Results

Metformin has minimal effects on metabolic parameters in PNA mice

Because metformin's primary application is as an anti-hyperglycemic in diabetic patients, we first assessed metabolic parameters in metformin-treated mice. Food intake and body mass were not different between control and PNA mice and were unaffected by metformin (n=11-12 per group, Figure 2A,B). Parametrial fat mass/body mass ratio (determined at euthanasia) was similar in control and PNA mice and unchanged by metformin (control, n=24; PNA, n=24; control metformin, n=21; PNA metformin, n=21, Figure 2C). Random-fed blood glucose levels did not differ between among groups, but

fasting glucose was reduced by metformin in PNA mice ($p < 0.05$, Figure 2D). This is consistent with the drug's primary mechanism of action to inhibit hepatic gluconeogenesis (283), and indicates that an effective dose was used. Insulin and glucose tolerance tests were performed after two and four weeks, respectively, of metformin administration. Metformin did not significantly alter either glucose disposal or insulin sensitivity in PNA mice ($n = 10$ mice per group, $p > 0.05$, Figure 2E,F). The glucose profiles for metformin-treated PNA and CON mice were intermediate between that of untreated PNA and CON, but not significantly different from either. No differences were noted among groups in the ITT. Importantly for the present study, metformin did not appear to alter peripheral insulin sensitivity in PNA animals. Further, an adipokine panel in serum collected from random-fed mice at the time of euthanasia showed no differences in adipokine or insulin levels ($n = 11-12$ mice per group, Table 1). The data used to generate ITT curves for untreated CON and PNA mice were reported previously (246), as were adiponectin values from untreated CON and PNA mice.

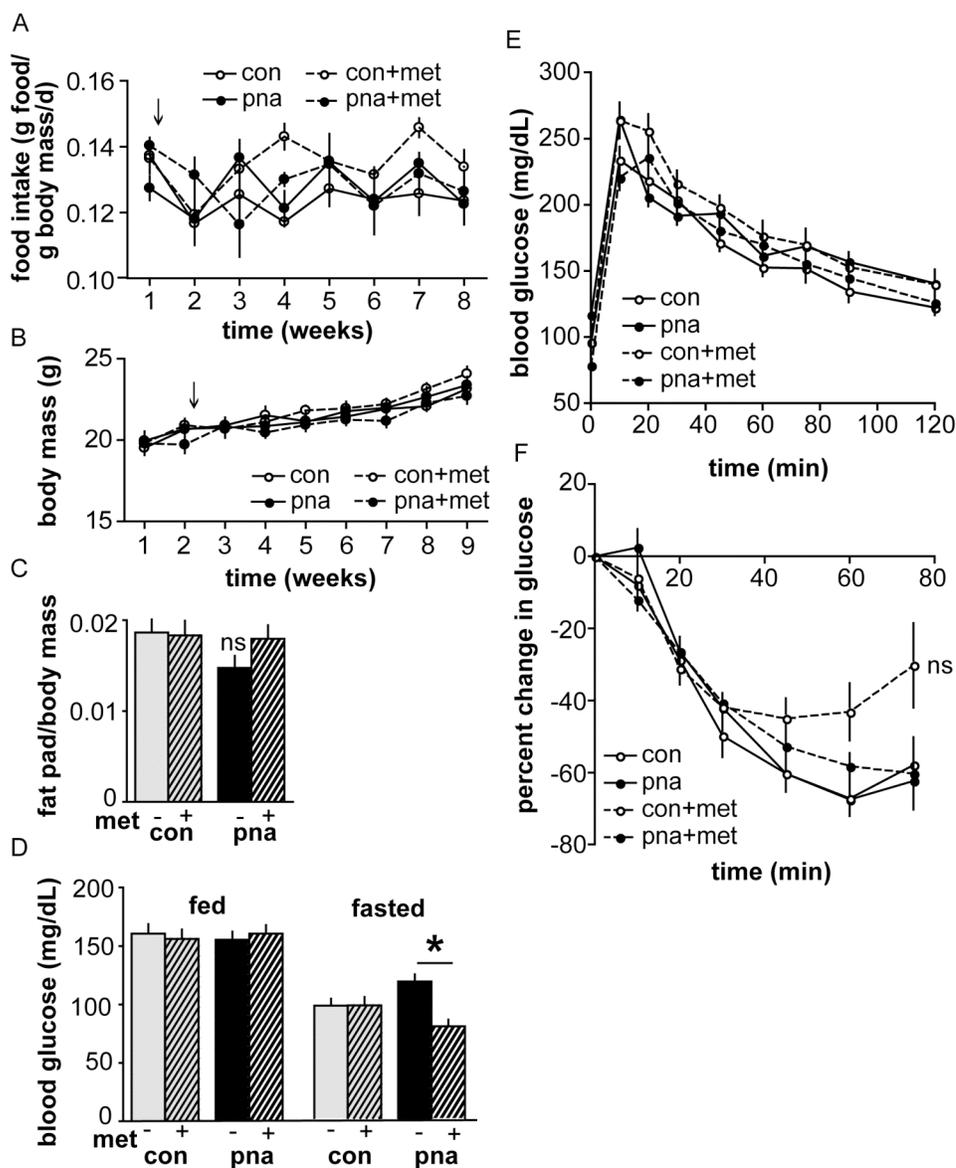


Figure 2. Metformin has minimal effects on metabolic parameters in PNA mice. A, Average daily food intake per unit body mass was similar in PNA and CON mice and unaffected by metformin (n=11-12 per group). Arrow indicates start of metformin administration to treated groups. B, Body mass was unaffected by metformin (n=11-12 per group). Arrow indicates start of treatment. C, Ratio of parametrial fat pad mass to body mass was not different among groups (n=21-24 per group). D, Blood glucose levels in random-fed (n=21-24 per group) and fasted (n=10 per group) mice. Metformin significantly decreased fasting blood glucose in PNA mice. E, Glucose tolerance test indicated no difference in glucose tolerance among groups (n=10 per group). F, Insulin tolerance testing showed no differences in insulin sensitivity (n=10 per group). *, p<0.05. ns, not significant. ITT curves from untreated CON and PNA mice were generated from data reported previously (246).

	CON	CON+met	PNA	PNA+met
Adiponectin (ng/mL)	16.2 ± 0.7	14.3 ± 1.2	14.0 ± 1.0	14.8 ± 0.2
Insulin (ng/mL)	1.3 ± 0.2	1.9 ± 0.4	1.7 ± 0.4	1.1 ± 0.2
Leptin (ng/mL)	2.5 ± 0.4	3.2 ± 0.6	1.7 ± 0.6	2.2 ± 0.5
TNF-α (pg/mL)	5.2 ± 0.4	5.3 ± 0.4	5.3 ± 0.7	4.1 ± 0.2
IL-6 (pg/mL)	16.8 ± 2.3	21.0 ± 3.0	28.2 ± 7.1	17 ± 2.7
Resistin (ng/mL)	2.0 ± 0.2	2.3 ± 0.2	1.7 ± 0.2	2.0 ± 0.1
PAI-1 (ng/mL)	10.2 ± 0.9	9.3 ± 0.8	8.7 ± 1.5	9.2 ± 0.9

Table 1. Mean \pm SEM serum insulin and adipokine levels in random-fed diestrous mice. Adiponectin levels in untreated CON and PNA mice were reported previously (246).

Metformin restores estrous cyclicity to PNA mice

Estrous cycles were monitored before and after metformin administration in individual mice and quantified over a 16-d period. In accordance with previous observations (36, 246), PNA mice exhibited abnormal estrous cycles prior to metformin administration (Figure 3A). Cycles were lengthened, with PNA mice rarely entering estrus (Figure 3B,C). After ten weeks of metformin administration, cycles in PNA mice were normalized, with mean cycle length and percent of time in estrus no longer different from control (Figure 3B-C, n=10 mice per group, p<0.05). In a subsequent study, cycles were monitored before and during metformin administration without interruption by metabolic testing. Significant differences in cycle duration and time in estrus were apparent by four weeks of treatment (n=11-12 mice per group, data not shown). Cycle parameters did not change over time in mice not treated with metformin (not shown).

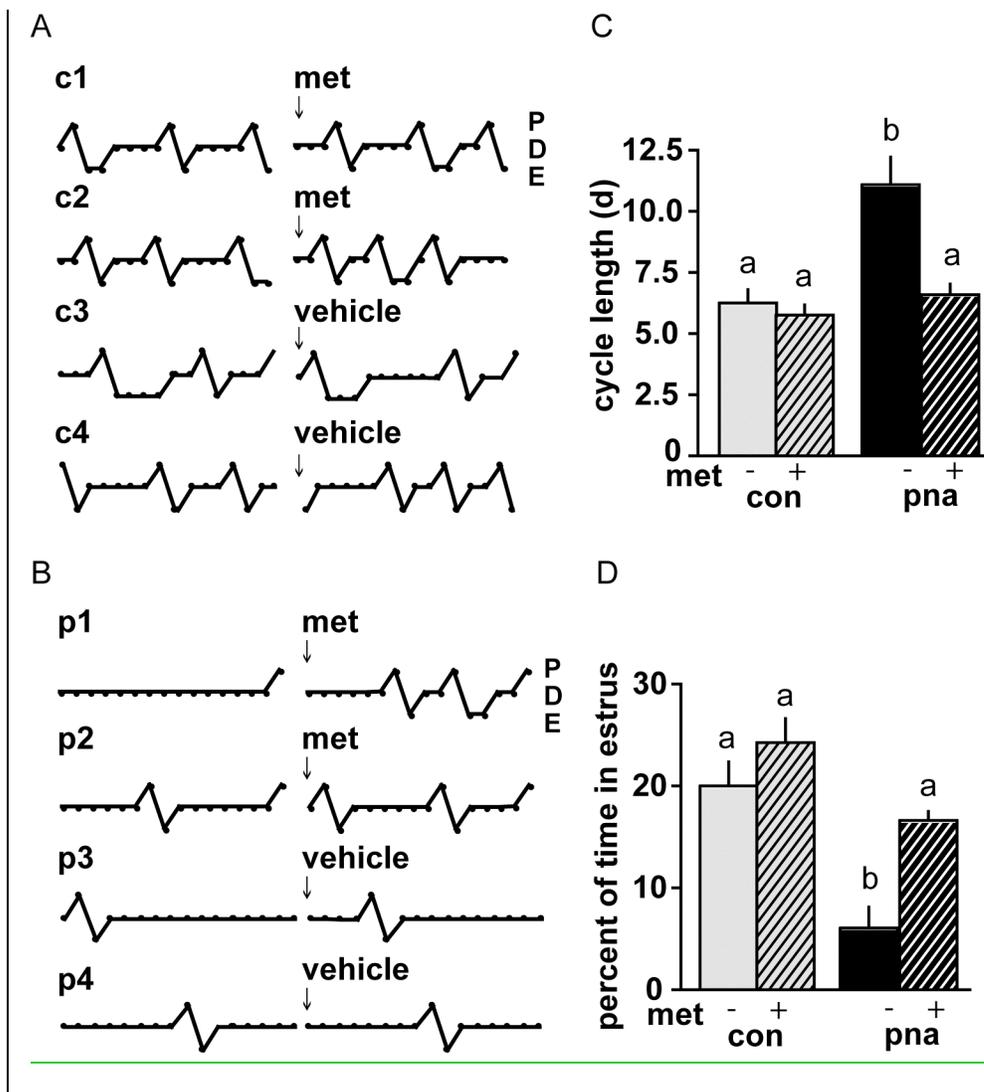
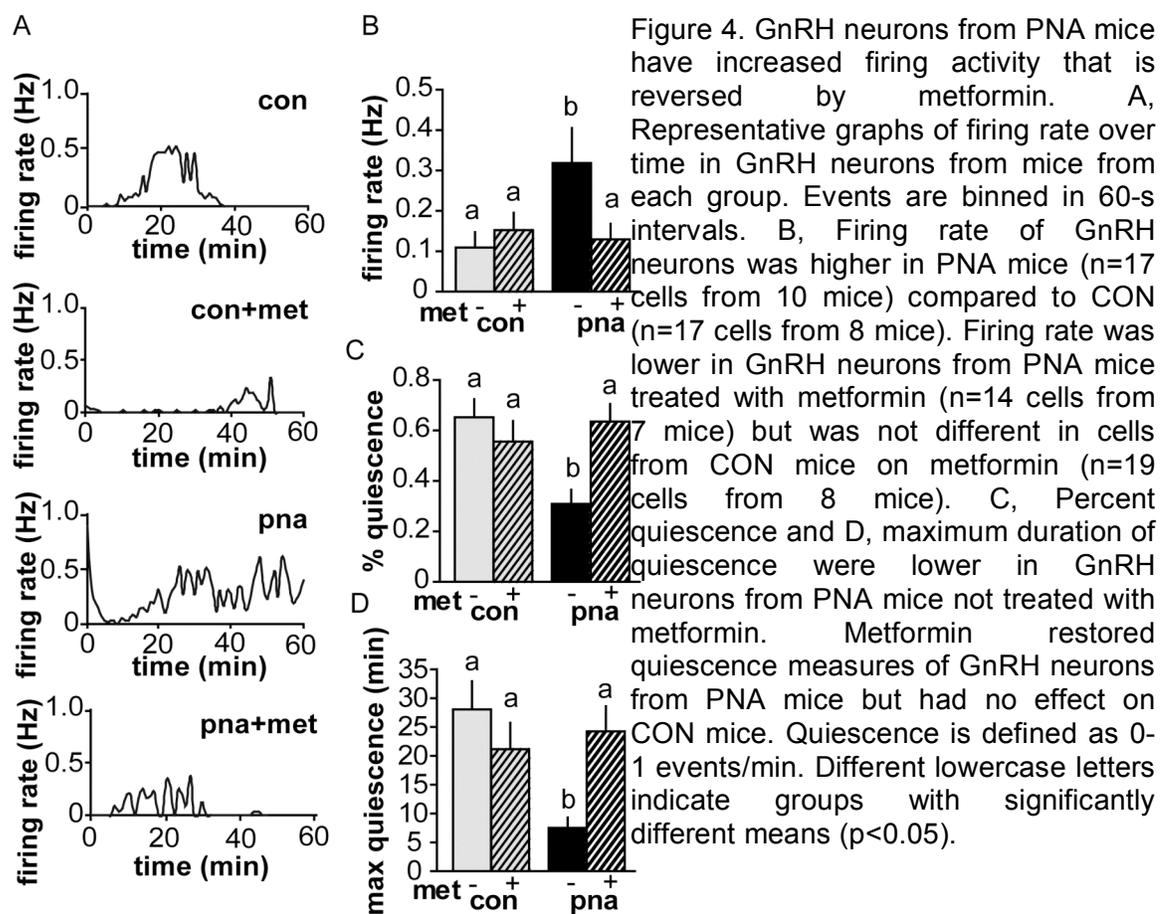


Figure 3. Metformin improves estrous cycles in PNA mice. A,B, Representative estrous cycle plots from met- and vehicle-treated CON (A) and PNA (B) mice. Daily cycle stage designated as diestrus (D), proestrus (P), or estrus (E). C, Estrous cycle length was significantly longer in PNA mice prior to metformin, but restored to control levels following treatment (n=10 per group). D, Percent of time in estrus was significantly lower in PNA than CON mice, but no longer different after metformin. Different lowercase letters indicate groups with significantly different means ($p < 0.05$).

PNA increases firing activity of GnRH neurons

In contrast to most mature neurons, in which GABA_A receptor activation is inhibitory, due to high intracellular chloride maintained by GnRH neurons, GABA_A receptor activation

excites these cells (13). Because GABAergic transmission to GnRH neurons and LH levels were previously shown to be increased in PNA mice (36), we hypothesized that firing activity of GnRH neurons from PNA mice would be elevated. Using extracellular recordings, we assessed firing activity of GnRH neurons from PNA mice. Mean firing rate was increased, and percent and maximum duration of quiescence were decreased in GnRH neurons from PNA mice (Figure 4, control n=17 cells from 8 mice, PNA n=17 cells from 10 mice, $p < 0.05$ frequency and $p < 0.01$ % and max duration quiescence). Firing activity is associated with hormone secretion in other neuroendocrine systems (284), suggesting that GnRH release may be elevated in PNA mice.



Metformin reverses the increase in firing activity of GnRH neurons from PNA mice

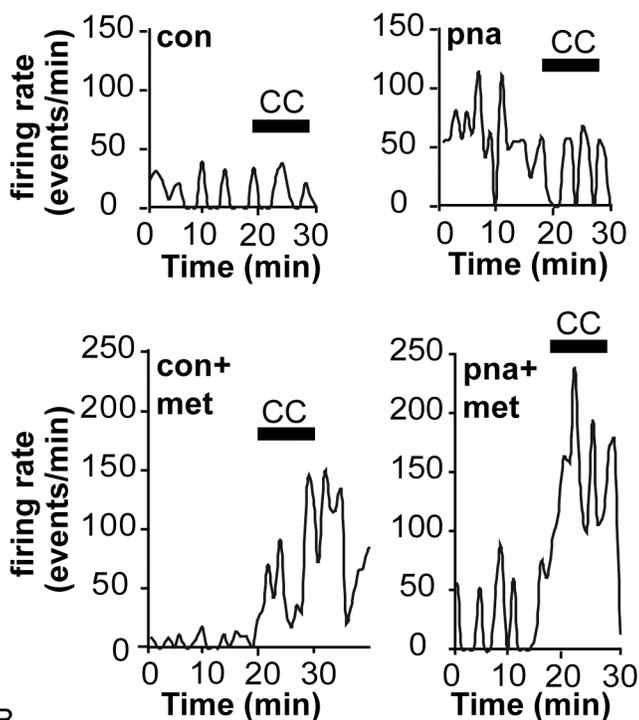
To test if the normalization of estrous cyclicity in PNA mice by metformin treatment could be attributed to altered GnRH neuron activity, we examined firing rate in animals treated with metformin. Metformin treatment normalized firing activity and quiescence measures in GnRH neurons from PNA mice, without altering firing parameters of GnRH neurons from control mice (Figure 4, metformin control n=19 cells from 8 mice; metformin PNA n=14 cells from 7 mice, $p<0.05$ frequency and $p<0.01$ quiescence measures vs PNA). This suggests that a normalization of GnRH neuron activity by metformin may have contributed to restoration of estrous cyclicity in PNA mice.

GnRH neurons from metformin-treated but not untreated mice respond to an AMPK antagonist

To test if AMPK is activated in GnRH neurons from metformin-treated mice, we used the specific AMPK antagonist compound C (CC). Bath application of CC caused an increase in firing in GnRH neurons from metformin-treated, but not untreated, CON and PNA mice (Figure 5, control n=10 cells from 5 mice; PNA n=8 cells from 3 mice; control metformin, n=10 cells from 4 mice; PNA metformin, n=11 cells from 5 mice; $p<0.05$ main effect of metformin by 2-way ANOVA). Although post-hoc testing did not find a difference in mean percent response to CC between untreated and metformin-treated PNA, the percent of cells that responded with a $>20\%$ increase in firing was statistically higher in the latter group (PNA 1/8 or 12.5% vs PNA metformin 10/11 or 90.9%, $p=0.001$ by Fisher's exact test). The increase in percent responders was also higher in metformin-treated vs untreated control (control 2/10 or 20% vs control metformin 8/10 or 80%, $p=0.023$). Combined with the main effect of metformin to enhance the response to CC, these data suggest that metformin renders GnRH neurons responsive to CC, consistent with AMPK

activation in GnRH neurons or their afferents as a result of metformin administration. AMPK activation may contribute to the reduced firing activity of GnRH neurons from metformin-treated mice, as AMPK activation inhibits GnRH neuronal activity (268).

A



B

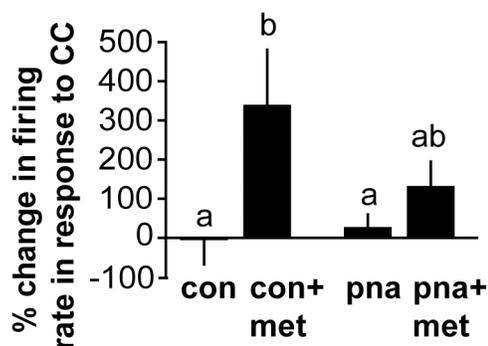


Figure 5. An AMPK antagonist stimulates firing in metformin-treated but not untreated mice. A, Representative plots of firing rate over time from each group. Black bar indicates time of compound C (CC) application. CC induces an acute increase in firing in GnRH neurons from metformin-treated mice. B, Percent change in firing rate in response to CC application. Firing was increased in GnRH neurons from both CON and PNA mice treated with metformin, but not untreated mice (CON n=10 cells from 5 mice; PNA n=8 cells from 3 mice; CON metformin, n=10 cells from 4 mice; PNA metformin, n=11 cells from 5 mice). Different lowercase letters indicate groups with significantly different means ($p < 0.05$).

Glucosensing is attenuated in GnRH neurons from metformin-treated mice

Recent work in our lab has demonstrated a critical role for AMPK in glucosensing by GnRH neurons (268). AMPK is activated by low glucose, inhibiting firing activity. We

hypothesized that in metformin-treated mice, AMPK is already activated; thus the inhibitory response to low glucose may be diminished. To test this hypothesis, firing activity was recorded for a control period in 5 mM glucose and following a switch to 0.2 mM glucose for ten minutes. All cells from untreated PNA and CON mice were markedly inhibited by low glucose (Figure 6, control, n=8 cells from 5 mice, p=0.003; PNA, n=8 cells from 4 mice, p=0.0001). However, cells from metformin-treated PNA and CON mice were less sensitive to low glucose, with the majority failing to show inhibition (Figure 6, metformin control, n=6 cells from 3 mice, p=0.4; metformin PNA n=8 cells from 3 mice, p=0.4). Basal firing rate in 5 mM glucose did not differ among groups in this experiment, although there was a trend for metformin to reduce firing rate (two-way ANOVA, p=0.08 main effect of metformin). It is important to point out that firing rates in this experiment differ from those in longer-term recordings (Figure 4) since here only cells that were actively firing at the onset of recording were used to enable us to see a potential suppression; previously all cells were included (if verified to be viable) to record the native firing activity. In conjunction with the above data using the AMPK antagonist, these experiments support the hypothesis that AMPK in GnRH neurons (or in the network controlling their activity) is activated by peripheral metformin administration.

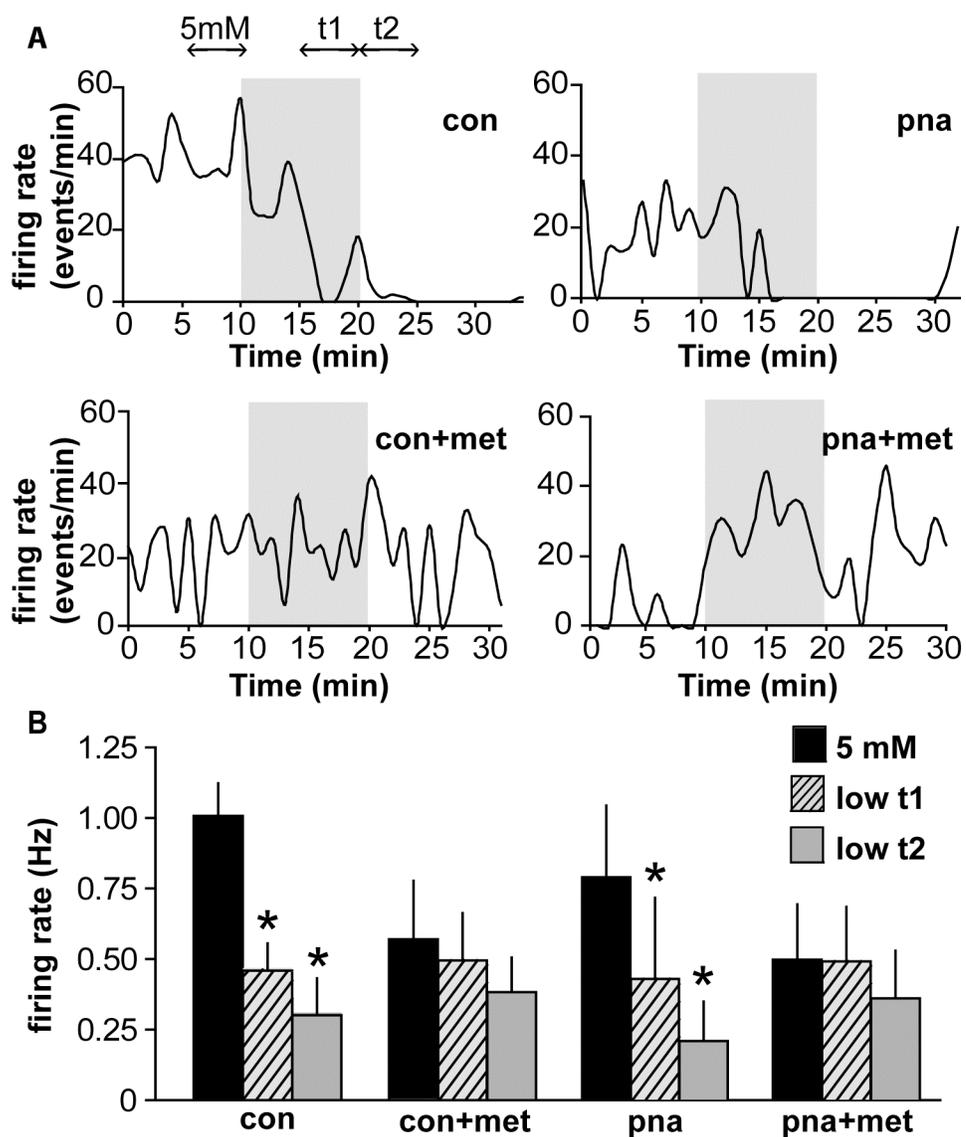


Figure 6. Glucosensing is attenuated in GnRH neurons from metformin-treated compared to untreated mice. A, Representative graphs of firing rate over time. Shaded region indicates period of low (0.2 mM) glucose application. Double-headed arrows in first graph indicate periods during which frequency was averaged for analysis. B, Firing rates in 5 mM glucose, and during time intervals t1 and t2 post low-glucose application. Firing rate was significantly reduced by low glucose in GnRH neurons from untreated CON and PNA mice, but not those treated with metformin (CON n=8 cells from 5 mice; PNA n=8 cells from 4 mice; CON metformin n=6 cells from 3 mice; PNA metformin n=8 cells from 3 mice). *, $p < 0.05$ vs 5 mM.

Metformin reduces LH in PNA mice but does not alter androgen levels

Hormones were measured in serum samples taken at the time of euthanasia on the day of recording from mice exhibiting diestrous vaginal cytology. Consistent with previous observations (36), LH was elevated in PNA mice (n=9-14 per group, $p<0.05$). Neither testosterone nor androstenedione were elevated, however (Figure 7B,C). The lack of elevation in testosterone in PNA mice may be due to the fact that levels were close to the lower limit of detection of the assay (7.5 ng/dL); biologically important changes in androgen levels could occur may be beyond the assay's ability to discern. Treatment with metformin restored LH levels to normal (PNA vs PNA metformin, $p<0.05$). Metformin increased testosterone levels (n=9-14 per group, Figure 7B, $p<0.05$ main effect of metformin); however post-hoc testing only found this increase significant in the control+metformin group (control vs control metformin, $p<0.05$). Metformin had no effect on androstenedione levels (Figure 7C, $p>0.05$). Uterine mass, a bioassay for estradiol levels (285), was similar among groups (n=19-23 per group, $p>0.5$, Figure 7D), suggesting neither PNA nor metformin markedly altered estradiol levels.

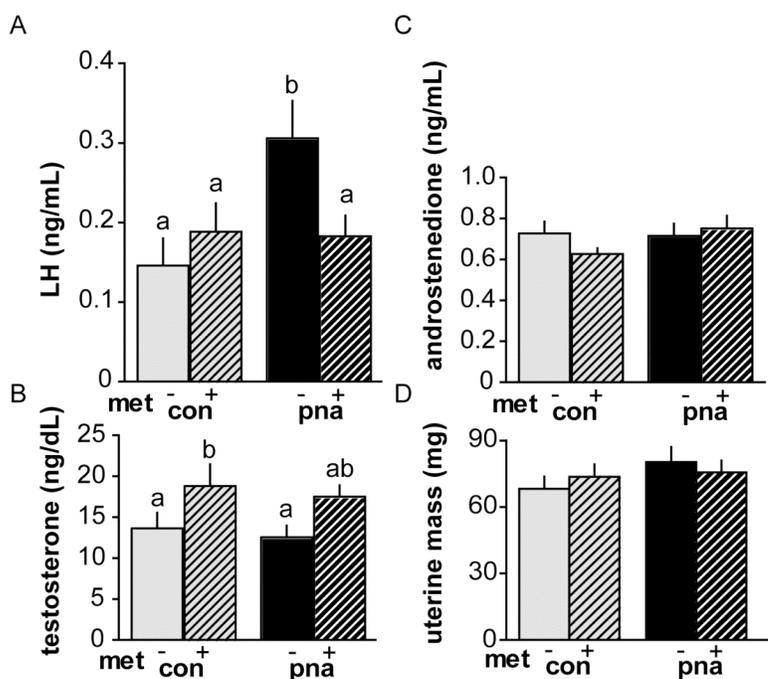


Figure 7. Serum reproductive hormone levels and uterine mass. A, LH levels were elevated in PNA mice. Metformin restored LH in PNA mice to the level of CON mice (n=9-14 per group). B and C, Testosterone and androstenedione levels were not different between PNA and CON mice; metformin increased testosterone, but had no effect on androstenedione levels (n=9-14 per group). D, Uterine mass was not different among groups (n=19-23 per group). Different lowercase letters indicate groups with significantly different means ($p < 0.05$).

Discussion

The antihyperglycemic drug metformin has been used clinically for decades and has demonstrated benefits in women with PCOS, but the mechanisms by which metformin improves reproductive cyclicity in this disorder are unclear, and potentially many. We hypothesize that metformin favorably affects the reproductive system by modulating the activity of central AMPK, which is emerging as an important regulator of reproduction. We tested the effects of metformin on reproductive parameters in a prenatally androgenized mouse model that recapitulates some aspects of PCOS. Metformin restored estrous cyclicity to PNA mice, concomitantly reducing hyperactivity of GnRH neurons and reducing LH levels. Importantly, metabolic parameters were largely

unchanged by metformin, and serum androgen levels were not reduced, suggesting that metformin's effectiveness to improve reproductive cyclicity was not dependent on reduction of circulating glucose, insulin, or androgens. GnRH neurons from metformin-treated mice showed changes in activity consistent with activation of AMPK, supporting a central site of metformin action.

The effectiveness of metformin to restore cyclicity to PNA mice is consistent with findings in normal weight, non-insulin-resistant PCOS patients, who demonstrate improved menstrual cycles and increased frequency of ovulation on metformin (24-27). In these studies, metformin improved various insulin sensitivity indices (HOMA, area under insulin curve during GTT) despite apparently normal metabolic function prior to administration, and androgen levels were reduced (24-27). Although ITT and random-fed insulin were unchanged by either PNA or metformin administration in the present study, it is possible that more sensitive measures may have revealed differences. The only apparent metabolic effect of metformin in this study was to reduce fasting blood glucose in PNA mice. Fasting hyperglycemia typically results from hepatic insulin resistance and enhanced fasting gluconeogenesis, suggesting fasting insulin levels may also have been reduced. However, it is unclear how metabolic abnormalities that are revealed only in response to a challenge -- such as a GTT in which a large bolus of glucose is given, or a 16-hour fast -- would impact on the functioning of the reproductive system under everyday conditions. Random-fed glucose and insulin levels, measured 5-7 hours after the time of lights-on when the active/feeding period of mice typically ends, were similar in PNA and CON mice and unaffected by metformin. This suggests that the observed changes in GnRH neuron function may be independent of changes in levels of circulating metabolic factors, at least in part.

The similarity of metabolic parameters among groups displaying altered reproductive parameters permitted assessment of other possible mechanisms of action of metformin in restoring reproductive cyclicity. We focused on central changes. Here we showed for the first time that GnRH neuron firing activity is abnormally high in PNA mice, extending our previous finding of increased excitatory GABAergic transmission to GnRH neurons in these mice (36). Firing rate has been correlated with hormone release in neuroendocrine systems (284), suggesting GnRH release is elevated in PNA mice, consistent with the observed elevation in LH levels. Metformin was able to restore GnRH neuron activity to normal; moreover, the changes in firing activity observed in PNA mice treated with metformin were consistent with effects of AMPK activation. A study in GT1-7 immortalized GnRH neurons (269) as well as recent work from our lab (268) indicate that central AMPK activation has an inhibitory effect on GnRH neurons. These findings are consistent with suppression of fertility by low energy availability, which is one stimulus for AMPK activation. Interestingly, measures of firing activity of GnRH neurons did not differ between untreated and metformin-treated control mice. This was surprising, as metformin would be expected to suppress firing in controls if it indeed activates AMPK. One possibility is that there could be tonic activation of AMPK in controls; however, this idea was not supported by the CC and glucosensing experiments. Another possible explanation is that chronic metformin administration may lead to compensatory mechanisms that maintain a basal level of firing, as AMPK can influence cellular metabolic processes such as glucose uptake (286) that may affect excitability. It is also possible that assessment of firing patterns over a period longer than one hour might reveal differences between untreated and metformin-treated controls.

AMPK activation is typically assessed using western blot to assay for phosphorylation; however, because of the scattered nature of GnRH neurons, this assay is not possible in this cell type. Instead we used both a pharmacological and physiological assay for AMPK activation. Acute application of the AMPK antagonist CC increased firing in GnRH neurons from metformin-treated mice, suggesting AMPK is activated in these neurons. This robust response to CC does not appear to be simply a relief of suppression, but instead an activation (i.e., GnRH neurons do not typically reach an average firing rate of 4 Hz when averaged over several minutes, which some did after CC). This could represent an unmasking of compensatory mechanisms induced by long-term metformin administration. Another possibility is that AMPK phosphorylates multiple intracellular targets, and by acute antagonism we are able to block only those with rapid effects, but other sustained excitatory effects remain. It is also possible that the observed effect of CC is nonspecific (non AMPK-mediated), but this would still be of interest as the effect of CC is only observed in cells from mice treated with metformin. Because the pharmacologic specificity of CC may not be limited to AMPK, we performed a second experiment to assess AMPK activation. Since AMPK plays a role in glucosensing in GnRH neurons (268), we tested the response to low glucose of GnRH neurons from untreated and metformin-treated mice. Consistent with enhanced basal AMPK activation, GnRH neurons from metformin-treated mice were less sensitive to the inhibitory effects of low glucose. It is important to note that mean basal firing rate in all groups in the glucosensing study (Figure 6) differed from that for long-term recordings of firing activity (Figure 4). This is explained by the fact that basal firing rate in Figure 6 represents cells that were selected on the basis of being tonically active at the time when they were patched. Quiescent cells, which occur more frequently in the control group, were excluded from these averages, as the absence of firing precludes the ability

to do the experiment shown in that figure, in which suppression of firing rate is the hypothesized outcome.

Although the small blood volume of mice makes serial sampling and thus the assessment of LH pulses very difficult, we did observe increased serum LH in single-point samples in PNA mice. This increase was reversed by metformin, consistent with the reduction in firing activity of GnRH neurons. Reduced LH could reflect reduced LH pulse frequency, amplitude, or both. Two studies in normal-weight women with PCOS demonstrated a decrease in LH pulse amplitude but no change in frequency following metformin administration (266, 267); another study in lean women with PCOS showed a decrease in overall serum LH levels after metformin (287). LH pulse amplitude is determined by the magnitude of the GnRH stimulus as well as the response of the pituitary to GnRH, either of which could be altered by metformin or PNA. Thus the observed post-metformin decrease in LH in this study could be due to the observed decrease in activity of GnRH neurons and subsequent reduction in GnRH release, diminished pituitary responsiveness mediated by direct effects of metformin on the pituitary (260), or both. However, a recent study of women with PCOS demonstrating a reduction in LH by metformin *in vivo* found no effect of metformin on LH release in the pituitary directly *in vitro* (265), suggesting the effects *in vivo* may have been centrally mediated.

Because these studies were performed in whole-animal models recapitulating aspects of human disease, we cannot rule out the possibility that observed central changes are secondary to peripheral changes. Studies have shown that metformin can have direct effects in the ovary to alter morphology (288, 289), vascularity (290), stromal blood flow

(289), and steroidogenic function (24, 256, 257, 291, 292). With regard to the latter, metformin has been shown to directly inhibit androgen production by theca cells in vitro (292). A reduction in androgens would be expected to reduce GnRH neuron activity (293). In this study metformin increased rather than decreased testosterone levels; this contradicts a direct effect of metformin to reduce ovarian androgen production, and suggests that lowered androgens result from reduced insulin levels in women with PCOS. Metformin may have altered levels of estradiol, progesterone, or other secreted ovarian factors (256, 257, 291); these were not evaluated due to limitations in blood volume in mice, but the lack of a difference in uterine mass among groups suggests estradiol levels were similar. Adipokines were evaluated because metformin affects adipocyte function (294, 295) and has been shown to alter secretion of adiponectin in vivo (296) and various adipokines in vitro (297-301). Similar to steroids, circulating adipokines can cross the blood brain barrier and influence neuronal function (302, 303). Of interest, adiponectin has been shown to activate AMPK in immortalized GnRH neurons (72) as well as in other cell types (260, 304-306). We found no changes in the circulating adipokines measured. It is possible that changes in levels of adipocyte-derived hormones or cytokines that were not assessed contributed to the observed changes in GnRH neuron activity.

Although the efficacy of metformin to improve live birth rate in PCOS has recently been challenged (307, 308), it remains an established tool in treating certain aspects of this disorder, and determining the mechanisms underlying its function is important to generate new therapeutics. The findings presented in this study raise a new possibility that metformin may alter function of the reproductive axis, at least in part, through central mechanisms to alter GnRH release.

Chapter 5
Conclusions

GnRH neurons regulate fertility centrally, and abnormal function of these neurons may contribute to dysregulation of the reproductive axis in polycystic ovary syndrome. The preceding chapters described metabolic characteristics in prenatally androgenized female mice, showed that glucose can alter the activity of GnRH neurons in a manner dependent on AMPK activation and inhibited by androgens, and used the PNA model to demonstrate that metformin may slow excessive GnRH pulse frequency in PCOS through a direct central effect on AMPK. These studies have broadened our understanding of metabolic regulation of GnRH neurons and helped to elucidate the mechanism of action of a commonly used therapeutic in PCOS.

Prenatal androgenization of female mice: an evolving model for the study of PCOS

In recent years, prenatal androgenization has received considerable attention as a potential etiology for PCOS. Our lab developed a mouse model in 2004 that recapitulated several aspects of PCOS by exposing mice to the non-aromatizable androgen DHT on days 16-18 of gestation. Female PNA mice exhibited elevated testosterone and LH levels and irregular estrous cycles. Electrophysiological examination of GFP-identified GnRH neurons in brain slices, a method unique to our PNA model, showed increased frequency and amplitude of GABAergic postsynaptic currents, consistent with increased central reproductive drive. A remaining question was whether prenatal androgenization could also induce the metabolic abnormalities that typify PCOS.

I began my graduate work by examining metabolic characteristics in female PNA mice. These findings were presented in Chapter 2. This began with assessment of whole-animal metabolic physiology, followed by examination at the cellular level of the

pancreatic islet and adipocyte. Glucose tolerance testing showed elevated fasting glucose and impaired glucose tolerance in PNA mice. Additional metabolic testing found no difference in insulin sensitivity, adiposity, or levels of circulating cytokines or adipocyte-derived hormones associated with visceral obesity and insulin resistance. These data suggested that unlike several other animal models of fetal androgen excess, PNA mice did not exhibit the visceral adiposity and insulin resistance that characterize PCOS. Assessment of pancreatic islet function using fura-2 imaging showed that islets from PNA mice failed to respond to increased glucose with an appropriate rise in intracellular calcium. These findings suggested that impaired glucose tolerance in PNA mice was the result of impaired islet function leading to attenuated glucose-stimulated insulin release. Lastly, PNA adipocytes were significantly larger than control adipocytes when assessed microscopically; however, the difference was small and did not cause overt changes in glucose uptake or insulin sensitivity.

Although this extensive metabolic characterization yielded primarily negative results, these findings added significantly to our understanding of prenatal androgen programming of PCOS phenotypes. A major difference between our model and others, besides the obvious difference in species, is the use of a non-aromatizable androgen. Testosterone, used in other models, can be converted to estradiol; thus it may be estradiol that causes some aspects of metabolic dysfunction in these models. This idea is supported by a recent study showing that testosterone treatment of pregnant sheep results in significant elevations in fetal estradiol and estrone, in addition to changes in maternal estradiol levels at term (309). These concepts should be considered in future model development. As PCOS encompasses a multitude of phenotypic presentations, multiple models exhibiting different aspects of the disorder could potentially be

generated by the administration of different androgens or hormonal combinations *in utero*. Further, the PNA mouse may be the only “lean” PCOS model to date. It would be of interest to determine how the PNA metabolic phenotype is affected by a high-fat diet, as the minor abnormalities in metabolism in this model appear to be predisposing factors for metabolic dysfunction in the context of obesity. An alternative dietary challenge would be a high glycemic index (GI) diet, as both high-fat and high-GI comprise the modern American diet and have been linked to metabolic dysregulation.

Because serum levels of metabolic factors that were measured (in the absence of a challenge) were not different between PNA and CON mice, these data also suggested that the previously observed reproductive neuroendocrine dysfunction was the result of androgen programming of the reproductive axis, and was not secondary to metabolic changes. This is consistent with the demonstrated role of prenatal androgen to masculinize the central GnRH pulse generator. A second possibility is that prenatal androgens program ovarian tissue to hypersecrete androgens, and the observed central changes resulted from positive central androgen feedback. Although this was suggested by the ability of the androgen-receptor antagonist flutamide to reverse abnormalities in our 2004 study, in the present studies we did not consistently observe elevated androgens. Further, a trial of flutamide did not normalize estrous cyclicity, as it did in the previous study; mice in this study were in persistent diestrus, as opposed to persistent estrus reported previously, which may explain the inability to reverse the phenotype. Regarding these differences, both caging conditions and food were changed following the initial characterization of PNA mice, with a switch from phytoestrogen-containing to phytoestrogen-free chow. While the inability to repeat previous findings imposed a difficulty in interpreting the present studies, it also raised an important question of

whether phytoestrogens in the mouse chow interacted with androgen to generate the previously observed phenotypes. Phytoestrogens are a component of some foods, including soy, which is the basis of many infant formulas. A potentially deleterious effect of phytoestrogen during development is certainly worthy of further study.

It is potentially confounding that some PNA mice exhibiting elevated LH levels did not exhibit elevated androgens, as high LH is thought to drive ovarian hyperandrogenism in PCOS. This may be a consequence of poor androgen assay sensitivity, or fluctuations in androgen levels throughout the day. If PNA mice indeed have high androgens, the question of which tissue, brain or ovary, is more important for the observed phenotype could be resolved using surgical manipulations in this model. Aspects of GnRH neuron function could be assessed in intact and ovariectomized mice. If removal of the ovaries fails to eliminate the observed differences between GnRH neuron activity in PNA and control mice, this would suggest that important programming events take place in the brain, and differences are not wholly dependent on ovarian factors. A limitation of this experiment is that the normal post-castration rise in GnRH neuron activity due to loss of steroid negative feedback might preclude observation of differences; alternatively, the rate of this rise could provide another potential source of observable differences between PNA and control mice. If GnRH neurons from PNA mice behave similarly to those from controls in the absence of steroids, they may still respond differently to steroidal feedback; this could be tested by administering steroids to the ovariectomized mice. A model of GnRH/LH surge generation in estradiol-treated ovariectomized animals was developed by our lab (210); it would be interesting to see if PNA mice are capable of generating surges under this paradigm. Lastly, a technically difficult but feasible study would be ovarian transplant of PNA ovaries to control mice to observe how reproductive

function is altered in the controls, and how ovarian function is altered in the transplanted ovary.

We also examined metabolic characteristics in male PNA mice, which is of interest as brothers of women with polycystic ovary syndrome often exhibit metabolic abnormalities, including insulin resistance, glucose tolerance, and beta cell dysfunction, which can be independent of BMI (310). We did not find differences between male PNA and control mice in body mass, fat percentage by DEXA, epididymal fat pad weight, seminal vesicle weight, testis weight, timing of prepuce separation (an outward marker of puberty onset), or adult glucose tolerance. PNA males did exhibit elevated fasting glucose and impaired glucose tolerance at one month of age, but these differences did not persist through later ages as in females (Figure 5-1). Serum collected from these mice could be assayed for reproductive hormones, which we have not yet assessed in males. Similar seminal vesicle and testis weights suggest that serum testosterone and gonadotropin levels are not likely to be grossly different. As overt reproductive phenotypes could not be observed in male animals, fertility could be assessed by mating studies. Mating studies are also needed to assess fertility in female PNA mice, as this is an important aspect of characterizing the model; however, this would have to be approached with caution, as the genitalia of these mice are somewhat masculinized, resulting in a smaller vaginal opening that could preclude mating.

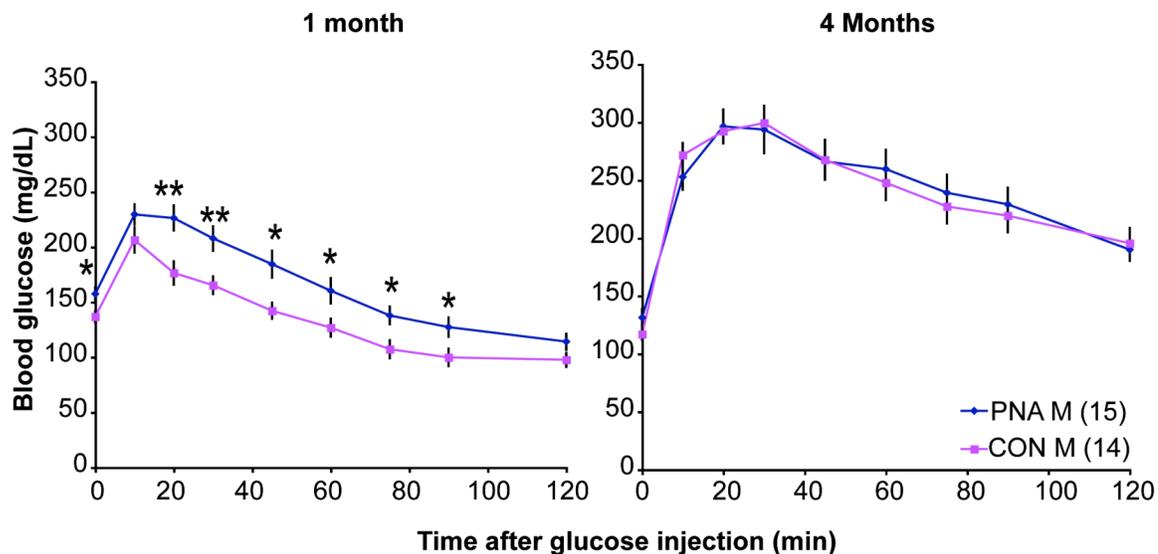


Fig 5-1. Glucose tolerance is impaired in male PNA mice at one month of age, but not at four months.

Another aspect of characterizing the PNA model is examination of the ovaries. With the assistance of collaborators at the University of Rochester, we have performed a preliminary histological analysis of ovarian morphology. These data will be discussed in conjunction with the metformin study toward the end of this chapter.

Glucosensing by GnRH neurons is mediated by AMPK and inhibited by androgens

Chapter 3 of this dissertation examined whether GnRH neurons could sense physiological changes in extracellular glucose by altering their firing rate, and if DHT and/or estradiol affected glucosensing. This study also began to divulge the underlying glucosensing mechanisms. As a number of *in vivo* physiological studies suggested that glucoprivation inhibits GnRH secretion, we hypothesized that GnRH neurons were glucose-excited -- stimulated by high glucose concentrations, and inhibited by low

glucose. We tested this hypothesis using an extracellular approach to monitor firing activity in response to a switch from 4.5 mM to 0.2 mM glucose, the theoretical maximal and minimal brain glucose concentrations (although levels are potentially higher in the hypothalamus). GnRH neurons were indeed sensitive to low glucose, which robustly inhibited firing activity in approximately 80% of neurons from ovariectomized mice. Glucosensing was significantly attenuated in GnRH neurons from OVX mice treated with DHT. Estradiol was able to overcome the effect of DHT and restored glucosensing to GnRH neurons, suggesting that estradiol amplifies glucosensing mechanisms. However, the effect of low glucose in GnRH neurons from OVX mice treated with estradiol was similar to that in neurons from OVX mice.

We hypothesized that K_{ATP} channel activation would be critical in mediating the inhibition of firing by low glucose. However, pharmacological closure of K_{ATP} channels with the sulfonylurea tolbutamide failed to attenuate the inhibitory response to low glucose. In contrast, the AMPK antagonist compound C significantly attenuated inhibition by low glucose, suggesting that AMPK activation is required for the response. This prompted us to hypothesize that pharmacological activation of AMPK using AICAR would mimic the effect of low glucose, similarly inhibiting firing activity. AICAR indeed inhibited firing; furthermore, AICAR was less effective in cells from DHT-treated mice, consistent with attenuated responses to low glucose in this steroid-treatment model. Using whole-cell voltage-clamp recordings, we found that low glucose and AICAR activated very similar currents in GnRH neurons, supporting the idea that glucose acts through an AMPK-dependent mechanism.

A control experiment was performed to see if compound C could block the effect of AICAR on firing. Unexpectedly, the antagonist failed to block the effect of the agonist, suggesting the possibility of a nonspecific effect (mediated by another AMP-sensitive enzyme). However, although compound C was able to antagonize physiological AMPK activation by low glucose, it may not be potent enough to overcome the effects of pharmacologic activation by AICAR. Published work showing this latter effect is primarily done in cell culture with long-term incubations in compound C or AICAR, sometimes as long as 12 hours; acutely prepared brain slices do not remain viable for this long. Given the sum of the data, and the similar inhibitory effect of the AMPK activator metformin (which likely activates AMPK through an indirect pathway), we favor the interpretation that the effects of AICAR are AMPK-mediated. Better antagonists are in development and could be utilized in the future. Additional experiments could substantiate a role for AMPK by utilizing neuronal AMPK knockout mice crossed with GnRH-GFP mice, in which we would expect to find impaired glucosensing. If AMPK is indeed involved, several questions arise: where is AMPK located, and what AMPK subunits constitute the form of AMPK that is involved in regulating GnRH neurons? AMPK is comprised of different isoforms of alpha, beta, and gamma subunits, and different subunit combinations convey differential sensitivities to AMP and ATP. Localization of AMPK could be assessed using immunohistochemistry, or by in situ hybridization or single-cell PCR to identify mRNA expression directly in GnRH neurons. Antibodies or primers specific for particular AMPK subunits could establish which subunits are present. Additionally, to determine if glucokinase plays any function in glucosensing in GnRH neurons, including possible K_{ATP} -independent roles, we could block glucokinase using the antagonist alloxan and repeat the test of low glucose on firing activity. Neuronal glucokinase and Kir6.2 knockout mice have also been generated and could be useful in

establishing what role, if any, these constituents play in GnRH neuron function. If AMPK is found to be expressed directly in the GnRH neuron, GnRH-Cre mice could be used to generate GnRH neuron-specific AMPK knockouts; this could also be done for glucokinase and/or Kir 6.2.

The findings of this study were novel with regard to AMPK, both as a mechanism of glucosensing and as a regulator of GnRH neuronal function. Although AMPK has been implicated in glucosensing in glucose-inhibited neurons, there is a paucity of evidence for a role in glucose-excited neurons, in which K_{ATP} channel-dependent glucosensing is thought to be particularly important. A difference in mechanism between GnRH neurons and VMN/ARC glucose-excited neurons was first suggested when we attempted to generate a dose-response curve, plotting the firing rate of GnRH neurons in response to graded reductions in extracellular glucose from 10 to 5 to 1 to 0.2 mM. Rather than exhibiting graded changes in response, which have been observed in VMN/ARC neurons, the response to declining glucose appeared to be an on/off phenomenon. That is, GnRH neurons were silenced upon reaching a particular threshold concentration, which differed somewhat from cell-to-cell. This suggests that subpopulations may have sensitivities that lie in different ranges of glucose concentration; here we focused on one particular range. DHT may alter the threshold for activation of inhibitory mechanisms. We performed preliminary studies examining GnRH neuron activity changes in response to a smaller switch from 5 mM to 1 mM glucose in OVX and OVX+DHT mice (Figure 5-2,3). Although a smaller subset of OVX cells were responsive to this less extreme reduction in glucose, the overall effect of 1 mM glucose was significant to reduce the firing rate in this group. Similar to the response to 0.2 mM glucose, DHT inhibited the response to 1 mM glucose.

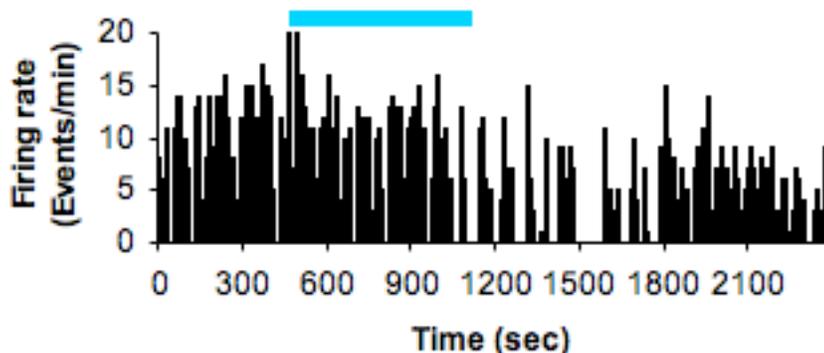


Figure 5-2. Representative plot of firing rate over time in a GnRH neuron from an OVX mouse in response to a switch from 5 mM to 1 mM (blue bar). This cell was inhibited by the reduction in glucose.

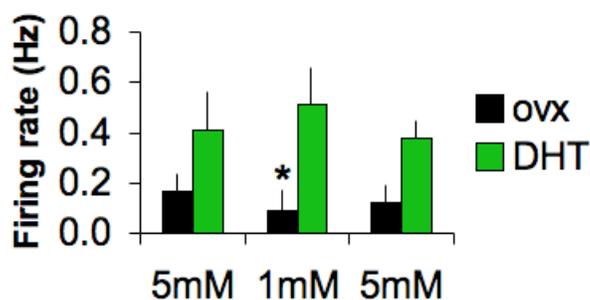


Figure 5-3. Summary of effects of 1 mM glucose exposure on firing rate in cells from OVX and OVX+DHT mice. Firing was inhibited only in cells from OVX mice . *, $p < 0.05$ vs OVX 5 mM.

We also tested the effect of low glucose in the presence of blockers of fast glutamatergic and GABAergic transmission, since synaptic transmission, particularly from GABAergic neurons, is thought to relay metabolic information to GnRH neurons. Surprisingly, GnRH neurons became more sensitive to changes in extracellular glucose in the presence of these antagonists, although this may simply reflect the fact that these blockers increased the basal firing rate, making a suppression easier to observe. This experiment indicated that glucosensing in GnRH neurons is either direct or mediated by neuromodulatory inputs maintained in the brain slice. The effect of blockers on glucosensing was also

tested in GnRH neurons from DHT-treated mice; blocking fast synaptic transmission unmasked glucosensing and also enabled AICAR to suppress firing activity in this group. Together, these experiments suggested that changes in the presynaptic network were the mechanism by which DHT attenuated glucosensing and responses to AICAR. This is consistent with the fact that androgen receptors have not been detected on GnRH neurons, suggesting that effects of androgens on GnRH neuron function are likely mediated by the network. As synaptic transmission remains intact *in vivo*, androgen should have an attenuating effect on glucosensing *in vivo*; this idea is supported by the observation that males are less sensitive than females to suppression of LH by central glucoprivation (47). Further, hyperandrogenemia in PCOS may reduce AMPK-mediated inhibition of GnRH neuronal activity, contributing to the elevation in pulse frequency. However, the effect of androgen may be mitigated by estradiol, also present in women with PCOS. The response may be determined by the ratio of androgen to estrogen in individual patients.

One confounding finding from these experiments was that recorded cells from DHT-treated mice on average tended to have lower basal firing rates than cells from other groups, although this difference was not statistically significant. This finding conflicts with previous observations in our lab that DHT excites GnRH neurons. Several differences between recording conditions may account for this discrepancy. First, in the present study we examined only actively firing cells in extracellular experiments, whereas previously data from quiescent cells were included in computed average firing rates. Second, previous studies assessed the effect of DHT only in the presence of estradiol. Third, previous studies were done in the morning, whereas these studies were done primarily in the afternoon, and steroids, particularly estradiol, can induce diurnal changes

in the activity of GnRH neurons. Fourth, basal firing rate in this study was assessed in 4.5 mM glucose, whereas all other electrophysiological studies in our laboratory are done in 10 mM glucose. In spite of these many differences, long-term firing activity in various steroid treatment models may need to be characterized in more physiological glucose concentrations, given that cells from DHT-treated mice respond differently to metabolic cues. However, determining what is truly “physiological” may be problematic, as the precise glucose concentrations to which GnRH neurons are exposed are unknown, given the anatomical proximity of these neurons to areas lacking a blood-brain barrier.

Another particularly perplexing finding was that the current activated by both low glucose and AICAR was not consistent with one that would be expected to inhibit GnRH neurons. The reversal potential was around -40 mV. This led us to assess the possibility that it was carried by chloride; however, changing the intracellular chloride concentration failed to alter the reversal potential of the current (Figure 5-4). We also attempted to block this current using ZD7288, a blocker of the hyperpolarization-activated cation current I_h , and SN-6, a blocker of the sodium/calcium exchanger, as these type of currents have reversal potentials around -40 mV. However, neither antagonist had an effect. This current did not resemble a K_{ATP} current, which we have previously identified in GnRH neurons using both agonists and antagonists. A control experiment also showed that current changes were not the result of long-term instability in recordings. The reversal potential and lack of effect of changing Cl_{in} suggest that the current is a mixed cation conductance. Activation of this type of conductance is almost uniformly excitatory in the literature. One possible explanation for the observed inhibition is shunting inhibition. The reduction in membrane resistance by AICAR or low glucose

would require increased current to generate an action potential. Another possible explanation is that this current may hyperpolarize cells in which the resting potential is positive to -40 mV. This was suggested by current-clamp experiments in which stimulating tonic firing in 4.5 mM glucose often required large depolarizations from the resting potential. Thus, the “active cells” targeted for recording in extracellular experiments may have been those with a resting potential positive to -40 mV. This would imply that neurons with a resting potential negative to -40 mV would respond to low glucose with excitation; however, we did not observe low-glucose excitation of any silent neurons tested in extracellular mode. Further, the observation that the majority of cells were not active at their native resting potential in current-clamp differs from what we typically observe under normal recording conditions of 10 mM glucose. In light of these studies, it is feasible that a lower glucose concentration of 4.5 mM might alter the threshold for action potential firing (and 0.2 mM may raise that threshold further). Assessing this possibility would require more detailed studies, but may shed light on the mechanism of cellular inhibition mediated by this current. In addition, ion-substitution experiments could be performed to elucidate the exact charge carriers of this current, which may help to identify the specific type of nonspecific cationic conductance that we have observed.

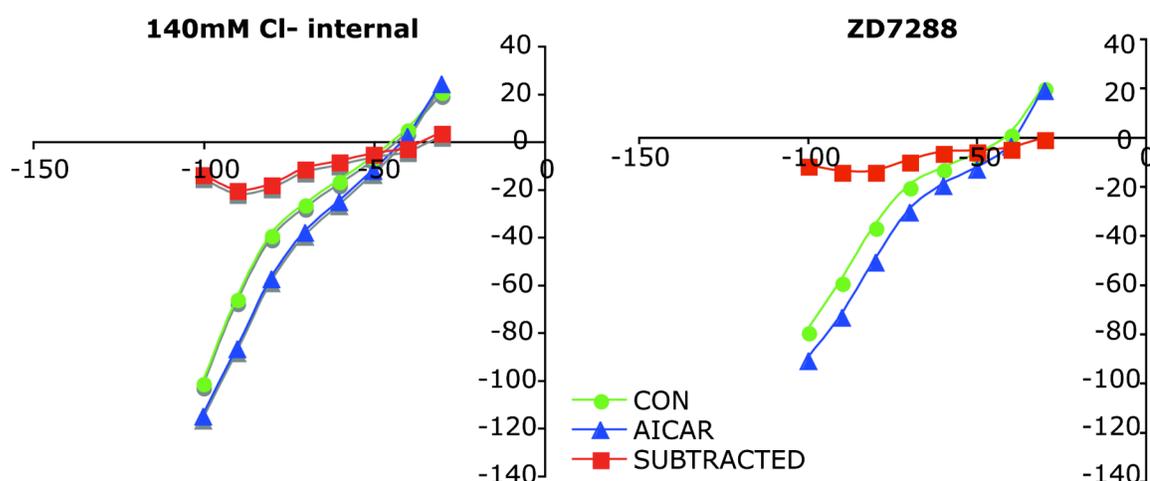


Figure 5-4. Neither high intracellular chloride nor the presence of the Ih antagonist ZD7288 modified the AICAR-induced current.

Originally we intended to compare glucosensing in coronal vs sagittal brain sections; the latter slice orientation could maintain inputs from more caudal hypothalamic nuclei shown to be involved in metabolic regulation of fertility in Chapter 1. However, we decided to focus on coronal sections only, as this would limit the influence of these synaptic contacts and approach studying direct effects. Studying glucosensing in sagittal sections is an interesting area for future research, if it can be established that the connections to relevant nuclei are maintained.

Although these findings suggested that K_{ATP} channels are not critical for glucosensing in GnRH neurons, which was supported by a 2007 study showing that K_{ATP} channels do not mediate the suppression of LH by fasting *in vivo* (226), these channels may have other functions in this cell type. We have unpublished data showing that K_{ATP} channels influence the activity of GABAergic afferents to GnRH neurons in a steroid-dependent manner. GABAergic postsynaptic currents (PSCs) were recorded from GnRH neurons in brain slices before and after application of the K_{ATP} channel opener diazoxide. Because steroid hormones also regulate GABA transmission to GnRH neurons, this was studied

in ovariectomized mice with estradiol and/or DHT implants. As previously observed in OVX mice treated with DHT plus estradiol (12), baseline GABA PSC frequency was higher ($p < 0.01$) in OVX+DHT mice compared to OVX, suggesting that DHT was the operative steroid. PSC frequency was reduced upon application of diazoxide in cells from OVX+DHT and OVX+DHT+E mice, but not in cells from OVX and OVX+E mice. This suggested that DHT conferred sensitivity to the drug in afferent GABA neurons, possibly by upregulating K_{ATP} channels or causing more channels to be closed (and thus susceptible to pharmacologic opening). PSC amplitude, an indicator of the postsynaptic response, was decreased by diazoxide only in cells from OVX and OVX+DHT mice, suggesting that estradiol attenuates the postsynaptic effects of the drug.

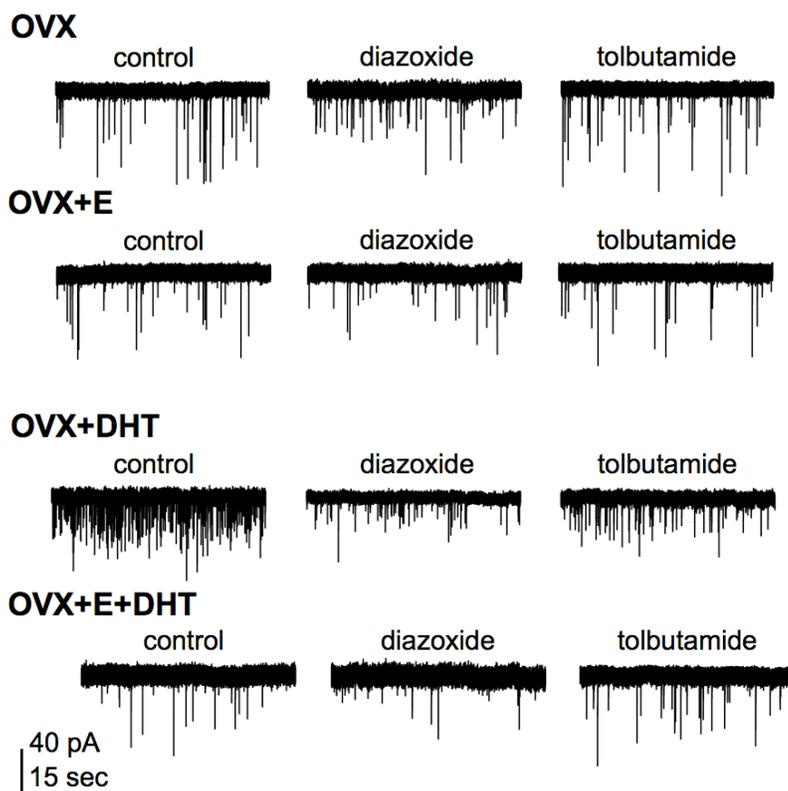


Figure 5-5. Representative traces of GABAergic postsynaptic currents in different steroid treatment groups. PSC traces are shown before drug treatment and after subsequent application of diazoxide, then tolbutamide. Diazoxide reduced PSC frequency; this effect was reversed by tolbutamide.

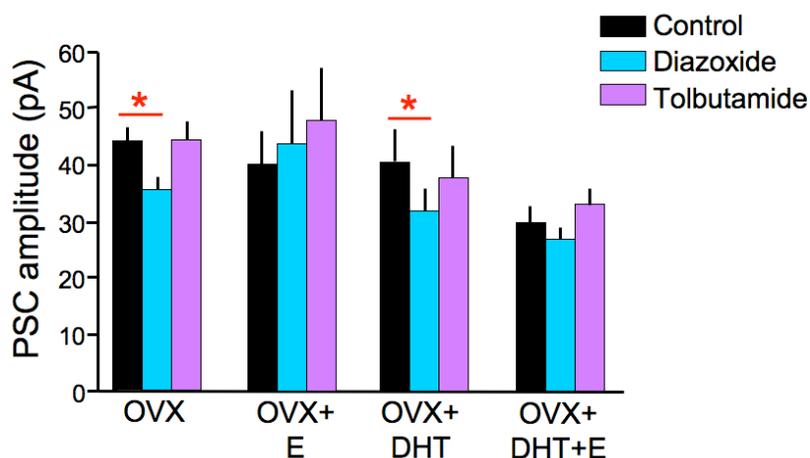


Figure 5-6. Opening K_{ATP} channels with diazoxide decreased GABA PSC frequency only in cells from mice treated with DHT. *, $p < 0.05$.

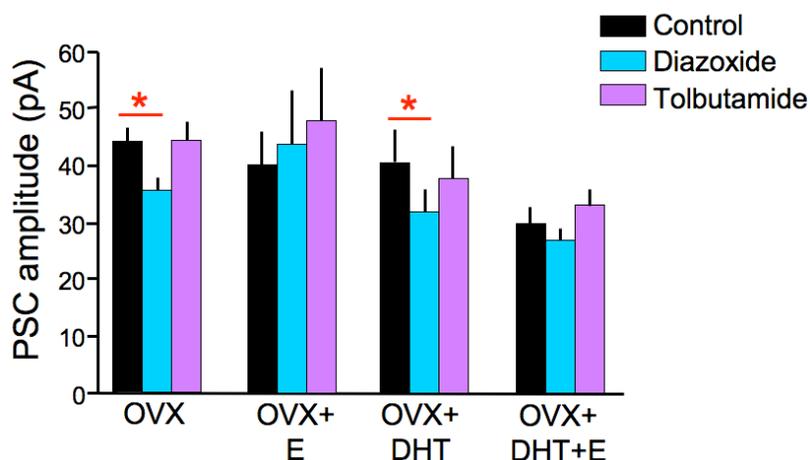


Figure 5-7. Opening K_{ATP} channels with diazoxide reduced GABA PSC amplitude only in the absence of estradiol. *, $p < 0.05$.

Central AMPK activation as a mediator of metformin's effects on reproductive cyclicity

Chapter 4 combined the findings of the previous two chapters to test a hypothesis regarding metformin's mechanism(s) of action in PCOS. The observation that AMPK activation by AICAR or metformin inhibited GnRH neurons *in vitro* suggested metformin may similarly inhibit GnRH neuron activity and thus hormone release *in vivo*. Since

metformin's mechanism of action in PCOS is generally thought to be reduction of insulin and thereby androgen levels, the absence of insulin resistance and compensatory hyperinsulinemia in PNA mice provided a model to assess insulin-independent effects of metformin in the context of enhanced central reproductive drive and acyclicity. An additional goal of this study was to establish whether PNA mice indeed had increased firing activity of GnRH neurons; although this was suggested by enhanced GABAergic neurotransmission, which is presumably excitatory, direct measurement of firing activity was not previously assessed.

This study was performed in two cohorts of mice. In the first cohort, estrous cycles were monitored before and after metformin, and then glucose and insulin tolerance tests were performed. In the second cohort, body mass, food intake, and estrous cyclicity were monitored without the potential disruption caused by the fasting and stress of metabolic testing. Examination of metabolic characteristics found little effect of metformin in either control or PNA mice treated with the drug. The one major observation was that fasting glucose in PNA mice, previously observed to be elevated, was reduced by metformin. This finding was consistent with the known effect of metformin to reduce fasting hepatic gluconeogenesis, and suggested that an effective dose was used. Glucose tolerance in PNA mice was different from controls when only these two groups were considered, similar to observations in Chapter 2; however, due to the statistical tests required to make multiple comparisons, this difference was not statistically significant as presented. This underscores the fact that the difference in glucose tolerance between PNA and CON mice is small. We have repeated the original GTT results in several groups of mice, including C57BL/6 mice.

Estrous cyclicity was monitored before and after metformin in individual PNA and control mice. Metformin initially worsened cyclicity in controls, but after approximately two weeks normal cyclicity was reestablished. This initial negative impact on cycles may reflect the fact that AMPK activation can signal low energy availability, which is disruptive to reproduction; compensatory mechanisms may attenuate this effect in the long-term. Metformin improvement of PNA cyclicity was apparent after two weeks of treatment and statistically significant after four. Prior to metformin treatment, PNA mice exhibited persistent leukocyte infiltration of the vaginal epithelium. Round and nucleated cells would appear, but few became cornified, suggesting the absence of an estradiol rise, ovulation, and estrus (the cycle stage during which mating normally occurs). Following metformin treatment, nearly all of the PNA mice began to transition regularly to estrus, in which there was near complete cornification of the vaginal epithelium. Despite this improvement, some degree of leukocyte infiltration was present in almost all vaginal smears, suggesting persistent hormonal or reproductive tract abnormalities that were not reversed by metformin, and may have been permanently programmed.

Electrophysiological recordings demonstrated increased frequency of action currents (which underlie action potentials) in GnRH neurons from PNA mice. Percent and maximum duration of quiescence, defined as a period in which 1 or fewer action currents occurred per minute, were lower in cells from PNA mice. GnRH neurons from control mice exhibited prolonged periods of quiescence, which were absent in cells from PNA mice. GnRH neurons from metformin-treated PNA and CON mice behaved similarly to control cells, at least in the activity parameters that were measured. These results confirmed the implications of our previous study, which showed that PNA increased GABAergic transmission to GnRH neurons and suggested GnRH neurons from these

mice would exhibit higher firing activity. Further, metformin was able to reverse the effects of PNA on firing activity, even in the absence of overt changes in metabolic function. The decrease in GnRH neuron activity by metformin was accompanied by a corresponding reduction in LH levels in PNA mice.

We performed two different experiments to assess AMPK activation in GnRH neurons. The first was acute application of the AMPK antagonist compound C to cells after a baseline recording period. Compound C evoked an acute increase in firing in the majority of cells from both control and PNA mice treated with metformin. In contrast, it had little effect on cells from untreated control and PNA mice, suggesting that metformin conferred sensitivity to compound C. A second experiment was the application of low (0.2 mM) glucose to cells. We hypothesized that if AMPK was already activated in these cells due to metformin, low glucose would be less effective at decreasing firing activity. In fact, the majority of cells from both control and PNA mice treated with metformin were not inhibited by low glucose. In contrast, nearly all cells from untreated mice responded with a decrease in firing rate. Together, these experiments suggested that AMPK was indeed activated in GnRH neurons as a result of metformin administration. As several adipocyte-derived hormones have been shown to have effects on AMPK activation, we considered the possibility of an indirect effect; metformin may have altered the levels of adipokines, which could have circulated to the brain to affect GnRH neuron function. However, we did not observe any changes in the adipokines that were measured. Further, androstenedione was unchanged by metformin, and a lack of change in uterine weight suggested estradiol levels were unchanged, suggesting that central changes were not mediated by changes in these hormones. Curiously, metformin increased testosterone levels; as testosterone activates GnRH neurons, this would be expected to

increase firing activity, although there could be negative feedback mediated by local conversion to estradiol. However, it is unlikely that these small changes in testosterone levels caused the significant changes in responsiveness to compound C and low glucose.

It was interesting that metformin did not affect firing activity parameters measured in control mice, as the other experiments suggested that AMPK was activated centrally in control mice in addition to PNA mice treated with metformin. We speculate that this is due to compensatory effects of long-term treatment, and this was suggested by the initial disruption of estrous cyclicity in control animals. AMPK may be a "low energy" signal to GnRH neurons, but it is probably one of many redundant signals as described in Chapter 1; thus, AMPK activation alone may not be sufficient to mediate the long-term effects of energy restriction on fertility.

We also assessed ovarian morphology in PNA and control mice with and without metformin. We hypothesized that the number of corpora lutea (CL) would be lower in PNA mice, as their disrupted cycles suggest impaired ovulation; however the number of CL appeared similar among groups. This could reflect persistence of CL in PNA mice. There was a significant reduction in antral follicle count in the PNA mice, suggesting an arrest of follicles at an earlier stage of follicular development; this abnormality was reversed by metformin.

Ideally, we would be able to demonstrate central AMPK activation after metformin treatment using more traditional methods to assay for phosphorylation. This was attempted using immunohistochemistry to stain for the phosphorylated form of AMPK;

however, the commercially available antibodies were inadequate for this purpose. Newer antibodies, if they become available, might yield better results. This method could also be used to assay for brain AMPK phosphorylation in GnRH neurons following hypoglycemia. Another possibility is the isolation of pools of GnRH neurons from metformin-treated and control mice using flow cytometry, extraction of protein, and subsequent analysis of AMPK phosphorylation state using western blot. A prerequisite is that the presence of AMPK must first be demonstrated in the GnRH neuron. A limitation is that this experiment could require large numbers of animals to generate a sufficient amount of protein, as there are only about 800 GnRH neurons per mouse. Further, sorting parameters for adult neurons may be difficult to establish.

Additional future directions for this study would require further characterization of the PNA model. If PNA mice fail to exhibit LH surges when ovariectomized and given a constant dose of estradiol, does metformin restore their ability to generate surges? If PNA mice are infertile, does metformin actually restore ovulation and/or fertility? Further, as our lab has recently undertaken a more detailed examination of the ovaries to assess changes in the ovarian matrix by PNA, ovaries leftover from these mice could be examined to determine if metformin reverses ovarian changes induced by PNA.

Final conclusions

These studies have provided novel insight into how metabolic and steroidal cues alter the function of GnRH neurons to regulate fertility centrally. Central AMPK appears to mediate the effects of altered extracellular glucose and metformin on GnRH neuronal activity. The novel finding that this kinase regulates GnRH neuronal activity has implications for other metabolic signals, which have been shown to regulate AMPK

activation. Through effects on afferent neurotransmission, androgens appear to attenuate the effects of AMPK activation. These findings form a foundation for many possible future studies, which may further elucidate the causes and molecular underpinnings of PCOS, potentially leading to novel preventative strategies and therapeutics.

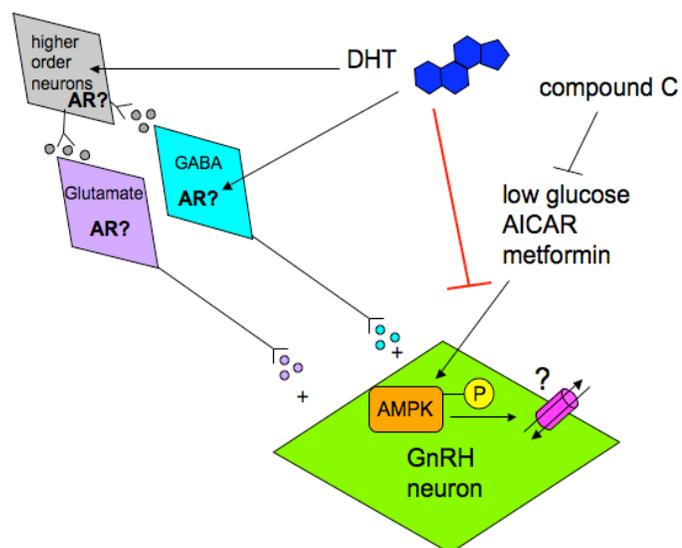
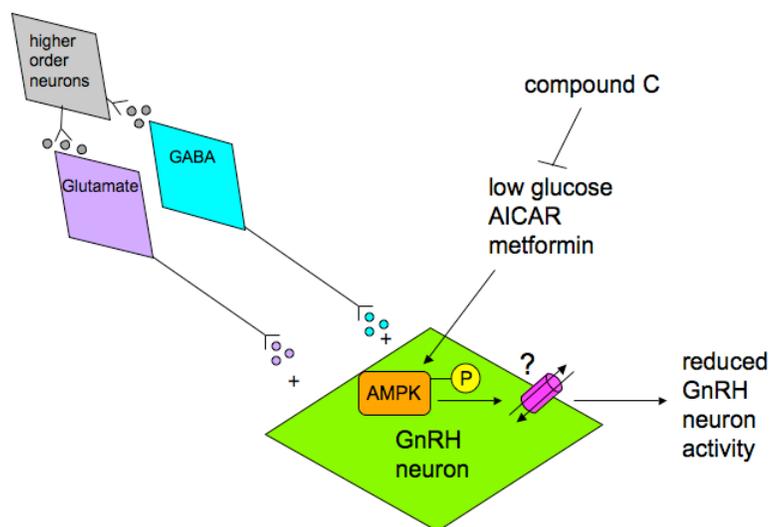


Figure 5-8. Model of glucosensing in GnRH neurons. Androgens inhibit glucosensing through effects on afferent neurotransmission.

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