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Studies of the Homeostatic and Metabolic Regulation of The GnRH Pulse Generator

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ABSTRACT

Studies of the Homeostatic and Metabolic Regulation of GnRH Pulse Generator

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Gonadotropin releasing hormone (GnRH) neurons comprise the final pathway through which the central nervous system exerts its control over the hypothalamic-pituitary-gonadal (HPG) axis. GnRH is released in a pulsatile manner, and conveyed to the anterior pituitary gland to stimulate synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A number of physiological factors, including gonadal steroids and energy balance, regulate this reproductive axis by modulation of GnRH pulsatility. In female mammals, ovarian steroids, i.e. estrogen and progesterone, inhibit GnRH/gonadotropin secretion via their homeostatic negative feedback actions. Despite extensive studies, the cellular and molecular mechanisms underlying these processes remain largely unknown. In addition, states of negative energy balance resulting from decreased food intake and/or increased energy expenditure, also lead to a suppression of the GnRH/gonadotropin secretion. However, the molecular mechanisms mediating this process are still poorly understood. Recent studies identified the ATP-sensitive potassium (K_{ATP}) channels in a variety of neurons in the hypothalamus, some of which have been implicated in energy homeostasis and regulation of GnRH neurons by ovarian steroids. I have postulated that K_{ATP} channels may convey the signals of ovarian steroids and negative energy balance to regulate GnRH secretion. This thesis tests this hypothesis by using molecular and integrative physiological approaches. In this thesis, the following evidence is provided: (1) K_{ATP} channels are involved in regulating activity of the GnRH pulse generator and the responsiveness of pulsatile GnRH release to central K_{ATP} channel blockade is conferred by the presence of both

estrogen and progesterone, (2) Estrogen and progesterone, but not either hormone alone, upregulate K_{ATP} channel expression in the preoptic area (POA) and mediobasal hypothalamus (MBH), (3) Short-term food deprivation suppresses pulsatile GnRH release and LH level in female mice, and (4) Central K_{ATP} channel modulation is not necessary for fasting-induced suppression of GnRH/LH release in female mice. Taken together, our data demonstrated that K_{ATP} channels are involved in mediating the negative feedback actions of ovarian steroids on GnRH secretion through the regulation of K_{ATP} channel gene expression, but do not play a role in mediating the effects of negative energy balance.

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taught me to enjoy the growing pain of becoming a good parent, just as I have learned to become a good scientist at Northwestern.

List of Abbreviations

2DG	2-deoxy-D-glucose
ABC	ATP-Binding Cassette
ADP	Adenosine Diphosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid
AP	Area Postrema
ATP	Adenosine Triphosphate
C	Control
Ca^{2+}	Calcium
cAMP	cyclic Adenosine Monophosphate
CCK	Cholecystokinin
cDNA	Complementary Deoxyribonucleic Acid
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
CREB	cAMP Response Element Binding Protein
CRF	Corticotropin-Releasing Factor
CV	Coefficient of Variance
DBH	Dopamine β-hydroxylase
DNA	Deoxyribonucleic acid
E_2	Estrogen
ER	Estrogen Receptor
ERKO	Estrogen Receptor Knock Out

ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
FFA	Free Fatty Acid
FSH	Follicle Stimulating Hormone
GABA	γ-Aminobutyric Acid
GIRK	G protein-gated Inwardly Rectifying Potassium channel
GnRH	Gonadotropin Releasing Hormone
HPG	Hypothalamic-Pituitary-Gonadal
i.c.v.	intracerebral ventricular
i.p.	intraperitoneal
Ih	Hyperpolarization-activated Cation Currents
IT	T-type calcium current
K_{ATP}	ATP Sensitive Potassium
KCO	K^+-channel openers
Kir	Inwardly Rectifying Potassium Channel
LH	Luteinizing Hormone
MAPK	Mitogen-Activated Protein Kinase
MBH	Mediobasal Hypothalamus
Mg^{2+}	Magnesium
mPR	membranous Progesterone Receptors
mRNA	messenger Ribonucleic Acid
MUA	Multiunit Activity

NBF	Nucleotide-Binding Fold
NE	Norepinephrine
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
NPY	Neuropeptide Y
OVX	Ovariectomy
P	Progesterone
PCOS	Polycystic Ovarian Syndrome
PHHI	Persistent Hyperinsulinemic Hypoglycemia of Infancy
PI3K	Phosphoinositide Kinase 3
PIP2	phosphatidylinositol-4, 5-bisphosphate
PIP3	phosphatidylinositol-triphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
POA	Preoptic Area
POMC	Pre-opiomelanocortin
PR	Progesterone Receptor
PRKO	Progesterone Receptor Knock Out
PVN	Periventricular Nucleus
RIA	Radioimmunoassay
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s.c.	subcutaneous

SEM	Standard Error of Mean
SUR	Sulfonylurea Receptor
TMD	Transmembrane Domain
VMN	Ventromedial nucleus

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Chapter I: Introduction and Literature Review

A. Introduction

In female mammals, reproductive hormone secretions and ovulatory cyclicity are controlled by GnRH neurons, which secrete the decapeptide in a pulsatile pattern into the hypophyseal portal vasculature (1). Pulsatile GnRH release stimulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn regulate steroidogenesis and ovulation (by LH) and folliculogenesis (by FSH). Fertility depends upon the normal functioning of this “hypothalamic-pituitary-gonadal axis” (HPG axis), A key process in the operation of the HPG axis is the homeostatic negative feedback regulation of GnRH release by ovarian steroids, i.e. estrogen and progesterone. How the feedback effects of these ovarian steroids are exerted is not clear. GnRH neurons are also subject to regulation by other physiological factors, including photoperiod (2), olfaction (3), and various types of stress (4, 5). These internal and external factors impinge upon GnRH neurons to regulate HPG axis and consequently fertility. Among them, energy balance is a very important factor (6). In natural settings, animals possess adaptive mechanisms that partition available energy to support or suppress reproduction, depending upon the circumstances. Under conditions of excessive energy resources, the availability of oxidizable fuels signals an activation of the HPG axis, thus enabling successful mating and breeding in a conducive environment. While under conditions of restricted energy resources, available energy is partitioned to ensure individual survival at the expense of the suppression of the reproductive axis through inhibition of gonadotropin and gonadal steroid secretions (7). The cellular and molecular mechanisms that mediate the dependency of GnRH release on energy balance are also relatively unknown. It has recently been found that some hypothalamic neurons, particularly those that are known to be synaptic afferents to GnRH neurons, express potassium channels that can act as metabolic sensors – the ATP sensitive

potassium (K_{ATP}) channels (8, 9). These same neuronal groups have been shown to express ovarian steroid hormone receptors (10-12), and have been implicated in mediating the negative feedback actions of steroids (13, 14), as well as the effects of negative energy balance on GnRH release (6, 15). Several physiological functions of K_{ATP} channels in the brain have been suggested (16, 17). However, the role of K_{ATP} channels in the regulation of GnRH secretion by ovarian steroids and negative energy balance has not been explored. This thesis will thus investigate whether K_{ATP} channels mediate the inhibitory signals of ovarian steroids and energy balance to alter GnRH secretion. Specifically, it serves the following purposes: (1) to determine the role of K_{ATP} channels in the negative feedback actions of estrogen and progesterone on GnRH neurons, and the mechanisms that may mediate any such effect, (2) to explore the effect of negative energy balance on pulsatile GnRH release in female mice, and (3) to assess the involvement K_{ATP} channels in the regulation of GnRH secretion by negative energy balance in female mice. In order to study the regulation of GnRH secretion in mice, microdialysis is developed to directly monitor *in vivo* GnRH pulses. The effect of ovarian steroids on pulsatile GnRH secretion is examined to validate this technique.

It has been well established that proper functioning of the HPG axis depends upon the pulsatile release of GnRH into the hypophyseal portal system. Continuous infusion of GnRH or a GnRH analogue tends to suppress, rather than stimulate the secretion of gonadotropins and gonadal steroids (18). To the contrary, pulsatile administration of GnRH rescues the reproductive deficits associated with hypogonadotropic hypogonadism (19). It is suggested that neurons located in the preoptic area and the hypothalamus, collectively named the “GnRH pulse generator”, controls the pulsatile secretion of GnRH (20). Extracellular recording in the hypothalamus reveals that multiunit activity (MUA) synchronizes with pulsatile LH release, a

good indicator of GnRH pulses (21, 22). While the exact cellular identity of the cells comprising the GnRH pulse generator is still unknown, it is believed to be composed of both GnRH neurons and their afferent neuronal network. However, the discovery of spontaneous and episodic electrical activity in GnRH neurons and GnRH neuronal cell lines (23, 24), together with the observed pulsatile release of GnRH from these neurons (25, 26), argues that GnRH neurons are possibly the GnRH pulse generator per se, although direct evidence needs to be provided to confirm this to be the case *in vivo*.

In females, GnRH release is subject to the negative feedback actions of estrogen and progesterone. Absence or disruption of these negative feedback actions leads to excess gonadotropin secretion and hyperstimulation of ovaries. For example, in polycystic ovarian syndrome (PCOS), LH levels are abnormally high as a result of resistance of hypothalamus and pituitary to the inhibitory effects of estrogen and progesterone (27). Although the inhibitory effect of estrogen and progesterone on gonadotropins secretion was documented decades ago (28), the mechanisms underlying these actions are poorly understood. Based on the earlier observations that GnRH neurons do not concentrate estradiol and are lack of immunostaining for estrogen receptors (later known as the receptor isoform, ERα) and progesterone receptors (29-31), direct effects of estrogen and progesterone on GnRH neurons have long been deemed unlikely. However, this idea has undergone renewed scrutiny since the discovery of functional estrogen receptor β (32) and membrane bound estrogen receptors (33) in GnRH neurons and GnRH neuronal cell line, GT1-7 cells. Estrogen and progesterone can also affect GnRH neurons via indirect mechanisms. These indirect mechanisms may involve glial cells (34) or neurons which send projections to GnRH neurons, such as those that produce the neurotransmitters neuropeptide Y (NPY) (35), melanocortins derived from the precursor protein

proopiomelanocortin (POMC) (36) and gamma amino butyric acid (GABA) (37). GnRH neurons express receptors for these neurotransmitters (35, 38, 39). In addition, estrogen and/or progesterone receptors are found in subsets of these neurons (10-12), suggesting their involvement in mediating the actions of ovarian steroids on GnRH neurons. Several lines of evidence have demonstrated that certain G-protein gated inwardly rectifying potassium (GIRK) channels mediate estrogen's inhibitory effect on GnRH neurons via GABAergic and opioidergic neurons (40). More recently, another type of inwardly rectifying potassium channels, K_{ATP} channels, are found in both GnRH neurons (Levine JE, unpublished data) and their afferent neurons, such as NPY, POMC, GABAergic neurons (8, 9). Their role in regulating GnRH secretion has yet been established.

Reproduction is also closely linked to energy status. Negative energy balance, as caused by reduced food intake or extreme energy expenditure, results in suppression of the HPG axis in a number of species (41-46). Food restriction or deprivation results in decrease in LH and gonadal steroids in both males and females. Likewise, excessive physical activity, such as seen in marathon runners, also causes reduction in LH secretion (47). The underlying mechanism is believed to be the disruption of pulsatile GnRH secretion by negative energy balance. In female sheep, dietary restriction increases the interpulse interval of both LH and GnRH pulses concomitantly detected in peripheral blood and pituitary portal blood respectively (48). Pulsatile administration of GnRH in men restores LH and testosterone levels inhibited by fasting, indicating that disturbed GnRH secretion underlie alterations in gonadotropin levels (49). However, this hypothesis has never been studied in mice due to the technical difficulty in monitoring GnRH/LH pulsatility or GnRH neuronal activity. A generally accepted model holds that negative energy balance first alters the availability of primary metabolic cue(s), possibly

glucose or/and free fatty acids. The primary metabolic cue(s) is/are then registered in certain brain regions either directly or indirectly via peripheral tissues. Signals from these brain regions, or from other peripheral tissues such as adipocytes or pancreas, then reach the hypothalamus to alter GnRH secretion (7). Several brain regions have been suggested as possible candidates for mediating these effects. The brain stem, especially the area postrema (AP), is critical in sensing the changes of metabolic cues and relaying these signals to GnRH neurons. Harboring both the glucose sensing mechanism and the GnRH pulse generator, hypothalamus may also serve as an integration site where changes in metabolic cues affect GnRH secretion. Other important metabolic signals include the adipocytokine leptin (50), and the pancreatic hormone insulin (51), both of which can exert actions on hypothalamic neurons through the activation of K_{ATP} channels (52, 53). The K_{ATP} channels mediate the effects of these hormones, are integral to the glucose sensing mechanism in the hypothalamus (54), and are also expressed in neurons which may regulate GnRH neurons (8, 9). Thus, these channels may play a role in mediating the effect of negative energy balance on GnRH secretion.

Interestingly, the signals of ovarian steroid feedback and energy balance may possibly converge to affect GnRH neurons. Evidence has accumulated that presence of estrogen enhances the ability of food restriction to suppress gonadotropin secretion (55, 56). Likewise, the negative feedback effect of estrogen on gonadotropin secretion is augmented by food deprivation (57). Signals from each pathway could converge either at the GnRH neurons or their afferent neurons.

K_{ATP} channels are composed of two types of subunits, inwardly rectifying potassium channels (Kir6.x) and sulfonylurea receptors (SURx) subunits, and couple cell metabolism with electrical activity (58). They are found in a number of organs including the central nervous system. Neuronal K_{ATP} channels serve a variety of functions, such as neuroprotection from

ischemia, hypoxia and excito-neurotoxins (16, 59). Hypothalamic K_{ATP} channels are mainly composed of Kir6.2 and SUR1 subunits, similar to those found in pancreatic β-cells (8). They are essential to the glucose sensing mechanism in both the pancreas and the hypothalamus. Kir6.2 knockout mice have impairments in food intake and glucagon secretion in response to neuroglucoprivation (54). As K_{ATP} channels are expressed in GnRH afferent neurons and also play a key role in energy homeostasis, it is possible that they may mediate the convergence of the signals of negative feedback of ovarian steroids and energy balance.

This dissertation attempts to identify the roles of K_{ATP} channels in the regulation of GnRH secretion by ovarian steroids and negative energy balance. Chapter II consists of experiments studying the effect of K_{ATP} channel blockade on LH pulses and the regulation of K_{ATP} channel expression in the preoptic area (POA) and mediobasal hypothalamus (MBH) by ovarian steroids in female rats. Results from these experiments will help to establish the role of K_{ATP} channels in the negative feedback action of ovarian steroids and possible molecular mechanism. Experiments in Chapter III investigate for the first time the effects of negative energy balance on GnRH secretion in female mice. A 48hr fast model is used to generate an acute state of negative energy balance in animals. As a preliminary experiment to monitor *in vivo* GnRH pulses in mice, microdialysis is developed and then validated by the known effect of ovariectomy on GnRH secretion. Chapter IV explores the role of K_{ATP} channels in mediating the signals of negative energy balance to GnRH neurons. It includes experiments examining the effect of a K_{ATP} channel blocker on LH levels in fed and fasted female mice and experiments studying the impact of 48 hours of fasting on LH secretion in SUR1 knockout female mice.

This dissertation will help to better understand the inhibition of GnRH secretion by ovarian steroids and negative energy balance. It has the following goals. The first of these is to gain

insight into the molecular mechanism of negative feedback actions of estrogen and progesterone on GnRH secretion. The second one is to study for the first time *in vivo* pulsatile GnRH secretion in mice as affected by negative energy balance. The final purpose is to ascertain the role of K_{ATP} channels in the regulation of GnRH/LH secretion by negative energy balance.

In accordance with the above goals, the following literature review will first address our current understanding of the female HPG axis, GnRH neurons and the mechanisms underlying and regulating pulsatile GnRH release. I will then discuss our present understanding of the negative feedback actions of estrogen and progesterone on GnRH secretion, and the possible mechanisms that mediate these effects. This will be followed by a discussion of the regulation of GnRH/LH secretion by negative energy balance. Finally, a detailed review of the molecular, cellular, and physiological aspects of K_{ATP} channels will be provided. A hypothesis for the involvement of K_{ATP} channels in the physiological regulation of GnRH release will then preface the succeeding chapters, which present my experimental observations.

Literature Review

B. The HPG axis and GnRH secretion

1. Overview of the Female Reproductive Cycles

GnRH neurons represent the final common pathway by which the central nervous system regulates the HPG axis. The human GnRH gene encodes an 92-amino-acid precursor protein called preproGnRH, which is later proteolytically processed to yield the mature decapeptide (1). GnRH synthesis occurs in the GnRH cell bodies located in the preoptic area and hypothalamus (60). After synthesis, GnRH is transported in secretory vesicles along the axons and then secreted into the hypophyseal portal system through the neurovascular junctions in the median eminence (61). After reaching the anterior pituitary, GnRH molecules diffuse into the extracellular space and bind to the G-protein coupled receptors on the plasma membrane of the gonadotropes and thereby stimulate the synthesis and secretion of LH and FSH. LH and FSH then act through their cognate receptors in the gonads to promote gametogenesis, steroidogenesis and secretion of polypeptide hormones such as inhibin.

In male mammals, GnRH and gonadotropin secretion remain at basal levels throughout the individual's reproductive lifespan. In sharp contrast, females exhibit reproductive/ovulatory cycles characterized by cyclic GnRH and gonadotropin secretion. The ovulatory cycles are of different lengths in different species, such as the 4 or 5-day estrous cycles in rodents and the month-long menstrual cycles in non-human primates and women. The central features of the female reproductive cycle are the preovulatory gonadotropin surges and ovulation. Towards mid cycle, preovulatory ovarian follicles release a surge of estrogen into the bloodstream, which triggers GnRH and subsequent LH and FSH surges via its positive feedback effects at both the

hypothalamus and pituitary. The LH surge results in the ovulation of mature oocytes, which are then ready to be fertilized.

Non-human primates and women have menstrual cycles. During the follicular phase of the ovaries, follicles undergo folliculogenesis. GnRH, LH and FSH surges occur at mid cycle. After LH induced-ovulation, if the oocyte(s) is/are not fertilized, ruptured follicles become endocrine organs known as corpus luteum, which defines the luteal phase. During the luteal phase, estrogen and progesterone are secreted from the corpus luteum and their levels are higher than those during the follicular phase. LH and FSH level are relatively low during luteal phase due to the negative feedback action of estrogen and progesterone on the hypothalamus and pituitary. At the end of the luteal phase, estrogen and progesterone levels fall due to lack of support for the corpus luteum, leading to shedding of the endometrium, namely menstruation. After menstruation the next reproductive cycle starts.

In contrast, rodents have estrous cycles. Metestrus and diestrus mark the phase of folliculogenesis, which is analogous to the follicular phase of the ovaries in primates. These two stages of estrous cycles precede the preovulatory LH and FSH surges which occur on afternoon of proestrus. Distinct from the primates, the FSH surges in rodents have a secondary phase on the morning of estrus, which is thought to play a role in recruiting follicles for the next estrous cycle. Ovulation is induced by proestrous LH surge and occurs in the early morning on estrus. Also different from primates, rodents do not have a real luteal phase.

2. LH and GnRH Pulsatility

It is very important to note that the LH is secreted from the anterior pituitary gland in a pulsatile manner, a secretory feature that has been demonstrated in all species studied, including

rats (62), monkeys (63) and humans of both sexes (64). This LH pulsatility differs in amplitude and/or frequency at different stages of the ovulatory cycle. For instance, in rats LH pulse frequency is around one pulse/hour on metestrus and diestrus (62, 65), but very infrequent or even absent on estrus (66).

LH pulses are driven by the pulsatile release of GnRH from the hypothalamus such that each LH pulse was preceded by a GnRH pulse. This has been very well demonstrated in ewes by using serial hypophyseal blood sampling (67) or push-pull perfusion (68) to successfully monitor the GnRH release *in vivo*. The pulsatile GnRH release is essential for the proper release of LH and FSH and hence the normal functioning of the reproductive axis. Continuous infusion of GnRH results in complete cessation of LH and FSH secretion in about two weeks (18). This feature of regulation of the HPG axis carries clinical significance. Continuous administration of GnRH analogs has been used effectively to treat patients with gonadal steroid-dependent malignancies, such as breast , ovarian and prostate cancers (69). To the contrary, infusion of GnRH at intervals comparable to the physiological levels restores LH pulses and ovulation in monkeys with defective GnRH secretion caused by hypothalamic lesions, and in women with hypothalamic amenorrhea and presumed endogenous GnRH deficiency (18, 19). The neuronal network which controls the pulsatile release of GnRH is referred as “GnRH pulse generator” (20, 70). Details of the GnRH pulse generator will be discussed below.

3. GnRH Neurons

a. Different Types of GnRH Neurons

Many forms of GnRH molecules have been discovered in various vertebrate species, namely the mammalian GnRH (GnRH I), chicken GnRH (GnRH II) and fish GnRH (GnRH III) (71-73).

GnRH I is the major molecule released into the hypophyseal portal vasculature and is responsible for the normal functioning of the HPG axis. Neurons expressing GnRH I molecules are termed GnRH I neurons. The GnRH II molecule was first identified in chicken hypothalamus (74), and later found in other species such as humans (75). It is primarily located outside the brain, such as kidney, bone marrow and prostate (1). In the central nervous system, it is mainly found in the midbrain (76), where it is important in the regulation of sex behavior (77). Whereas, GnRH III molecules were first discovered in fish brains (78). Later studies also found GnRH III expression in the hypothalamus of humans (79) and rats (80) .

Although there are a few lines of evidence suggesting their involvement of in modulating gonadotropin secretion and/or steroidogenesis, GnRH II (81) and GnRH III (82) molecules are not generally considered the predominant GnRH molecules in the HPG axis. Therefore, for convenience, all the usage of “GnRH” in this dissertation will refer to GnRH I unless otherwise specified.

GnRH neurons are oval or fusiform-shaped, with few dendritic branches (83). They are distributed rostrally from the diagonal band of Broca to the premamillary region and extend to up to 2mm on either side of the midline of preoptic area and medial forebrain bundle in rats (84, 85). GnRH neurons which project to the median eminence are mainly located at the midline region of medial preoptic area rostrally and through the retrochiasmatic area caudally (86). Interestingly, in the hypothalamus, GnRH neurons do not form a distinct nucleus, but instead they are distributed in a scattered, network-like pattern. In addition, GnRH neurons are of remarkably few numbers compared to other types of neurons in the brain: there are only about 1200-1600 GnRH neurons. This number is quite consistent among many species, including rodents and primates (87, 88).

b. Origins and Migration of GnRH Neurons

Although GnRH neurons are localized to the septum, preoptic area and hypothalamus, they are embryonically derived from outside the central nervous system. Over two decades ago, it was discovered in mice (89, 90) and primates (91) that GnRH neurons originate from nasal placode. Later studies corroborate this finding in other species (92, 93). GnRH-containing neurons can be detected as early as embryonic day 11.0 (E11.0) in the nasal placode in mice. They then traverse the nasal placode and penetrate the cribriform plate into the forebrain. After entering into the forebrain, they assume a more caudal and ventral route into the basal forebrain and finally reach their destination in the preoptic area and hypothalamus (94, 95). Failure of GnRH-containing neurons to migrate to the hypothalamus leads to deficient GnRH release, which then causes hypogonadotropic hypogonadism. Such a disorder in humans is called Kallmann's syndrome (96). Several molecules have been implicated in directing GnRH neurons to their final destinations. Neuronal fibers containing peripherin, which extend from the nasal placode to the basal forebrain before the beginning of GnRH neuron migration, are thought to serve as the axophilic pathway for the GnRH neurons to migrate to their final locations (97). In addition, cell adhesion or soluble molecules, such as tenascin (98), gamma-aminobutyric acid (GABA) (99), are also proposed to play a role in regulating GnRH neuronal migration.

4. GnRH Pulse Generator

Pulsatile GnRH release is determined by a neural GnRH pulse generator located in the preoptic area and the hypothalamus. Despite extensive research, the true cellular identity of the GnRH pulse generator is still unknown. It has been demonstrated that the hypothalamic MUA recorded extracellularly was tightly coupled to the pulsatile LH secretion in several species, such

as monkeys (21), goats (100), and rats (22). Subsequent studies using cultured GnRH neurons (25) and GT1-7 cells (26, 101), showed pulsatile GnRH release from these cells with pulse intervals similar to that of physiological GnRH release. It points to the possibility that GnRH neurons themselves may comprise the GnRH pulse generators, although this remains to be directly demonstrated.

a. Electrical Activities of GnRH Neurons

It has been established in many peptide hormone-releasing neurons that hormone release is associated with an acceleration of firing rates of the neurons (102, 103). Interestingly, GnRH neurons, such as GT1-7 cells, spontaneously fire high-frequency volleys of action potentials, which can been sustained without extrinsic cues (23, 24, 104). These spontaneous firings of action potentials are usually grouped into bursts, with 5-10 action potentials in one burst. The bursts can be rhythmic or random. For the rhythmic bursts, the intervals between bursts are about 5-10 seconds. Moreover, GT1-7 cells and GnRH neurons derived from olfactory placodes of rhesus monkeys both show spontaneous intracellular calcium oscillations (24, 105). The latter observation provides convincing evidence that firing spontaneous action potentials is an intrinsic, but not a later acquired, property of GnRH neurons.

The electrical activities of GnRH neurons are determined by the ion channels present on the cell membrane. These ion channels have been shown to be very important in either maintaining the intrinsic electrical activity of GnRH neurons or mediating the regulation of GnRH neuronal activities (106).

Two cation channels, hyperpolarization-activated cation currents (Ih) and transient, T-type calcium current (I_T) have been demonstrated to be crucial for the episodic electrical activity of GnRH neurons (107, 108). They are both activated by hyperpolarization. Once activated, they

permit inward cation currents and depolarize the membrane potential to threshold for firing the next action potential.

Potassium channels in GnRH neurons, as in other excitable cells, are important for maintaining the membrane potential and regulating neuronal firing rates. Several types of potassium channels have been identified in GnRH neurons. G-protein coupled inwardly rectifying potassium channels are linked to G-proteins on the membrane and have been suggested to play a role in mediating the effects of opioids on GnRH neurons (108). Several forms of voltage-dependent potassium channels including A-type potassium channels and slowly inactivating potassium channels, have been suggested to be involved in estrogen's effects on the electrical activity of GnRH neurons (109). Small conductance calcium-activated potassium channels are stimulated by an increase of intracellular calcium due to calcium influx. The subsequent potassium efflux after the channel activation hyperpolarizes the cells and contributes to the afterpolarization of the neurons following action potential firing. It is believed these channels play a key role in inhibits cell firing and limits the frequency of repetitive action potentials (110).

Voltage-gated calcium channels are important in neurosecretion (24). Firing of action potentials depolarizes the cell and activates voltage-gated calcium channels, enabling calcium to enter the cell. Once inside the cell, the calcium molecules interact with intracellular machinery and initiate fusion of secretory granules with presynaptic membrane and hence neurosecretion. Numerous studies have shown that depletion of extracellular calcium can abolish or greatly reduce the secretion of GnRH (111).

b. Calcium Oscillations

Oscillations of intracellular calcium are coupled to changes in membrane potential and the firing of action potentials in many excitable cells. One study disclosed that the periodic calcium waves were initiated by spontaneous action potentials in the GT1-1 cells (112). The increase in intracellular calcium can either result from calcium influx or from intracellular storage, such as smooth endoplasmic reticulum and mitochondria, or both. The calcium oscillations observed in the GnRH neurons are believed to be largely due to the calcium influx through the voltage-gated calcium channels activated by depolarization of the neurons, although in a small part due to the recruitment of mitochondrial calcium pool (24). The calcium oscillations exhibit an interpulse interval of 8 minutes and a duration of around 90 seconds. Interestingly, up to 85% of the cultured embryonic GnRH neurons showed synchronicity at an interval of about 60 minutes, which is similar to the pulse intervals of observed GnRH pulses. In a recent study, synchronization of calcium oscillation in GT1-7 cells is found concomitant with an increase in plasma membrane area, indicating its association with neurosecretion (113). However, the relationship of the synchronization of calcium oscillation with GnRH release requires further investigation.

c. Different Time Domains of GnRH Neuronal Firing Pattern

A recent study using Fourier spectral analysis identified three time domains of the firing patterns of green fluorescence protein-tagged GnRH neurons recorded extracellularly in mouse hypothalamus (114). The three time domains are defined by the authors as bursts (<100s), clusters (100-1000s), and episodes (>1000s) according to the intervals between adjacent events. The bursts are the high-frequency rhythm and composed of several action currents with an interval of less than 20 seconds, which are comparable to what has been observed in GT1-7 cells and cultured embryonic GnRH neurons (23, 24, 104). Further analysis of the peaks and nadirs in

firing rate of action currents identified two low-frequency rhythms, clusters and episodes. The clusters occur at intervals between 100-1000 seconds, with most of them being around 500 seconds (8 minutes), which is interestingly similar to the interpulse interval of calcium oscillation observed in cultured embryonic GnRH neurons (105). Episodes occur even more infrequently with intervals between adjacent episodes more than 1000 seconds. Most of the episodes are around 1600 seconds. Whether and how the latter two low-frequency components, clusters and episodes, relate to the pulsatile GnRH secretion is not known and clearly requires additional exploration.

d. Synchronization of GnRH Secretion

Although GnRH neurons show intrinsic and episodic firing of action potentials, secretion of GnRH from one or a few neurons can not explain the pulsatility of GnRH secretion documented *in vivo* and *in vitro*. In fact, if the firing of action potentials is completely random across the GnRH neuronal population, no coordinated pulses will be generated. In order to form a GnRH pulse, a number of, if not a large portion of, GnRH neurons have to be recruited at the same time to secrete GnRH into the hypophyseal portal system.

A few lines of evidence supporting the existence of synchronization have been obtained from studies of calcium oscillations in embryonic GnRH neurons (105, 115). It has been found, for example, that calcium oscillations in several GnRH neurons in a single culture system tends to synchronize at an interval around 50-60 minutes, which is similar to the *in vivo* interpulse interval of GnRH pulses (63, 116).

In cultured GT1-7 cells, it has been discovered that the electrical stimulation of one GT1-7 cell can be propagated to its neighboring cells. A majority of GT1-7 cells situated in close contact with each other exhibit synchronous electrical activities (117). In addition, a more recent

study, in which a few groups of GT1-7 cells were recorded at the same time in multimicroelectrodes plates, showed that there is a coordinated activity between independently active cell groups. This coordination leads to episodic changes in the network electrical activity (118). Whereas, how this coordination of action potential firings of individual GnRH neurons relates to the calcium oscillation remains undetermined.

i. Models of Synchronization

Several models of synchronization of GnRH network have been proposed to explain for the pulsatile release of GnRH (119).

The first is the "independent" model. In this model system, the GnRH neurons fire independently. There is actually no synchronization between them. It is the coincidence of GnRH secretion from a number of GnRH neurons that contribute to the GnRH pulses observed in the hypophyseal portal system.

The "coupled" model suggests that GnRH neurons are coupled with each other through either gap junctions (101) or intercellular factors released from GnRH neurons themselves or non-GnRH cells. The coupling of the GnRH neurons then leads to the synchronized secretion of GnRH.

The "triggered" model proposes that there is master pulse generator outside the GnRH neuronal network, which dictates the GnRH neurons to fire action potentials and secrete GnRH synchronously (120).

ii. Mechanisms of Synchronization

The distribution of GnRH neurons as a scattered population instead of as a discrete nucleus in the hypothalamus poses extreme difficulty in studying the synchronization of the electrical activity of GnRH neurons. Up to now, the exact mechanisms underlying the synchronization of

the GnRH neurons are still unknown. There are a number of possible mechanisms which have been proposed so far.

Direct connections between GnRH neurons via chemical synapses have been observed under electron microscope and thought to play a role in mediating the synchronous activity of GnRH neurons (121, 122). It is proposed that GnRH molecules released into these synapses bind to GnRH receptors on the postsynaptic membranes and transmit the signal of neuronal activation. Several lines of evidence support the presence of GnRH receptors on the plasma membranes of GnRH neuron (123, 124). One recent study using embryonic GnRH neurons reported that application of GnRH causes increase in both electrical activity and pulsatile GnRH secretion, thus indicating the role of GnRH molecules in its pulsatile secretion.

In addition to the chemical synapses, GnRH neurons may form gap junctions among each other (101). Recently, two components of gap junctions, connexin-26 (101, 125) and connexin-32 (126), were found in the GT1-7 cells and rat hypothalamic GnRH neurons respectively. The presence of gap junctions between GnRH neurons makes it possible that electrical couplings between GnRH neurons may play a role in the synchronization of GnRH neurons.

Non-neuronal cells have also been suggested to be involved in GnRH synchronization (127). Perikarya of GnRH neurons are ensheathed by processes of glial cells (128). In a culture system derived from rhesus monkey embryo which included both the olfactory placodes and the ventral GnRH neuronal migratory pathway, the glial cells exhibit episodic calcium oscillations. They not only occur at a frequency similar to that of GnRH pulses, but also are synchronous with the calcium oscillations in the GnRH neurons (129). Moreover, the calcium waves spread between the population of cells composed of neurons and glial cells.

In addition to the neuron-neuronal and neuron-glial contact, diffusable intercellular molecules are another possible mechanism to mediate GnRH synchronization. Several molecules have been suggested to play such a role, such as nitric oxide (NO) (130, 131), extracellular ATP (132). NO stimulates GnRH release from median eminence or mediobasal hypothalamus fragments (133) . NO from the vascular origin is released from the rat median eminence in a pulsatile pattern. Furthermore, its release is also cyclic across the estrous cycle, with the highest levels on proestrus. GnRH release from the same isolated median eminence correlates with NO production. Inhibition of endothelial NO synthase decreases GnRH release on proestrus, indicating the coupling of NO and GnRH secretion (130, 131). Extracellular ATP has also been postulated to be involved in GnRH synchronization. Although little is known about the exact sources of extracellular ATP, it is suggested that ATPs bind to the P2X receptors on GnRH neurons, open voltage-gated L-type calcium channels and induce calcium oscillations. Depletion of extracellular ATP, antagonist to P2X receptor and blockade of the calcium channel all abolish the ATP-induced calcium oscillations in GnRH neurons (132).

e. Neuronal Inputs to GnRH Neurons and Regulation of GnRH Secretion

As the central mechanism controlling the reproductive axis, GnRH neurons receive synaptic inputs from various types of neurons. Below are a brief summary of some well characterized afferent neurons that have been implicated in mediating the physiological signals to GnRH neurons. By no means is this list comprehensive. In fact, with development of more sensitive imaging techniques, this list will surely expand in the future.

Glutamatergic neurons

Glutamate is the major excitatory neurotransmitter in the CNS. It has been shown to stimulate GnRH secretion. Glutamate treatment resulted in N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptor activation, which in turn leads to inward currents and subsequently increased firing activity of isolated GnRH neurons (119, 134). Glutamate and its agonists cause elevation in LH secretion in animals. Both NMDA and non-NMDA receptor antagonists inhibit pulsatile LH release in castrated animals (135). Accordingly, various types of glutamate receptors have been found in GnRH neurons, including NMDA receptor (136, 137) and non-NMDA receptors (134, 137), indicating a direct mechanism of glutamate on GnRH neurons.

GABAergic neurons

As the major inhibitory neurotransmitters in the central nervous system, GABA has also been implicated in regulating GnRH secretion. Blockade of $GABA_A$ activation by bicuculline induced a large and prompt increase in GnRH release in prepubertal monkeys, in comparison to a slight increase in GnRH release in pubertal monkeys (138). In contrast, GABA suppresses GnRH release in pubertal, but not prepubertal, monkeys (138), possibly due to the high endogenous level of GABA in the prepubertal monkeys. Moreover, bicuculline, a $GABA_A$ receptor antagonist, is also able to advance the LH surge in rats without affecting their circadian rhythm (139). Interestingly, excitatory effects of GABA on GnRH neurons have also been strongly suggested to be dominant, especially through $GABA_A$ receptors. Depolarizing GABAergic synaptic activity were documented in embryonic GnRH neurons derived from the olfactory placode (140). Direct application of GABA to GnRH neurons causes depolarization and subsequent action potentials (141). The discrepancy between the inhibitory and excitatory effects of GABA on GnRH neurons is proposed to be due to the difference in intracellular chloride

concentration. High intracellular chloride concentration due to chloride-accumulating cotransporters may explain the excitatory effect of $GABA_A$ activation on GnRH neurons (141).

Norepinephrine (NE) neurons

NE signaling has been functionally linked to GnRH secretion. Locus ceruleus lesion blocks the preovulatory surge of LH, but not the basal level, in female rats (142). NE molecules, especially those from the brain stem is important in the regulation of GnRH secretion (142, 143). Blockade of NE signaling by α-adrenergic antagonist suppresses LH pulsatility in guinea pig (144). In addition, neuroanatomical studies provided more evidence of the involvement of NE in the regulation of GnRH neurons. NE neurons in the caudal brain stem have been demonstrated to project to GnRH perikarya at rostral preoptic area in a retrograde tracing study (145). Accordingly, α-adrenergic receptor immunoreactivity was also found in GnRH neurons (146), suggesting direct effects of NE on GnRH neurons.

NPY neurons

The effect of NPY on GnRH neurons is complex and may depend on the hormonal milieu. It has been suggested to contribute to the initiation of the preovulatory LH surge. NPY stimulated GnRH secretion from the median eminence harvested from rats on proestrus afternoon (147). Moreover, in cultured GT1-7 cells, NPY is stimulatory to GnRH release (148). However, NPY can also inhibit GnRH and LH secretion. Third ventricle infusion of NPY reduced LH pulse frequency and pulse amplitude in ovariectomized rats, which could be restored by subsequent GnRH treatment (149). Chronic infusion of NPY inhibited LH, FSH and testosterone levels in male rats (150). Consistent with the *in vivo* studies, synaptic connections between GnRH and NPY neurons has been documented (151). In addition, NPY Y1 (35) and Y5 receptors (152)

have also been found in nerve terminals and cell bodies of GnRH neurons and were shown to regulate GnRH secretion.

POMC neurons

POMC neurons are mainly located in the arcuate nucleus of the hypothalamus. They have been found to send projections to GnRH neurons (36, 153). Accordingly, different types of opioid receptors have been identified in GnRH neurons in a number of species. Although one study reported no expression of either μ, δ, or κ opioid receptor mRNA in GnRH neurons in rats (154), a more recent study using double-labelling immunofluorescence and confocal microscopy revealed the presence of δ-opioid receptor in a subset of GnRH neurons in rats (38) . Moreover, δ-opioid receptors have also been found in immortalized GnRH cell lines, such as GT1-1 and GT1-7 cells (38, 155). In guinea pig, μ-opioid receptors were localized in subpopulations of GnRH neurons (156). Physiologically, different endogenous opioids, such as enkephalin and dynorphin, have been showed to inhibit GnRH secretion (157, 158).

Kisspeptin neurons

Kisspeptin is an extraordinarily potent GnRH/LH secretagogue at doses as low as 1 fmol (159, 160). Deficiency of kisspeptin receptor, a G-protein coupled receptor named GPR54, causes hypogonadotropic hypogonadism (161). The underlying mechanism is an impairment in GnRH secretion, distinct from the defective GnRH neuronal migration found in the Kallmann's syndrome (162). Kisspeptin neurons have been shown to be localized in areas important in regulating GnRH neurons, such as anteroventral periventricular nucleus (AVPV), POA and arcuate nucleus (163) and kisspeptin fibers are found in close approximation to GnRH neurons (164). Accordingly, GnRH neurons express GPR54 (160) and kisspeptin treatment results in

rapid induction of Fos in GnRH neurons (165), indicating a direct effect of kisspeptin on GnRH neurons.

Summary

GnRH pulses are generated by a complex mechanism involving episodic, spontaneous electrical activity of GnRH neurons, synchronization of GnRH release and possible interaction between GnRH neurons and their afferent neurons.

Being the final common pathway through which CNS regulates reproductive axis, GnRH neurons are regulated by a number of physiological factors, internal or external. Among them are ovarian steroids and states of energy balance. Both ovarian steroids and negative energy balance are able to suppress GnRH secretion and the HPG axis. The following reviews will shed light on our current understanding of the effects of these two factors on GnRH secretion and possible mechanisms involved in these processes.

C. Negative Feedback Actions of Ovarian Steroids on GnRH Secretion

GnRH is released in a pulsatile manner from the median eminence into the hypophyseal portal system (166). This pulsatile pattern of GnRH release is believed to be dictated by a neural GnRH pulse generator located in the preoptic area and hypothalamus (167). Currently, the activity of the GnRH pulse generator is believed to result from the spontaneous and episodic electrical activities of GnRH neurons (68). In order to maintain proper homeostasis of the HPG axis, GnRH pulse generator is under strict regulation by gonadal steroid hormones, such as estrogen and progesterone in female. In the female, there exist both positive and negative feedback of estrogen and progesterone on GnRH and gonadotropin secretion. The negative feedback actions of estrogen and progesterone are manifested as reduced GnRH pulsatility and/or decreased response of gonadotropes to GnRH. Except before ovulation when sustained high level of estrogen evokes a unique positive feedback action, these negative feedback actions of estrogen and progesterone are maintained throughout the female reproductive cycle.

1. Estrogen's Negative Feedback Actions on GnRH Secretion

In spite of numerous investigations, the mechanisms mediating the negative feedback actions of estrogen still remain unknown. Evidence accumulates that estrogen may have multiple effects on pulsatile GnRH and LH secretion. One study showed that 24hr estrogen treatment immediately after ovariectomy on diestrus 1 had no effect on the increase of LH induced by ovariectomy in rats (168). The same group also reported that estrogen reduced both pulse frequency and pulse amplitude of LH pulses if it was given immediately after ovariectomy on diestrus 2 and lasted for 24hr (169). Similarly, another study showed that estrogen decreased both LH pulse frequency and pulse amplitude if given two weeks after ovariectomy in rats (170).

However, a three-day estrogen treatment given three months after ovariectomy decreases LH level mainly by reducing its pulse frequency (171).

Nevertheless, most studies seem to agree that estrogen mainly decreases the amplitude of LH pulses (172). In addition, studies monitoring *in vivo* GnRH pulsatility found that estrogen reduces GnRH pulse amplitude, but not GnRH pulse frequency in both female monkeys and sheep (173-175). Although the relationship of GnRH pulse amplitude to LH pulses is not clear, these findings at least argue against the idea that estrogen decreases LH pulse frequency by reducing GnRH pulse frequency. Interestingly, the ability of estrogen to suppress LH levels is enhanced by food restriction (57). As food restriction is also inhibitory to LH secretion. This observation points to the possibility that there is interaction of these two pathways.

More recently, single unit extracellular recording of GnRH neurons, which were identified in slices from the GnRH-enhanced green fluorescence protein (EGFP) mice demonstrated that estrogen alters the firing patterns of GnRH neurons (176, 177). Specifically, when estrogen was given to the female GnRH-EGFP mice to mimic its negative feedback, it increased the duration when GnRH neurons remained silent. After presynaptic inputs to GnRH neurons were pharmacologically eliminated by blockade of the AMPA, NMDA and $GABA_A$ receptors, there was no change in the GnRH neuron firing pattern in tissues from OVX animals. However, in half of the estrogen treated animals, the estrogen-induced lengthening of intervals between firing episodes was abolished by the blockade of presynaptic inputs, indicating that the inhibitory effect of estrogen may involve afferent synaptic signals to the GnRH neurons. The same group also classified the observed GnRH electrical activity into three time domains after Fourier spectral analysis: bursts (<100s), clusters (100-1000s), episodes (>1000s). The episode is actually in accordance with the time frame of GnRH pulsatility. It was demonstrated that estrogen does not

affect the intrinsic properties of bursts, but is able to affect the patterning of the bursts, thus increasing the episode period (32, 34, 178). The authors then went one step further to examine the effects of estradiol on voltage-gated potassium channels found in the GnRH neurons (109). It was determined that estradiol results in a decrease in current amplitude and a slower inactivation. Interestingly, when the firing properties of GnRH neurons were investigated in the same study, estradiol causes a shorter latency and a hyperpolarized threshold for firing action potentials. The above two effects of estrogen actually will make it easier for GnRH neurons to fire action potentials, which seems to be contradictory to the negative feedback actions of estrogen. The author proposed that there may be two modes of direct feedback mechanisms of estrogen on GnRH neurons. One is excitatory, while the other is inhibitory. Shifting between these two modes of actions determines when estrogen exerts negative or positive feedback actions on GnRH neurons.

Three potential pathways have been proposed to explain the negative feedback of estrogen on GnRH secretion (32, 34). The first one suggests direct actions of estrogen on GnRH neurons. The second pathway involves trans-synaptic regulation of GnRH neurons through intervening neurons. The third pathway is associated with the interaction between the GnRH neurons and glial cells (Fig. 1).

Figure 1

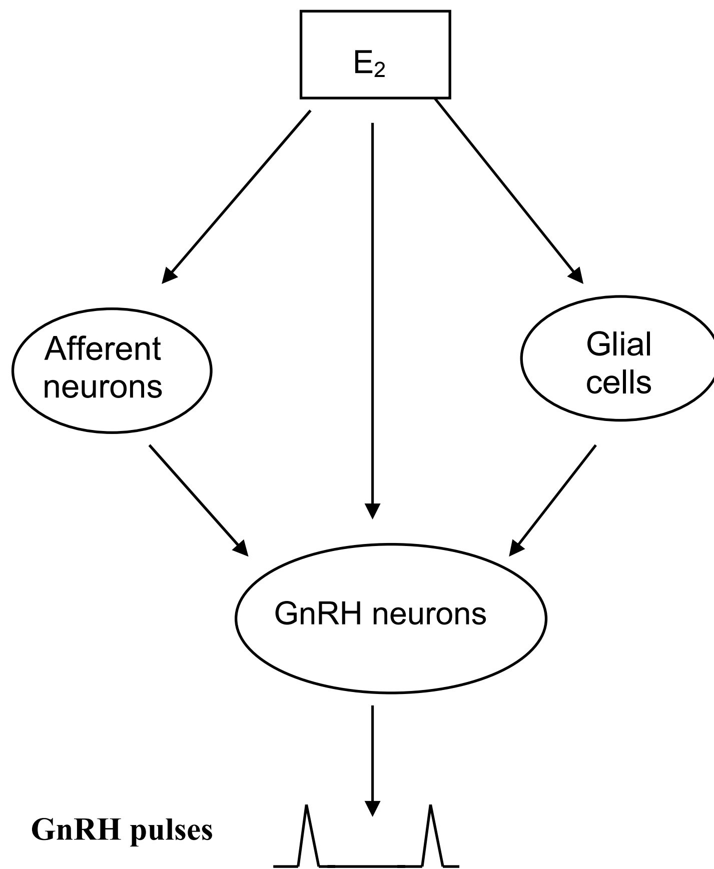

Fig. 1. Multiple pathways of the negative feedback actions of E_2 on GnRH neurons. E_2 can directly regulate GnRH neurons via the genomic or non-genomic effects. E_2 can also affect GnRH neurons through a trans-synaptic approach with afferent neurons as mediators. Moreover, glial cells can also transmit the effect of E_2 to GnRH neurons.

a. Direct Actions of Estrogen on GnRH Neurons

It has long been believed that ERs are not expressed in GnRH neurons, thus making impossible the direct effects of estrogen on GnRH neurons. The evidence first stemmed from the study showing that GnRH neurons do not concentrate estradiol (29). Then this observation was further corroborated by studies using double-label immunohistochemistry (30). However, a new type of ER, termed ER_{β}, was discovered more recently (179, 180) and was found to be expressed in a subset of GnRH neurons (32, 181), thus reviving the possibility that estrogen has direct effects on GnRH neurons. At present, although ER_{β} is generally believed to exist in GnRH neurons, whether another isoform of ER, i.e. ER_{α}, is expressed in GnRH neurons is still under debate. A recent study using RT-PCR and Western blot revealed that both ER_{α} and ER_{β} are expressed in GT1-7 cells, and estrogen directly downregulates GnRH mRNA expression (182). Another line of evidence came from a study using immunoprecipitation and double-label immunohistochemistry showing that GnRH neurons within the rat preoptic area possesses ER_{α}-immunoreactivity (183). A more recent study also demonstrated that ER_{α}, ER_{β} and progesterone receptor A (PR_{A}) were expressed in GT1-7 cells and isolated hypothalamic neurons from fetal rats (33). Moreover, it showed that estrogen and progesterone, when given to these cells, were able to alter the pulsatile pattern of GnRH release and cAMP level through a Gi dependent mechanism. In contrary to these studies, other studies have continued to fail to detect ER_{α} in GnRH neurons (184, 185).

Both ER_{α} and ER_{β} are members of the superfamily of classic nuclear receptors. They function as ligand-activated transcriptional factors to regulate target gene expression. However, ample evidence suggests that there are also membrane-bound ERs (186, 187). Two possible locations of these plasma membrane ERs have been proposed. One speculation is that ER_{α} and/or

ER_β can undergo post-translational lipid modification in the endoplasmic reticulum, which then facilitates the insertion of the receptors into the plasma membrane (188). The other one, which is more promising, is that ERs may exist entirely within the membrane phospholipid bilayer, probably in small compartments known as caveolae (189). It has been shown that after binding to those membrane receptors, estrogen is capable of initiating a number of signaling pathways, such as mobilizing intracellular calcium (190), stimulating adenylate cyclase activity and cAMP production (191), activating phosphatidylinositol 3-kinase (PI3 kinase) (192) and mitogen-activated protein kinase (MAPK) signaling pathway (193) and stimulating endothelial nitric oxide synthase (194).

Estrogen may impact upon GnRH neurons either through the classic genomic effect, or through the non-genomic effect. The genomic effect usually requires at least 45 minutes for protein synthesis, and even longer time to initiate corresponding cellular functions (195). After estrogen binds to its nuclear ER, the ligand-receptor complex can either directly bind to its target genes and activate their expression as a transcriptional factor, or can interact with other transcriptional factors and regulate gene expression (196). The genomic effect of estrogen can also been accomplished by binding of estrogen to its membrane receptors, with the cAMP response element binding protein (CREB) as the downstream signal to mediate estrogen's genomic effect (197). Besides the genomic effects, there is a large body of evidence supporting that estrogen has non-genomic effects (198-200). Compared to the genomic effect, the non-genomic effect of estrogen is more rapid, always happening in a few minutes and involving membrane bound ERs. An electrophysiological experiment clearly showed that estrogen could directly and rapidly hyperpolarize GnRH neurons by opening ligand-gated potassium channels (108). Moreover, estrogen also contributes through the plasma membrane ERs to the uncoupling

of G proteins with potassium channels or activation of the second messenger systems, such as the phosphatidylinositol (PI) pathway (201-203), cyclic adenosine monophosphate (cAMP) pathway (204), and cyclic guanosine monophosphate (cGMP) pathway (205). A more recent studies demonstrated that ER_{β} mediates estrogen's rapid phosphorylation of CREB in GnRH neurons (206), thus may directly affect GnRH neurons.

b. Actions of Estrogen on GnRH Neurons through Intervening Neurons

In addition to its direct actions, estrogen may exert its negative feedback actions on GnRH neurons via a trans-synaptic mechanism. A number of neurotransmitters have been shown to be involved in this pathway, such as GABA (207) and opioids (34), two major inhibitory neurotransmitters in the brain. GABA is most likely the main mediator of estrogen's negative feedback. GABA neurons synapse on GnRH neurons (37) and GnRH neurons express both $GABA_A$ (208) and $GABA_B$ (39) receptors. In addition, studies have shown that estrogen influenced both GABA release (207) and reuptake (209). ERs are also found in GABAergic neurons (13, 210). All the above observations suggest that estrogen may act upon GnRH neurons by affecting the GABA input. More convincingly, there exists an inverse profile of GABA level and GnRH secretion in the prepubertal and pubertal monkey (138). While in rats, a similar inverse relationship between preoptic GABA levels and mean LH release was also found (207, 211). Blockade of $GABA_A$ receptor led to increase of LH secretion in ovariectomized, estradiol–treated rats (212). More direct evidence of estradiol's effect on GABAergic neurons was obtained from electrophysiological studies, which showed that 100nM estrogen given to the guinea pig brain slices rapidly uncoupled $GABA_B$ receptor with G-protein gated inwardly rectifying K^+ (GIRK) channels in GABAergic neurons through a protein kinase A (PKA)- and protein kinase C (PKC)- dependent pathway. This uncoupling thus released the autoinhibition of

these GABAergic neurons and permitted more inhibitory inputs to GnRH neurons (213). Opioids, such as enkephalin, β-endorphin, are the second major inhibitory neurotransmitters in the brain. β- and μ-opioid receptor agonists have potent inhibitory action of GnRH and LH release in ovariectomized rats and naloxone, a μ-opioid receptor antagonists, blocked this inhibition, indicating they may subserve estrogen's feedback on GnRH neurons (158). However, subsequent studies revealed that this blocking effect of naloxone was independent of sex steroid status (157). So, endorphin neurons may not mediate the estrogen's inhibitory effect on GnRH neurons. To the contrary, electrophysiological study showed that like in GABAergic neurons, estrogen also uncoupled the μ-opioid receptor with GIRK channels, thus reopening the issue that opioids may be involved in mediating the negative feedback of estrogen (40, 108).

Kisspeptin may also be involved in the negative feedback actions of estrogen. As stated earlier, kisspeptin stimulates LH secretion primarily via a GnRH-dependent pathway (159, 160). Both ERα and ER_{β} (214) have been be localized in kisspeptin neurons. ERα appears to be dominant as it is expressed in majority of GnRH neurons, while ER_{β} is found only a 20% of GnRH neurons (215). In addition, kisspeptin expression was demonstrated to be regulated by E_2 (215). Specifically, kisspeptin mRNA level in the arcuate nucleus is decreased by ovariectomy but increased by estrogen. Since arcuate nucleus is believed to be pivotal to the negative feedback action on GnRH neurons, it is thus possible that kisspeptin neurons mediate the inhibitory effects of estrogen on GnRH neurons (216, 217).

c. Actions of Estrogen on GnRH Neurons through Glial Cells

Estrogen may also act through glial cells to inhibit GnRH neurons. There are astrocytes in the GnRH neuronal network. They ensheathe the soma and/or nerve terminals of GnRH neurons and may affect synaptic inputs to the GnRH neurons (218). A few epidermal growth factors, such as

transforming growth factor β, have been shown to be secreted by astrocytes to influence the differentiation and activity of GnRH neurons (219). Interestingly, estrogen was demonstrated to have impacts on astrocytes. Astrocytes in the rat hypothalamus exhibit cyclic morphological changes in accordance to the different stages of estrous cycle, indicating the strong association of astrocyte morphology with gonadal steroid environment (220). A more direct evidence of estrogen's effect on astrocytes was provided by an important observation (221) that astrocytes in the guinea pig hypothalamus express ER_{α}. Therefore, alteration to the astrocytes under estrogen's action may underlie some of the changes of GnRH secretion (34).

By and large, all these three pathways may be involved in the negative feedback action of estrogen on GnRH neurons. However, possible involvement of each pathway may depend on the species, the stage of reproductive cycle and the hormone milieu. Besides estrogen, the other ovarian steroid, progesterone, also plays an important role in regulating GnRH secretion.

2. Progesterone's Negative Feedback on GnRH Secretion

Compared to the enormous efforts that have been devoted to the study of estrogen's feedback actions on GnRH and LH secretion, much less has been carried out to understand the effects of progesterone. Moreover, the studies of progesterone are more focused on the positive feedback actions of progesterone during the preovulatory LH surge (222) instead of on its inhibitory actions.

Progesterone's inhibitory actions on the HPG axis have been studied relatively extensively in the ewes compared to other species (14, 223). It is now generally believed that progesterone decreases GnRH and LH pulse frequency in the luteal phase (224). While in rodents, the inhibitory effect of progesterone seems to be dependent on the stage of estrous cycle. One group

has shown that progesterone treatment immediately after ovariectomy on diestrus 1 reduces the LH pulse amplitude (168). Whereas if progesterone was given immediately after ovariectomy on diestrus 2 or more than one week after ovariectomy, it has no effects on LH pulsatility (169, 172). More recently, studies of the progesterone receptor knockout (PRKO) female mice from our laboratory demonstrated that PRKO female has elevated basal LH level compared to the wild type female, indicating that progesterone alone may have negative feedback through progesterone receptors (225). It is interesting to note that after ovariectomy there was a further increase in LH in the PRKO mice, which agrees with the observations that if progesterone and estrogen are given together, they have more pronounced inhibitory effect than either one of them alone (169). The exact mechanisms of progesterone's negative feedback effects are still unknown. However, in studies using ewes as subjects, a large body of evidence suggests that the endogenous opioids mediate progesterone's inhibitory effects on GnRH and LH pulses (226, 227). $GABA_A$ receptor is also considered as the mediator of progesterone inhibitory action, since the metabolite of progesterone (228), 3α-hydroxy-5α-pregnan-20-one (3α, 5α-THP, allopregnenolone), has been shown to be a potent modulator of $GABA_A$ receptors (229). GnRH neurons receive synaptic inputs from GABAergic neurons (140). So, it is possible that by modulating the activity of $GABA_A$ receptor, 3α, 5α-THP can indirectly regulate GnRH neurons. Besides the indirect actions, direct effects may also be possible as well. It has been shown that PRs are expressed in GT1-7 cells (230) and a small fraction of GnRH neurons in guinea pigs (231). A recent study in our laboratory has shown that 1μM progesterone alone, when given to the GT1-7 cells for 5 minutes, significantly reduces the production of cAMP, which has been shown to be positively associated with GnRH secretion. The molecular mechanisms utilized in progesterone signaling are largely unknown. One study demonstrated in ewes that acute

progesterone implant withdrawal results in rapid increase in both GnRH and LH pulse frequency, while re-insertion of the progesterone capsules decreases GnRH and LH pulsatility (232). Moreover, the above effects of progesterone are blocked by RU486, a PR antagonist, indicating the involvement of the classic nuclear PRs. However, non-genomic effects of progesterone have also been recognized. Progesterone was demonstrated to bind to uterine oxytocin receptor, thus blocking oxytocin signaling and subsequent uterine contraction (233). This discovery opens the question whether the inhibitory effects could be mediated by membrane-associated receptors. Interestingly, a recent study has cloned and characterized a type of membranous progesterone receptors (mPR) (234). These mPRs are G protein coupled receptors with seven transmembrane domains. Some preliminary studies in our lab showed that these mPRs are expressed in GT1-7 cells and in the rat hypothalamus. In addition, like the classic nuclear PRs, they are upregulated by estrogen.

It is worth to note that estrogen may also play a role in the negative feedback actions of progesterone. Estrogen has long been shown to induce the expression of nuclear PRs (235, 236), thus it is possible that after the induction of PRs by estrogen, progesterone can bind to its receptors and exert its inhibitory effects. In support of this, it has been shown in ewes that progesterone is without effects on GnRH and LH pulsatility in ovariectomized animals which were devoid of estrogen for 4 months. However, if estrogen were given back to the animal for just two weeks, the negative feedback of progesterone were restored (232).

3. The Combined Negative Feedback of Estrogen and Progesterone on GnRH Secretion

Combined treatment with E_2 and P reduces LH pulse frequency, an indicator of GnRH pulse generator activity (169). Direct monitoring of *in vivo* GnRH release into the hypophyseal portal

blood in ewes demonstrated that only when P was given on an E_2 background could it reduce GnRH pulse frequency, while P alone had no effect on GnRH pulses (232). This observation suggests the necessary roles of both E_2 and P in the negative feedback actions on GnRH pulse generator. The estrogen receptor knockout mouse (ERKO) and PRKO models provide additional evidence for their genomic mechanisms. ER_α and ER_β single and double knockout mice (αERKO, βERKO and αβERKO) and PRKO mice have been generated and their phenotypes were extensively studied (237-239). Female αERKO, αβERKO and PRKO mice have elevated LH levels compared to the wild type animals (225, 240), while βERKO mice have normal gonadotropin level (241). Those observations indicate that ER, at least ER_α and PR have negative feedback actions on gonadotropin secretion. However, whether this elevated LH level is a result of increased GnRH release from the hypothalamus or enhanced sensitivity of pituitary gonadotropes to GnRH stimulation or both is still unknown. This is largely due to the difficulty in monitoring *in vivo* GnRH release in mice. Interestingly, ovariectomy of αERKO or αβERKO mice causes no change in their LH levels (240). Failure of ovariectomy to further increase LH level in the ERKO mice indicates the necessity of at least ER_α in P's negative feedback on LH secretion. In contrast, ovariectomy of PRKO mice resulted in a further increase of LH (225), which is consistent with the previous observation that estrogen reduces LH secretion at least partly by decreasing the sensitivity of gonadotropes to GnRH stimulation (172).

In spite of extensive investigation, the mechanism underlying the negative feedback action of estrogen and progesterone is largely unknown. A genomic action was proposed since RU486, a putative antagonist of nuclear PR, could restore the GnRH pulse frequency inhibited by P in ovariectomized, estrogen-primed ewes (232). The induction of PR by estrogen further

corroborates this possible mechanism (235, 236). Nevertheless, nongemonic mechanisms were also reported to be involved in regulating GnRH neuron activity (108).

The immediate targets of E_2 and P's negative actions are yet unknown. A direct effect on GnRH neuron has long been disfavored, mostly due to the failure to detect ER and PR in GnRH neurons (29, 30). However, with the recent discovery of ER_β and PR in GnRH neurons, the direct effects of E_2 and P on GnRH neurons seems possible (179, 180, 231). In contrast to the direct effects, several neurotransmitter systems have been proposed to mediate the inhibitory effects of E_2 and P, such as GABA (207, 228), β-endorphin (34, 226). They both regulate GnRH secretion (223) and express ERs and PRs (210, 227, 242), therefore may play a role in the negative feedback actions of E_2 and P on GnRH secretion. Other ovarian steroid responsive neurons, such as NPY (10) and POMC (11) neurons, may also be involved.

Summary

Ovarian steroids exert their negative feedback actions on GnRH neurons through complicated mechanisms involving various types of neuronal populations. These mechanisms include alterations of multiple cellular activities, including gene expression, neurosecretion and ion channel activities. Both genomic and non-genomic effects are implicated. In addition, GnRH neurons are also regulated by other factors, such as negative energy balance. In fact, the negative feedback actions of ovarian steroids are enhanced by food restriction and the inhibitory effects of negative energy balance are enhanced by ovarian steroids, indicating an interaction between ovarian steroids and negative energy balance in regulating GnRH secretion.

The next literature review will focus on the inhibitory effects of negative energy balance on GnRH secretion and the HPG axis. Possible pathways and involved humoral and neural components will also be discussed.

D. Negative Energy Balance and GnRH secretion

1. Effects of Negative Energy Balance on the HPG Axis

Reproduction has long been known to be closely associated with physiological energy status. In the wild, when food resources are adequate and basic survival needs are met, energy is partitioned to ensure successful reproduction. To the contrary, when the energy sources are limited, reproductive activity and processes are diminished to allow the survival of individual. Energy homeostasis is composed of energy intake, energy expenditure and energy storage. When energy intake is lower than energy expended and retrieved from energy stored, a state of negative energy balance is reached. The effect of such negative energy balance on reproduction depends on the interaction among energy intake, energy expenditure and energy storage. Food restriction or deprivation results in decreased level of LH, impairment in follicular development and ovulation in a number of species including rats (41, 42), sheep (43, 44), monkeys (45), and humans (46). There have been extensive studies on the effect of negative energy balance on human reproduction involving both men and women, although the latter are more susceptible and thus receive more clinical attention. In men, a 48hr fast reduces LH, FSH and testosterone levels. The reduction of LH is a result of an decrease in basal level and pulse frequency, but not pulse amplitude (243). Likely, dietary restriction to 1/5 to 1/4 of normal daily needs in young menstruating women decreases LH pulsatility (244). A mental disease state, anorexia nervosa, in which patients restrict their food intake, is also associated with suppression of LH secretion and gonadal steroids (245). In addition to less energy intake, excessive energy expenditure similarly results in negative energy balance, which in turn affects GnRH and gonadotropin secretion. In adolescent ballet dancers who maintain high physical activity since early adolescence, a marked delay in the onset of menarche was documented although they have a normal body weight and

body fat content 4-8 months before menarche (246). In cycling athletes, regardless of their menstrual status, their LH pulse frequency is lower compared to the non-athletic cycling women (47). As an important component of energy homeostasis, energy storage also plays a critical role in maintaining energy balance. Loss of body weight can cause a state of negative energy balance similar to less energy intake. When severe, body weight loss can render unresponsiveness of LH to GnRH stimulation, while weight gain can reinstate the responsiveness once a threshold of body weight of 15% below ideal body weight is reached (247). In Syrian hamsters, the ability of a 48hr-fast to inhibit estrous cycles seems to depend on the body fat content before fasting, as the fasting-induced inhibition was observed only in lean, but not fat, hamsters (248).

The reduction of LH can be due to less GnRH input from hypothalamus or lower sensitivity of gonadotropes to GnRH stimulation or both. A large body of evidence supports the idea that the inhibition of LH secretion induced by negative energy balance predominantly results from suppression of the activity of GnRH pulse generator. The reduced LH pulsatility observed in animals under negative energy balance is usually indicative of the suppression of pulsatile GnRH secretion. In men, pulsatile administration of GnRH restores the LH and testosterone levels inhibited by fasting, denoting central mechanism is underlying the changes in gonadotropin release (49). In female sheep, dietary restriction increases the interpulse interval of both LH and GnRH pulses as detected concomitantly in peripheral blood and pituitary portal blood respectively (48). Interestingly, in the same study, it was also found that there are small, low amplitude GnRH pulses which were not followed by LH pulses.

How the signal of energy status is transmitted to the central nervous system to regulate GnRH secretion is still controversial. A generally accepted model is as follows. The negative energy status is represented by one or a few primary metabolic cue(s), which is (are) registered

by metabolic cue detectors. The latter then convey the secondary signals, neural and/or humoral, to the hypothalamus to modulate GnRH secretion (Fig. 2).

Figure 2

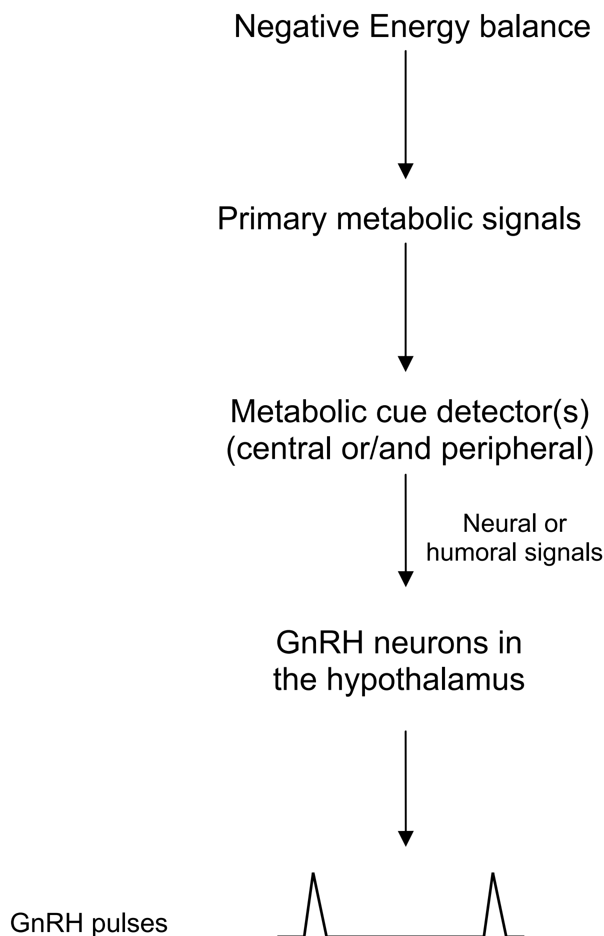

Fig. 2. Current model of the effects of negative energy balance on GnRH secretion. Factors causing negative energy balance first result in changes in primary metabolic cues such as glucose and FFA, which are then detected by central or/and peripheral detectors. Secondary signals from these detectors, either neural or humoral or both, are then registered in the hypothalamus to affect GnRH neurons.

2. Primary Metabolic Cues

The primary metabolic cues are molecules that are the basic forms of cellular metabolism, easily transported throughout the body and respond reliably to changes in energy intake, energy expenditure and energy storage. Glucose, free fatty acids (FFA) and amino acids are the major metabolic cues. Among them, glucose and FFA have been extensively studied and believed to the primary metabolic cues involved in regulation the HPG axis by negative energy balance.

a. Glucose as A Primary Metabolic Cue

As the primary product of carbohydrate metabolism, glucose has long been thought as the only primary metabolic cue as its level drops when the animal is food restricted or deprived. Hypoglycemia induced by insulin decreases LH pulsatility in a number of species such as rats (249), sheep (250) and monkeys (251). Moreover, direct monitoring of the GnRH pulse generator activity by MUA revealed that insulin-induced hypoglycemia led to decreased activity of GnRH pulse generator in monkeys (252). The same study showed that the underlying mechanism may involve corticotrophin releasing factor (CRF) but not arginine vasopressin (AVP) as intracerebral ventricular administration of CRF delayed the inhibition of pulse generator frequency in response to insulin-induced hypoglycemia. Similar suppression of GnRH pulse generator activity was also observed in female ovariectomized rats in response to insulin-induced hypoglycemia. The suppression can be reversed by peripheral infusion of glucose, indicating a direct role of glucose instead of insulin (253). Inhibition of intracellular glucose utilization and subsequent energy production can also lead to negative energy balance. 2-deoxy-D-glucose (2DG) blocks glycolysis as a competitive inhibitor of hexokinase, a key enzyme in glycolysis. As a result, pyruvate level drops, which in turn causes reduction in oxidizable fuel entering the citric acid cycle and eventually a drop of intracellular ATP level (254). Systemic

2DG treatment results in increase in food intake and decrease in LH release in both male and female rats. Females tend to be more sensitive to the inhibitory effect of 2DG on LH levels, probably as a result of estrogen (55, 56), although the presence of gonadal steroids potentiate the effect of 2DG in both sexes (56, 248, 255-257).

In sheep, 2DG given both systemically (higher dose) and centrally (lateral ventricle, 1/10 of peripheral dose) caused a decrease in LH pulse frequency, but not pulse amplitude, indicating the involvement of GnRH pulse generator. This effect on GnRH secretion was further confirmed by the observation that the inhibition on LH pulsatility can be reversed by giving GnRH or GnRH secretagogue (258). Interestingly, there was no difference in the decrease in LH pulsatilty when 2DG was infused into either lateral or fourth ventricle, suggesting the role of brain stem structures surrounding the fourth ventricle in response to 2DG. (259)

b. Free Fatty Acid (FFA) as A Primary Metabolic Cue

Although glucose is an important primary metabolic cue, several studies have shown that it may not be necessary in signaling the states of negative energy balance. In adult male monkeys, although the fasting-induced increase in growth hormone and cortisol were blocked by parenteral glucose infusion, it only partially restore the fasting-induced reduction in LH, indicating the role of other metabolic fuels (260). In monkey, fasting-induced reduction in LH can be restored not only by carbohydrates or mixed nutrients which increase blood glucose level, but also by nutrient composed of only protein and fat which does not affect blood glucose level (261, 262).

Ample evidence suggests that FFA may also serve as the primary metabolic cue in transmitting the signal of energy balance status. Blockade of FFA oxidation by mercaptoacetate (MA) gave rise to an increase in food intake in the absence of increase in glucose level (43, 263). Combination of glucoprivation and lipoprivation by using both 2DG and MA resulted in

suppression of estrous cyclicity in Syrian hamster similarly to what was observed if these animals were fasted for 48 hours (264).

The mechanisms underlying the effect of FFA oxidation in regulating food intake are not clear but are proposed as due to either FFA per se (265) or an indirect action through intracellular metabolic cues, such as ATP (266).

3. Metabolic Cue Detectors

How the metabolic signals are detected is largely controversial. So are the secondary signals that are sent from the metabolic cue detector to GnRH neurons, the central effector. The metabolic cue detectors are categorized as peripheral or central, according to their location. The secondary signals are classified as neural or humoral according to their characteristics.

a. Peripheral Metabolic Cue Detectors and Their Secondary Signals

i. Liver /Intestinal Tract via Vagal Inputs:

It has been shown in both Syrian hamsters and rats that subdiaphragmatic vagatomy can reverse the fasting-induced reduction in LH pulsatility (264, 267, 268). The proposed mechanism is that the primary metabolic cue is first registered in liver and/or intestinal tract, which is then transformed into changes in activity of vagal nerves innervating these organs. These vagus nerves then send signals to the CNS to affect GnRH pulse generator, consequently LH pulsatility. However, several lines of evidence disfavor the role of liver and intestinal tract in transmitting the primary metabolic signal to the CNS via the vagal input. Fasting-induced reduction in LH pulsatility is reversed by total subdiaphragmatic vagotomy, or its gastric branch, but not hepatic or celiac branch (264, 267, 268). 2DG given at the same dose to hepatic portal system or jugular vein had similar effect in reducing LH frequency, while when given to the

hepatic vein in a lesser dose there was no effect of 2DG on LH secretion, indicating the absence of a specific glucose sensor in the liver (259).

ii. Adipose Tissue via Leptin

Leptin is secreted by adipocytes and responds to a number of factors, such as fasting, glucose, glucocorticoid, etc (269). Leptin receptors are expressed in NPY, POMC neurons in the arcuate nucleus of the hypothalamus (270). In addition, leptin signaling is found in immortalized GnRH neuronal cell line, GT1-7 cells (271). All these neurons are implicated in regulating GnRH pulse generator activity. Thus, leptin may act as a mediator in transmitting the metabolic cues to the CNS effector. Moreover, leptin is also capable of regulating cellular metabolism of glucose and FFA (269, 272). For example, leptin stimulates gluconeogenesis and glucose metabolism in rodents (273, 274). Leptin also enhances lipolysis, increases fatty acid synthesis in the liver (275). However, it is not clear whether these effects result from leptin's action in the central nervous system or in the targeted peripheral organs.

As the major signal of body adipose deposition, leptin has been associated with regulation of the HPG axis. Adult female leptin-deficient (ob/ob) mice have prepubertal gonadotropin level and are infertile (276). Similar endocrine defects are also found in leptin receptor knockout (db/db) mice (277-279). Leptin treatment in female ob/ob mice rescues their LH levels (278) and fertility (280). Since leptin signals the CNS of the body fat content and play an important role in energy homeostasis, it has been implicated in relaying the status of negative energy balance to the brain, thereby suppressing the HPG axis. Food restriction or deprivation decreases leptin level in both rats and humans (281, 282), and leptin improves fasting-induced decrease in LH level in a number of species including sheep (283), rats (284) and monkeys (285). In mice, leptin partially restore the LH level suppressed by 48 hours of fasting to 40% of the fed mice (286).

This occurrs without any change in body weight, blood glucose, insulin and ketones. Besides its effect in acute states of negative energy balance, leptin is also responsible for the increase in LH level in sheep under chronic food restriction (287, 288). Recently, a study provides evidence for a possible cellular mechanism through which leptin may exert its effects on GnRH neurons. Leptin was shown to reverse the 48hr fast-induced decrease in excitatory $GABA_A$ input to GnRH neurons. Moreover, leptin also modulates the postsynaptic signaling by potentiating the response of GnRH neurons to $GABA_A$ receptor activation, indicating that the state of negative energy balance may transmit its signals to GnRH neurons through leptin's modulation of $GABA_A$ signaling (289). In addition to its possible role as a mediator between the metabolic cues and central effector, leptin may simply serve as a regulator of intracellular metabolism of oxidizable fuels. Leptin affects glucose metabolism by increasing glucose turnover and glucose uptake and decreasing hepatic glycogen content without causing changes in blood glucose and insulin levels after systemic and central administration (274). It also affects FFA metabolism. Leptin directly inhibits de novo synthesis of fatty acids and increases release and oxidation of fatty acids in isolated rat adipocytes (290). Therefore, it is possible that negative energy balance leads to changes in leptin levels, which in turn interferes with neuronal activity by altering the intracellular metabolism of oxidizable fuels.

Certain neurons are possible mediators of leptin's effects on GnRH neurons. Both NPY and POMC neurons in the arcuate nucleus express high level of leptin receptors (270). Moreover, both of them have been implicated in regulating GnRH secretion (149, 157, 158). Short-term fasting fails to suppress LH secretion in NPY knockout mice, indicating an essential role of NPY in mediating the effects of negative energy balance on GnRH neurons (291). Kisspeptin is another possible mediator for the regulation of GnRH secretion by leptin. A majority of

kisspeptin neurons in the arcuate nucleus express leptin receptor and leptin deficient ob/ob mice have low level of kisspeptin in the hypothalamus which can be rescued by leptin treatment (292). Moreover, food deprivation induces a decrease in hypothalamic kisspeptin expression and central administration of kisspeptin is able to restore the LH secretion suppressed by fasting (293), indicating that kisspeptin may transmit the signal of negative energy balance to GnRH neurons (51, 294). Although GT1-7 cells express leptin receptors, whether GnRH neurons in situ are the target of the direct leptin's action remains unclear.

Despite the above evidence, several studies have suggested that leptin may not be necessary in mediating the effect of negative energy balance on HPG axis. It has been observed in ewes that reduction in LH pulsatility occurs without any change in plasma leptin level after food deprivation (295). After refeeding, LH level is restored before leptin level returns to normal (296, 297) and it occurs in the absence of any change in body fat content (45, 250, 298-300). In addition, leptin has no effect on GnRH secretion from cultured hypothalamic neurons (301). In one study investigating the effect of leptin on puberty onset, leptin treatment can fully reverse the fasting-induced delay of puberty in female rats fed with 80% of their *ad libitum* food, but only partially reverse the delay of puberty onset in those fed with 70% of their *ad libitum* food, indicating that leptin may play a permissive, instead of a necessary role, in puberty onset (302).

iii. Pancreas via Insulin

Secreted by pancreatic β-cells, insulin is an important metabolic hormone in regulating energy homeostasis as central administration of insulin increases food intake (303). It can also affect GnRH/ LH secretion (253, 304). Thus, insulin may mediate in part the effect of negative energy balance on gonadotropin secretion. The effect of insulin can be due to its direct action on the CNS or insulin-induced hypoglycemia. There are lines of evidence supporting each

mechanism. In rats, a decrease in LH secretion caused by systemic insulin administration can be reversed by simultaneous infusion of glucose, arguing against a direct role of insulin in transmitting metabolic cues to the central effector (250, 253). However, mice deficient in neuron-specific insulin receptors have markedly decreased LH level and a more robust LH response to GnRH despite mild insulin resistance, indicative of a central role of insulin in regulating gonadotropin secretion (305). A recent study provided more evidence for the direct role of insulin. Using hyperinsulinemic clamping in male mice, the study showed that insulin stimulates GnRH synthesis and secretion when glucose is clamped at physiological level (301). Interestingly, both leptin (52) and insulin (53) are capable of activating hypothalamic K_{ATP} channels, a key component of the glucose sensing mechanism in the hypothalamus. Moreover, leptin and insulin share a common signaling pathway via the activation of PI3 kinase (306) , indicating that leptin and insulin signaling may converge in the hypothalamus to impact upon GnRH neurons.

iv. Stomach via Ghrelin

Ghrelin is a hormone predominantly secreted by stomach to regulate GH secretion from pituitary. It is inhibited by meals (307), stimulated by fasting (308) and increases gradually during intermeal intervals. Systemic or central administration of ghrelin decreases LH pulsatility in prepubertal and adult animals in a few species, such as sheep (309), rats (310-312), and monkeys (313). However, due to the limited knowledge of ghrelin, it is not clear whether it plays a role in mediating the effect of negative energy balance on GnRH/gonadotropin secretion.

v. Duodenum via Cholecystokinin (CCK)

It is also possible the effect of food restriction or deprivation is detected by duodenum and transmitted to CNS via CCK. Studies have shown that CCK stimulates LH secretion centrally

(314) and peripherally (315, 316). However, conflicting results also revealed that CCK antagonists failed to affect LH level increased by refeeding in monkeys (316).

vi. Adrenal Glands via Glucocorticoid or Catecholamine

Adrenal glands are proposed to play a role in transmitting the signal of energy balance to the central effector. Food deprivation or restriction causes increase in both glucocorticoid and catecholamine levels. Adrenalectomy has been shown to block the inhibitory effect of insulin-induced hypoglycemia on LH secretion (249). However, opposing evidence also showed that adrenalectomy or glucocorticoid antagonist has no effect on estrous cyclicity in Syrian hamster suppressed by food deprivation (317). Moreover, one recent study revealed that in rats with central glucoprivation caused by 2DG infusion, although leptin restored the increased level of corticosterone, it did not restore the reduced LH levels associated with the metabolic challenge, suggesting that glucocorticoids may not play a role in mediating the effect of negative energy balance on gonadotropin secretion (318).

b. Central Metabolic Cue Detectors and Their Secondary Signals

i. Central Metabolic Cue Detectors

(1) Brain Stem

The brain stem, especially the area postrema (AP) has been implicated in detecting the metabolic cues and relaying these signals to the central effector to regulate gonadotropin secretion. Lesion of the AP led to abolishment of the suppression of LH by insulin-induced hypoglycemia (319). It also blocks the effect of systemically administered 2DG on estrous cycle (320) or sexual behavior (321). Infusion of 2DG directly into the 4th ventricle causes reduction in LH pulsatility and increase in food intake (257). Interestingly, one study compared the effect of

different routes of central 2DG infusion on LH secretion and found that 4th ventricle infusion reduces LH level to the similar extent as lateral ventricle infusion (259), indicating that structure(s) in the brain stem may mediate the effect of neuroglucoprivation.

(2) Hypothalamus

The hypothalamus plays an important role in energy homeostasis and regulation of the HPG axis. It also harbors the central glucose-sensing mechanism. NPY and POMC neurons in the arcuate nucleus respond to changes in circulating glucose level (9). They are also the targets of insulin and leptin, two important hormones in energy homeostasis (270). Studies have shown that both NPY and POMC can affect GnRH release (158, 322). Therefore, hypothalamus is possible to play a role in sensing primary or secondary metabolic cues, such as glucose or leptin or insulin.

However, a large body of evidence has demonstrated that despite having an intact hypothalamus, animals with AP lesion lose LH response to 2DG, thus questioning the necessity of the involvement of hypothalamus in mediating the signals of metabolic perturbation to the central effector (264, 323). Yet, the role of hypothalamus in food restriction/deprivation-induced suppression has yet been established.

ii. Signals from Central Detectors to the Central Effector

Pulsatile GnRH release is believed to be dictated by neural GnRH pulse generator in the preoptic area and hypothalamus. Signals of negative energy balance, either thorough peripheral or central detectors, are ultimately conveyed to the GnRH pulse generator, i.e. the central effector, to affect GnRH secretion. Although its cellular identity remains to be determined, the GnRH pulse generator must contain GnRH neurons. In fact, since GnRH neurons exhibit

episodic firings spontaneously even without any extrinsic cues, they themselves are considered to be the GnRH pulse generator per se. In addition to being regulated by the humoral factors as discussed above, GnRH neurons also receive inputs from other intervening neurons including those neurons from the area involved in sensing energy status, such as the brain stem.

NPY/NE (norepinephrine) neurons in the hindbrain are thought to relay the signals detected in the brain stem to the GnRH pulse generator in the hypothalamus. Food deprivation and systemic 2DG infusion increased release of NPY (324) and NE (325) in the periventricular nucleus (PVN) of the hypothalamus respectively. NPY/NE neurons are shown to send projections from AP to PVN (326) and also directly to the GnRH neurons (327). Infusion of NPY (322) and NE (328) into the forebrain results in a decrease in LH. Targeted destruction of NPY/NE fibers in PVN by using toxin conjugated to monoclonal dopamine beta-hydroxylase (DBH) antibody prevents the 2DG-induced delay of the next expected estrous cycle (323). Injection of NE synthesis inhibitor in the PVN prevents fasting-induced reduction in LH (329). Third ventricle infusion of an α-adrenergic blocker in fasted female rats blocked the suppressive effect of food deprivation on LH (328). In addition to the above observations, NPY knockout mice provide more information regarding the effect of NPY on GnRH secretion. LH is not suppressed by food deprivation in the NPY knockout mice (291). Moreover, when they were treated with insulin or 2DG, no increase in food intake was demonstrated (330). However, in the DBH knockout mice in which NE is deficient, there is normal increase in feeding in response to glucoprivation (331), indicating NPY and NE may play different role in relaying the signals of the status of negative energy balance.

Another group of neurons, CRF neurons in the hypothalamus, may also be involved in mediating the effect of negative energy balance on the GnRH pulse generator. CRF neurons are

mainly located in the PVN of the hypothalamus and receive projections from NPY/NE neurons in the AP (332). Study using electron microscopic double-label immunostaining demonstrated that they synapse with GnRH neurons in the preoptic area (333). Functionally, the release of CRF is increased by NE treatment or electrical stimulation of the catecholaminergic fibers arising from the brainstem (334). The above effect was prevented by pretreatment with α1-adrenergic receptor antagonist. CRF also causes reduction in LH by inhibiting hypothalamic GnRH release (335). Central administration of CRF antagonist blocked the suppression of LH by food deprivation (329), 2DG (336), and NE infusion at PVN (337).

Summary

States of negative energy balance result in changes in metabolic signals, most likely glucose and FFA. These signals are then detected by or conveyed to brain circuitries that regulate GnRH neurons. Both humoral and neural pathways may be implicated in this regulation.

Recently, K_{ATP} channels are found in ovarian steroids-sensitive neurons implicated in regulating GnRH secretion. They are also critical to glucose homeostasis, possibly energy homeostasis, controlled by hypothalamus. Taken together with our current knowledge about the negative feedback actions of ovarian steroids on GnRH neurons, it is thus possible that K_{ATP} channels may mediate the effects of ovarian steroids and/or negative energy balance on GnRH neurons. Therefore, the next review will discuss in details about K_{ATP} channels and their biological functions.

E. K_{ATP} Channels

1. Overview

K_{ATP} channel was first discovered as a potassium channel inhibited by ATP applied to the cytoplasmic surface of the patch-clamps of pancreatic β-cells (338). Ever since then, this channel has been extensively studied. It is now clear that K_{ATP} channels couple the electrical activity of the cell with its intracellular metabolism and are found in many tissues, including the pancreas (338) , brain (339), heart (340), and blood vessels (341).

K_{ATP} channels are hetero-octamers composed of two types of subunits, inwardly rectifying potassium channels 6 (Kir6.x) and sulfonylurea receptors (SURx). When complexed to form a functional K_{ATP} channel, the Kir6.x/SUR_X shows a 1:1 stoichiometry (342). Kir6.x subunits are believed to form the potassium-selective pore of the channel, while SURx subunits are considered as the regulatory component of the channel (343). The composition of K_{ATP} channels by Kir6.x and SURx subunits shows an interesting tissue-specific pattern. For example, K_{ATP} channels composed of Kir6.2 and SUR1 are mainly found in pancreatic β-cells and neurons (344), while Kir6.2 and SUR2A are the predominant subunits to form K_{ATP} channels in the cardiomyocytes (341).

K_{ATP} channels are both potassium selective and inwardly rectifying. The latter means the channels conduct potassium ions better in the inward direction than the outward direction. The degree of rectification is dependent on intracellular magnesium (Mg^{2+}) concentration (345). When membrane potentials are higher than the equilibrium potential of potassium, Mg^{2+} molecules enter the pore of the channels and slow the efflux of potassium. The Kir6.x subunits determine the potassium selectivity, inward rectification and unitary conductance, while SUR_X subunits dictate the nucleotide selectivity and channel pharmacology. Normally, the channels

exhibit bursting activity during which they switch very quickly between open and closed states. Opening of the channel causes potassium efflux, which results in hyperpolarization of the cell. While closing of the channel blocks potassium efflux, thus depolarizing the cell. Between the bursts are silent gaps when the cell remains dormant.

It is now generally accepted that the ATP/ADP ratio in the cell is the major regulator of the channel activity (58). ATP binds to the SUR_X subunit at the cytoplasmic side and closes the channel independent of intracellular Mg^{2+} (346). Consequently, the duration of the burst is shortened and the time of the gaps is prolonged (347). To the contrary, ADP binds to SUR_X subunits but opens the channel, which occurs only in the presence of Mg^{2+}. However, without being complexed with Mg^{2+}, ADP is inhibitory to the channel (348). As a result of channel blockade by ATP, the cell depolarizes and the voltage-dependent calcium channels open, results in Ca^{2+} influx and increase in intracellular Ca^{2+} concentration, which in turn may recruit intracellular Ca^{2+} pools from intracellular organelles and cause further increase in intracellular Ca^{2+} concentration. The increase in intracellular Ca^{2+} concentration then triggers the downstream pathways, such as insulin secretion from pancreatic β-cells and neurotransmitter release from synapses of neurons. Besides ATP/ADP ration, K_{ATP} channel activity is also regulated by a number of factors including free fatty acid, leptin and insulin, which will be discussed later in details.

2. Kir6.x Subunits

Kir6.x subunits belong to the inwardly rectifying potassium channel superfamily, and are thought to form the pore of the K_{ATP} channels. Two mammalian Kir6.x genes have been cloned, namely Kir6.1 and Kir6.2 (349, 350). Kir6.1 mRNA is ubiquitously expressed in mouse tissues

(349), while Kir6.2 mRNA is more abundant in pancreatic β cells, neurons, heart, skeletal muscles, etc (58). Kir6.1 and Kir6.2 genes share about 70% similarity in their coding regions. Interestingly, Kir6.2 gene does not have any intron, making it relatively easy to study. The protein products of both genes are approximately 38-40 kDa, as revealed by [^{125}I] azidoglibenclamide labeling studies on COS cells expressing the cloned subunits (342).

Analysis of the amino acid sequence suggested that Kir6.x subunit has two transmembrane domains (TMD), M1 and M2, a pore forming loop, and intracellular N- and C- terminals. The C-terminus contains an endoplasmic reticulum retention signal, preventing the trafficking of Kir6.x subunit to the plasma membrane to form functional channels. However, truncated forms of Kir6.x subunits, lacking amino acid 16-35 from the C-terminal ends, form active potassium channels. This clearly lends support to the view that the Kir6.x subunits constitute the pore of the channels (351). In addition, the M2 domain, especially its cytoplasmic loop, is thought to convey the potassium selectivity due to its structural similarity to the pore forming part of voltage-gated potassium channel family (352).

It is interesting to note that a new type of Kir6.x subunit, namely zKir6.3, is recently identified and cloned in zebrafish (353). Although it has 66% homology with mammalian Kir6.2, they differ significantly at the C-terminal region. Its mRNAs are found in the embryo brain of zebrafish. The zKir6.3 subunits couple with SUR1 subunits to form functional K_{ATP} channels on the plasma membrane. However, the physiological functions of K_{ATP} channels composed of Kir6.3 subunits have not been examined.

3. SUR_X Subunits

SUR_X subunit is a member of the ATP-binding cassette (ABC) superfamily and is able to bind to ATP with high affinity. It is first discovered as a 140kDa polypeptide with high affinity to sulfonylurea at nanomolar range (354). Later, another form with a 150-170kDa molecular weight was found in a binding study using [^{125}I] iodoglibenclamide to identify SUR_X subunits (355). Currently, it is believed that the 140kDa species is a simply glycosylated core with the basic mannose-containing glycosyl groups, while the 150-170 kDa species is a complexed, glycosylated form which is the major form in pancreatic islets and brain tissues (356, 357). Two isoforms of SUR_X subunits have been discovered so far, SUR1 and SUR2. The latter has two splice variants, namely SUR2A and SUR2B.

Genes encoding human SUR1 and SUR2 have been cloned and mapped to chromosome 11 and 12 respectively. Interestingly, human SUR_X genes are found pairing with Kir6.x genes. SUR1 gene is upstream and next to Kir6.2 on chromosome 11, while SUR2 is adjacent to Kir6.1 on chromosome 12. Moreover, there are only 4900bps between the stop codon of SUR1 and the start codon of Kir6.2, indicating that they may evolve from a large ancestor gene (343). The product of the SUR_X gene is a large trans-membrane protein. Recent studies based on hydrophobicity analysis of the amino acid sequence predict that the SUR_X subunit is composed of nine TMDs linked with nucleotide-binding fold 1 (NBF1), which is then followed by six TMDs linked with NBF2 (9TMD-NBF1-6TMD-NBF2) (358, 359). The two NBFs, as the name implies, are the nucleotide binding sites. They both have Walker A and Walker B consensus motifs, which have high similarity to the corresponding motifs in other members of the ABC superfamily (360). Photolabelling study suggested that ATP binds to NBF1 with high affinity, while MgADP binds to NBF2 and reduces the affinity of ATP binding to NBF1 (361). Mutation of the NBF2 motif results in failure of MgADP and diazoxide to activate channel via the SUR_X

subunit , while channels with mutated of NBF1 can still be activated but with an altered kinetics (362, 363).

4. Trafficking and Assembly of Kir6.x and SUR_X Subunits into K_{ATP} Channels

Exactly how the Kir6.x and SUR_X subunits are trafficked and assembled into K_{ATP} channel is still not clear. Co-expression of cDNAs from the two subunit genes results in a potassium channel with characteristics similar to native K_{ATP} channel, such as ATP sensitivity, while expression of either one alone fails to produce similar potassium channels (350). Photo-labeling of the SUR_X subunit shows a 38 kDa mass later identified as the Kir6.x subunit (342). The above findings suggest that Kir6.x and SUR_X subunits are physically associated. More recently, stoichiometry studies by co-expressing Kir6.2 and SUR1 cDNA at different ratios demonstrated that four Kir6.x and four SUR_X subunits complex to form a functional K_{ATP} channel (342). In the same study, it was proposed that the N-terminal of Kir6.2 subunit is close to the C-terminus of SUR1 subunits. This physical adjacency may enable the transmission of the signal of receptor conformational change induced by nucleotide binding to the pore of the channel, thus conferring the responsiveness of the whole channel to ATP.

The mechanism underlying the trafficking of the channel subunits to the plasma membrane is less understood. Both Kir6.2 and SUR1 subunit have endoplasmic reticulum retention signals, which prevent the exit of the subunits from the quality control mechanism in the endoplasmic reticulum and cis-Golgi complex (364). Proper interaction of two subunits shield this retrograde signal and allow the normal trafficking of the channel to the plasma membrane. However, removal of these retention signals enables the subunits to escape from the quality control mechanism in endoplasmic reticulum and eventually form channels on the plasma membrane

(351). How the interaction of the two subunit lead to the normal trafficking of the whole K_{ATP} channel is not clear and even controversial. One study found that the C-terminus of SUR1 contains an anterograde signal which helps the Kir6.2 and SUR1 subunits exit the endoplasmic reticulum and Golgi complex (365). Deletion of a few amino acids from the C-terminus of the SUR1 subunit results in reduced expression of K_{ATP} channel on the cell membrane. Nonetheless, a more recent study co-expressed Kir6.2 subunit with different truncated forms of SUR1 C-terminus and demonstrated that these mutant K_{ATP} channels are able to show normal expression on the plasma membrane, normal channel activity and ATP sensitivity, but their responses to MgADP, tolbutamide and diazoxide are disrupted (366).

5. Physiological Functions of K_{ATP} Channels

K_{ATP} channels serve a broad range of physiological functions depending largely on their distribution at specific tissues. Functions of K_{ATP} channel in pancreatic β-cell , which are composed of Kir6.2 and SUR1 subunits, have been extensively investigated (367). The channels act as a glucose-sensing mechanism. Once the extracellular glucose concentration increases, it enters the β-cell and generates more ATP through glycolysis and citrate acid cycle, leading to an increase in ATP/ADP ratio. The increased ATP/ADP ratio then leads to blockade of most of the channels, depolarizes the cell and results in calcium influx and eventually insulin secretion. The opposite events happen when the extracellular glucose is reduced, causing reduction in insulin secretion. Mutation of either subunits results in a disruption of the glucose-sensing mechanism, and supersecretion of insulin regardless of extracellular glucose concentration (54, 368). In human, such mutations cause a serious medical condition known as "Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI) (369, 370). In these patients, K_{ATP} channels lose their normal

response to hypoglycemia. So, insulin is constantly secreted and stays at abnormally high level despite a dangerously low blood glucose level. A number of defects in proper channel functioning, e.g. physical uncoupling of the two subunits, impaired MgADP activation, abnormal trafficking and potassium permeation, are caused by mutations of the Kir6.2 or SUR1 subunits and are responsible for the above clinical manifestation in PHHI patients (371).

K_{ATP} channels are also found in the central nervous system, such as hippocampus, hypothalamus, cerebellum, etc (8). The major neuronal form of K_{ATP} channel is Kir6.2 and SUR1 (372), while the combination of Kir6.1 and SUR1 has also been reported (373). Since the potassium efflux through the channel hyperpolarizes the cell, it is thought K_{ATP} channel may play a role in neuroprotection by reducing the detrimental effects of cerebral ischemia and the toxicity of excitatory neurotransmitters, such as glutamate (16, 374, 375). Overexpression of SUR1 in the forebrain in mice renders the animal resistant to seizure induction and excitotoxic neuronal death (376). Moreover, K_{ATP} channels found in the arcuate nucleus and ventromedial nucleus of the hypothalamus are believed to be involved in central glucose sensing (377, 378). In the hypothalamus, a group of neurons are identified with increased action potential frequency upon glucose application, and are named glucose-responsive neurons. These neurons utilize K_{ATP} channels to sense glucose (379) and are involved in glucose homeostasis, regulation of food intake, counterregulatory response of hypoglycemia and even energy homeostasis. Mice lacking Kir6.2 gene exhibit complete loss of glucagon secretion and reduced food intake in response to neuroglycopenia, indicating the important role of hypothalamic K_{ATP} channels in glucose homeostasis (54).

K_{ATP} channels are also expressed in cardiomyocytes as the combination of Kir6.2 and SUR2A (341). Numerous studies have been carried out to study the role of K_{ATP} channels in pre-

conditioning of cardiomyocytes upon ischemia. Pre-conditioning is a phenomenon that when the cardiac muscle cells are challenged with a short-period ischemia, they are more resistant to the injury incurred by subsequent, long-lasing ischemia or reperfusion. Opening of the K_{ATP} channels by the pre-conditioning ischemia is thought to quiet the cardiomyocytes by hyperpolarization, thus decreasing their demand for oxygen and making them less vulnerable to future ischemic challenges (380, 381). However, closing the channel by glibenclamide abolishes this protective effect of K_{ATP} channels, causing an increase in infarct size after long-term ischemia (382). Although still not conclusive, such impairment of the pre-conditioning mechanism may explain the higher incidence of cardiovascular accidents in diabetic patients taking tolbutamide, a glucose lowering drug of the sulfonylurea class (383).

Furthermore, K_{ATP} channels are localized to vascular smooth muscle cells (384), in which they are mainly composed of Kir6.1 and SUR2B subunits (385). It has been proposed that K_{ATP} channels play a role in regulating vascular tone, especially during metabolic stresses. It is believed that they are at least partially responsible for the hypoxia-induced dilation of coronary and cerebral arterioles (386, 387). The dilation of those arterioles may serve to compensate the tissue hypoxia by allowing more blood flow to the affected area. Application of a K_{ATP} channel blocker, glibenclamide, to vascular muscle cells thus block this protective vaso-dilatory effects (388, 389).

It is also worth mentioning that K_{ATP} channels are found not only on plasma membrane, but also on the membrane of some intracellular organelles, such as mitochondria (390, 391). However, the identity of these intracellular K_{ATP} channels remains undefined. Several lines of evidence support the role of mitochondrial K_{ATP} channels in regulating oxidative phosphorylation (392). It has been suggested the mitochondrial K_{ATP} channels in cardiomyocytes

has a protective role against ischemia and reperfusion injury (393). Moreover, in pancreatic β-cells, K_{ATP} channels are also found in several organelles. The expression of K_{ATP} channels in these organelles may accounts for the plasma membrane K_{ATP} channel-independent insulin secretion upon tolbutamide application (394).

6. Regulation of K_{ATP} Channel Activity

Given its wide distribution and various physiological functions, it is not surprising that the activity of K_{ATP} channel is regulated by a number of factors. ATP and ADP are thought to be the most important intracellular regulator of channel activity. ATP binds to SUR_X subunits and closes the channel, while ADP binds the SUR_X subunits and opens the channel after complexed with Mg^{2+} (58). The half-maximal inhibitory concentration (IC_{50}) of ATP is at the range of 10-50μM, well below the physiological intracellular ATP level at a few millimolar range (395). Such great discrepancy between the ATP sensitivity and intracellular ATP concentration triggered a number of theories trying to explain the difference, such as compartmentalization of ATP (338), spare channel model (396). The subsequent discovery that MgADP opens the channel inhibited by ATP initiates the currently accepted model that ATP/ADP ratio is the major variable that controls the channel activity (397). Besides intracellular ATP and MgADP, intracellular signaling molecules, such as phosphatidylinositol-4, 5-bisphosphate (PIP2), PKA and PI3 kinase, have also been shown to affect K_{ATP} channel activity (306, 398, 399).

In addition to the intracellular regulators, there are quite a few extracellular factors that can modulate channel activity by either directly acting on the channels or changing the intracellular availability of ATP and ADP or the channel sensitivity to the intracellular ATP and ADP. Metabolic cues, such as glucose and FFA, have been shown to affect opening and closing of the

channel (400, 401). Regulation of channel activity by glucose in pancreatic β-cells is a classic model where glucose enters the β-cell, produces ATP via glycolysis and citrate acid cycle, closes the channel, allows calcium influx and subsequent insulin secretion.

Hormones, such as insulin (53), leptin (52), galanin (402), and calcitonin gene-related peptide (CGRP) (403) have also been implicated in altering K_{ATP} channel activity. The physiological significance of such regulation is probably determined by the local distribution of K_{ATP} channels and relevant functions of the regulators. For example, leptin hyperpolarizes hypothalamic glucose-responsive neurons in lean rat by activating K_{ATP} channels, which is in agreement with the effect of leptin on energy homeostasis.

In addition to the physiological factors listed above, there are a few pharmacological agents that can either close or open K_{ATP} channels. Given the various functions of K_{ATP} channels, these agents apparently carry a lot of research and clinical significance. These agents are classified into two major types according to their effects on the channel activity: (1) sulfonylurea compounds, e.g. glibenclamide, tolbutamide, which bind to SUR_X subunits and close the channel (58), (2) K^+-channel openers (KCOs) such as diazoxide and pinacidil which bind to SUR_X subunits and open the channel (345). Interestingly, different composition of K_{ATP} channels determines the channel sensitivity to different pharmacological agents. For example, the IC_{50} of Kir6.2/SUR1 by tolbutamide is 3-20μM (404), well below that of Kir6.1/SUR2B at 380μM (405).

7. Regulation of K_{ATP} Channel Expression

K_{ATP} channels are regulated not only of their activity but also of their expression levels. The expression of K_{ATP} channel subunits can be affected by a number of factors. This regulation mostly happens at transcriptional level and appears to be related to the function of the individual

regulator at specific tissue. In pancreatic β-cells, a 72hr exposure to high glucose (>10mM) decreases both Kir6.2 and SUR1 mRNA levels, which can be re-induced by subsequent exposure to low glucose (5mM). It is postulated that this transcriptional regulation may affect the responsiveness of pancreatic β-cells to future challenges, thus may explain the observation that long-term exposure to high glucose impairs β-cell functions (406). Glucocorticoids also decrease Kir6.2 and SUR1 transcription in pancreatic β-cells, but have no effect on the stability of each mRNA (407). Since glucocorticoids increase glucose level, the downregulation of K_{ATP} channel expression may help to increase insulin secretion to counteract the effect of glucocorticoids on glucose metabolism. It has long been proposed that estrogen has a protective action on cardiovascular system and there is a marked difference in the incidence of cardiovascular accident between men and pre-menopausal women (408). The above observation may be partially explained by the fact that estrogen upregulates K_{ATP} channel expression at both mRNA and protein levels in heart cells (409). More K_{ATP} channels on the cardiac cell membrane may quiet the heart cells and lower their metabolic requirement, thus protecting them from ischemic stress. In addition, a gender-specific difference is also found in expression of cardiac K_{ATP} channels. Specifically, women have a higher level of cardiac K_{ATP} channel than men (410). Interestingly, in both studies only SUR2A, but not Kir6.2, mRNA level was upregulated by estrogen, while at the protein level, both subunits were increased to similar degree.

The differential regulation of Kir6.2 and SUR2A mRNA expression by estrogen in heart cells and the downregulation of both Kir6.2 and SUR1 mRNA by glucose in pancreatic β-cells may suggest the existence of different transcription regulatory mechanisms. The regulation of K_{ATP} channel expression may depend on both the tissues and the regulators under study.

The promoter regions of human Kir6.2 and SUR1 gene have been cloned and investigated (411). Interestingly, both promoter regions are similar to those of the “house keeping” genes as they both lack the “TATA” box in the minimal promoter region and are “G+C” rich. Additionally, SUR1 promoter region has a few E-boxes, SP1 sites and G-boxes, while Kir6.2 has several E-boxes, SP1 sites, G-boxes and possible AP-2 sites. For the SUR1 gene, a short flanking fragment (173bps) is enough for maximal promoter activity. Whereas, for Kir6.2 gene, at least a 900bp 5’-flanking region is needed for its full expression.

Foxa2, an winged-helix transcriptional factor important in pancreas development and insulin signaling has recently been associated with the expression of both genes, as conditional knockout of Foxa2 gene in pancreatic β-cells showed a 75% decrease in both Kir6.2 and SUR1 mRNA level and a severe phenotype of hyperinsulinemic hypoglycemia similar to human PHHI (412). However, the exact mechanisms of the transcriptional regulation are not clear.

8. K_{ATP} Channels and GnRH Secretion

Aside from other locations in the brain, K_{ATP} channels are also found in the hypothalamus, which is the key regulatory center of the HPG axis. The mRNAs of channel subunits have been shown to be co-localized with NPY (8) and POMC (9) in the arcuate nucleus and glutamic acid decarboxylase isoform, GAD65 (marker for GABAergic neurons) in ventromedial nucleus (VMN) (8). A subset of these neurons has been demonstrated to be estrogen and/or progesterone sensitive. Our recent work has identified functional K_{ATP} channels composed of Kir6.2 and SUR1 subunits in GnRH neurons (Levine JE, unpublished data). Interestingly, all these neurons are implicated in regulating GnRH release (207, 413, 414). Moreover, K_{ATP} channels in the hypothalamus are essential to glucose homeostasis, and possibly energy homeostasis (54). They

are also subject to regulation by a number of metabolic signals, such as glucose (54), FFA (401), leptin (52) and insulin (53). Therefore, the expression of K_{ATP} channels in these hypothalamic neurons may suggest a new role of K_{ATP} channel in mediating the regulation of GnRH secretion by ovarian steroids and negative energy balance.

F. Questions for Thesis Studies.

- **Chapter II: To determine if K_{ATP} channels are involved in the negative feedback actions of ovarian steroids on GnRH secretion.**

  A number of hypothalamic neurons express receptors for ovarian steroids and are implicated in regulating GnRH pulse generator activity, such as NPY, POMC and GABAergic neurons. Therefore, they may have potential roles in mediating the negative feedback action of ovarian steroids. These neurons also express K_{ATP} channels, which points to the possibility that modulation of K_{ATP} channel may underlie this process. Chapter II will attempt to identify the role of K_{ATP} channel in the inhibition of GnRH neurons by ovarian steroids. A K_{ATP} channel blocker, tolbutamide, will be intracerebroventricularly (i.c.v.) infused to test whether central blockade of K_{ATP} channels affects pulsatile GnRH secretion, as indirectly reflected by LH pulsatility and whether ovarian steroids confer the responsiveness of GnRH secretion to K_{ATP} channel blockade. Finally, semi-quantitative RT-PCR will be employed to study the genomic effects of ovarian steroids on K_{ATP} channel subunit expression in brain regions important to the regulation of GnRH secretion, i.e. POA and MBH.

- **Chapter III: To determine the effects of short-term food deprivation on GnRH/LH secretion.**

  Negative energy balance, such as caused by decreased energy intake and/or increased energy expenditure, have been closely associated with suppression of the HPG axis. It is manifested as hypogonadotropic hypogonadism and infertility. Evidence from a number of species has

suggested that the reduction in gonadotropin levels results from suppression of pulsatile GnRH secretion from the hypothalamus. However, this hypothesis has never been proved in the mouse. Therefore, Chapter III investigates this question using microdialysis to monitor *in vivo* GnRH secretion in female mice and studies the effects of 48 hours of fasting on GnRH secretion. The feasibility of microdialysis to monitor *in vivo* GnRH release in mice will first be tested by using an OVX mouse model. As has been known decades, removal of ovaries results in augmentation of gonadotropin secretion. This process is believed to at least partially result from removal of the inhibitory effect of ovarian steroids on GnRH secretion. In this chapter, comparison of GnRH pulsatility as revealed by microdialysis will be made between OVX and gonadally intact female mice to confirm the effects of ovariectomy on GnRH secretion. Thereafter, the effects of a 48hr fast on GnRH/LH secretion will be assessed in female mice.

- **Chapter IV: To determine whether K_{ATP} channels mediates the effects of short-term food deprivation on GnRH/LH secretion.**

  K_{ATP} channels link intracellular metabolism with membrane potential and are essential to nutrient sensing in the hypothalamus and maintenance of energy homeostasis. These channels are found in a variety of neurons involved in regulating GnRH secretion. Therefore, this chapter will attempt to study the role of K_{ATP} channels in the suppression of GnRH/LH secretion by negative energy balance. First, tolbutamide will be i.c.v. administered in fed and fasted female mice to test whether central blockade of K_{ATP} channel affects LH secretion in an energy status-dependent manner. Then, SUR1 knockout mice which are deficient in

functional K_{ATP} channels will be utilized to investigate whether 48 hours of fasting inhibits LH secretion in these mice.

Chapter II: Role of K_{ATP} Channels in the Negative Feedback Actions of Estrogen and Progesterone on GnRH secretion: Steroid-dependent Response of GnRH Pulse Generator to K_{ATP} Channel Blockade and Up-regulation of K_{ATP} Channel Expression by Estrogen and Progesterone

Abstract.

K_{ATP} channels couple the metabolic state of excitable cells to their membrane potentials, and are expressed in various tissues including the brain. They are composed of two types of subunits, Kir6.x and SUR, and are expressed in hypothalamic neurons that provide afferent inputs to GnRH neurons. Their roles in regulating reproductive neurohormone secretion, however, have not been determined. The present study first tested whether K_{ATP} channels play a role in regulating pulsatile GnRH secretion, as indirectly reflected by pulsatile LH secretion. Ovariectomized rats received sc. capsules containing either oil, estradiol-17β (E_2), or E2 and progesterone (P) at 24h prior to blood sampling experiments. Infusion of the K_{ATP} channel blocker, tolbutamide, into the 3rd ventricle resulted in increased LH pulse frequency in animals treated with E_2+P but was without effect in all other groups. Effects of ovarian steroids on Kir6.2 and SUR1 mRNA expression in the POA and MBH were then evaluated. After 24hr treatment, E_2+P treatment produced a modest but significant increase in Kir6.2 mRNA expression in the POA, which was reversed by RU486. Neither SUR1 in the POA nor both subunits in the MBH were altered by any steroid treatment. After 8d of steroid or vehicle treatment, Kir6.2 mRNA levels were again enhanced, but to a greater extent by E_2+P in the POA. Our findings demonstrate that 1) blockade of preoptic/hypothalamic K_{ATP} channels produces an acceleration of the GnRH pulse generator in a steroid-dependent manner, and 2) combined E_2 and P treatments stimulate Kir6.2 gene expression in the POA. These observations are consistent with the hypothesis that the negative feedback actions of ovarian steroids on the GnRH pulse generator are mediated, in part, by their ability to upregulate K_{ATP} channel subunit mRNA expression in neurons in the POA.

Introduction.

GnRH is released in a pulsatile manner into the hypophyseal portal vessels and conveyed to the anterior pituitary gland, where it directs synthesis and release of LH and FSH (166). In the female reproductive axis, the activity of the neural GnRH pulse generating mechanism is subject, in turn, to regulation by the homeostatic negative feedback actions of the ovarian steroids, E_2 and P. Significant progress has been made in the characterization of the neural circuitries that may mediate ovarian steroid feedback, yet the molecular and cellular pathways through which they are exerted have remained ill-defined. In the present studies, I have explored the hypothesis that the suppressive effects of ovarian steroids on the GnRH pulse generator are mediated by regulation of ion channels that control the excitability of hypothalamic neurons, and hence the neurosecretory activity of GnRH neurons.

In females of several species it has been shown that the replacement of E_2 and P reverses ovariectomy (OVX)-induced increases in pulsatile GnRH/LH release, with E_2 exerting its primary effects upon GnRH/LH pulse amplitude (172), and P being most effective in retarding GnRH pulse frequency (224). Combined treatment with both hormones is required to fully restore LH secretion to levels found in ovary-intact animals (169, 232). Studies of ER null mutant mice have revealed that the ER_{α} isoform is probably most important in mediating E_2 negative feedback actions on LH secretion (240). Pharmacological experiments in sheep (232) and analysis of PRKO mice have also provided evidence for the involvement of PR_A and/or PR_B in P's inhibitory actions (225). It has been proposed that ER_{α}- and $PR_{A/B}$-expressing afferents to GnRH neurons (34, 216), and possibly a subset of GnRH neurons themselves (181, 183), are the targets of these steroid hormones that mediate their negative feedback actions.

Irrespective of which neuronal populations may mediate steroid negative feedback, an elucidation of the post- receptor mechanisms mediating these processes has remained elusive. Previous studies have revealed that steroid hormones can rapidly alter the electrophysiological properties of GnRH neurons (108, 109), as well as putative GnRH afferent neuronal populations including GABAergic (177) and POMC neurons (415). Among the downstream signaling mechanisms identified in these neurons are the uncoupling of μ-opioid receptors from GIRK channels in POMC neurons, and $GABA_B$ receptors from GIRK channels in GABAergic neurons (40). It is not known if these rapid non-genomic effects are accompanied by more slowly developing genomic actions that culminate in altered ion channel gene expression. Whether these or other effects of steroids on ion channel function are relevant to *in vivo* steroid hormone feedback mechanisms also remains to be confirmed.

The present studies explore the possibility that steroid hormones can inhibit GnRH pulse generation by altering the activity of another set of inwardly-rectifying potassium channels, the K_{ATP} channels. The K_{ATP} channels function as cellular metabolic sensors, mediating glucose sensing in pancreatic β-cells and hypothalamic glucose responsive neurons by linking intracellular metabolism with electrical excitability (58). Activation of K_{ATP} channels is also believed to mediate hypothalamic actions of insulin (53), as well as some of those of leptin (52). More broadly, K_{ATP} serve cellular protective roles, limiting effects of cardiac ischemia (381) and restraining hypoxia-induced seizure propagation (375). In the present studies I have focused on the role of K_{ATP} channels in ovarian negative feedback because they are expressed in hypothalamic neurons implicated in regulating GnRH release, such as NPY (8), POMC (9), and GABAergic neurons (8), as well as in a subset of GnRH neurons themselves (Levine JE, unpublished data). Moreover, gonadal steroids have been shown to exert physiological effects

via activation of K_{ATP} channels in other tissues, such as the myocardium (416). I hypothesized that E_2 and P stimulate the activity and/or expression of K_{ATP} channels, thereby altering the level of excitability of GnRH neurons or the afferent neuronal groups that control GnRH neurosecretion. To assess this hypothesis, I sought to determine if E_2 and P stimulate the expression of genes encoding the K_{ATP} channel subunit proteins, and to assess whether the pharmacological closure of K_{ATP} channels produces an acceleration of GnRH pulsatility (as reflected by LH pulsatility) in steroid replete animals.

Methods.

Animals

Female Sprague-Dawley rats (Charles River laboratory, Wilmington, MA) (200-220g) were housed in temperature-controlled facilities (23-25°C) with a 14:10 light cycle (Lights on: 0500-1900). They were fed standard lab chow and had access to water *ad libitum.* All surgical and experimental procedures were used in strict accordance with protocols approved by the Institutional Animal Care and Use Committee at Northwestern University (Evanston, IL).

Experiment1: Effects of 24 hour steroid treatments on pulsatile LH responses to K_{ATP} channel blockade.

On day 0, all animals were anesthetized with 75mg/kg, ip. ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 5mg/kg, ip. xylazine (Burns Veterinart Supply, Inc. Rockville Center, NY) and bilaterally ovariectomized (OVX). At the same time, they received stereotaxic guide cannulae implants fitted with stainless steel obdurators targeted to the anterior extremity of the third ventricle (coordinates 1mm caudal to bregma, 7.5mm ventral to the skull, 0.2mm lateral) (417). On day 7 following cannula implantation (1000-1200h), animals were anesthetized by halothane inhalation and an indwelling atrial catheter (PE-50, Becton Dickinson, Sparks, MD) was surgically implanted through the right jugular vein as previously described (418).

The animals were then divided into three treatment groups. Control (C), E_2 and E_2+P groups received sc. Silastic capsule implants containing vehicle (sesame oil), E_2, or both E_2 and P, respectively. E_2 capsules were prepared by using a 15mm Silastic tubing (ID:0.16cm, 0.062in; OD: 0.38cm, 0.125in; Dow-Corning, Midland, MI) containing 100µg 17β-estradiol (Sigma, St. Louis, MO)/ml sesame oil sealed off at both ends with medical grade adhesive. P capsules

consisted of one 20mm long capsule (ID: 0.16cm (0.062in); OD: 0.38cm (0.125in), Dow-Corning, Midland, MI) containing crystalline P (Sigma, St. Louis, MO). These treatments have been shown to sustain peripheral E_2 levels of 15-25 pg/ml and P levels of 30-40 ng/ml (168), these ranges being confirmed by us in preliminary experiments.

Blood sampling sessions were initiated 24h following the sc. capsule implantations, with 100μl being withdrawn from the atrial catheters every 5 minutes for a total of three hours. An equivalent volume of 0.9% saline was infused back into the catheter after each blood withdrawal. Blood samples were centrifuged at 2,000g for 15 minutes, and plasma was collected and stored at –20°C for subsequent LH RIA.

At 90min prior to the start of sampling, the obdurator was removed from the i.c.v. guide cannula and an infusion cannula (0.64mm o.d.) was inserted. The animal was then left undisturbed throughout the remainder of the experiment. A CMA400 Microdialysis pump (CMA Microdialysis AB, North Chelmsford, MA) and a liquid switch (CMA Microdialysis AB, North Chelmsford, MA) were used to deliver artificial cerebrospinal fluid (aCSF) through the cannula. Blank aCSF was infused during the first hour, followed by infusion of aCSF containing the K_{ATP} channel blocker, tolbutamide (Sigma, St. Louis, MO), for the second hour. Blank aCSF was again infused during the third hour of blood sampling sessions. Tolbutamide is a K_{ATP} channel blocker that is relatively specific for the SUR1 subunit isoforms (384), which is the predominant SUR expressed in hypothalamic and preoptic area neurons (8, 419). The dose of tolbutamide was determined on the basis of preliminary experiments in gonad-intact animals, in which a total of 6.5 μg/h in the anterior third ventricle was found to produce a significant acceleration of LH pulsatility. No grossly observable behavioral alterations, sensorimotor deficits, or neurohistological damage were produced by the tolbutamide infusions when carried out at the

rate of 2µl/min. For comparison, I note that in a previous study (420) a dose of 162 µg/h tolbutamide was similarly infused into the lateral ventricle and found to suppress counterregulatory (epinephrine and glucagon) responses to brain glycopenia or systemic hypoglycemia. For infusions, tolbutamide was first dissolved in a small volume of DMSO, and then diluted in aCSF to 200µM. The components of aCSF were (in mM): NaCl, 124; KCl, 5; $NaHCO_3$, 26; NaH_2PO_4, 2.6; dextrose, 10; HEPES, 10; $MgSO_4$, 2; $CaCl_2$, 2. Final concentration of DMSO in aCSF was 0.1%.

Experiment 2: Effects of 24 hour steroid treatments on K_{ATP} channel subunit mRNA expression

Rats were OVX on day 0. At 1100h on day 7, the animals received sc. capsule implants containing oil vehicle treatment (C), E_2, P, or E_2+P as described in the foregoing experiment. An additional E_2+P-treated group was pretreated with the progesterone receptor antagonist, RU486, at 0800 on day 7, and then again at 0800 on day 8 (4mg sc. in 0.2ml benzyl benzoate/sesame oil). At 1100h on day 8, all animals were anesthetized by halothane inhalation and decapitated, and brains were immediately removed and POA and MBH tissues were dissected. The POA was defined as a 2mm cubic tissue block extending from -1.3mm to 0.7mm anteroposterior, -9.5mm to -7.5mm dorsoventral, and 1 mm bilaterally from the midline (417).The MBH was delineated rostrally by the posterior margin of optic chiasm, laterally by the hypothalamic sulci and caudally by the mammillary body, and extending 2mm ventrally into the brain (417). These two areas were chosen to include regions including GnRH cell bodies (POA) and the soma of neuronal populations known to regulate pulsatile GnRH release (MBH), such as POMC (414) and NPY-producing neurons (413).

Total RNA from POA and MBH were extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with 10U RNase-free DNase (Promega, Madison, WI). A 2.4μg total RNA was reversely transcribed in a 20μl reaction mixture with final concentrations of each reagent as 1mM dNTP, 110u MMLV, 22u RNasin (Promega, Madison, WI), 25μg/ml random hexamer (Genosys, The woodlands, TX), 5mM $MgCl_2$, 1xPCR buffer (Roche, Indianapolis, IN). After first strand synthesis, 5μl cDNA was used for PCR amplification of either Kir6.2 or SUR1 subunit. Primers were Kir6.2 (sense: 5'-GCTGCATCTTCATGAAAACG-3'; antisense: 5'-TTGGAGTCGATGACGTGGTA-3'; 298bp, accession no. AB043638), SUR1 (sense: 5'-TGGGGAACGGGGCATCAACT-3'; antisense: 5'-GGCTCTGGGGCTTTTCTC-3'; 388bp, accession no. L40624). The expression level of Kir6.2 or SUR1 was normalized to that of RPL19 (421), which was amplified by primers (sense: 5'-CTGAAGGTCAAAGGGAATGTG-3'; antisense: 5'-GGACAGAGTCTTGATGATCTC-3'; 195bp, accession no. XM_235216) in the same tube with each of the two subunits. All primers were obtained from Genosys (The woodlands, TX). PCR was performed in a 45μl reaction mixture containing 2mM $MgCl_2$, 1xPCR buffer, 1.25u Taq DNA polymerase (Roche, Indianapolis, IN), 300nM each primer, 110μM dNTP and ^{32}P-dCTP (Amersham Biosciences Corp.,Piscataway, NJ). The mixture was first incubated at 94°C for 4.5 minutes, and then cycled through denaturing at 94°C for 30 seconds, annealing at 58°C (59°C for SUR1) for 1minute and extension at 72°C for 1 minute. Finally, 10-minute extension was carried out at 72°C. The number of cycles for Kir6.2 and SUR1 amplification were 31 and 32 respectively, and 23 cycles (with Kir6.2) or 24 cycles (with SUR1) for rRPL19. The cycle number for each PCR amplification was tested to ensure that the results were obtained from the linear region of the amplification curve. After PCR, the products were separated by polyacrylamide gel electrophoresis (PAGE), and the autoradiography images were

obtained by using phosphoimager (STORM 860, Molecular Dynamics, Sunnyvale, CA). The density of each band was be analyzed by ImageQuant (Molecular Dynamics, Piscataway, NJ). Both RT control samples in which no MMLV-RT was added to the RT reaction mixture and PCR control sample in which Milli-Q H_2O instead of cDNA was added to the PCR mixture were included in PCR. The expected sequences of the PCR products were confirmed by DNA sequence analysis with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

Experiment 3: Effects of 8-day steroid treatments on K_{ATP} channel subunit mRNA expression

Animals were OVX and received sc. Silastic capsule implants containing C, E_2, P and E_2+P. The implants used in these experiments were designed after those used by Dubal et al. (422) and Goodman et al. (172) which have been shown to produce physiological E_2 and P levels over the course of at least 8d of treatment. The E_2 capsules consisted of a 30mm length of Silastic tubing (ID:0.16cm, 0.062in; OD: 0.38cm, 0.125in; Dow-Corning, Midland, MI) containing 180μg 17β-estradiol (Sigma, St. Louis, MO)/ml sesame oil which was sealed off at both ends with medical grade adhesive. The P capsules were made of a 20mm length of Silastic tubing (ID: 0.33cm, 0.132in; OD: 0.46cm, 0.183in, Dow-Corning, Midland, MI) containing crystalline P. Vehicle capsules were capsules prepared as the above E_2 or P capsules but filled with only sesame oil. On day 8, all the animals were anesthetized and decapitated. POA and MBH were immediately dissected and stored on dry ice. Total RNA was harvested and DNAase-treated, and 4.8μg RNA was reverse transcribed. The samples were then processed and analyzed by RT-PCR as described in Experiment 2.

Radioimmunoassays (RIAs)

Plasma LH levels were determined by using RIA reagents obtained from the NIDDK, including LH reference (RP-3) and anti rat LH antibody (S-11). The assay had a lower limit of detection of 0.2ng/ml. Intraassay and interassay coefficients of variance (CV) of LH assay were 8.28%, and 9.66% respectively. Steroid hormone RIAs were performed using kits purchased from Diagnostic Products Corp., Los Angeles, CA (estrogen RIA) and MP Biomedicals, Costa Mesa, CA (progesterone RIA). The estrogen assay had a lower limit of detection of 2pg/ml, and the intraassay and interassay CV were 2.7% and 2.85% respectively. The progesterone RIA had a lower limit of detection of 0.15ng/ml, and the intraassay and interassay CV were 12.0% and 16.5% respectively.

Statistical Analysis

All the reported values were presented as mean ± SEM. In experiment 1, plasma LH pulses were analyzed using ULTRAGUIDE pulse analysis software originated by Dr. Eve Van Cauter, University of Chicago, Chicago, IL (423). A threshold of two times the CV in the corresponding ranges of values in the LH RIAs was used in the algorithm program to identify significant LH pulses. In each treatment group, LH pulse frequency, pulse amplitude and mean LH level before and after i.c.v. tolbutamide infusion were compared to values observed during the infusion by using one way ANOVA with repeated measures, followed by Bonferroni's multiple comparisons post-ho*c* test (GraphPad Software Inc., San Diego, CA).

In experiment 2 and 3, comparison among treatment groups of Kir6.2 and SUR1 mRNA expression in the POA and MBH were made using one-way ANOVA, followed by Bonferroni's

multiple comparisons post-hoc test (GraphPad Software Inc., San Diego, CA). For all statistical analyses, differences were considered statistically significant if $p < 0.05$.

Results.

Experiment 1: Effects of 24 hour steroid treatments on pulsatile LH responses to K_{ATP} channel blockade.

The effects of 24-hr C, E_2, or E_2+P treatments on pulsatile LH responses to i.c.v. tolbutamide infusions are depicted in Figure 3-8. Representative LH profile of each treatment group was shown in Figure 3 for vehicle treatment, Figure 5 for E_2 treatment and Figure 7 for E_2+P treatment. In OVX, vehicle-treated rats, LH pulse frequency, pulse amplitude and mean level were not significantly altered by i.c.v. infusion of tolbutamide (Figure 4). Similarly, no significant alterations of those parameters were observed in OVX, E_2-treated rats (Figure 6). In contrast, after the animals were treated with E_2+P for 24 hours, LH pulse frequency was significantly increased by i.c.v. tolbutamide infusion (1.80 ± 0.37, 3.60 ± 0.24 and 1.40 ± 0.40 pulses/hr before, during and after infusion respectively; $p<0.05$, Figure 8). The LH pulse amplitude and LH mean level were not significantly affected by i.c.v. tolbutamide infusions (Figure 8).

Figure 3

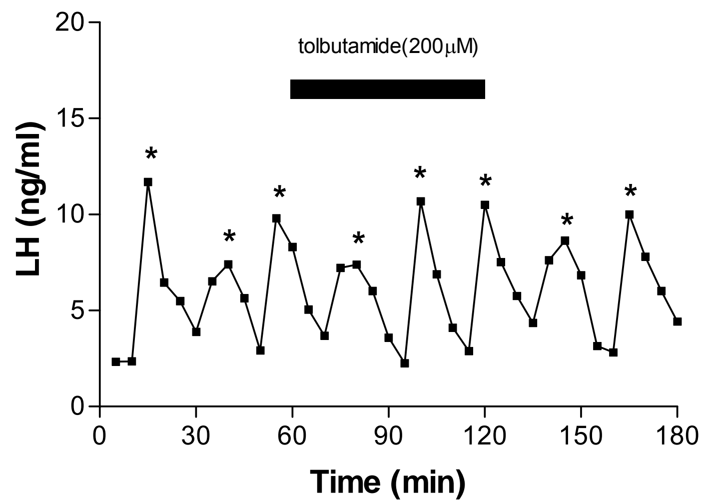

Fig. 3. Representative profile of pulsatile LH release in OVX, 24hr vehicle treated female rats. Asterisks denote pulses determined by ULTRAGUIDE pulse analysis software. The black bar indicated the time when tolbutamide was infused into the 3rd ventricle.

Figure 4

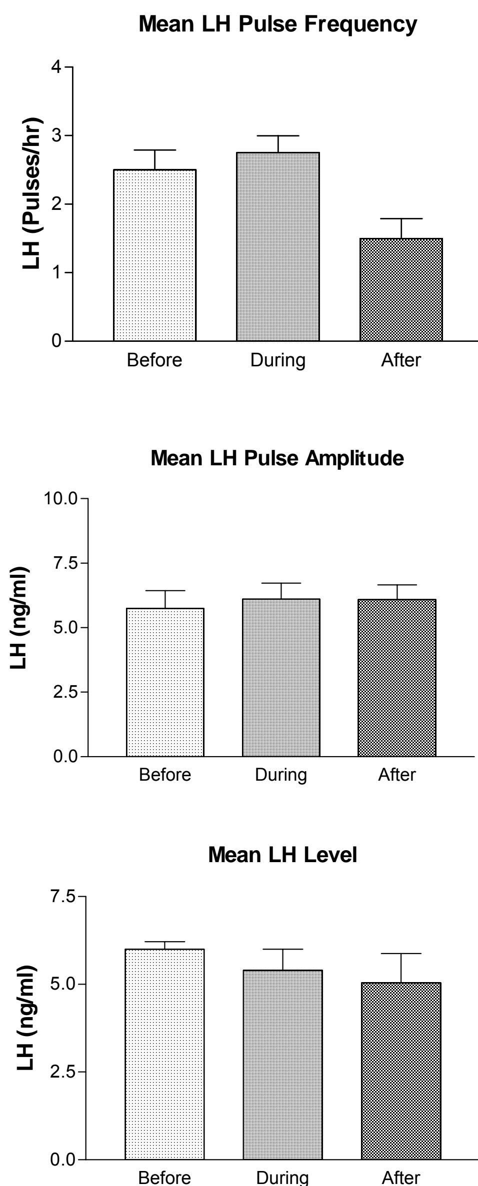

Fig. 4. Characteristics of LH pulses before, during and after i.c.v. infusion of 200µM tolbutamide (n=4) in OVX, 24hr vehicle treated rats. There is no significant difference in pulse frequency, pulse amplitude and mean LH levels of LH pulses during i.c.v. tolbutamide infusion compared to those before and after infusion.

Figure 5

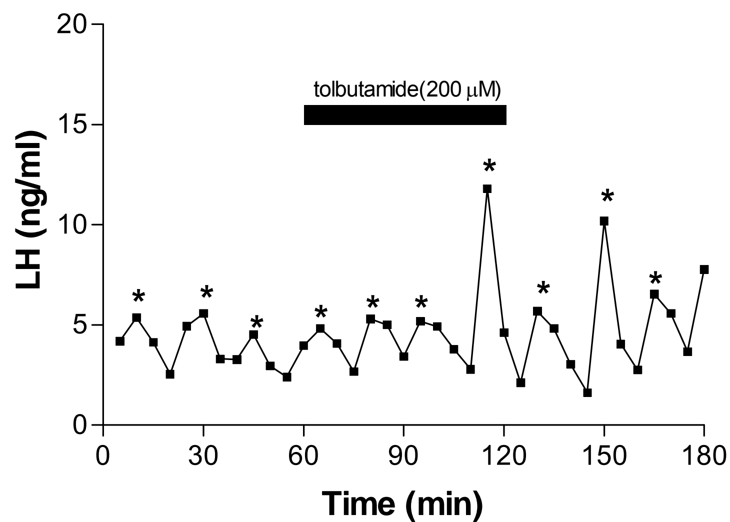

Fig. 5. Representative profile of pulsatile LH release in OVX, 24hr E_2 treated rats.

Asterisks denote pulses determined by ULTRAGUIDE pulse analysis software. The black bar indicated the time when tolbutamide was infused into the 3rd ventricle.

Figure 6

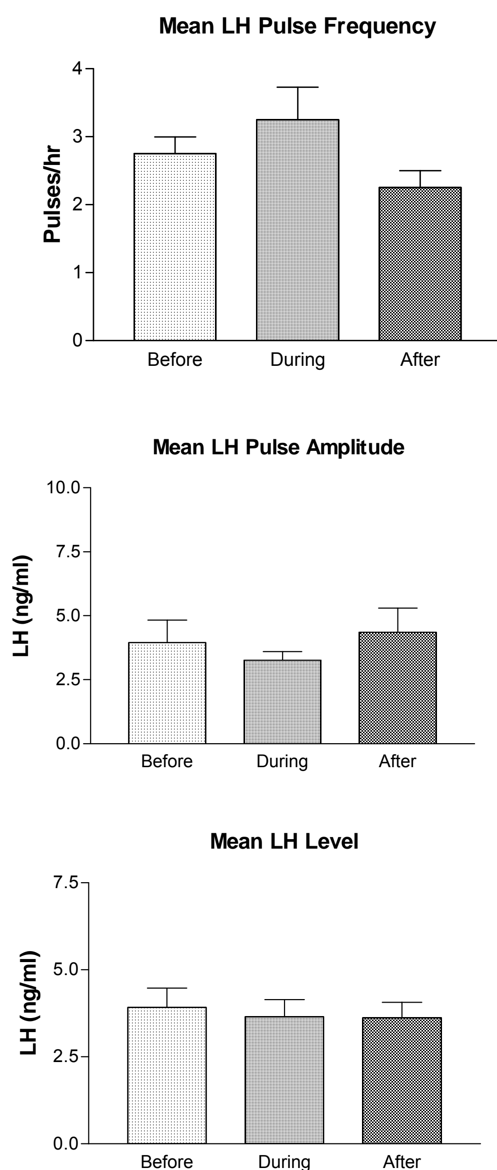

Fig. 6. Characteristics of LH pulses before, during and after i.c.v. infusion of 200µM tolbutamide in OVX, 24hr E_2 treated rats (n=5). There is no significant difference in pulse frequency, pulse amplitude and mean LH levels of LH pulses during i.c.v. tolbutamide infusion compared to those before and after infusion.

Figure 7

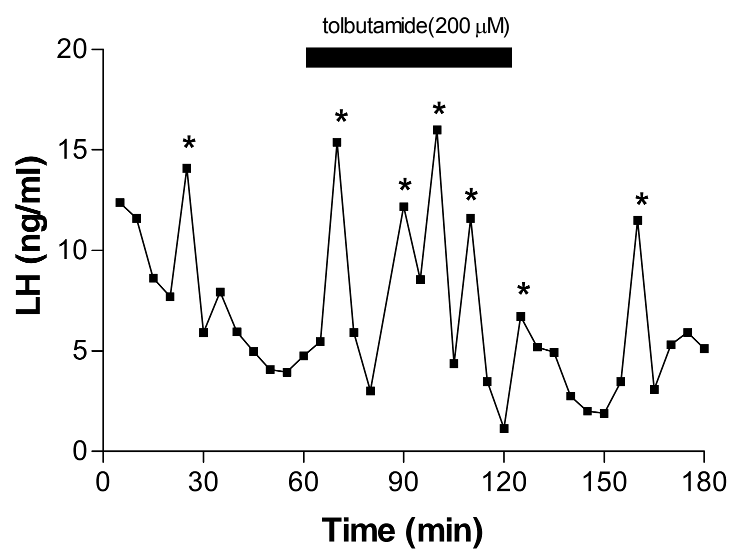

Fig. 7. Representative profile of pulsatile LH release in OVX, 24hr E_2+P treated rats. Asterisks denote pulses determined by ULTRAGUIDE pulse analysis software. The black bar indicated the time when tolbutamide was infused into the 3rd ventricle.

Figure 8

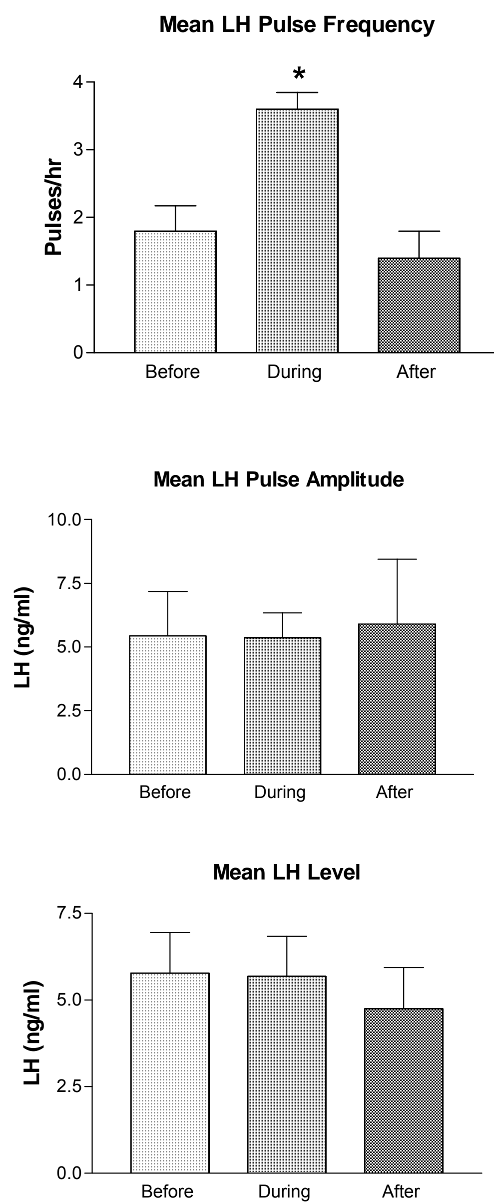

Fig. 8. Characteristics of LH pulses before, during and after i.c.v. infusion of 200µM tolbutamide in OVX, 24hr E_2+P treated rats (n=5). Tolbutamide infusion into the 3rd ventricle significantly increased LH pulse frequency compared to before and during infusion (*, $p<0.05$).

Experiment 2: Effects of 24h steroid treatments on K_{ATP} channel subunit mRNA expression.

The expression levels of Kir6.2 and SUR1 mRNA in the POA and MBH after 24hr treatment with vehicle, E_2, P, or E_2+P are depicted in Figure 9-10. The E_2+P treatment significantly upregulated Kir6.2 mRNA expression in the POA by 25% compared to vehicle (1.72 ± 0.09 in vehicle group vs. 2.15 ± 0.04 in E_2+P group; *, $p<0.05$, Figure 9), while E_2 or P alone had no effect on Kir6.2 expression. The stimulatory effect of E_2+P on Kir6.2 mRNA expression in the POA was abolished by pretreatment with RU486 (Figure 9). SUR1 mRNA expression levels in the POA were not significantly altered by any of the steroid treatments (Figure 9). Expression levels for both subunits in the MBH tissues were not affected by any steroid hormone treatment (Figure 10).

Experiment 3: Effects of 8-day steroid treatments on K_{ATP} channel subunit mRNA expression

The expression levels of Kir6.2 and SUR1 mRNA in the POA and MBH after 8-day treatment with different ovarian steroids are depicted in Figure 11-12. In the POA, Kir6.2, but not SUR1, subunit expression was significantly upregulated by 78% after E_2+P treatment (4.94 ± 0.51 in vehicle group vs. 8.81 ± 1.30 in E_2+P group; $p<0.05$) (Figure 11). In the MBH, SUR1 instead of Kir6.2 mRNA expression was significantly increased by E_2+P compared to vehicle and E_2 (1.30 ± 0.14 in C group and 1.44 ± 0.25 in E_2 group vs. 2.61 ± 0.26 in E_2+P group; $p<0.05$) (Figure 12). The 8-day treatments with E_2 or P alone did not change the expression level of either subunit in both POA and MBH.

Effects of steroid treatments on serum estrogen, progesterone, and LH levels

Trunk blood samples from animals in experiments 2 and 3 were assayed for estrogen and progesterone by respective RIAs, and the results of these analyses are provided in Table 1. The estrogen and progesterone levels in rats receiving estrogen or progesterone treatments for 24h or 8d were determined to be in the normal physiological range for these hormones in intact female rats (Table 1). For the long-term (8d) treatment group, the relative LH levels were determined to be highest in the vehicle treated OVX animals, intermediate in the animals treated with either steroid hormone alone, and lowest in animals treated with the combination of E_2+P. These measurements confirm that the steroid treatments produced the desired, physiological hormone levels in the steroid-treated animals, and that the combined E_2+P treatments effectively provided a negative feedback suppression of LH levels equivalent to that present in ovarian-intact animals (Figure 13).

Figure 9

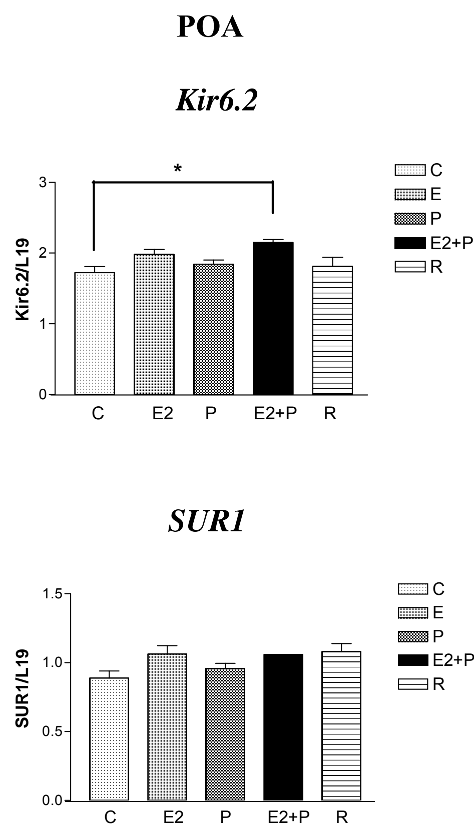

Fig. 9. Kir6.2 and SUR1 mRNA expression in the POA in OVX, 24hr vehicle (C), E_2, P, E_2+P and E_2+P+RU486 (R) treated rats. E_2+P significantly upregulated Kir6.2 mRNA expression in the POA (*, $p<0.05$), which was reversed by RU486. E_2 or P alone was without effect on Kr6.2 mRNA expression. SUR1 mRNA expression in the POA was not affected by any steroid treatment (n=5 or 6 for each group).

Figure 10

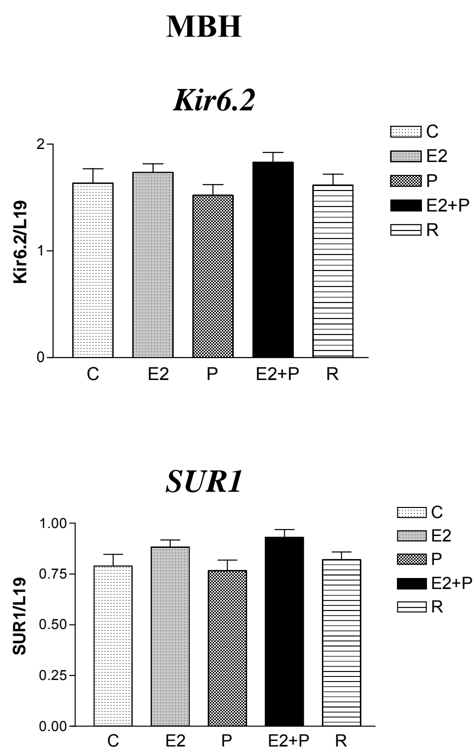

Fig. 10. Kir6.2 and SUR1 subunit mRNA expression in the MBH in OVX, 24hr vehicle (C), E_2, P, E_2+P and E_2+P+RU486 (R) treated rats. Neither Kir6.2 nor SUR1 mRNA was affected by any steroid treatment in the MBH (n=5 or 6 for each group).

Figure 11

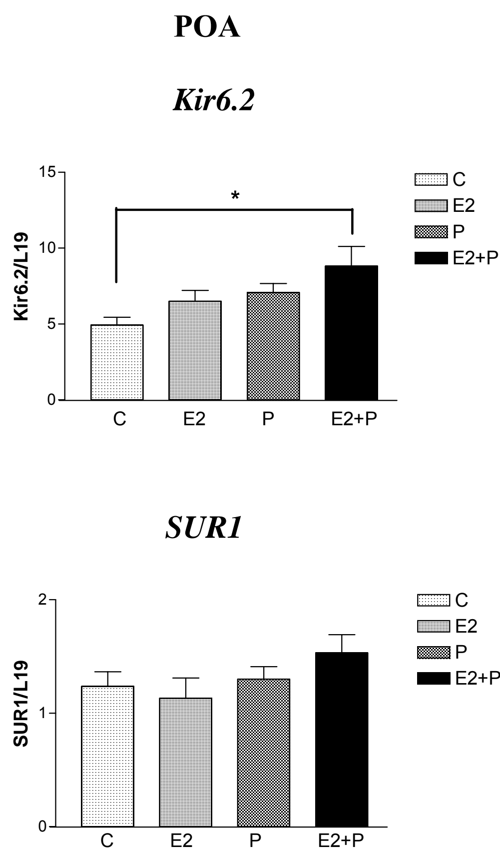

Fig. 11. Kir6.2 and SUR1 mRNA expression in the POA in OVX, 8d vehicle (C), E_2, P or E_2+P treated rats. In the POA, E_2+P significantly increased Kir6.2 subunit mRNA expression compared to vehicle (*P<0.05), while E_2 or P alone had no significant effects on Kir6.2 mRNA expression. SUR1 subunit mRNA expression in the POA demonstrated no difference among different treatment groups (n=8 for each group).

Figure 12

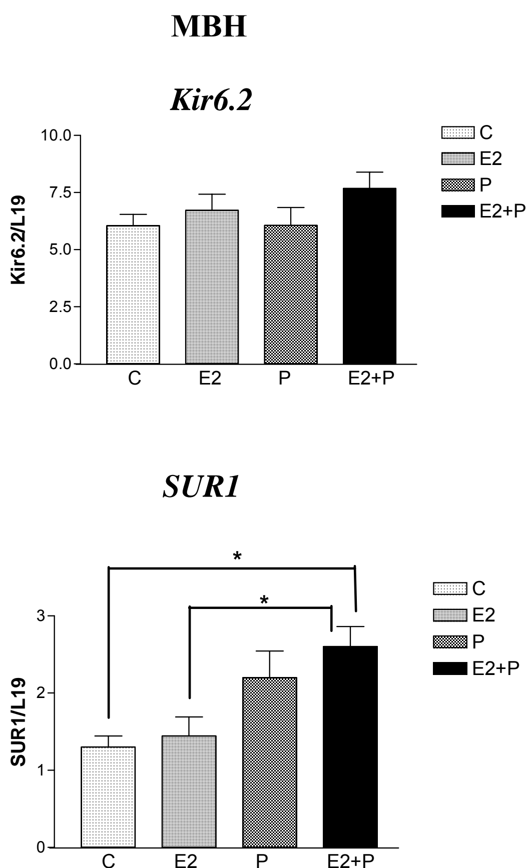

Fig. 12. Kir6.2 and SUR1 mRNA expression in the MBH in OVX, 8d vehicle (C), E_2, P or E_2+P treated rats. In the MBH, E_2+P significantly increased (*$P<0.05$) SUR1 subunit expression compared to vehicle, and E_2. Kir6.2 subunit expression in MBH was not altered by any steroid treatment (n=8 for each group).

Figure 13

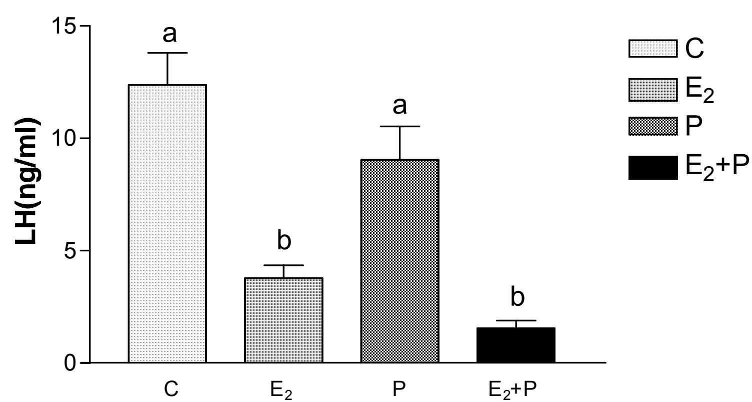

Fig. 13. LH levels after 8-day steroid treatments. E_2 and E_2+P treatment significantly reduced LH levels compared to vehicle and P treatment. Groups with different letters are significantly different from each other. ($p<0.05$: E_2 vs. P; $p<0.001$ for C vs. E_2, C vs. E_2+P, P vs. E_2+P).

Table 1: Hormone levels after steroid treatments

Hormone levels	Capsules	24 hr treatment	8d treatment
Estrogen (pg/ml)	Vehicle for E_2	3.47 ± 0.34	14.7 ± 3.77
	E_2	8.15 ± 1.14	29.2 ± 4.59
Progesterone (ng/ml)	Vehicle for P	7.04 ± 1.14	4.83 ± 0.78
	P	23.36 ± 1.36	10.23 ± 0.99

Discussion.

The K_{ATP} channels function as metabolic sensors, playing critically important roles in protective responses to metabolic stresses such as hypoglycemia (389), ischemia (380, 381), and hypoxia (384). In hypothalamic glucose-responsive neuronal populations, they mediate neurophysiological responses to hypoglycemia, such as feeding behavior and sympathetic activation of pancreatic glucagon secretion (54). However, recent studies have demonstrated that K_{ATP} channels also mediate hypothalamic responses to hormonal signals under normal physiological conditions, such as those conveyed by insulin (53), and leptin (52). In these experiments we have assessed the roles that K_{ATP} channels may play in the actions of gonadal steroid hormones on neuroendocrine circuitries that govern GnRH release. Our studies were prompted by the observations that both K_{ATP} channels and steroid hormone receptors are expressed in subsets of POMC (8, 9, 11), NPY (8, 10, 424), and GABAergic (8, 12, 13, 210) neurons, and that these same cell groups may mediate the feedback actions of ovarian steroids on GnRH neurosecretion (35-37, 138, 413, 414). Our results reveal that treatment of OVX rats with physiological levels of E_2 and P confers responsiveness of the GnRH pulse generator to K_{ATP} channel modulation, while it also stimulates expression of mRNAs encoding K_{ATP} channel subunit, Kir6.2, in the POA. These data are consistent with the hypothesis that the suppression of GnRH pulsatility by ovarian hormones is mediated, in part, via the stimulation of K_{ATP} channel expression and/or activation.

The expression of Kir6.2 mRNA in the POA is most enhanced in response to combined treatment with E_2 and P, compared to responses to either hormone administered alone. Similarly, the blockade of K_{ATP} channels by tolbutamide results in an enhancement in the frequency of pulsatile LH secretion, and hence the frequency of pulsatile GnRH release, only in rats treated

with a combination of the two steroids. Presence of E_2 is important for the full manifestation of P's negative feedback effects on gonadotropin secretion in female rats (169), and subsequent reports have confirmed that E_2 treatments induce expression of nuclear P receptors in the POA and MBH (425-427). Taken together, these observations suggest that the negative feedback actions of P are transduced by the activation of E_2-induced PRs, and that these feedback effects are manifest in part via PR-mediated up-regulation of K_{ATP} channel expression. This idea is further reinforced by the finding that nuclear PR antagonism with RU486 treatments blocks the ability of the combined E_2 and P treatment regimen to increase Kir6.2 mRNA expression (present study) and to suppress LH secretion (232).

Previous studies have also provided evidence for steroid-dependency of K_{ATP} channel subunit expression or activity in non-neural tissues. Estrogen has been shown to reduce myocardial injury in ischemia-reperfusion through the activation of K_{ATP} channels (416), and in heart-derived H9c2 cells, E_2 increases K_{ATP} channel formation and thereby protects cardiac cells from hypoxia-reoxygenation (409). In a canine model of myocardial infarction, E_2 was found exert an antiarrhythmic effect mediated by activation of sarcolemmal K_{ATP} channels (428). More relevant to the present studies would be information on the effects of P on K_{ATP} channel function, however we are not aware of any studies prior to the present ones to have addressed this issue. Nevertheless, the P-dominated hormonal milieu of pregnancy has been shown to be associated with enhanced K_{ATP} channel activity in uterine, cerebral and renal vascular beds (429). Moreover, it was recently reported that Kir6.2 expression levels in the aorta and kidney are increased in pregnant versus non-pregnant Wistar rats (430), and that K_{ATP} channel blockade reverses the decrease in systolic blood pressure (SBP) that normally occurs in pregnant animals, but does not alter SBP in non-pregnant animals (430). These findings suggest that increases in

circulating P during pregnancy are associated with an up-regulation of K_{ATP} channel expression and activity, although a causal relationship remains to be demonstrated in these peripheral tissues.

I have found that ovarian steroids confer LH responsiveness to a K_{ATP} channel blocker, tolbutamide, administered in the third cerebroventricle. It is reasonable to assume that these drug effects are exerted within the brain and not on pituitary gonadotropes, since it is generally accepted that modulation of LH pulse frequency, as was observed in these experiments, reflects an antecedent alteration in the frequency of GnRH pulse generation. The locus of tolbutamide's effects within preoptico-hypothalamic tissues, however, cannot be determined from these experiments. Both the 24h and 8d E2 and P treatments stimulated Kir6.2 mRNA expression in the POA, but not in the MBH, and increased Kir6.2 expression has been shown in other studies to mediate increased responses to K_{ATP} channel modulators (431). The effects of E_2 and P may therefore be mediated by an induction of Kir6.2 expression in the POA. It also remains possible, however, that steroid-induced alterations in K_{ATP} channel subunit expression are dissociable from those that alter responses to K_{ATP} channel modulation, and thus an MBH action can not be ruled out.

Thus, the effects of ovarian steroids may be mediated by their actions in either the preoptic cell groups that form afferent circuitries that govern GnRH neurosecretion, or GnRH neurons themselves. The latter possibility is a viable one given the recent demonstration of ERα associated with the membranes of GT1-7 cells (33), and the immunohistochemical demonstration of ERs in a subset of GnRH neurons from adult animals (181). It is also a strong possibility that E_2 and P exert their effects on preoptic GABAergic and/or glutamatergic neurons. Recent studies have implicated GABAergic and glutamatergic neurons as E_2-sensitive afferents to GnRH

neurons that regulate their episodic firing patterns (177). Both neuronal types have been shown to co-express ovarian steroid receptors (13, 210, 432), and GABAergic cells in several brain regions have been shown to express Kir6.2 (8) and exhibit altered electrical activity and GABA release in response to K_{ATP} channel modulators (433). NPY and POMC neurons in the MBH are also candidates as targets of these steroid actions, as they have both been implicated in mediating inhibitory effects on GnRH neurosecretion (158, 322), and subsets of both types of peptidergic neurons express steroid receptors (10, 11, 424) and K_{ATP} channels (8, 9).

I observed a modest increase in Kir6.2 mRNA levels at 24h following steroid treatments, and a relatively robust enhancement after 8d of treatment. While expression of the predominant sulfonylurea receptor subtype in the brain, SUR1, tended to be higher in the POA of steroid treated rats, this trend did not reach statistical significance. Expression of SUR1 mRNA was significantly increased by the 8d E_2 and P treatment in the MBH. It is possible that our measurements reflect an underestimate of the net effects of steroids on channel subunit expression, since only a subset of neurons expressing Kir6.2 and SUR1 in the POA and MBH also express steroid receptors and would be responsive to the steroid treatments. It is also possible that the stronger effect of E_2 and P is exerted on the expression of Kir6.2, and that the pool of SUR1 is not limiting in the formation of the channel complex. In cardiac tissue, the opposite appears to be true, as E_2 stimulates formation of new channels by stimulating expression of SUR2A in the context of a non-limiting pool of Kir6.2. However, overexpression of Kir6.2 alone in the forebrain results in increased protection against hypoxic-ischemic injury, suggesting that the total pool of SURx available for coupling is not limiting (374). It has also been suggested that the pore-forming Kir6.2 subunit alone may be localized to the plasma membrane (434) and function independently of the regulatory subunit.

Ovarian steroids may additionally exert non-genomic effects that increase the activation of K_{ATP} channels. Apart from their role in glucose sensing, neuronal K_{ATP} channels are known to be activated by circulating metabolic hormones, specifically insulin (53) and leptin (52). They have therefore been suggested to act as integrators of physiological signals that impact energy homeostasis. The actions of insulin, and to a lesser extent leptin, are mediated in part by the activation of phosphoinositide kinase 3 (PI3 kinase), production of phosphatidylinositol-triphosphate (PIP3), and consequent activation of K_{ATP} channels (306). Recent studies have demonstrated that estrogen and other steroid hormones can activate plasma membrane-associated receptors, and thereby initiate intracellular signaling pathways that culminate in relatively rapid, non-genomic effects on neuronal function (108, 203). Several of these effects have also been shown to be mediated by the stimulation of PI3 kinase activity (435, 436). It is therefore possible that the ability of E_2 and P to upregulate LH responsiveness to K_{ATP} channel modulation may be mediated, in part, by the activation of PI3 kinase in target neurons.

In most mammals, sustained activity of the GnRH pulse generator appears to be dependent upon the availability of oxidizable metabolic fuels, thereby coupling fertility to a state of energy reserve that is sufficiently permissive for reproductive success. Thus, food restriction (41, 43), excessive energy expenditure (246), or other states of negative energy balance have been shown to produce varying degrees of inhibition of GnRH pulsatility. It is likely that this adaptive mechanism is collectively mediated by a number of hormones, neuropeptides, and metabolic signals that exert integrated actions via metabolic sensors in the periphery, brain stem, and preoptic-hypothalamic tissues (6). There is some evidence to suggest that at least some of these pathways conduct signals that converge with steroid hormone feedback mechanisms that regulate GnRH neurosecretion. It has been demonstrated, for example, that food restriction increases the

efficacy of estrogen's negative feedback effects on LH secretion (57). In the present studies, I have provided evidence that the expression and/or activation of K_{ATP} channels in the POA may contribute to the negative feedback actions of ovarian steroids on GnRH pulsatility. It is therefore possible that K_{ATP} channel activation may represent one common pathway by which ovarian steroids and hypoglycemia, and perhaps also sustained negative energy balance, may suppress GnRH pulsatility.

In summary, I have demonstrated that combined treatment with E_2 and P stimulates expression of the mRNA encoding the K_{ATP} channel subunit, Kir6.2, and confers responsiveness of the GnRH pulse generator to stimulation by tolbutamide, a K_{ATP} channel blocker. Our observations are consistent with the hypothesis that ovarian steroids can exert negative feedback effects on GnRH pulsatility by stimulating the expression K_{ATP} channels and/or modulating their activity (Figure 14). This idea is more generally consistent with the previous findings that steroids can exert suppressive effects on GnRH neurons, or on the circuitries controlling GnRH neurosecretion, by modulating the activity of other members of the inwardly-rectifying K^+ channel superfamily (437). It remains to be determined if the regulation of K^+ channel gene expression is a common physiological mechanism by which other steroid hormones may regulate hormone-sensitive neurons throughout the brain.

Besides ovarian steroids, GnRH secretion is also subject to regulation by states of negative energy balance. Negative energy balance has been associated with reduced activity of the HPG axis in a number of species. Interestingly, as previously stated, there may exists a converging pathway that mediates the effects of both ovarian steroids and states of negative energy balance on GnRH neurons. As K_{ATP} channels are critical in energy homeostasis, the establishment of their role in the negative feedback actions of ovarian steroids on GnRH secretion in this chapter

thus prompts the possibility that they may be also involved in the regulation of GnRH secretion by negative energy balance. The following chapters will test this hypothesis by first identifying the effect of 48 hours of fasting on pulsatile GnRH secretion and then investigate the role of K_{ATP} channels in this regulation.

Figure 14

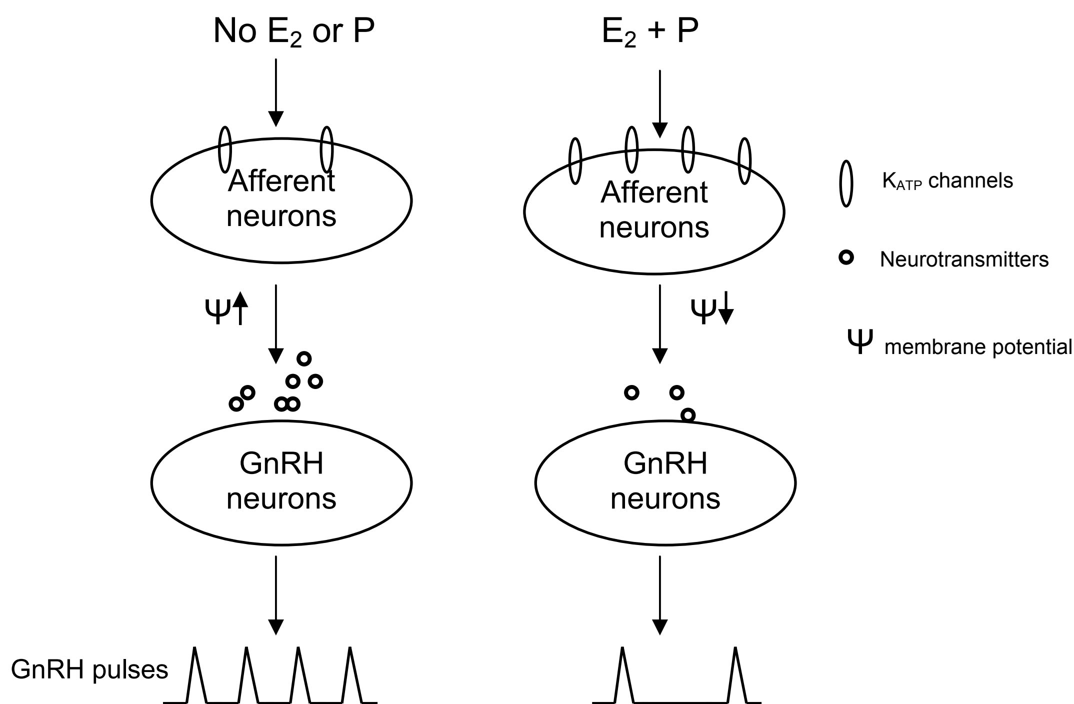

Fig. 14. Proposed model : the role of K_{ATP} channels in the negative feedback actions of E_2 and P on GnRH secretion. Without E_2 and P, K_{ATP} channels expression level in the afferent neurons are low, which leads to less potassium outflux, a higher membrane potential and subsequent more action potential firing and neurotransmitter release. GnRH neurons are thus stimulated and release more GnRH. In the presence of E_2 and P, K_{ATP} channels expression is upregulated, causing more potassium outflux, a lower membrane potential and subsequent less action potential firing and neurotransmitter release. GnRH neurons are thus inhibited and release GnRH less frequently.

Chapter III: Effects of Ovariectomy and 48 Hours of Fasting on Pulsatile GnRH Secretion in Mice as Revealed by Microdialysis: GnRH Pulsatility is Accelerated by Ovariectomy, but Suppressed by 48 Hours of Fasting.

Abstract.

Negative energy balance, a condition that can result from food restriction or deprivation, often causes suppression of the HPG axis. In a number of species such as sheep and rats, this process involves perturbation of LH secretion, most likely by the antecedent disruption of pulsatile GnRH release. However, the effects of an acute state of negative energy balance on GnRH release have never been directly demonstrated. In order to study the impact of energy balance on GnRH release in mice, I first developed microdialysis to monitor pulsatile GnRH secretion in free-moving female animals, and used an OVX model to validate the technique. I demonstrated that OVX significantly increases GnRH pulse frequency, pulse amplitude and mean GnRH levels, which is consistent with the removal of negative feedback actions of ovarian steroids on GnRH secretion. I then sought to test my hypothesis that negative energy balance inhibits pulsatile GnRH release in female mice by using microdialysis to study GnRH release in OVX mice which were either fed *ad libitum* or fasted for 48 hours. Our results show that mean GnRH levels significantly correlate with the LH levels, further corroborating the close relationship of GnRH and LH release. In addition, there is a bimodal response of LH secretion to 48 hours of fasting. Sixty percent of the fasted mice have reduced GnRH pulsatility and LH levels. LH secretion decreases in parallel with reduction in GnRH pulsatility. A subset of the mice are resistant to this inhibitory effect of fasting. The characteristics of their GnRH pulses and their LH levels are comparable to those of fed mice. I conclude that microdialysis is an effective method to monitor *in vivo* GnRH release in mice and that the suppression of LH secretion caused by 48 hours of fasting is mediated by the suppression of GnRH pulsatility. The cellular basis for the resistance of some mice to fasting-induced GnRH and LH suppression is currently under study.

Introduction.

It has long been known that the level of activity in the reproductive axes of both sexes is closely linked with the status of energy balance. Positive energy balance is permissive for successful mating and reproduction. To the contrary, negative energy balance is associated with suppression of the HPG axis, manifested by decreased levels of gonadotropins, gonadal steroids and infertility (6). Effects of negative energy balance on the HPG axis is determined by the interaction among energy intake, energy expenditure, and energy storage. Decreased energy intake, such as food restriction or deprivation, causes reductions in LH secretion in a number of species including rats (41, 42), sheep (43, 44), monkeys (45) and humans (46). Similarly, excessive energy expenditure, such as that seen in female ballet dancers and marathon runners, results in a delay in menarche, amenorrhea and low LH levels (47, 246). Energy storage also plays an important role in regulating the HPG axis. In Syrian hamsters, the ability of a 48hr fast to inhibit estrous cycles seems to depend on the body fat content before fasting, as the fasting-induced inhibition was observed only in lean, but not obese, Syrian hamsters (248).

It has been suggested that suppression of the gonadotropin secretion by negative energy balance is mainly due to the reduction of GnRH secretion from the hypothalamus. In young female rats, pubertal development and an adult LH secretory pattern are prevented by food restriction, and re-feeding results in a rapid elevation of LH pulse frequency and resumption of pubertal development, indicating that the disruption of pubertal development by food restriction involves suppression of the GnRH pulse generator (300). When dietary restriction was used to retard growth in lambs, GnRH interpulse intervals were lengthened as compared to normally growing lambs (48), thus providing direct evidence that GnRH secretion is disrupted by negative

energy balance. In agreement with above observations, pulsatile GnRH infusion in men completely abolished the fasting-induced decline in LH pulsatility (49).

Although the suppression of LH secretion by negative energy balance has also been documented in mice (286), it has never been established whether this reduction in LH secretion results from the disruption of hypothalamic GnRH release. I hypothesized that negative energy balance directly results in suppression of hypothalamic GnRH secretion. Therefore, I first sought to develop a novel technique, microdialysis of the MBH in free-moving mice, to monitor pulsatile GnRH release *in vivo* and validate the technique by studying the effects of ovariectomy on GnRH secretion. I then tested my hypothesis by examining the effect of an acute state of negative energy balance, as a result of 48 hours of fasting, on GnRH secretion in female mice.

Methods.

Animals

Female C57BL/6 mice (Charles River laboratory, Wilmington, MA) (3 months old) were used in this study. They were housed in temperature-controlled facilities (23-25°C) with a 12:12 light cycle (0500-1700). The animals were fed standard lab chow and had access to water *ad libitum.* All surgical and experimental procedures were used in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Northwestern University (Evanston, IL).

Experiment1: Effects of ovariectomy on GnRH pulsatility in female mice

On day 0, half of the animals were bilaterally ovariectomized and the other half were laparotomized (sham-ovariectomized). At the same time, animals were stereotaxically fitted with CMA/7 guide cannula with obdurators (CMA/Microdialysis AB, North Chelmsford, MA) using a stereotaxic neurosurgical instrument equipped with a mouse adaptor (Stoelting, Wood Dale, IL). The coordinates for placement of the guide cannula were 1.2mm posterior to bregma, 4.9mm ventral to the skull and 0.2mm laterally (417). All surgical procedures were performed after anesthesia was inducted in the animal with 80mg/kg, ip. ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 32mg/kg, ip. xylazine (Burns Veterinart Supply, Inc. Rockville Center, NY). The ovariectomized mice (OVX group) were then given 7 days to recover before microdialysis sessions were conducted. After exhibiting two complete, consecutive estrous cycles, the sham-ovariectomized mice (metestrus group) were selected on the day of metestrus for microdialysis sampling experiments. On the day of microdialysis, a CMA/7 microdialysis probe (CMA/Microdialysis AB, North Chelmsford, MA) was connected with FEP tubes (ID: 0.12 mm;

internal volume: 1.2 µL/100 mm length) via its inlet and outlet ends. The probe and the tubes were then filled with aCSF. The components of aCSF were (in mM): NaCl, 124; KCl, 5; $NaHCO_3$, 26; NaH_2PO_4, 2.6; dextrose, 10; HEPES, 10; $MgSO_4$, 2; $CaCl_2$, 2. Unless otherwise stated, all the chemicals were purchased from Sigma (St. Louis, MO). Prior to insertion into the guide cannula, the probe was first equilibrated in aCSF for 1 hour with aCSF being delivered through the tubes and probe by a microdialysis microsyringe pump (CMA/400, CMA/Microdialysis AB). After equilibration, the animals were briefly anesthetized by isoflurane inhalation and the probe was inserted into the guide cannula, enabling the semipermeable dialysis membrane at the tip of the probe to be positioned at the level of the median eminence (1.2mm posterior to bregma, 5.9mm ventral to the skull and 0.2mm laterally) (417). aCSF was then pumped through the probe for 30-40 minutes after probe insertion. During this time, dialysate was discarded. Microdialysis was initiated afterwards and lasted for three hours. During microdialysis, a flow rate of 1.6 µl/min was maintained by the pump, which was comparable to the flow rates of several previous studies utilizing intracranial microdialysis in mice (438, 439). The dialysate samples were collected every five minutes directly into glass assay tubes, mixed with 92µl of phosphate buffered saline with 0.1% gelatin, snap-frozen in a dry ice-ethanol bath and stored at -80°C for subsequent GnRH RIAs. After completion of experiments, mice were anesthetized and killed by decapitation. Brains were frozen on dry ice, and stored for subsequent sectioning and histological verification of cannula placement.

Experiment 2: Effects of 48-hour fasting on GnRH pulsatile secretion.

On day 0, all animals were bilaterally ovariectomized under anesthetized same as in experiment 1. At the same time, they were also stereotaxically fitted with CMA/7 guide cannulae

with the coordinates identical to those used in experiment 1. Five days later, 10 mice were fasted beginning at 11am (Fasted group) while the remaining 8 mice continued to have access to food *ad libitum* (Fed group). Both groups of animals received water *ad libitum* throughout experiments. The microdialysis sessions were initiated 48 hours after the onset of fasting or at a comparable time of day and post-ovariectomy interval in the non-fasted group. The microdialysis sessions were performed in the same manner as in experiment 1. After microdialysis, the animals were anesthetized by isoflurane inhalation and sacrificed. Blood samples were collected by cardiac puncture, centrifuged at 4°C and stored at -20°C for LH, corticosterone and leptin RIA. All the animals were weighed on day 5 immediately before fasting, on day 6 and on day 7 before they were killed.

RIAs:

The GnRH assay used GnRH antibody (R1245) generously provided by Terry Nett and had a sensitivity of 1.0 pg/ml. For the LH assay, LH reference (RP-3) and anti rat LH antibody (S-11) obtained from NIDDK were used and the assay had a sensitivity of 0.2 ng/ml. Intraassay and interassay CV were 6.15%, 14.2% for the GnRH assay and 8.28%, 9.66% for the LH assay respectively. For corticosterone assay, we used a double antibody RIA kit (MP Biomedicals, Orgageburg, NY) with a sensitivity of 7.7ng/ml. Leptin was measured using a leptin RIA Kit from Linco Research (St. Charles, MO). The sensitivity of the leptin assay is 0.5ng/ml. The intraassay CV for corticosterone and leptin assays were 0.32% and 1.48% respectively.

Statistical analysis

Significant GnRH pulses were determined by using ULTRAGUIDE pulse analysis program as previous reported (440). GnRH pulse frequency is presented as pulses/hour. GnRH pulse amplitude is defined as the difference between the peak value of a pulse and the lowest value immediately before that pulse. All values were reported as mean ± SEM.

Correlation of LH level with mean GnRH level was performed using standard linear regression analysis. Comparisons of GnRH pulse frequency, pulse amplitude and mean GnRH levels between different groups were conducted using unpaired Student's t-tests. Following an initial analysis of GnRH release in all animals, a second analysis was performed in which these same parameters were compared between fed animals and only those fasted animals in which LH levels were found to be equal to or below 0.2 ng/ml, a criterion that was chosen as the lowest LH level observed in the fed animals. For this analysis, these mice were defined as "responders" on the basis of this marker. Similarly, the same parameters of GnRH pulses were also compared between fed animals and those fasted animals with LH values above 0.2 ng/ml which were defined as "non-responders". Comparisons of initial body weight, weight loss, corticosterone, and leptin levels after 48 hours of fasting between fasting-responsive and fasting non-responsive mice were similarly analyzed by unpaired Student's t-tests. For all the statistical analyses, differences were considered significant if $p < 0.05$.

Results.

Measurement of pulsatile GnRH release in MBH microdialysates

RIAs of mouse MBH microdialysates revealed detectable levels of GnRH ranging from 12.5 pg/ml to 177.5 pg/ml (Figure 15). This reflects a range of secretory rate from 0.1 pg/5min to 1.42 pg/5min. These values are comparable to the GnRH release rates previously measured in female rats (441) and male rats (442) by push-pull perfusion and microdialysis methods. In all GnRH release profiles that were assessed, the pattern of release was determined to be pulsatile, with pulse frequencies also similar to those observed in rats under similar experimental circumstances (166, 441).

Effect of Ovariectomy on GnRH pulsatility

Ovariectomy significantly enhanced GnRH pulsatility (Figure 16). GnRH pulse frequency was 0.99 ± 0.34 pulse/hr in metestrus mice compared to 2.69 ± 0.29 pulses/hr in OVX mice ($p< 0.01$). GnRH pulse amplitude was elevated by 2.7 fold from 10.61 ± 3.51 pg/ml in metestrus mice to 27.71 ± 3.54 pg/ml in OVX mice ($p< 0.01$). Mean GnRH levels also exhibited an increase from 15.62 ± 1.95 pg/ml in the metestrus group to 24.91 ± 2.31 pg/ml in the OVX group ($p< 0.01$).

Figure 15

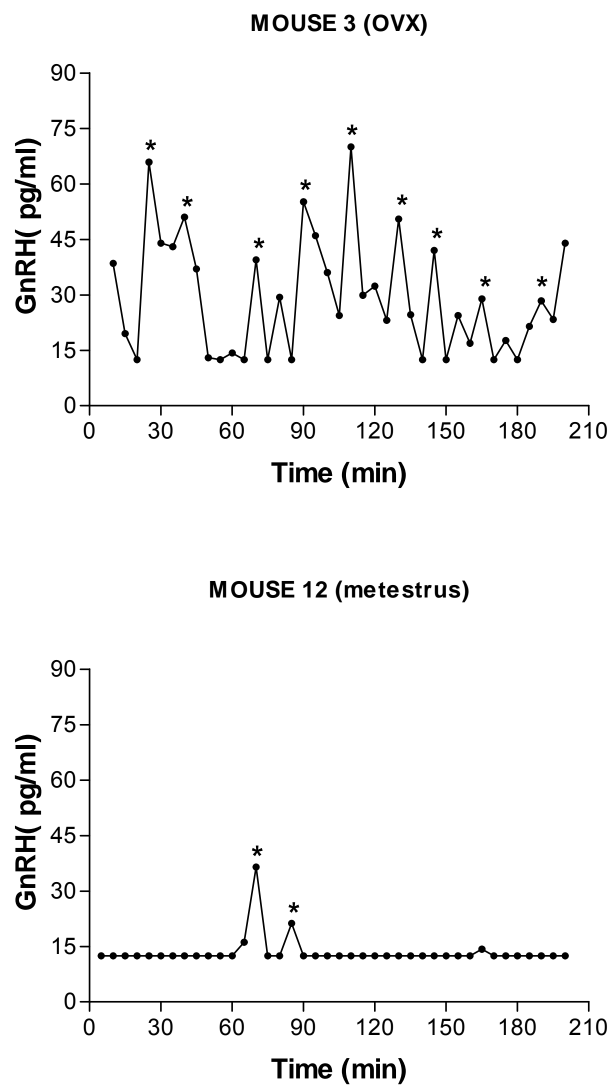

Fig. 15. Representative profiles of GnRH pulses in OVX mice and metestrus mice. Asterisks denote pulses detected by ULTRAGUIDE pulse analysis software.

Figure 16

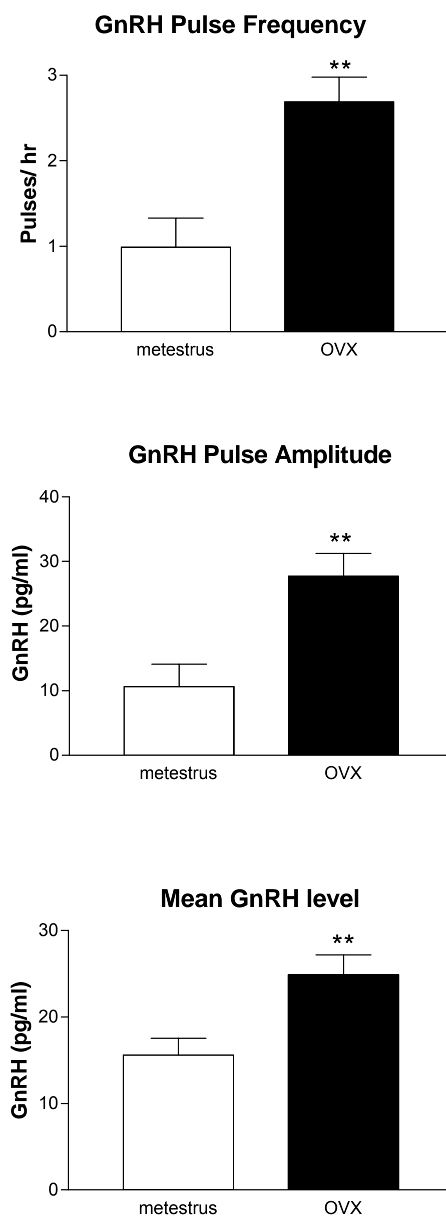

Fig. 16. Effect of OVX on GnRH pulsatility. OVX increased all GnRH pulse parameters including GnRH pulse frequency (0.99 ± 0.34 pulse/hr and 2.69 ± 0.29 pulses/hr for metestrus and OVX respectively), GnRH pulse amplitude (10.61 ± 3.51 pg/ml and 27.71 ± 3.54 pg/ml for metestrus and OVX respectively) and mean GnRH level (15.62 ± 1.95 pg/ml and 24.91 ± 2.31

pg/ml for metestrus and OVX respectively). Metestrus (n=7) and OVX (n=6). **, $P<0.01$ vs. metestrus

Effects of the 48hr fast on GnRH/LH secretion:

Correlation between mean LH and GnRH levels

Linear regression analysis of pooled data from all animals revealed a significant correlation between LH levels as measured in the peripheral blood and mean GnRH levels as measured in the microdialysis dialysate after 48hr food manipulation ($r^2=0.63$, $p<0.0001$) (Figure 17).

Comparison of LH levels and GnRH pulsatility in fasted mice versed fed mice

The basal LH levels in the fasted mice were not significantly different from the values observed in the fed mice (Figure 4). Similarly, there were no overall differences in GnRH pulse frequency, pulse amplitude or mean GnRH level between fasted mice and fed mice (Figure 18).

Comparison of GnRH pulsatility in fasting-responsive and fed mice

There was a bimodal distribution of LH levels in the 48hr-fasted mice (Figure 19). Six of 10 fasted mice had LH levels equal or less than 0.2ng/ml, which is the lowest LH level observed in the fed animals, while the others had LH levels higher than 0.2 ng/ml. I thus defined the mice with fasting LH level equal to or lower than 0.2 ng/ml as "fasting-responsive", while those with LH level higher than 0.2 ng/ml as "fasting non-responsive". Shown are representative GnRH pulse profiles from fed, fasting-responsive and fasting non-responsive mice (Figure 20). GnRH pulsatility of fasting-responsive mice was significantly lower than that of fed mice. (GnRH pulse frequency: 1.45 ± 0.27 pulses/hr in fasting-responsive mice vs. 2.52 ± 0.33 pulses/hr in fed mice, $p<0.05$; GnRH pulse amplitude: 9.85 ± 0.64 pg/ml in fasting-responsive vs. 14.4 ± 2.37 pg/ml in fed mice, $p<0.05$ and mean GnRH level: 14.40 ± 0.51 pg/ml in fasting-responsive vs. 17.12 ± 1.08 pg/ml in fed mice, $p<0.05$) (Figure 21).

Comparison of GnRH pulsatility in fasting non-responsive and fed mice

In contrast to the above findings, GnRH pulse frequency, pulse amplitude and mean GnRH level were not different between fasting non-responsive and fed mice (Figure 22).

Initial body weight and weight loss in fasting-responsive and fasting non-responsive mice

Before food manipulation, fasting-responsive and fasting non-responsive mice had similar body weight. Again, after 48h fasting, no difference in weight loss was revealed between the two groups of mice (Figure 23).

Corticosterone and leptin levels in fasting-responsive and fasting non-responsive mice

Fasting-responsive and fasting non-responsive mice did not differ in corticosterone and leptin levels after 48hr food manipulation (Figure 24).

Figure 17

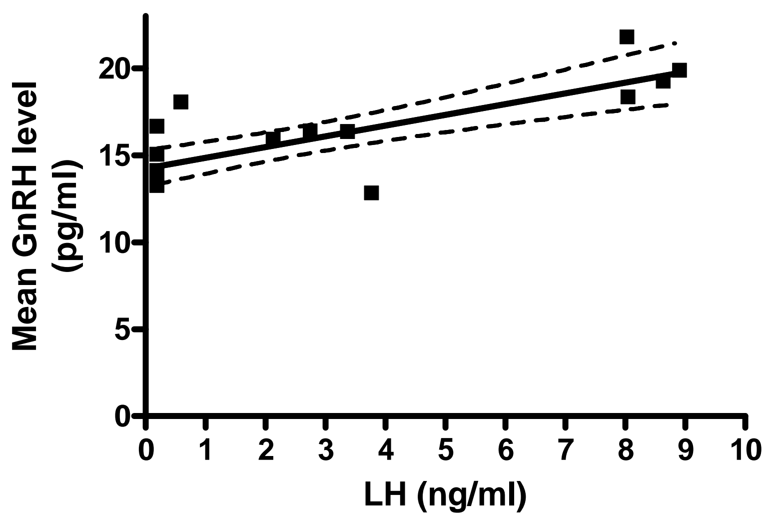

Fig. 17. Correlation of basal LH levels with mean GnRH levels. Basal LH levels, as measured in peripheral blood, are significantly correlated with mean GnRH levels as measured in microdialysis dialysate ($r^2 = 0.63$, $p<0.0001$). The dotted line showed the 95% confidence limits.

Figure 18

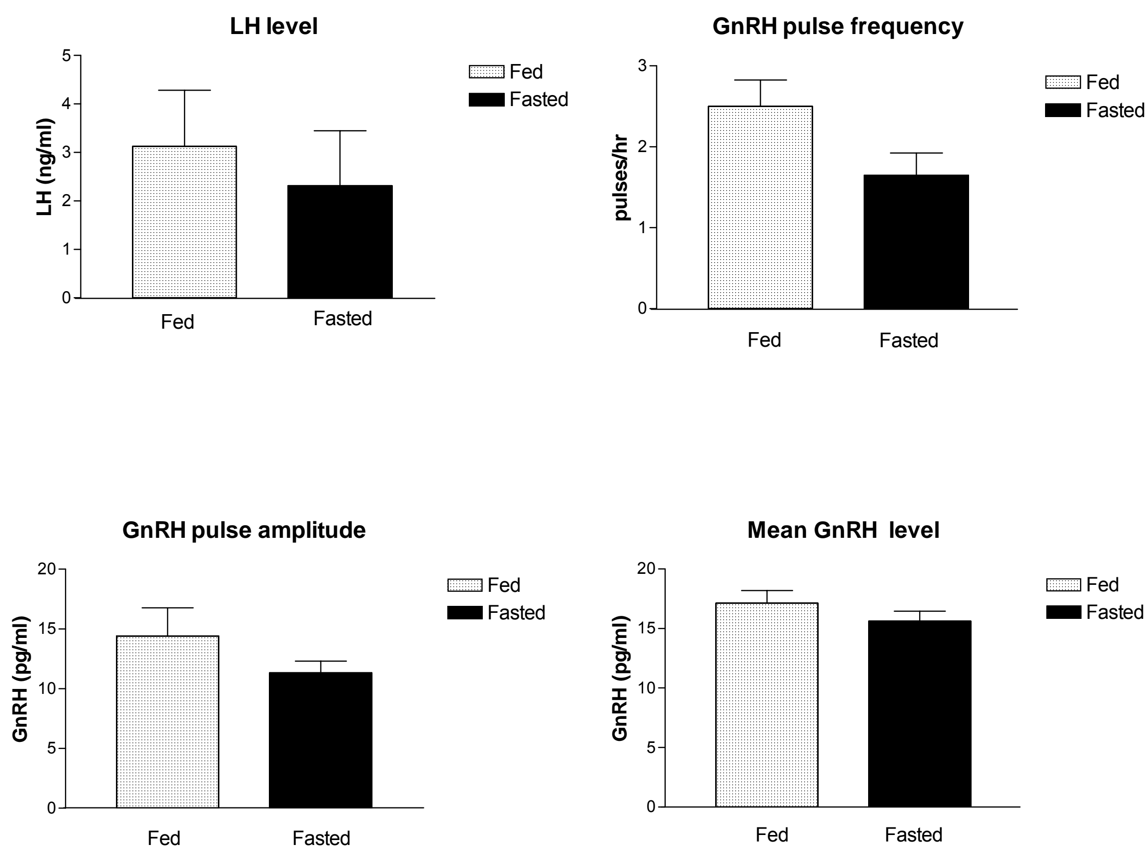

Fig. 18. Effects of 48 hours of fasting on LH level and GnRH pulsatility. Comparison of LH levels and characteristics of GnRH pulses including pulse frequency, pulse amplitude and mean GnRH level showed no significant difference between fed and fasted mice.

Figure 19

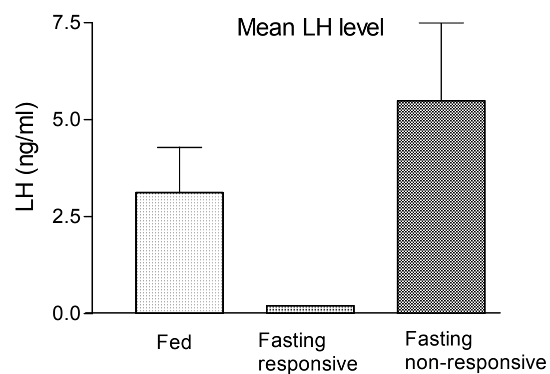

Fig. 19. Bimodal distribution of LH response to 48 hours of fasting. According to their LH levels, fasted mice were divided into fasting-responsive (LH ≤ 0.2 ng/ml) and fasting non-responsive groups (LH > 0.2 ng/ml). Shown are LH levels in fed, fasting-responsive and fasting non-responsive female mice.

Figure 20

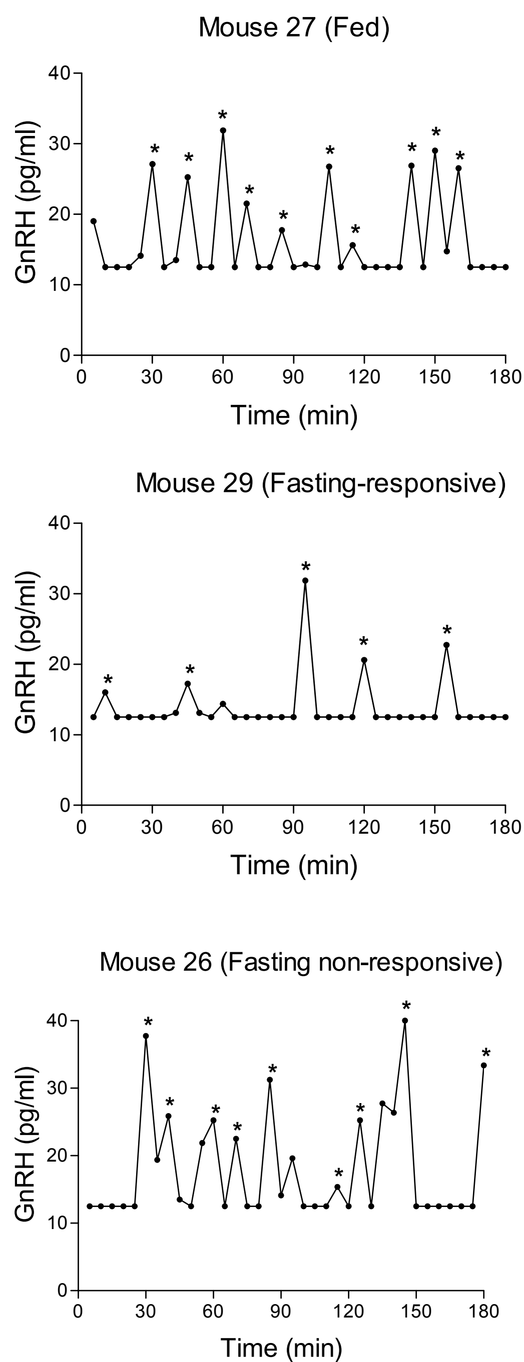

Fig. 20. Representative profiles of GnRH pulses in fed, fasting-responsive and fasting non-responsive female mice. LH concentrations were 2.14 ng/ml, 0.2 ng/ml and 0.6 ng/ml for

Mouse 27, Mouse 29 and Mouse 26 respectively. Asterisks denote pulses detected by ULTRAGUIDE pulse analysis software.

Figure 21

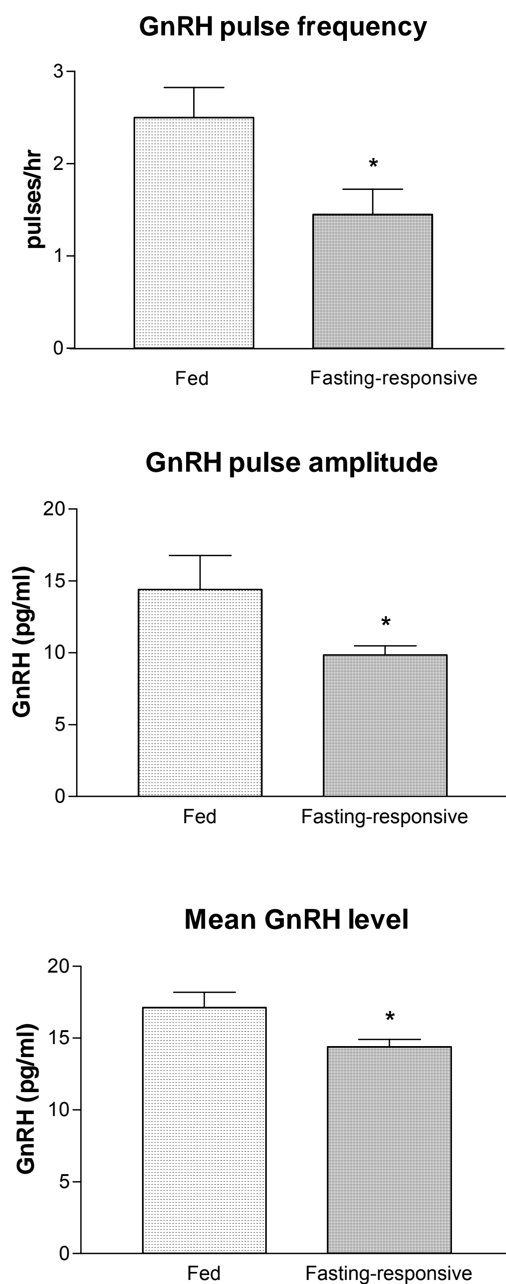

Fig. 21. Effect of 48 hours of fasting on GnRH pulses in fasting-responsive mice. All GnRH pulse characteristics including GnRH pulse frequency, pulse amplitude and mean GnRH level in fasting-responsive mice (n=6) were significantly lower than those of the fed mice (n=8) (GnRH

pulse frequency. 1.45 ± 0.27 pulses/hr in fasting-responsive mice vs. 2.52 ± 0.33 pulses/hr in fed mice; GnRH pulse amplitude. 9.85 ± 0.64 pg/ml in fasting-responsive vs. 14.4 ± 2.37 pg/ml in fed mice and GnRH mean level. 14.40 ± 0.51 pg/ml in fasting-responsive vs. 17.12 ± 1.08 pg/ml in fed mice.) * , $p<0.05$ vs. fed mice.

Figure 22

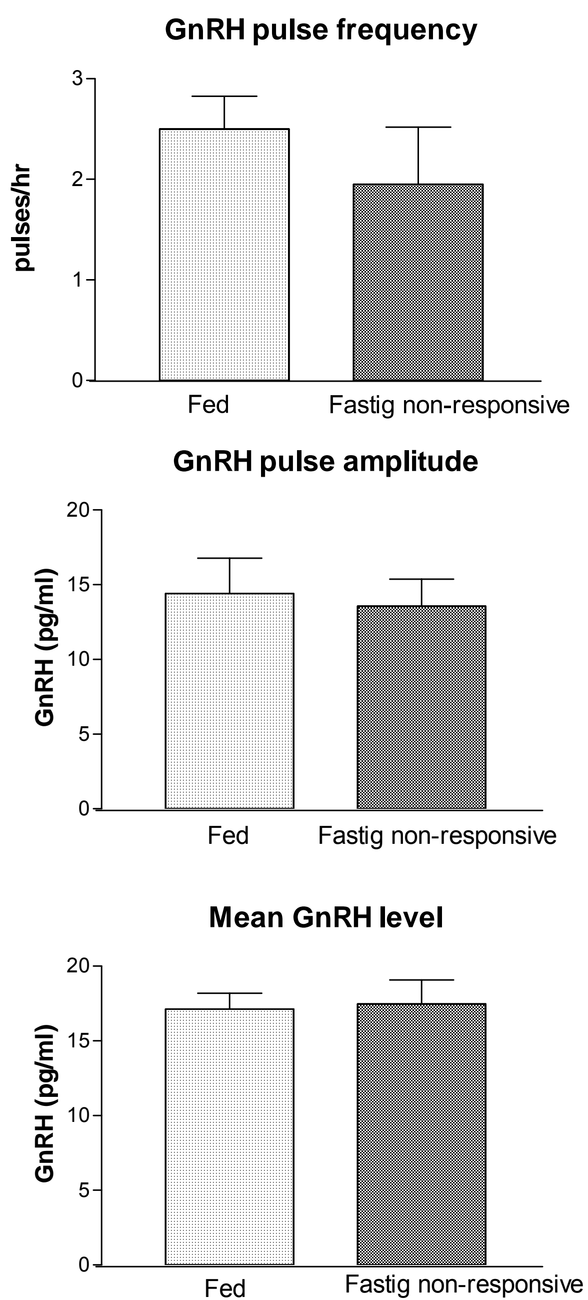

Fig. 22. Effect of 48 hours of fasting on GnRH pulses in fasting non-responsive mice. GnRH pulse frequency, pulse amplitude and GnRH mean level in fasting non-responsive mice (n=4) were not different from those of fed mice (n=8).

Figure 23

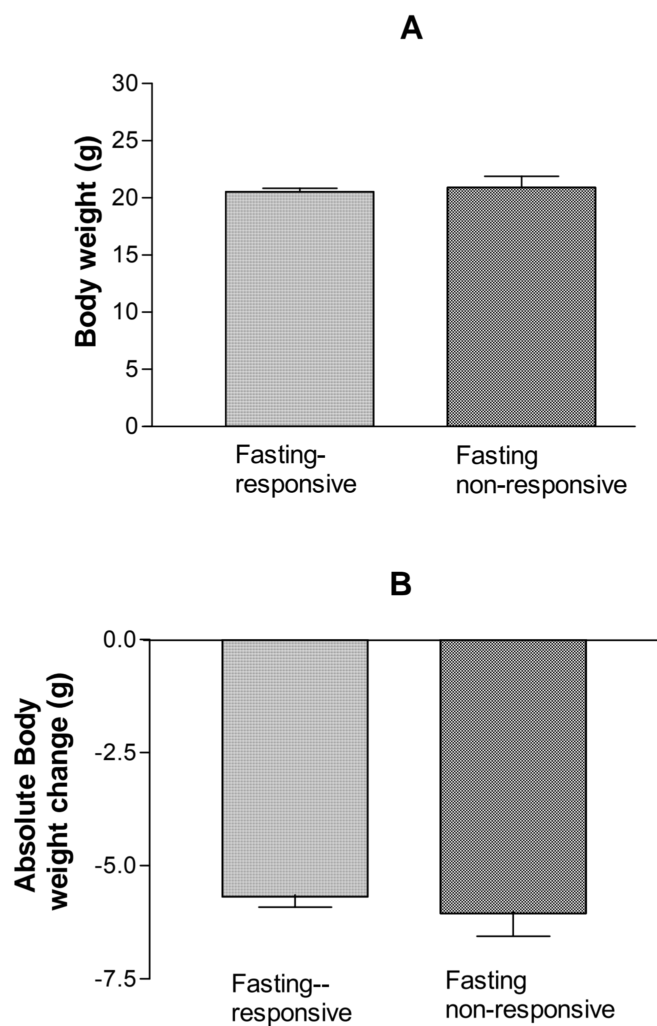

Fig. 23. Initial body weight and body weight change in fasting- responsive and fasting non-responsive mice. Initial body weight before fasting (A) and absolute body weight change after the 48hr fast (B) revealed no difference between fasting-responsive and fasting non-responsive mice.

Figure 24

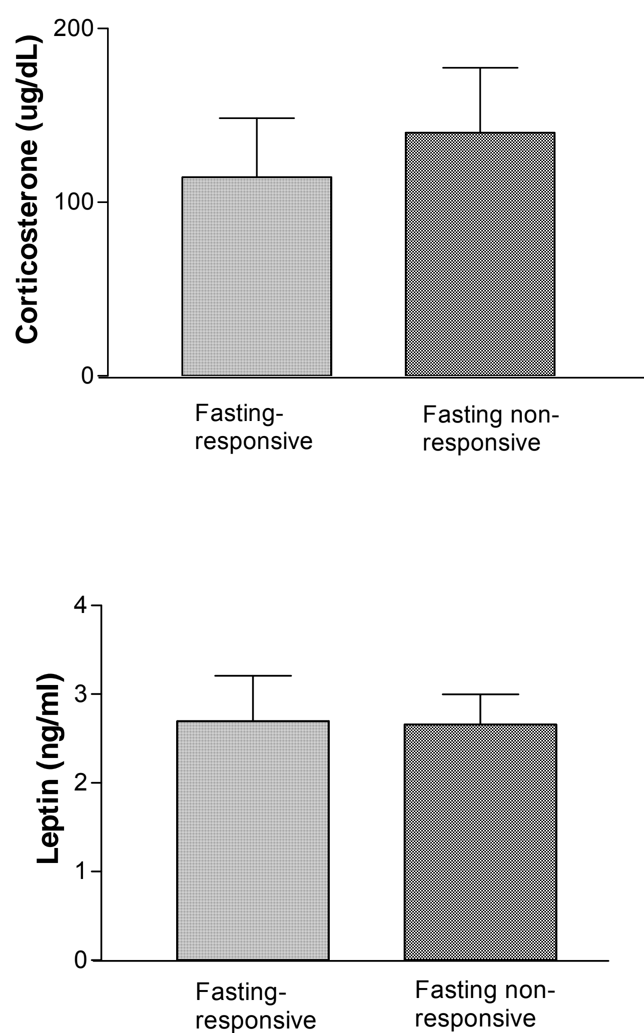

Fig. 24. Corticosterone and leptin levels after 48 hours of fasting in fasting-responsive and fasting non-responsive mice. There is no significant difference in corticosterone or leptin levels between the fasting-responsive and fasting non-responsive mice.

Discussion.

Energy balance is an important factor in the regulation of gonadotropin secretion (6, 7). Negative energy balance, as a result of decreased energy intake or increased energy expenditure, often causes reduction in LH secretion in a number of species such as rats (41, 42), sheep (43, 44), monkeys (45) and humans (46). A large body of evidence suggests that this decrease in LH secretion is due to suppression of the pulsatile GnRH release from the hypothalamus. In female rats, food restriction prevents pubertal development (300). Re-feeding reverses the LH suppression in these animals by increasing LH pulse frequency without affecting GnRH content and responsiveness of gonadotropes to GnRH, indicating that food restriction suppresses GnRH secretion from the hypothalamus (300). In addition, pulsatile administration of GnRH in men restored the LH and testosterone level inhibited by fasting, suggesting central mechanism is underlying the changes in gonadotropin release (49).More direct evidence of the effects of negative energy balance on GnRH secretion has come from studies of larger animals. In female sheep, dietary restriction increases the interpulse interval of both LH and GnRH pulses measured simultaneously in peripheral blood and hypophyseal portal blood respectively (48).When MUA of the GnRH pulse generator was recorded in female goats, the volley interval was significantly prolonged after 4 to 5-day fasting, indicating suppression of the GnRH pulse generator activity (443). It has previously been demonstrated that food deprivation suppresses LH secretion in mice (286). However, whether the GnRH pulse generator is involved in this process has yet been determined. In this study, we first developed microdialysis to monitor pulsatile GnRH release *in vivo* in female mice. I showed that GnRH is released in a pulsatile manner. In addition, as a validation of my microdialysis method, I demonstrated that ovariectomy enhances GnRH pulsatility, which agrees with the idea that the negative feedback actions of ovarian steroids are

exerted at least in part by suppression of GnRH neurosecretion. Furthermore, by using microdialysis to study the effect of negative energy balance on GnRH secretion, I revealed for the first time that 48 hours of food deprivation in female mice causes suppression of pulsatile GnRH secretion.

GnRH is released from the hypothalamus into the hypophyseal portal system in a pulsatile manner (444). This pulsatile release of GnRH underlies the physiological functions of the HPG axis and disruption of the GnRH pulsatility leads to hypogonadotropic hypogonadism. Several methods have been developed to monitor GnRH pulsatile secretion *in vivo* either directly or indirectly. Among them are hypophyseal portal blood sampling, push-pull perfusion, and microdialysis, as well as high-resolution measurements of LH pulsatility as a reflection of GnRH pulsatility. Hypophyseal portal blood sampling collects serial blood samples from the portal veins located at the pituitary stalk and is able to detect pulsatile GnRH release (67). However, due to the difficulty of the neurosurgical procedures, size of the hypophyseal portal system and volume of blood required for GnRH assays, this technique is largely reserved for big animals, such as sheep (67). Push-pull perfusion was first reported to study *in vivo* GnRH release in rats (445), then was used for similar purpose in other species including monkeys (446) and rabbits (447). It utilizes concentric cannulae composed of an inner tube and an outer tube (tube within a tube) with the perfusion solution pumped through the inner tube and the extracellular fluid aspirated from the outer tube (448). GnRH molecules at the extracellular space can thus be collected and measured. This technique has the advantage of higher recovery rate compared to microdialysis. However, it causes a net flow of solution outside the tube and thus can potentially result in damage of the surrounding tissue, making it less appealing to study *in vivo* GnRH release (449). In addition to the above two methods, LH pulsatility detected in serial peripheral

blood sampling can be used to indirectly monitor GnRH pulsatility (63, 68). However, due to the requirement of frequent blood sampling for pulse detection and the requirement on blood sample volume for LH assays, study of LH pulsatility in mouse appears to be implausible at this point. In this study, we chose to use microdialysis to monitor GnRH secretion. This technique involves using a probe to collect interested molecules from the extracellular space (450). The probe has at its tip a semi-permeable membrane with certain cut-off value for molecular weight. As a semi-permeable membrane, it allows free passage of molecules with molecular weight lower than the cut-off value, but excludes molecules with higher molecular weight. The species under study determines the size of the probe to be used. Up to now, this method has been successfully employed to study a number of molecules in rats (418), mice (451) and monkeys (452). To study GnRH release, the tip of the probe is placed at the median eminence. A carrier solution is constantly pumped through the probe to carry away GnRH molecules which enter the probe through the semi-permeable membrane. Microdialysis has only been applied to study *in vivo* GnRH secretion in rats (418), rabbits (453) and monkeys (Levine JE, unpublished data). In this study, I employed this method to study *in vivo* GnRH pulse in mice for the following reasons: (1) push-pull perfusion, hypophyseal portal blood sampling and frequent blood sampling all have technical limitations, as such they are generally used in animals bigger than mice, (2) microdialysis has been used in rats to study GnRH release and the microdialysis probes are commercially available and proven to be reliable, and (3) microdialysis has been used in mice to study other molecules in the brain such as NE (171) and DA (451).

Until recently, no direct monitoring of GnRH release in free moving, conscious mice has ever been reported. The study of *in vivo* GnRH secretion in mice provides new opportunities to study the regulation of HPG axis due to the vast availability of transgenic and gene knockout

mice. By using this methodology in mice, we can better understand the physiological functions of the manipulated genes. In the transgenic and gene knockout mice that were generated to specifically study HPG axis, such as ERKO and PRKO mice, increases in LH and/or FSH levels were documented (225, 240). However, the exact mechanism leading to these changes has not been explored. The increase in gonadotropins could result from alterations in either hypothalamic GnRH secretion or responsiveness of gonadotropes to GnRH input or both. Moreover, alterations in gonadotropin secretion might result from changes in GnRH pulse frequency, amplitude, or both parameters. In order to discern real alterations in GnRH release, monitoring of the *in vivo* GnRH release over time appears critical.

As a validation for this technique, I first tested the effects of ovariectomy on GnRH pulsatility. The negative feedback of ovarian steroids on gonadotropin secretion has been known for many decades. Removal of ovaries results in elevation of both LH and FSH (28), an effect that likely results in part from an increase in GnRH pulsatility. In the present study, I showed for the first time that GnRH is released in a pulsatile manner in female mice and that ovariectomy causes significant increase in GnRH pulse frequency, pulse amplitude and mean GnRH levels.

I have further investigated the effect of negative energy balance on pulsatile GnRH secretion. Our results revealed two distinct groups in the fasted mice in regard to their response to 48 hours of fasting. Although the majority of them had low LH levels near the limits of the assay, similar to what has been observed in other studies (286, 291), some mice exhibited relatively high LH levels which were comparable to those of the fed mice. If these two groups of mice were considered together as a single group, their LH level and GnRH pulsatility after for 48 hours of fasting were not significantly different from those of the fed mice. Because LH is released in a pulsatile manner, I first sought to determine whether this discrepancy in LH levels was an artifact

of detecting the peak LH values in mice with high LH levels and the nadir LH values in those with low LH levels after food manipulation. Linear regression of mean GnRH and LH levels from the pooled data revealed that these two variables are highly correlated, thus arguing against that possibility that the high LH levels in some of the fasted mice was an artifact introduced by the pulsatile pattern of LH release.

The two groups of mice were then reanalyzed, designating mice as being fasting-responsive and fasting non-responsive according to their LH levels. Mice with LH concentrations equal to or lower than 0.2 ng/ml were defined as fasting-responsive, while those with LH concentrations higher than 0.2 ng/ml were defined as fasting non-responsive. Comparison of the GnRH pulse frequency, pulse amplitude and mean GnRH levels in the fasting-responsive mice with those in the fed mice clearly demonstrated that GnRH pulsatility is reduced by 48 hours of fasting. In sharp contrast to the above finding, the GnRH pulsatility in the fasting non-responsive mice was of no difference from that in the fed mice. It is worth to note that this bimodal distribution of LH concentrations in response to negative energy balance has also been documented in a recent study using human subjects. The study investigated the effect of energy availability on LH pulsatility in young, menstruating women by restricting their dietary intake. When the energy availability was limited to 10 or 20 kcal/kg lean body mass·day (normally 45 kcal/kg lean body mass·day), a bimodal distribution of LH pulsatility was observed. The two groups had entirely non-overlapping in LH pulsatility response (454) similar to what we have observed in this study. It is also worth mentioning that chronic food restriction can cause an increase in mean serum LH concentrations. This finding has been confirmed in ovariectomized female rats (300), castrated male rats (455) and male sheep (456). The increase in mean serum LH after chronic food restriction is believed to result from the increase in LH pulse amplitude and pulse length. The

increase in LH pulse amplitude is probably a result of an increased responsiveness of gonadotropes to GnRH under chronic food restriction (455). In the present study, fasting non-responsive mice exhibit GnRH pulsatility comparable to that of fed mice. However, their LH concentrations showed a trend of being higher than those of fed mice, which may possibly be explained by the increased sensitivity of gonadotropes to GnRH stimulation. However, I did not assess response of LH to exogenous GnRH in these animals, thus would not be able to test this possibility.

Since body weight is a reflection of body energy storage, I then compared the initial body weight and weight loss between fasting-responsive and fasting non-responsive mice. Body energy storage, like energy intake and energy expenditure, is a critical factor in determining the responsiveness of gonadotropin to negative energy balance. Even in a condition of negative energy balance, fuels released from energy storage such as liver and fat tissues, may compensate for the deficiency of energy intake. One study demonstrated that in women there exists a threshold in regard to the ability of low energy availability to inhibit LH secretion. Above that threshold, even when the energy availability is lower than what is normally required, i.e. in a state of negative energy balance, normal LH pulsatility is preserved. When energy availability is lower than the threshold, LH pulsatility is abruptly disrupted, manifested as decrease in LH pulse frequency and increase in LH pulse amplitude (454). A similar study in female monkeys showed that a body weight loss of 2-11% was able to inhibit ovulation in normal weight monkeys. Whereas, a weight loss of 46% was required to inhibit ovulation in obese monkeys (457). However, in the present studies, I found no difference in the initial body weight and weight loss after the 48hr fast between the fasting-responsive and fasting non-responsive mice.

Because glucocorticoids and leptin (281, 282) have been implicated in mediating the effect of negative energy balance on the GnRH/LH secretion, I further examined whether the fasting-responsive and fasting non-responsive groups differ in their corticosterone and leptin levels. Our results showed that there was no difference in the levels of these two hormones between the two groups of fasted mice. Interestingly, in the aforementioned human study in which a bimodal distribution of LH responses to negative energy availability was found, the authors also sought to identify the differences between the two distinct groups of women which correlate with their LH response. A number of factors were compared between the two groups including plasma glucose, β-hydroxybutyrate, insulin, cortisol, growth hormone, and IGF-1. None except the luteal phase length before food restriction was found different. The subjects whose LH was inhibited by dietary restriction had luteal phases less than11 days, while those who did not respond had luteal phases longer than 11 days (454). In this study, since all the animals were ovariectomized, it was difficult to address whether the fasting-responsive group and the fasting non-responsive group had difference in the length of their estrous cycles. Moreover, since mice do not have distinct luteal phases, it is less likely that luteal phase length is the major characteristic that distinguishes between the fasting-responsive and fasting non-responsive mice in regard to their LH response to fasting.

The bimodal distribution of LH response to fasting may be attributed to difference in individual susceptibility at a state resistant to fasting-induced suppression, such as OVX. It has been demonstrated that estrogen enhances the inhibitory effect of food restriction on gonadotropin secretion (43, 57). In ovariectomized female sheep, chronic undernourishment causes virtually no LH pulses when the animals were treated with estrogen compared to a few discernable LH pulses when the animals were treated with vehicle (43). In the present study, all

the animals were OVX first, and then treated with food manipulation. Thus it is possible that the absence of estrogen may render some of the animals resistant to the inhibitory effects of food deprivation.

In summary, I have demonstrated for the first time that 48 hours of fasting decreases GnRH pulsatility in majority of the female mice and that reduction in GnRH secretion correlates with reduction in LH levels. The mice that were responsive to fasting and the mice that were resistant to fasting can not be distinguished by their initial body weight, weight loss, glucocorticoids and leptin levels after fasting. I conclude that in female mice negative energy balance inhibits LH secretion by suppressing hypothalamic GnRH pulse generator activity. Whereas, the physiological parameters that distinguish animals in which LH levels responded to fasting from those in which LH resists fasting-induced suppression remain to be determined.

K_{ATP} channels can signal the brain of the current status of energy availability and have been closely associated with energy homeostasis. In Chapter II, I also demonstrated the K_{ATP} channel modulation is able to regulate GnRH pulse generator activity. Therefore, it is possible that K_{ATP} channels mediate the inhibitory effects of negative energy balance on GnRH secretion. The next chapter will attempt to investigate the role of K_{ATP} channels in the regulation of GnRH secretion by 48 hours of fasting. Pharmacological approaches and an SUR1 subunit gene knockout mouse model with deficiency of K_{ATP} channels will be employed to address this question.

Chapter IV: Role of K_{ATP} Channels in the Effects of Negative Energy Balance on GnRH/LH Secretion: K_{ATP} Channels are not Necessary in Mediating the Effects of Negative Energy Balance on GnRH/LH Secretion.

Abstract.

Our previous studies demonstrated that 48 hours of fasting suppresses both GnRH and LH secretion in female mice. In this study, I assessed whether modulation of K_{ATP} channels is involved in fasting-induced reduction in GnRH and LH secretion. Vehicle or a K_{ATP} channel blocker, tolbutamide, was infused into the right lateral ventricle of ovariectomized mice which were either fed or fasted for 48 hours. The 48hr fast suppressed LH secretion, while i.c.v. tolbutamide infusion resulted in approximately two-fold increase in LH levels in both fed and fasted mice compared to vehicle. However, tolbutamide was not able to restore the LH levels of the fasted mice to those of the fed mice, thus arguing against the idea that K_{ATP} channels may play a necessary role in the effects of negative energy balance on GnRH/LH secretion. I further investigated the effects of 48 hours of fasting on LH secretion in SUR1 null mice, which are deficient in functional K_{ATP} channels comprised of the Kir6.2-SUR1 subunit combination found in neurons and pancreatic β cells. Wild type (WT) and knockout ($SUR1^{-/-}$) SUR1 mice were ovariectomized on day 0. Five days later, blood samples were collected by tail bleeding before the animals were fasted. The fast lasted for 48 hours before the animals were killed. Our results showed that before fasting, there was no significant difference in body weight, ovary weight, basal LH levels and basal glucose levels between WT and $SUR1^{-/-}$ mice. Upon challenge of 48 hours of fasting, both groups of animals showed significant body weight loss and suppression of LH secretion. Serum glucose levels were significantly reduced by fasting in the $SUR1^{-/-}$, and a trend that did not reach significance in the WT mice. In addition, after 48 hours of fasting, $SUR1^{-/-}$ mice had significantly higher insulin levels and lower glucose/insulin ratio, but similar leptin levels and uterine weights compared to WT mice. The present study demonstrates that (1) central blockade of K_{ATP} channels causes an elevation in LH secretion regardless of states of energy

balance , but is not able to fully restore LH secretion to levels exhibited prior to food deprivation; (2) WT and SUR1$^{-/-}$ mice do not differ in body weight, ovary weight, uterine weight, basal LH and basal glucose levels; and (3) short-term food deprivation results in similar inhibition of LH secretion in SUR1$^{-/-}$ and WT mice. In summary, the present studies support the idea that K_{ATP} channels are linked to the regulation of GnRH release, but they do not play an obligatory role in mediating the effects of negative energy balance on GnRH/LH secretion.

Introduction.

GnRH neurons serve as the final common neural pathway that regulates the HPG axis. A number of physiological factors have been implicated in regulating GnRH neurosecretion, including the states of energy balance of an animal (6, 7). Negative energy balance, a condition that can be caused by decreased food intake or increased energy expenditure, results in suppression of both gonadotropin and gonadal steroid levels in a number of species including rats (41, 42), sheep (43, 44), monkeys (45) and humans (46). Recent studies have suggested that the reduction in gonadotropins, especially LH, is a result of inhibition of the GnRH pulse generator in the hypothalamus (48).

A large body of evidence has suggested that the hindbrain, particularly the AP, plays an important role in relaying signals representing states of negative energy balance to the GnRH pulse generator in the hypothalamus. AP lesions have been shown to lead to abolishment of hypoglycemia-induced reduction in LH secretion (319). Neuroanatomical studies also provide relevant evidence that neurons in the hindbrain, especially NPY/NE neurons, send projections to the GnRH neurons (327) and affect GnRH/LH secretion (322, 328). However, as an integration site for the regulation of both energy homeostasis and reproduction, hypothalamus may also be involved in signaling the brain of the status of energy balance and affecting GnRH pulse generator activity. The role of hypothalamus in the regulation of GnRH secretion by negative energy balance is prompted by the following important observations. Glucose has been shown to be an important primary metabolic cue that transmits the signals of negative energy balance to affect GnRH and gonadotropin secretion (6). Studies have demonstrated that there are glucose-sensing neurons in the hypothalamus, e.g. NPY and POMC neurons in the arcuate nucleus (9). Alteration in blood glucose level leads to changes in their electrical activity and subsequently

neuropeptide secretion from these neurons. As an important metabolic hormone, leptin has also been shown to account for at least part of the effects of negative energy balance on gonadotropin secretion. Leptin treatment improves LH secretion that is inhibited by food restriction or food deprivation (283, 284). In addition, high levels of leptin receptors are expressed in NPY and POMC neurons in the arcuate nucleus (270). NPY and POMC neurons have been found to have synaptic contacts with GnRH neurons (35, 36). Moreover, NPY and POMC treatment result in changes in GnRH/LH secretion (158, 322). Recently, leptin has been shown to hyperpolarizes hypothalamic neurons via activation of K_{ATP} channels, which are composed of Kir6.2 and SUR1 subunits (52). These channels have been found to be expressed in a subset of NPY and POMC neurons (8, 9) and are thought to be essential to the central mechanism of glucose homeostasis, even energy homeostasis. Kir6.2 knockout mice have an impairment in the increase of food intake and glucagon secretion upon challenge of food deprivation (54).

Taken together, the above evidence supports the idea the K_{ATP} channels are expressed in hypothalamic neurons which can both detect peripheral metabolic signals and affect GnRH neurosecretions. Therefore, I sought to determine whether K_{ATP} channels play a role in mediating the effect of negative energy balance on GnRH/LH secretion. In the present study, I first investigated whether lateral ventricular infusion of tolbutamide, a K_{ATP} channel blocker, affects LH secretion in female mice under different states of energy balance. I then studied the HPG axis and the response of LH secretion to a short-term fast in the K_{ATP} channel-deficient mice, the SUR1 knockout mice which were generated by targeted disruption of the gene encoding the SUR1 subunit of K_{ATP} channel.

Methods.

Animals

Female C57BL/6 mice (Charles River laboratory, Wilmington, MA) were used in experiment 1, while female WT and SUR1$^{-/-}$ mice were used in experiment 2. SUR1$^{-/-}$ male and female mice (C57BL/6 X 129 SvJ) were generously provided by Mark A. Magnuson (Vanderbilt University, Nashville, TN) (368). Because mating of the SUR1$^{-/-}$ male and SUR1$^{-/-}$ female mice failed to produce offspring, I used C57BL/6 mice to breed with SUR1$^{-/-}$ mice to generate heterozygous SUR1 knockout mice. Subsequent mating of the heterozygous SUR1 knockout mice then produced both WT and SUR1$^{-/-}$ mice. In the present studies, age-matched isogenic adult WT and SUR1$^{-/-}$ mice were used. All animals were housed in temperature-controlled facilities (23-25 ^{o}C) with a 12:12 light cycle (0500-1700). The animals were fed standard lab chow and had access to water *ad libitum.* All surgical and experimental procedures were used in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Northwestern University (Evanston, IL).

Experiment 1: Effects of i.c.v. tolbutamide infusion on LH secretion in fed and fasted female mice

On day 0, the animals were anesthetized with 80mg/kg, i.p. ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 32mg/kg, i.p. xylazine (Burns Veterinart Supply, Inc. Rockville Center, NY) and bilaterally ovariectomized (OVX). At the same time, all of them received stereotaxic CMA/7 guide cannulae implants (CMA Microdialysis AB, North Chelmsford, MA) fitted with obdurators aimed at the right lateral ventricle (coordinates 0.2 mm caudal to bregma, 2.0 mm ventral to the skull, 1.0 mm lateral) (458). Five days later, a subset of

the mice were fasted, while the rest received food *ad libitum*. All animals had free access to water. Forty eight hours after fasting, the animals were briefly anesthetized by isoflurane inhalation and an infusion cannula was fitted into the guide cannula. After insertion, either vehicle (0.1% DMSO in aCSF) or tolbutamide (500 μM in 0.1% DMSO) was infused through the cannula at a rate of 1μl/min (equal to 8.1 μg/hr infusion rate for tolbutamide). The components of aCSF were (in mM): NaCl, 124; KCl, 5; $NaHCO_3$, 26; NaH_2PO_4, 2.6; dextrose, 10; HEPES, 10; $MgSO_4$, 2; $CaCl_2$, 2. The infusion lasted for two minutes. After infusion, the probe was maintained in place for one additional minute and then replaced with the obdurator. Four minutes later, the animals were anesthetized by CO_2 inhalation and terminal blood samples were collected by cardiac puncture and centrifuged at 13000rpm at 4°C for 10min. Plasma was then harvested and stored at -20°C for subsequent LH RIAs. All animals were weighed on day 5 immediately before being fasted, on day 6 during fasting and on day 7 before they were killed.

Experiment 2: Effects of 48 hours of fasting on LH secretion in female SUR1 knockout mice

On day 0, both WT and $SUR1^{-/-}$ mice were bilaterally ovariectomized under anesthesia by isoflurane inhalation (Baxter, Deerfield, IL). Ovaries were weighed and initial body weight was recorded. Five days later, all animals were tail bled in the morning under brief anesthesia with isoflurane inhalation and around 80-100μl blood was collected. Blood glucose levels were measured using Prestige Smart System Glucose Monitor (Home Diagnostics, Inc,. Fort Lauderdale, FL). Blood samples were centrifuged at 13000rpm at 4°C for 10min. Plasma was then collected and stored at -20°C for subsequent LH RIAs. Fasting started after tail blood collection for all WT and $SUR1^{-/-}$ animals. During fasting, the animals received water *ad libitum.*

After 48 hours of fasting, the animals were anesthetized by CO_2 and killed by cervical dislocation. Blood samples were collected by cardiac puncture and again blood glucose was determined using Prestige Smart System Glucose Monitor (Home Diagnostics, Inc,. Fort Lauderdale, FL). Blood samples were centrifuged at 13000rpm at $4^{o}C$ for 10min. Plasma was harvested afterwards and stored at $-20^{o}C$ for LH, insulin and leptin RIAs. Bilateral uterine tissues were removed and weighed. All the animals were weighed on day 5 immediately before being fasted, on day 6 during fasting and on day 7 before they were killed.

RIAs

Plasma LH levels were determined by using RIA reagents obtained from the NIDDK, including LH reference (RP-3) and anti rat LH antibody (S-11). The assay had a lower limit of detection of 0.2ng/ml. Leptin was measured using a leptin RIA Kit from Linco Research (St. Charles, MO). The sensitivity of the leptin assay is 0.5ng/ml. Insulin assays were conducted by using an insulin RIA kit from Linco Research (St. Charles, MO) with a sensitivity of 0.2ng/ml. The intraassay and interassay CV for LH assays were 7.64% and 11.5% respectively. The intraassay CV for leptin and insulin assays were 1.48% and 2.7% respectively.

Statistical analysis

All reported values were presented as mean ± SEM. The effects of i.c.v. vehicle or tolbutamide infusion on LH levels in fed and fasted mice were assessed by two-way ANOVA, followed by Bonferroni's multiple comparison post-hoc test (GraphPad Software Inc., San Diego, CA).

Comparison of body weight, LH and glucose levels before and after 48 hours of fasting in WT and $SUR1^{-/-}$ mice were performed by two-way ANOVA with repeated measures, followed by Bonferroni's multiple comparison post-hoc test (GraphPad Software Inc., San Diego, CA). Ovary weight before fasting, uteri weight, leptin levels, insulin levels and glucose/insulin ratio after fasting were compared between WT and $SUR1^{-/-}$ mice by unpaired t-tests. For all statistical analysis, significant difference was reported at $p<0.05$.

Results.

Experiment 1: Effect of i.c.v. tolbutamide on LH secretion in fed and fasted female mice.

Infusion of 270 ng tolbutamide in two minutes into the right lateral ventricle resulted in a two-fold increase in plasma LH levels in both fed (1.43 ± 0.16 ng/ml for vehicle vs. 2.68 ± 0.64 ng/ml for tolbutamide, $p<0.05$) and 48hr-fasted mice (0.23 ± 0.03 ng/ml for vehicle vs. 0.45 ± 0.08 ng/ml for tolbutamide, $p<0.05$) (Figure 25). Tolbutamide did not restore the LH levels of fasted mice to those of the fed mice treated with vehicle.

Figure 25

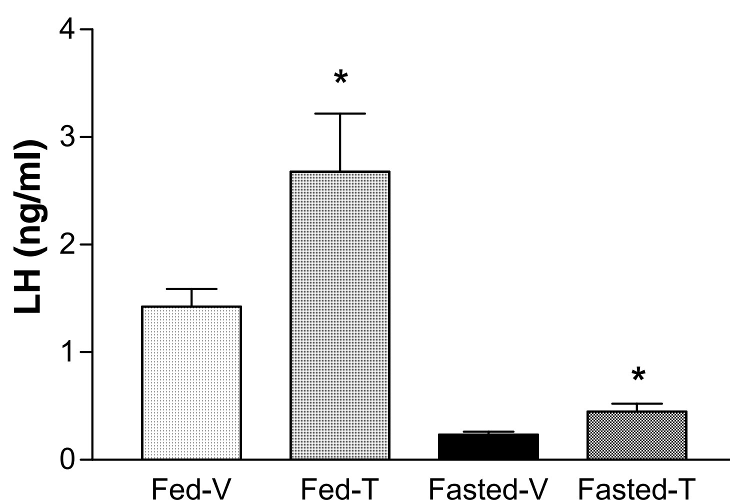

Fig. 25. Response of LH secretion to i.c.v. tolbutamide (500µM) or vehicle (0.1% DMSO) infusion in fed and 48hr-fasted female mice. There were 8, 10, 9 and 15 mice in fed and vehicle infused (Fed-V), fed and tolbutamide infused (Fed-T), fasted and vehicle infused (Fasted-V) and fasted and tolbutamide infused (Fasted-T) groups respectively. Tolbutamide infusion into the lateral ventricle significantly increased LH levels in both fed and 48hr fasted female mice (*, $p<0.05$ vs. corresponding vehicle respectively).

Experiment 2: Effect of 48 hours of fasting in WT and SUR1$^{-/-}$ female mice

Body and tissue weights

There was no significant difference in initial body weight between WT and SUR1$^{-/-}$ female mice. The 48hr fast caused similar and significant weight loss in both WT and SUR1$^{-/-}$ mice (20.96 ± 0.88 g before fasting vs. 15.93 ± 0.68 g after fasting in WT mice; 21.94 ± 0.92 g before fasting vs. 16.69 ± 0.82 g after fasting in SUR1$^{-/-}$mice) (Figure 26). The weights of ovaries prior to fasting, and the weights of uteri post-mortem, did not differ between the WT and SUR1$^{-/-}$ mice (Table 2).

Glucose levels

Comparison of basal glucose levels before fasting revealed no difference between WT and SUR1$^{-/-}$ mice. The 48hr fast led to a significant decrease in glucose levels in SUR1$^{-/-}$ mice (141.2 ± 10.79 mg/dL before fasting vs. 86.7 ± 12.45 mg/dL after fasting). In WT mice, 48 hours of fasting produced serum glucose levels that tended to be reduced but this trend did not reach statistical significance (Figure 27).

Insulin levels, Glucose/insulin ratio and leptin levels after fasting

After 48 hours of fasting, insulin levels were significantly higher in SUR1$^{-/-}$ mice than in WT mice after fasting (0.37 ± 0.04 ng/ml and 0.59 ± 0.07 ng/ml in WT and SUR1$^{-/-}$ respectively) (Figure 28A), while the glucose/insulin ratio in SUR1$^{-/-}$ mice was significantly lower than that in WT mice (16.7 ± 1.88 and 7.46 ± 1.81 of WT and SUR1$^{-/}$ respectively) (Figure 28B). However, leptin levels were not significantly different between the two genotypes (Figure 29).

Figure 26

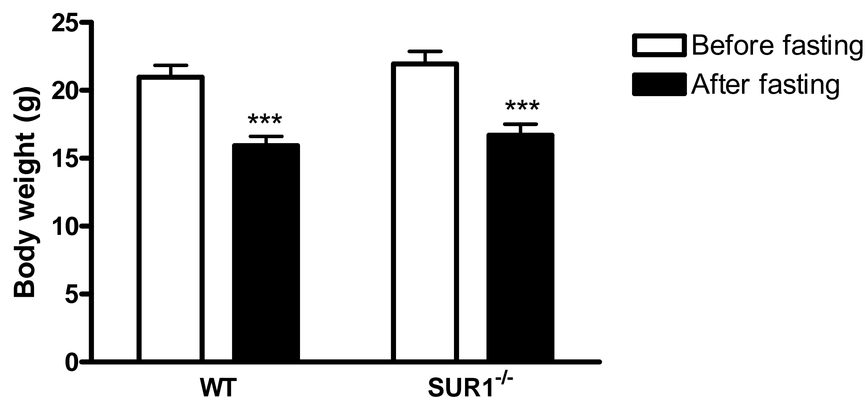

Fig. 26. Body weight in WT and SUR1$^{-/-}$ female mice before and after 48 hours of fasting. Two-way ANOVA with repeated measures revealed that there is significant effect of food manipulation on body weight. The 48hr fast resulted in significant body weight loss in both WT (n=11) and SUR1$^{-/-}$ (n=10) female mice (***, $p<0.001$). However, there is no significant effect of either genotype or the interaction between food manipulation and genotype.

Figure 27

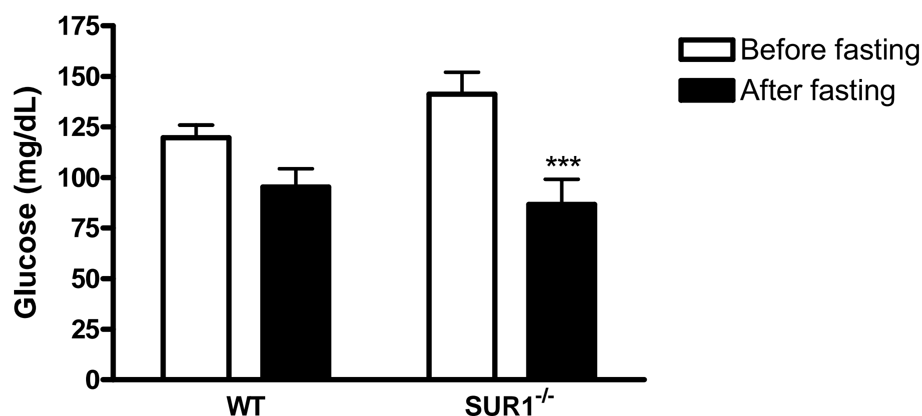

Fig. 27. Glucose levels in WT and SUR1$^{-/-}$ female mice before and after 48 hours of fasting. Two-way ANOVA with repeated measures revealed that there is significant effect of food manipulation on glucose levels ($p<0.0001$). Bonferroni's post-hoc analysis demonstrated that glucose levels in the SUR1$^{-/-}$ mice were significantly lowered by the 48hr fast (***, $p < 0.001$). Although glucose levels also showed a decline in WT mice, it did not reach statistical significance. However, there is no significant effect of either genotype or the interaction between food manipulation and genotype.

Figure 28

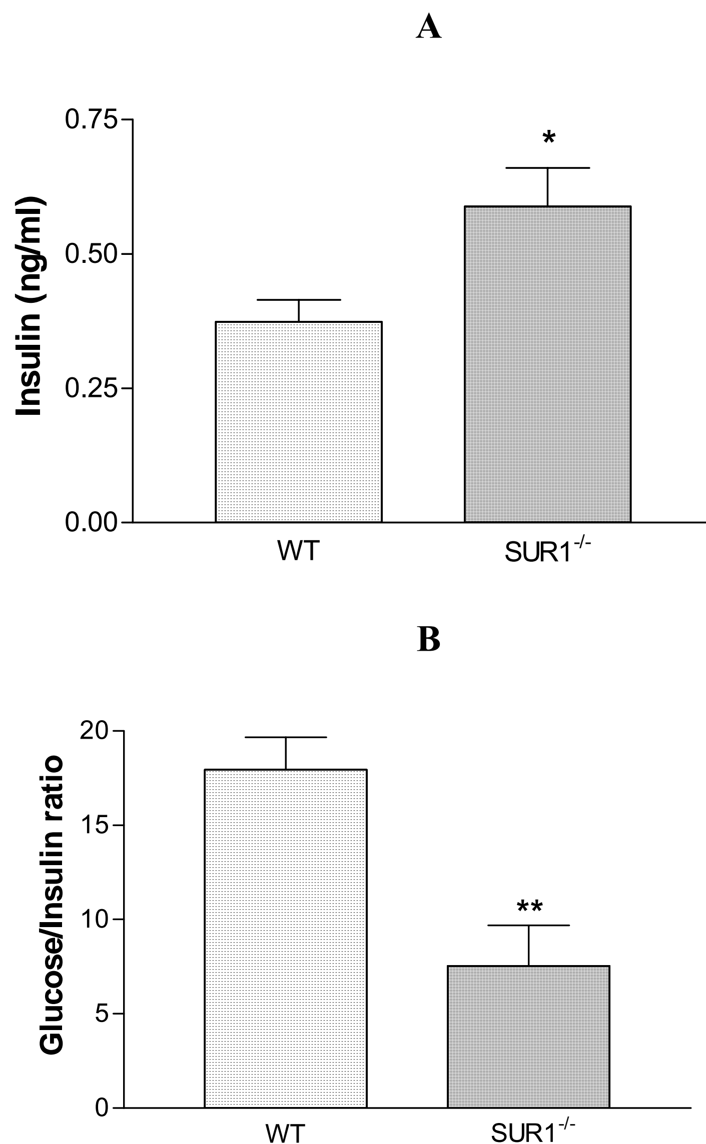

Fig. 28. Insulin levels and glucose/insulin ratio in WT and SUR1$^{-/-}$ female mice after fasting. (A) Insulin levels are significantly higher in the SUR1$^{-/-}$ mice than in the WT mice (0.37 ± 0.04 ng/ml and 0.59 ± 0.07 ng/ml in WT and SUR1$^{-/-}$ mice respectively, *, $p<0.05$). (B) Glucose/insulin ratio of SUR1$^{-/-}$ mice was significantly lower than that of WT mice (16.7 ± 1.88 and 7.46 ± 1.81 of WT and SUR1$^{-/-}$ mice respectively, **, $p<0.01$).

Figure 29

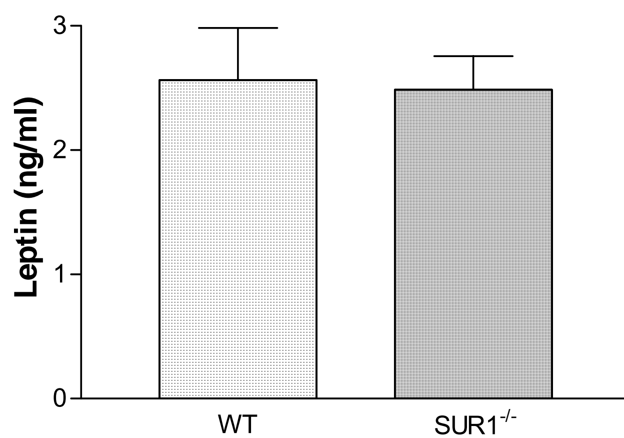

Fig. 29. Leptin levels in WT and SUR1$^{-/-}$ female mice after fasting. Leptin levels after fasting did not differ between the WT and SUR1$^{-/-}$ female mice.

LH levels

LH levels before and after fasting in each individual animal were shown in Figure 30A. Basal LH levels before fasting did not differ between WT and SUR1$^{-/-}$ mice. After 48 hours of fasting, there was a significant reduction in LH levels in both WT and SUR1$^{-/-}$ mice (1.85 ± 0.37 ng/ml before fasting vs. 0.55 ± 0.13 ng/ml after fasting in WT mice, $p<0.01$; 1.27 ± 0.30 ng/ml before fasting vs. 0.27 ± 0.05 ng/ml after fasting in SUR1$^{-/-}$ mice, $p<0.05$) (Figure 30B).

Figure 30

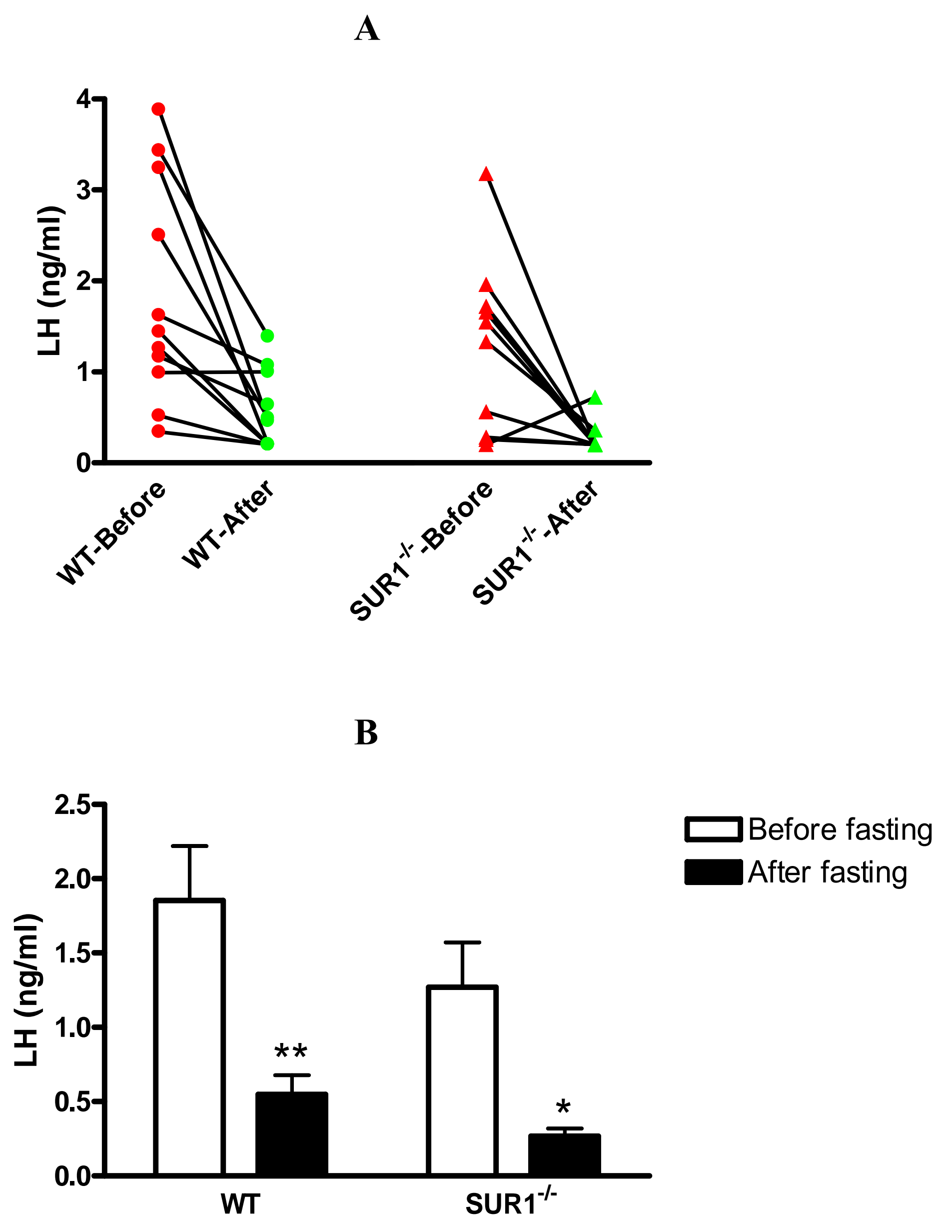

Fig. 30. LH levels in WT and SUR1$^{-/-}$ female mice before and after fasting. (A) LH levels in each individual animal before and after fasting were plotted and connected by lines (WT, n=11; SUR1$^{-/-}$, n=10). (B) LH levels in WT and SUR1$^{-/-}$ female mice before and after fasting. Two-way

ANOVA with repeated measures found a significant effect of food manipulation on LH levels. Bonferroni's post-hoc test revealed that 48 hours of fasting significantly reduced LH levels in both WT (**, $p<0.01$) and SUR1$^{-/-}$ (*, $p<0.05$) mice. There is no significant effect of genotype or the interaction between food manipulation and genotype.

Table 2. Ovarian weights before fasting and uterine weights after fasting in WT and $SUR1^{-/-}$ female mice

	WT	$SUR1^{-/-}$	Statistics
Ovary weight before fasting (mg)	12.18 ± 0.65	12.10 ± 1.03	$p > 0.05$
Uteri weight after fasting (mg)	36.64 ± 3.79	32.85 ± 2.40	$p > 0.05$

Discussion.

Energy balance is an important factor in regulating the HPG axis. Negative energy balance such as caused by food deprivation or excessive physical activity causes reduction in LH secretion in a number of species including rodents (41, 42), sheep (43, 44) and monkeys (45). The reduction in LH induced by negative energy balance has been shown to result from suppression of the hypothalamic GnRH pulse generator (48, 49). However, the underlying mechanisms that mediate this suppression have yet to be determined. Because the hypothalamus is important for both energy homeostasis and regulation of HPG axis, it may play a role in the integration of metabolic signals related to negative energy balance with the GnRH pulse generator activity. The present studies evaluated the role of K_{ATP} channels in mediating the effects of negative energy balance on GnRH and LH secretion. Our data demonstrated that lateral ventricle infusion of tolbutamide, a K_{ATP} channel blocker, caused approximately two-fold increase in LH levels in both fed and 48hr-fasted OVX mice. However, tolbutamide was not able to fully restore the LH levels in the fasted mice to the level at the fed state. In addition, 48 hours of fasting resulted in similar suppression of LH secretion in both WT and $SUR1^{-/-}$ female mice. Taken together, our results strongly support the idea that K_{ATP} channels are involved in the regulation of GnRH neurosecretion, but do not appear to be necessary in mediating the effect of negative energy balance on GnRH/LH secretion. Thus, K_{ATP} channel modulation may serve some other functions in GnRH neurons or their afferent circuitries, such as mediation of steroid hormone negative feedback actions, neuroprotection, or other as yet undetermined regulatory mechanisms.

In conditions of negative energy balance, changes in the magnitude of metabolic cues are thought to be the primary event for the regulation of GnRH secretion (262). Glucose has been suggested to be a key metabolic cue mediating the effect of negative energy balance on the GnRH pulse generator (253, 459). A number of studies have identified glucose-sensing mechanisms in the hindbrain, especially the AP, which are important for relaying the signal of negative energy balance to the hypothalamus (257, 460). Neuronal projections from the hindbrain play a critical role in transmitting the signal of negative energy balance to the hypothalamic GnRH pulse generator (6). Destruction of hindbrain structures, including the AP, resulted in abolishment of hypoglycemia-induced suppression of LH secretion (319). However, a large body of evidence has demonstrated that glucose-sensing neurons are also present in the hypothalamus, such as the NPY and POMC neurons in the arcuate nucleus (9, 461). These neurons not only respond to change in extracellular glucose concentration by altering their electrical activities, but also have been implicated in regulating both energy homeostasis and GnRH secretion (158, 322). Therefore, they may serve as mediators in the regulation of GnRH secretion by negative energy balance.

It has recently been found that K_{ATP} channels are expressed in hypothalamic glucose-sensing neurons (462). The hypothalamic K_{ATP} channels are composed of Kir6.2 and SUR1 subunits (372), and play a critical role in glucose sensing in the hypothalamus. Kir6.2 knockout mice demonstrate impaired glucose sensing in the hypothalamus, as manifested by complete loss of glucagon secretion and smaller increase in food intake in response to neuroglycopenia (54). Intracerebroventricular infusion of K_{ATP} channels blockers abolishes the counterregulatory secretion of glucagon and epinephrine in response to both systemic hypoglycemia and brain glycopenia, indicating a role of K_{ATP} channels in hypothalamic sensing and response to

perturbation of available glucose (420). In addition, two important hormones in regulating energy homeostasis, leptin (52) and insulin (53), have also been shown to activate hypothalamic K_{ATP} channels. The effects of leptin to regulate food intake is believed to be mediated by activation of the K_{ATP} channels expressed in its target neurons, such as NPY neurons (463). Since leptin and insulin are also involved in regulating GnRH/LH secretion, it is thus possible that K_{ATP} channels may convey the signal of energy balance to GnRH secretion. However, my observations that central K_{ATP} channel blockade by tolbutamide is not able to fully restore the LH levels in the fasted mice and that a 48hr fast results in similar reduction in LH levels in both $SUR1^{-/-}$ and WT mice argue against a necessary role of K_{ATP} channel in mediating the effect of negative energy balance on GnRH secretion

Based on my observation, I postulate that the signals of negative energy balance may be transmitted through a K_{ATP} channel-independent pathway. The ability of K_{ATP} channel modulation to affect GnRH/LH secretion indicates that K_{ATP} channels may be involved in other mechanisms regulating GnRH pulse generator, such as negative feedback actions of ovarian steroids. In the state of negative energy balance, GnRH pulse generator activity is suppressed by this K_{ATP} channel-independent pathway. Since modulation of K_{ATP} channel activity can lead to changes in the membrane potentials and consequently in the excitability of neurons (368, 464, 465), the blockade of K_{ATP} channels by tolbutamide can still alter the activity of GnRH neurons via a mechanism which does not overlap with that involved in the effects of negative energy balance on GnRH neurons. This may explain why tolbutamide infusion caused elevation of LH level to a similar extent in both WT and $SUR1^{-/-}$ mice.

It is also possible that a K_{ATP} channel-dependent pathway represents only one of many parallel pathways that mediating the signals of energy balance to the GnRH neurons. And these

parallel pathways are K_{ATP} channel-independent. Therefore, blockade of K_{ATP} channels in the state of negative energy balance is sufficient to alter the activity of GnRH pulse generators. However, lack of this K_{ATP} channel-dependent pathway in the $SUR1^{-/-}$ mice is not able to abolish the effects of fasting on GnRH secretion due to the presence of its parallel pathways. Moreover, like in other gene knockout animal models (466), there is also a possibility that compensatory mechanisms operate via the parallel pathways to affect GnRH release. These compensatory mechanisms may explain that, despite of a deficiency in K_{ATP} channel-dependent pathway, the $SUR1^{-/-}$ mice have similar basal LH levels, body weights and ovarian weights compared to the WT mice,.

K_{ATP} channels in the afferent neurons or GnRH neurons themselves may serve other functions such as mediating the negative feedback action of ovarian steroids and neuroprotection. In Chapter II, I demonstrated that estrogen and progesterone confer the responsiveness of GnRH pulse generator to K_{ATP} channel modulation, and also upregulate K_{ATP} channel expression in the POA, indicating the role of K_{ATP} channel in mediating the negative feedback actions of ovarian steroids on GnRH secretion. Moreover, K_{ATP} channels have been suggested to confer neuroprotection under the conditions of various stresses, such as ischemia and hypoxia (16). This neuroprotective function may also apply to those channels expressed in GnRH neurons or their afferent neurons.

I also found that 48 hours of fasting caused significant weight loss in both WT and $SUR1^{-/-}$ mice. Glucose levels before fasting was not different between the two genotypes, which is consistent with previous studies (368, 467). However, upon challenge of a 48hr fast, the decrease in glucose levels was more pronounced in the knockout mice, which was in agreement with observations from pervious studies (467)**.** It is also interesting to note that after 48 hours of

fasting, insulin levels were significantly higher and glucose/insulin ratio was significantly lower in SUR1$^{-/-}$ mice compared to the WT mice, but glucose levels were similar between the two genotypes. These observations are compatible with the inability of pancreatic β-cells to respond to the fasting induced-reduction in glucose levels in the SUR1$^{-/-}$ mice (467). Insulin has been suggested to transmit the signals of negative energy balance to the GnRH pulse generator either through its direct effect on the CNS or indirectly through its associated-hypoglycemia (7). In this study, the comparable glucose levels after 48 hours of fasting between WT and SUR1$^{-/-}$ mice enabled us to explore the direct effect of insulin on GnRH secretion. I demonstrated that despite higher level of insulin in the SUR1$^{-/-}$ mice, their LH levels after fasting were similar to those of the WT mice. Thus, my data suggest that at least in a state of negative energy balance in this experiment setting either there is no direct effect of insulin on GnRH neurons or this direct effect of insulin on GnRH secretion is K_{ATP} channel dependent. In the latter model, lack of the K_{ATP} channel-dependent mechanism in the SUR1$^{-/-}$ mice disrupts normal insulin signaling. Therefore, even in the presence of high insulin level, the LH secretion is still suppressed by food deprivation.

In summary, the present studies demonstrated that central K_{ATP} channel blockade elicits a similar elevation in LH levels in both fed and fasted female mice. In addition, 48 hours of fasting inhibits LH secretion in both WT and SUR1$^{-/-}$ mice. Our results are consistent with the hypothesis that K_{ATP} channels are linked to the regulation of GnRH release, but they do not play an obligatory role in mediating the effect of negative energy balance on GnRH secretion.

Chapter V: Summary and Discussion

Overview

Proper functioning of the HPG axis depends upon the pulsatile release of GnRH from nerve terminals in the median eminence, and the delivery of the GnRH decapeptide from the hypothalamus to the anterior pituitary via the portal vasculature (444). Disruption of the pulsatile secretion of GnRH leads to hypogonadotropic hypogonadism, which is manifested by low LH and FSH, low gonadal steroids and infertility (96, 468). As the final common pathway through which the HPG axis is regulated by the central nervous system, GnRH neurons are subject to regulation by a variety of intrinsic and extrinsic factors, such as photoperiod (2), olfaction (3), and stress(4). The experiments described in this thesis have focused on two physiological variables that are perhaps most important in the regulation of the GnRH pulse generator: *homeostatic negative feedback actions of ovarian steroids* and *energy balance*. Homeostatic regulation of GnRH and LH secretion by both estrogen and progesterone has been extensively characterized in women and in a wide variety of experimental animals. The release of GnRH is also known to be sensitive to the prevailing state of energy balance, as any prolonged decrease in energy intake and/or increase in energy expenditure has been shown in a variety of species to be accompanied by a suppression of GnRH release. The overall hypothesis that was formulated and tested in these dissertation studies held that both of these critically important regulatory processes are mediated by a common cellular mechanism; the proposed mechanism was one in which both metabolic cues and ovarian steroids influence the expression and/or activation of K_{ATP} channels in GnRH neurons or, more likely, in the afferent neurons that control GnRH pulsatility. On the basis of my results, I conclude that the negative feedback actions of gonadal steroids may indeed be mediated, at least in part, via the modulation of K_{ATP} channel expression and/or function. However, the effects of negative energy balance on GnRH release, at least in the

context of these experimental circumstances, do not appear to be dependent upon the activation of K_{ATP} channels. While it is clear that K_{ATP} channel modulation does lead to alterations in GnRH release, suggesting some physiological role for K_{ATP} channels in this regard, it appears not to be the case that they function as reproductive “sensors” of energy status. My amended model for the regulation of GnRH release by the ovarian steroids and energy balance is depicted in Figure 31.

Figure 31

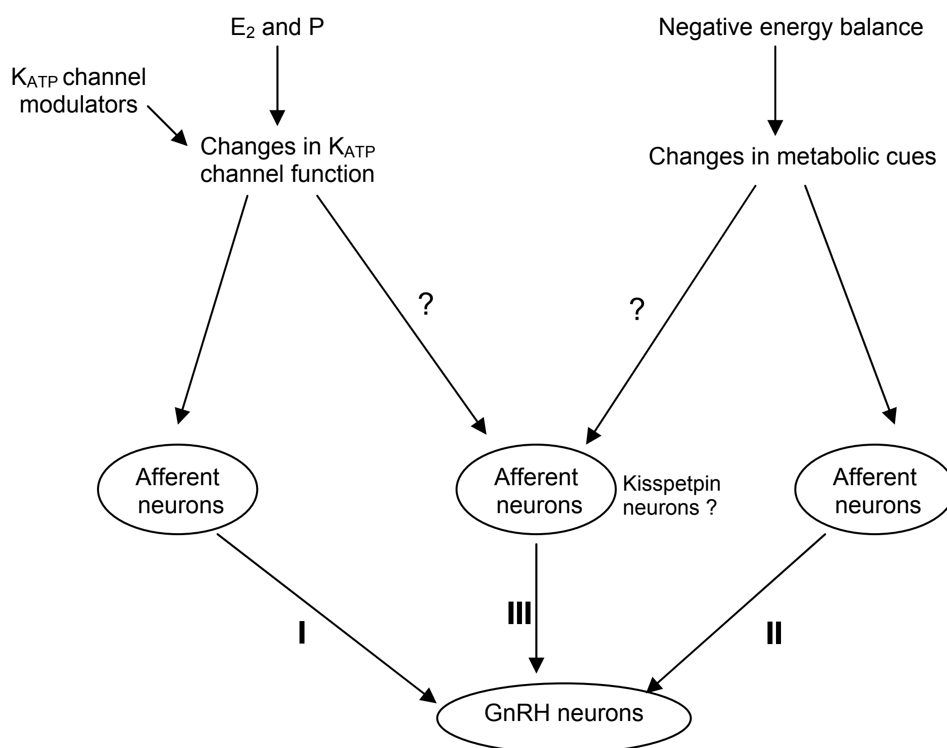

Fig. 31. Proposed model of the regulation of GnRH neurons by ovarian steroids and negative energy balance. Ovarian steroids and states of negative energy balance may utilize different pathways to affect GnRH neurons. E_2 and P inhibit GnRH neurons by altering the function of K_{ATP} channels which in turn leads to changes in the activity of the afferent neurons (Pathway I). In contrast, state of negative energy balance results in changes in metabolic cues, which in turn signal to a different population of afferent neurons via a K_{ATP} channel-independent mechanism (Pathway II). Alternatively or additively, ovarian steroids and negative energy balance may signal through a common population of afferent neurons (Pathway III). Kisspeptin neuron is a possible candidate (see text for detail). The presence of K_{ATP} channels in kisspeptin neurons has not been explored. However, if the channels are expressed in kisspeptin neurons,

two distinct mechanisms should exist in regulating this common pathway. E_2 and P affect these neurons in a K_{ATP} channel-dependent manner, while K_{ATP} channels are not critical to the regulation of these neurons by states of negative energy balance. Also note that K_{ATP} channel modulators may affect GnRH secretion through the afferent neurons by opening or closing channel.

K_{ATP} channels in the negative feedback actions of ovarian steroids on GnRH secretion

Chapter II of this dissertation established for the first time that K_{ATP} channels are involved in the negative feedback actions of E_2 and P on GnRH secretion. I demonstrated that central blockade of K_{ATP} channels enhances the activity of GnRH pulse generator in an ovarian steroid-dependent manner. Specifically, the presence of both E_2 and P renders the responsiveness of pulsatile GnRH/LH secretion to K_{ATP} channel modulation. These findings, taken together with evidence from previous studies, suggest that the involvement of K_{ATP} channels in the regulation of GnRH neurons may reflect a more general role for members of the related class of inwardly rectifying potassium channels in regulating GnRH secretion. K_{ATP} channels belong to the superfamily of inwardly rectifying potassium channel (469). Besides K_{ATP} channels, other members of the same superfamily have also been implicated in the regulation of GnRH neurons. GIRK channels which are associated with $GABA_B$ and μ-opioid receptors have been shown to be rapidly uncoupled by estrogen, a non-genomic mechanism believed to be mediated by membrane bound estrogen receptors. These $GABA_B$ and μ-opioid receptors are localized in GABAergic and POMC neurons respectively and serve as an autoinhibitory mechanism of the corresponding neurons. Uncoupling of the GIRK channels with the receptors by estrogen releases the autoinhibition on these neurons, thus causing more GABA and opioid release which in turn impact upon GnRH neurons to inhibit GnRH secretion (437, 470). More recently, a specific GIRK channel was found to be linked to LH receptors expressed in GT1-7 cells. Activation of these channels by LH causes suppression of the membrane excitability, action potential firing and consequently GnRH secretion (471). In addition, another type of inwardly rectifying potassium channel is also found in GT1-1 cells, a GnRH cell line (472). This current is activated by hyperpolarization in the presence of elevated extracellular K^+. Together with a persistent

inward current, it is believed to generate an oscillating membrane pacemaker potential which contributes to the spontaneous electrical activity of the GnRH neurons. Moreover, modulation of this current could result in a sustained increase in Ca^{2+} oscillation frequency and sustained pulsatile release of neurohormone. Therefore, it is possible that the effect of K_{ATP} channels on GnRH secretion is one of several additive effects on resting membrane potential and responsiveness to excitation, through which the superfamily of inwardly rectifying potassium channels impact upon GnRH neurons. Actually, an interaction of inwardly rectifying potassium channels in regulating neuronal activity has been reported in a recent study. The study demonstrated that substance P excites neurons at nucleus basalis by inhibiting two different types of inwardly rectifying potassium channels. One is a constitutively active inwardly rectifying potassium channel, while the other is GIRK channel (473).

It is also demonstrated in Chapter II that the expression of Kir6.2 mRNA in the POA is most enhanced in response to combined treatment with E_2 and P, compared to responses to either hormone administered alone. Previous studies have also provided evidence for steroid-dependency of K_{ATP} channel subunit expression or activity in non-neural tissues. Estrogen has been shown to reduce myocardial injury in ischemia-reperfusion through the activation of K_{ATP} channels (416), and in heart-derived H9c2 cells, E_2 increases K_{ATP} channel formation and thereby protects cardiac cells from hypoxia-reoxygenation (409). Although to our knowledge, the direct effect of progesterone on K_{ATP} channel has never been studied, it has been shown that Kir6.2 expression levels in the aorta and kidney are increased in pregnant versus non-pregnant Wistar rats (430). In addition, K_{ATP} channel blockade reverses the decrease in systolic blood pressure (SBP) that normally occurs in pregnant animals, but does not alter SBP in non-pregnant animals (430). However, the molecular mechanisms of the regulation of K_{ATP} channel expression

have yet been determined. E_2 and P can alter K_{ATP} channel expression either directly or indirectly through regulation of other unknown genes which in turn affect channel expression. Kir6.2 and SUR1 genes have been cloned in mouse and human (343, 358, 407, 411). Interestingly, in both species, Kir6.2 and SUR1 genes are adjacent to each other on the same chromosome, suggesting that both genes possibly derive from a single ancestor gene. The promoter regions of the two genes have been shown to share several characteristics (407, 411). Both promoter regions are similar to those of the “house keeping” genes as they both lack the “TATA” box in the minimal promoter region and are “G+C” rich. Additionally, SUR1 promoter region has a few E-boxes, SP1 sites and G-boxes, while Kir6.2 has several E-boxes, SP1 sites, G-boxes and possible AP-2 sites. In particular, the SP1 binding sites are suggested to mediate transcription initiation of the SUR1 gene (407) and to increase Kir6.2 gene promoter activity (474). Since both estrogen (475) and progesterone (476) have been shown to signal through interaction with SP1 sites, it is possible that the SP1 sites of the Kri6.2 and SUR1 gene promoters are the target of the upregulatory effect of estrogen and progesterone. However, it is also possible that estrogen and progesterone interact with other regions of the promoter to affect K_{ATP} channel subunit expression. A recent study showed that dexamethasone inhibits both Kir6.2 and SUR1 mRNA expression. However, even though the proximal region of the SUR1 promoter is sufficient for significant transcriptional activity, a screening from the proximal promoter region up to -4500bp upstream failed to identify the dexamethasone response element, indicating that the response element may reside outside the screened region (407).

Besides genomic effects, ovarian steroids may additionally exert non-genomic effects that increase the activation of K_{ATP} channels. Apart from their role in glucose sensing, neuronal K_{ATP} channels are known to be activated by circulating metabolic hormones, specifically insulin (53)

and leptin (52). They have therefore been suggested to act as integrators of physiological signals that impact energy homeostasis. Many of the actions of insulin, and to a lesser extent leptin, are mediated in part by the activation of PI3 kinase, production of PIP3, and consequent activation of K_{ATP} channels (306). Recent studies have demonstrated that estrogen and other steroid hormones can activate plasma membrane-associated receptors, and thereby initiate intracellular signaling pathways that culminate in relatively rapid, non-genomic effects on neuronal function (108, 203). Several of these effects have also been shown to be mediated by the stimulation of PI3 kinase activity (435, 436). It is therefore possible that the ability of E_2 and P to upregulate LH responsiveness to K_{ATP} channel modulation may be mediated, in part, by the activation of PI3 kinase in target neurons. Furthermore, it has been demonstrated in pancreatic β-cells that estrogen has a rapid insulinotropic by inhibiting K_{ATP} channel activity through cGMP dependent pathway, which is thought to be mediated by membrane bound estrogen receptors (477). The non-genomic effects of E_2 and P may involve multiple signaling pathways.

K_{ATP} channels and the effects of altered energy homeostasis on GnRH secretion

Chapters III and IV of this dissertation have assessed the mechanisms that may mediate the effects of states of energy balance on GnRH neurosecretion (6, 7). In most mammals, sustained activity of the GnRH pulse generator appears to be dependent upon the availability of oxidizable metabolic fuels, thereby coupling fertility to a state of energy reserve that is sufficiently permissive for reproductive success. Thus, food restriction (41-46), excessive energy expenditure (246), or other states of negative energy balance have been shown to produce varying degrees of inhibition of LH secretion. Compelling evidence supports the idea that the decrease in LH secretion is a result of suppression of GnRH pulse generator in the hypothalamus. In female rats,

food restriction prevents pubertal development (300). This suppressive effect on LH secretion is reversed by re-feeding with an increase in LH pulse frequency, but without effect on GnRH content and responsiveness of gonadotropes to GnRH, indicating that food restriction leads to a reduction in GnRH secretion (300). More direct evidence of the effects of negative energy balance on GnRH secretion has come from studies of larger animals. In female sheep, dietary restriction increases the interpulse interval of both LH and GnRH pulses measured simultaneously in peripheral blood and hypophyseal portal blood respectively (48). Since the interpulse interval is negatively correlated with pulse frequency, the above finding confirms the diminution of GnRH pulse frequency under negative energy balance. In female goats, the volley interval of MUA, which is an indicator of the GnRH pulse generator activity, was significantly prolonged after 4 to 5-day fasting, indicating suppression of the GnRH pulse generator (443). Moreover, pulsatile administration of GnRH restored the LH and testosterone levels inhibited by fasting in men, suggesting central mechanism is underlying the changes in gonadotropin release (49).

In mice, a similar drop of LH level after food deprivation has also been documented (286). However, whether the GnRH pulse generator is involved in this process has yet been determined. Experiments in Chapter III were designed to address this question. In order to assess the effect of negative energy balance on GnRH secretion in female mice, I first developed microdialysis to monitor pulsatile GnRH release *in vivo*. I showed that GnRH is released from the MBH in a pulsatile manner with release rate similar to what have been reported in rats (441, 442). In addition, as a validation of my microdialysis method, I demonstrated that ovariectomy enhances GnRH pulsatility, which agrees with the idea that the negative feedback actions of ovarian steroids are exerted at least in part by suppression of GnRH neurosecretion. Furthermore, by

using microdialysis to study the effect of negative energy balance on GnRH secretion, I revealed for the first time that 48 hours of food deprivation in female mice causes suppression of pulsatile GnRH secretion.

A large body of evidence points to the possibility that the adaptive mechanism underlying the effect of negative energy balance on GnRH secretion may be collectively mediated by a number of hormones, neuropeptides, and metabolic signals that exert integrated actions via metabolic sensors in the periphery, brain stem, and preoptic-hypothalamic tissues (6). There is some evidence to suggest that at least some of these pathways conduct signals that converge with steroid hormone feedback mechanisms that regulate GnRH neurosecretion. It has been demonstrated, for example, that food restriction increases the efficacy of estrogen's negative feedback effects on LH secretion (57). Likewise, ovarian steroids render a higher sensitivity of GnRH/LH pulsatile secretion to the inhibition by food restriction or deprivation. In ovariectomized female sheep, chronic undernourishment causes virtually no LH pulses when the animals were treated with estrogen compared to a few discernable LH pulses when the animals were treated with vehicle (43). In Chapter II, I have provided evidence that the expression and/or activation of K_{ATP} channels in the POA may contribute to the negative feedback actions of ovarian steroids on GnRH pulsatility. It is therefore possible that K_{ATP} channel activation may represent one common pathway by which ovarian steroids and sustained negative energy balance, may suppress GnRH pulsatility.

Experiments in Chapter IV further tested the possible role of K_{ATP} channels in the effect of acute state of negative energy balance on GnRH/LH secretion. Our result in Chapter IV demonstrated that lateral ventricle infusion of tolbutamide, a K_{ATP} channel blocker, causes approximately two-fold increase in LH levels in both fed and 48hr-fasted OVX mice. However,

tolbutamide was not able to fully restore the LH levels in the fasted mice to the level at the fed state. In addition, a 48hr fast results in similar suppression of LH secretion in both WT and $SUR1^{-/-}$ female mice. Taken together, the data in Chapter IV strongly support the idea that K_{ATP} channels are involved in the regulation of GnRH neurosecretion, but do not appear to be necessary in mediating the effect of negative energy balance on GnRH/LH secretion.

Although K_{ATP} channels in the afferent neurons or GnRH neurons themselves may not be involved in signaling the GnRH neurons of the state of energy balance, they may serve other functions such as mediating the negative feedback action of ovarian steroids, rendering neuroprotection and maintaining energy homeostasis. In Chapter II, I demonstrated that estrogen and progesterone confer the responsiveness of GnRH pulse generator to K_{ATP} channel modulation, and also upregulate K_{ATP} channel expression in the POA, indicating the role of K_{ATP} channel in mediating the negative feedback actions of ovarian steroids on GnRH secretion. Moreover, K_{ATP} channels expressed in cortical and hippocampal neurons have been suggested to confer neuroprotection under the conditions of various stresses, such as ischemia and hypoxia (16). Overexpression of SUR1 in the forebrain in mice renders the animal resistant to seizure induction and excitotoxic neuronal death (376). The neuroprotective functions of K_{ATP} channels found in these neurons may also extend to the channels expressed in GnRH neurons or their afferent neurons. K_{ATP} channels in the hypothalamus also play a critical role in glucose homeostasis, even energy homeostasis, such as regulation of food intake, glucose and lipid metabolism. Deficiency in K_{ATP} channels, as seen in Kir6.2 knockout mice, leads to impaired response of food intake to insulin-induced systemic hypoglycemia (54). Activation of K_{ATP} channels in the MBH is sufficient to lower blood glucose levels by inhibiting hepatic gluconeogenesis via the hepatic branch of vagal nerve (478). Peripheral glucose metabolism is

also regulated by central fatty acid sensing through K_{ATP} channels in the CNS. Central inhibition of fatty acid oxidation leads to selective activation of brainstem neurons and a marked decrease in liver gluconeogenesis and glucose production via a K_{ATP} channel-dependent pathway (479). Furthermore, the effects of central administration of glucose or lactate to lower peripheral glucose and triglyceride concentration can be negated by central blockade of K_{ATP} channels (480, 481). Taken together, K_{ATP} channels in the hypothalamus appear to play an integrative role in the regulation of peripheral metabolism of the energy fuels by the central nervous system.

Interestingly, results from Chapter IV also revealed that although the LH secretion was substantially suppressed, the glucose levels after a 48hr fast only exhibited mild reduction. Previous studies have also provided similar evidence in other species such as goats (482) and monkeys (260, 261) that short-term fasting results in a reduction in LH secretion which is concomitant with only lower level euglycemia or mild hypoglycemia. The above observations argue against the essential role of severe hypoglycemia in the suppression of GnRH/LH secretion by short-term food deprivation. In fact, glucose has been shown to be only partially effective in reversing the effect of short-term fasting on LH secretion (260). In contrast, when insulin or 2DG was used to suppress GnRH/LH secretion, they either cause a severe hypoglycemia (insulin) or a considerable restriction of glucose availability (2DG). Under the above two states of negative energy balance, short-term fasting vs. insulin/2DG, GnRH/LH secretion is similarly inhibited. However, the glucose availability under the two conditions is largely different. It suggests that different mechanisms, such as different humoral or neural pathways, may be involved in the LH suppression caused by mild hypoglycemia compared to that caused by severe hypoglycemia. Interestingly, a previous study has demonstrated that although 2DG given systemically is able to suppress LH secretion, 2DG plus fasting is even more effective in inhibiting LH secretion,

suggesting that distinct pathways may be involved in the effects of 2DG and short-term fasting on GnRH secretion (256). Several lines of evidence now suggest that 2DG or insulin may exert this effects on GnRH neurons via the hindbrain structures, especially the AP. Lesion of the AP led to abolishment of the insulin-induced LH suppression (319). It also blocks the effect of systemically administered 2DG on estrous cycles (320) or sexual behavior (321). Infusion of 2DG directly into the 4th ventricle causes reduction in LH pulsatility and increase in food intake (257). The NPY/NE neuronal populations in the hindbrain are currently believed to act as the mediators between the glucose-sensing mechanism in the hindbrain and GnRH neurons in the hypothalamus (7). However, what neural structures and/or homeostatic hormones are involved in the inhibition of LH secretion by short-term fasting is less defined and warrant further investigation.

Is there a common pathway of the negative feedback mechanism and negative energy balance mechanism in the regulation of GnRH neurons?

As stated earlier, I hypothesized that the pathway via which ovarian steroids exert their negative feedback actions on GnRH neurons may converge with that utilized by the states of energy balance to regulate GnRH secretion. Our results showed that K_{ATP} channels may not be a converging point of these two pathways. However, compelling evidence supports the idea that kisspeptin may serve this role. Kisspeptin is a very potent GnRH/LH secretagogue at doses as low as 1 fmol (159, 160). Deficiency of kisspeptin receptor, a G-protein coupled receptor named GPR54, causes hypogonadotropic hypogonadism (161). The underlying mechanism is an impairment in GnRH secretion as GnRH antagonist, acyline, can abolish kisspeptin-induced LH release (159), which is distinct from the defective GnRH neuronal migration found in the

Kallmann's syndrome (162). Kisspeptin neurons have been shown to be localized in areas important in regulating GnRH neurons, such as anteroventral periventricular nucleus (AVPV), POA and arcuate nucleus and they send projections to GnRH neurons (163). Accordingly, GnRH neurons express GPR54, indicating a direct effect of kisspeptin on GnRH neurons (160). Moreover, both ER (214) and PR (483) have been found in kisspeptin neurons and kisspeptin expression is regulated by E_2 (215) and P (483). Therefore, it is suggested that kisspeptin may play a pivotal role in the negative feedback action of ovarian steroids on GnRH neurons (216, 217). Importantly, kisspeptin is also involved in the regulation of GnRH secretion by negative energy balance. Food deprivation induces a decrease in hypothalamic kisspeptin expression and central administration of kisspeptin is able to restore the LH secretion suppressed by fasting (293), indicating that kisspeptin may mediate the signal of negative energy balance to GnRH neurons (51, 294). Taken together, kisspeptin may be an integration site for the mechanisms of ovarian steroids and states of energy balance to affect GnRH neurons. Interestingly, whether K_{ATP} channels are expressed in kisspeptin neurons has never been studied so far. Given the results from my thesis studies, I postulate that K_{ATP} channels are not expressed in kisspeptin neurons. Or alternatively, they are localized in kisspeptin neurons, but they respond only to ovarian steroid regulation, but not to short-term food deprivation.

Summary

This dissertation expands our current understanding of the molecular mechanisms of the negative feedback action of ovarian steroids on GnRH secretion. Previous studies have proposed the involvement of GABAergic and opioidergic neurons, glial cells and direct effect of ER and PR in the negative feedback regulation of GnRH neurons. This thesis established for the first

time the role of K_{ATP} channels as a mediator of E_2 and P's inhibitory effects on GnRH neurons. Abnormal negative feedback of ovarian steroids is seen in a few disorders such as PCOS. In PCOS patients, the LH levels are unusually high despite relatively normal levels of E_2 and P, suggestive of decreased sensitivity of GnRH neurons to their negative feedback (484, 485). Based on the results of my thesis studies, it is possible that disruption of normal expression and/or activity of K_{ATP} channels may contribute to the pathogenesis of the resistance of GnRH neurons to the suppression by E_2 and P. In addition to their reproductive phenotype, PCOS patients also have marked metabolic phenotypes such as insulin resistance which is characterized by hyperinsulinemia and hyperglycemia (27). Their insulin resistance may also be related to K_{ATP} channels. K_{ATP} channels are expressed at high levels in pancreatic β–cells and skeletal muscles, which determine insulin secretion and insulin sensitivity respectively. Deficiency in K_{ATP} channels has been shown to lead to hyperinsulinemia and altered insulin sensitivity (465, 486). Therefore, a defect in K_{ATP} channel functions may also underlie the metabolic manifestations in PCOS patients. Although relevant evidence needs to be provided, I am thus bold to propose that an abnormal regulation of K_{ATP} channels may explain both the reproductive and metabolic phenotypes of PCOS patients. In addition, medications of the sulfonylurea class which are widely used to treat Type II diabetes mellitus may be associated with changes in LH. In one clinical study, it was demonstrated that patients treated with glibenclamide, a sulfonylurea, had elevated LH levels compared to those patients treated with insulin (487), indicating that sulfonylurea may affect the gonadotropin secretion in human.

In this dissertation, I established microdialysis as a useful tool to monitor GnRH pulsatile release *in vivo* in mice and showed that removal of E_2 and P by ovariectomy stimulates GnRH pulse generator. Surprisingly, few studies have ever been done to address the effect of E_2 and P

on pulsatile GnRH secretion in rodents. Using microdialysis to evaluate GnRH secretion in gene knockout animal models such as ERKO and PRKO mice will help us better understand the negative feedback mechanisms of ovarian steroids. More interestingly, with the generation of conditional knockout mouse models, such as brain insulin receptor knockout mouse (305), a detailed neural pathway and related neuronal populations involved in regulating GnRH neurons will be identified and mapped, which will enable us to further investigate the pathogenesis of the neuroendocrine disorders associated the HPG axis such as PCOS and eventually help us to treat these disorders.

I also demonstrated that negative energy balance as a result of short-term food deprivation suppresses GnRH secretion via a K_{ATP} channel-independent mechanism. Also associated with states of negative energy balance are changes in energy homeostasis, such as increase in food intake (463). Accumulating evidence suggests that K_{ATP} channels expressed in the hypothalamus, especially in neurons critical to regulation of energy homeostasis such as NPY and POMC neurons, are important in sensing the current status of energy balance and signaling to the CNS (463). My observations that K_{ATP} channels do not play an obligatory role in mediating the effects of negative energy balance on GnRH neurons may point to the possibility that although changes in states of energy balance are associated with changes in both energy homeostasis and GnRH/LH secretion, different central sensing mechanisms may be involved. This idea is important since the opposite end of negative energy balance, i.e. positive energy balance especially as seen in obese patients, have been associated with defects in both central nutrient-sensing (488) and GnRH/LH secretion (489). Abnormal K_{ATP} channel regulation in the hypothalamus has been suggested to account for at least part of the defective central glucose- and lipid-sensing mechanisms in the hypothalamus (481, 490). Possible correction of this K_{ATP}

channel-dependent central glucose-sensing mechanism may benefit the metabolic abnormalities of the obese patients (481), but possibly not the reproductive phenotypes of these patients.

In summary, the regulation of GnRH neurosecretion involves complex mechanisms. In this dissertation, I provide evidence that K_{ATP} channel modulation regulates GnRH pulse generator activity. Additionally, K_{ATP} channels may play an important role in the negative feedback actions of ovarian steroids on GnRH secretion through its expression regulation in the POA by the ovarian steroids. However, they do not appear to be necessary in relaying the signals of negative energy balance to the GnRH neurons.

Future directions may be targeted to understand the molecular mechanisms underlying the upregulatory effects of ovarian steroids on K_{ATP} channel expression. Specifically, Kir6.2 and/or SUR1 promoters driving expression of a reporter gene such as luciferase can be used to locate response segments of estrogen and progesterone in each promoter region. Also, future studies can be directed to identify neuronal populations whose K_{ATP} channels respond to E_2 and P. Combination of immunohistochemistry and in situ hybridization can be used to address this question. In addition, since kisspeptin neurons appear to be a promising converging point of the two pathways, whether K_{ATP} channels are expressed in these neurons and how it is regulated by ovarian steroids and negative energy balance can be studied using RT-PCR and in situ hybridization. Furthermore, neuronal populations and pathways underlying the effects of short-term food deprivation on GnRH secretion can be further pursued using knockout animal models and in vivo pharmacological approaches.

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