Kiss 1 neurons and metabolic sensing

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The University of Toledo
A Dissertation

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Kiss1 neurons and Metabolic Sensing

by

Xiaoliang Qiu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Science

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August, 2013
An Abstract of
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The onset of puberty occurs when GnRH neurons are released from the suppression
of the prepubertal period. The neuropeptide kisspeptin modifies GnRH neuronal activity
to initiate puberty and maintain fertility, but the factors that regulate Kiss1 neurons and
permit pubertal maturation remain to be clarified. A sophisticated network of regulatory
signals linking metabolism and reproduction has evolved to ensure appropriate regulation
of GnRH secretion. One such metabolic signal is the pancreatic hormone insulin. Insulin
circulates in proportion to adipose tissue stores in most mammals and is important in
initiation of puberty and maintaining fertility. To determine whether insulin sensing
plays an important role in Kiss1 neuron function, we generated mice lacking insulin
receptors in Kiss1 neurons (IRΔKiss mice) to look at their pubertal, reproductive and
metabolic phenotype. A second metabolic signal with an important role in initiation of
puberty and maintaining normal reproduction is the adipokine leptin. Given the
overlapping signaling pathways of leptin and insulin, these two hormones may interact
with each other in the regulation of Kiss1 neurons. To determine whether insulin and
leptin sensing interact to play an important role in regulation of Kiss1 neurons, we
generated mice lacking both insulin and leptin receptors in Kiss1 neurons (IR/LepRΔKiss mice) to look at their pubertal, reproductive and metabolic phenotype. IRΔKiss females showed a delay in vaginal opening and in first estrus, while IRΔKiss males also exhibited late sexual maturation. Correspondingly, LH levels in IRΔKiss mice were reduced in early puberty in both sexes. Adult reproductive capacity, body weight, fat composition, food intake, and glucose regulation were comparable between two groups. These data suggest that impaired insulin sensing by Kiss1 neurons delays the initiation of puberty but does not affect adult fertility. However, in IR/LepRΔKiss mice, both female and male knockout mice showed normal puberty, adult metabolism and reproduction.

In sum, insulin and leptin do not regulate metabolism through Kiss1 neurons. Insulin sensing by Kiss1 neurons plays a positive permissive role in puberty, but is not required for adult fertility. Leptin may counteract the effect of insulin in Kiss1 neurons during puberty; their interacting signaling pathways in Kiss1 neurons remain to be elucidated. These studies provide insight into the mechanisms regulating pubertal timing in anabolic states.
Dedicated to those who I love and those who chase their dreams and pursue
truth, liberty and justice
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List of Abbreviations

AGD ....................... Anogenital distance  
AgRP ........................ Agouti-related protein  
AMPK ........................ 5'-adenosine monophosphate-activated protein kinase  
ARC .......................... Arcuate nucleus  
AVPV ........................ Anteroventral periventricular nucleus  
BPS ............................ Balanopreputial separation  
CART .......................... Cocaine- and amphetamine-regulated transcript  
CNS ............................ Central nervous system  
Cre .............................. Recombinase  
Crtc1.......................... cAMP response element-binding protein regulated transcription coactivator-1  
CV .............................. Coefficients of variance  
DAG .......................... Diacylglycerol  
EB .............................. Estradiol benzoate  
FACS .......................... Fluorescence-activated cell sorting  
FOXO1 ........................ Forkhead box protein O 1  
FSH ............................ Follicle-stimulating hormone  
GFP ............................ Green fluorescent protein  
GPCRs .......................... G protein-coupled receptor  
GPR54 ........................ G protein-coupled receptor 54  
GSK3 .......................... Glycogen synthase kinase 3  
GTT ............................ Glucose tolerance test  
HFD .......................... High fat diet  
HPG ............................ Hypothalamus-pituitary-gonad axis  
IGF-1 .......................... Insulin-like growth factor-1  
IGFR-I .......................... Insulin-like growth factor receptor-I  
ITT ............................. Insulin tolerance test  
IR .............................. Insulin receptor  
IRS .............................. Insulin receptor substrate  
IP3 .............................. Inositol triphosphate  
*Kiss1* ........................ Kisspeptin  
JAK ............................ Janus kinase  
KNDy .......................... Kisspeptin-Neurokinin B-Dynorphin neurons (*Kiss1* neurons)  
LepR (ObR) ........................ Leptin receptor  
LH ............................. Luteinizing hormone  
loxP site ........................ Locus of crossover P1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBH</td>
<td>Medial basal hypothalamus</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin 4 receptor</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MCH</td>
<td>Melanin-concentrating hormone</td>
</tr>
<tr>
<td>NIRKO</td>
<td>Insulin receptor knockout in the brain</td>
</tr>
<tr>
<td>NK3R</td>
<td>Neurokinin B receptor</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid receptors</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomy</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PeN</td>
<td>Periventricular nucleus</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLCβ</td>
<td>Phospholipase Cβ</td>
</tr>
<tr>
<td>PMV</td>
<td>Ventral premammillary nucleus</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein Tyrosine Phosphatase 1B</td>
</tr>
<tr>
<td>RP3V</td>
<td>Rostral periventricular region of the third ventricle</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 3</td>
</tr>
<tr>
<td>STAT</td>
<td>Transducer and activator of transcription</td>
</tr>
<tr>
<td>TRPC</td>
<td>Transient receptor potential canonical</td>
</tr>
<tr>
<td>vGLUT2</td>
<td>Vesicular glutamate transporter-2</td>
</tr>
</tbody>
</table>
List of Symbols

°C ............................... Degrees celsius
Δ ................................. Deletion
µg ............................... Microgram
µm .............................. Micrometer
µl ............................... Microliter
µmol ........................... Micromole
dl ................................. Deciliter
i.p. .............................. Intraperitoneal injection
kg ............................... Kilogram
bp ............................... Base pair
mg ............................... Milligram
ml ............................... Milliliter
n ................................. Sample size
ng ............................... Nanogram
pg ............................... Picogram
IR^{AKiss} ........................ IR deletion in Kiss1 neurons
IR/LepR^{AKiss} .................. IR and LepR deletion in Kiss1 neurons
Chapter 1

Introduction

Kisspeptin

Kisspeptin (a product of the \textit{Kiss1} gene) is a key hypothalamic neuropeptide involved in initiating puberty and maintaining reproductive function (Navarro et al., 2004a). However, kisspeptin was originally discovered as a metastasis-suppressor gene in 1996 (Lee et al., 1996), named for its role as a suppressor sequence (ss). The letters “Kl” were appended to the prefix “SS” to form “KISS” in honor of the location of its discovery: Hershey, Pennsylvania, home of the famous “Hershey Chocolate Kisses.” Metastin was the name coined for the 54-amino acid product of the \textit{Kiss1} gene, whereas another group named it kisspeptin (Kotani et al., 2001). Use of both terms continues to this day, with cancer biologists largely preferring the name metastin, whereas investigators from other fields have favored the term kisspeptin (Oakley et al., 2009).

\textit{Kiss1} gene product initially is a 145 amino acid peptide, from which is cleaved to a 54 amino acid peptide. This product is not stable and is degraded into several short but active peptides, kisspeptin-10, kisspeptin-14 and kisspeptin-15, all of which have the
same affinity and efficacy in binding its receptor, Kiss1r (Figure 1.1).

Figure 1.1. Products of the Kiss1 genes. RF-NH2, Arg-Phe-NH2. Modified from (Popa et al., 2008; Oakley et al., 2009).

**Kisspeptin receptor and signaling**

Kisspeptin isoforms have a common RFamide (Arg-Phe-NH2) motif that binds the receptor Kiss1r, also known as GPR54 (G protein-coupled receptor 54) with high-affinity (Figure 1.1 and Figure 1.2). G protein-coupled receptors (GPCRs) mediate diverse physiological functions such as perception of sensory information, modulation of synaptic transmission, hormone release and actions, regulation of cell contraction and migration, or cell growth and differentiation (Wettschureck and Offermanns, 2005). Due to the importance of this class of receptors, its discoverers Robert Lefkowitz and Brian Kobilka were awarded the 2012 Nobel Prize in Chemistry. The GPCR superfamily can be classified into three subdivisions, rhodopsin-, secretin-, and metabotropic glutamate receptor-like families (Marchese et al., 1999). Typical of the rhodopsin family of GPCRs, Kiss1r contains seven transmembrane domains, with three glycosylation sites at the N
terminus (Clements et al., 2001). The binding of Kiss1r by Kiss1 peptide leads to the activation of G protein-activated phospholipase C (PLCβ), suggesting a Gaq/11-mediated signaling pathway. PLCβ activation leads to phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis and generation of the intracellular second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG), which in turn mediate intracellular Ca2+ release and activation of protein kinase C, respectively (Oakley et al., 2009). Kisspeptin is thought to stimulate GnRH secretion by activating transient receptor potential canonical (TRPC)-like channels and inhibiting inwardly rectifying potassium channels (Zhang et al., 2008), likely mediated by DAG and/or Ca2+ (Figure 1.2).

![Figure 1.2. Proposed mechanism of neuronal depolarization by kisspeptin binding to its receptor. From (Oakley et al., 2009).](image)

**Kisspeptin physiology**

In 2003, two independent research groups nearly simultaneously reported that mutations in kiss1r were associated with the idiopathic hypothalamic hypogonadism and impaired pubertal maturation found in their patients (de Roux et al., 2003; Seminara et al., 2003). Since that time, Kiss1 has emerged as a star neuropeptide in neuroendocrinology and reproductive physiology. One of the most important roles of Kiss1 is to stimulate GnRH secretion by a direct action on GnRH neurons, most of which
express the Kiss1r (although location, developmental and patterns of Kiss1 expression differ among species). The detailed distribution of Kiss1 in mice has been mapped in the hypothalamus and some other areas like cerebral cortex, medial amygdala, the bed nucleus of the stria terminalis, and periaqueductal gray (Clarkson et al., 2009; Cravo et al., 2011). Using in situ hybridization and immunohistochemical staining, Kiss1 has been located in the hypothalamus of mice and rats, including the rostral periventricular region of the third ventricle [RP3V, including the anteroventral periventricular nucleus (AVPV) and periventricular nucleus (PeN)], and the arcuate nucleus (ARC) (Oakley et al., 2009). The former is sexually dimorphic and much more abundant in female than male adults (Kauffman et al., 2007b). In sheep and primates, including monkeys and humans, Kiss1 is mainly expressed in the ARC (Pompolo et al., 2006; Rometo et al., 2007).

Kiss1 has been regarded as the key upstream signal in conveying the gonadal steroids on GnRH activity during puberty, the estrous cycle, and seasonal reproductive transitions (Lehman et al., 2010). Kiss1 neurons in mice have different physiological functions in different regions (Oakley et al., 2009). However, there is also a species-specific role in the regulation of GnRH upon sex steroids stimulation. Kiss1 neurons in the ARC appear to be involved in the negative feedback regulation of GnRH/LH by sex steroids in mammals. The expression of Kiss1 mRNA in the ARC is inhibited by estradiol, progesterone, and testosterone. In rodents, these hormones induce Kiss1 mRNA expression in the AVPV, where Kiss1 neurons are thought to be involved in the positive feedback regulation of GnRH/LH, producing the preovulatory surge of GnRH and thus LH (Figure 1.3). In ewe and primates, there is no homolog of the AVPV as in rodents, so the positive feedback of steroids to Kiss1 neuron in AVPV and GnRH does not exist.
(Oakley et al., 2009). In ewe, however, \textit{Kiss1} neurons in ARC play both negative and positive feedback in response to sex steroids (Estrada et al., 2006). For humans, the mechanism of the formation of the LH surge appears to be independent of hypothalamic control (Plant, 2012).

Kiss1 neurons in the ARC of several species have been found to coexpress neurokinin B and dynorphin, each of which has been shown to play a critical role in the central control of reproduction (Lehman et al., 2010). Therefore, experts in this area are prone to calling these neurons KNDy neurons. Neurokinin B (NKB) and its receptor NK3R (also known as TACR3) signaling have been placed in the spotlight after mutations were found to be associated with hypogonadotropic hypogonadism in humans (Guran et al., 2009; Topaloglu et al., 2009). Recent work suggests that NKB-NK3R signaling plays a role in pubertal maturation and that its alteration may contribute to

**Figure 1.3.** A schematic representation proposed mode of Kiss1 signaling in the forebrain of the mouse in response to steroids. From (Gottsch et al., 2006).
pubertal disorders linked to metabolic stress and negative energy balance (Navarro et al., 2012). This overlap in the expression of neurokinin B, kisspeptin, and dynorphin may not occur in humans, however (Hrabovszky et al., 2012).

The exact mechanism underlying how KNDy neurons in the ARC participate in the regulation of pulsatile GnRH secretion is under intense investigation. Recent work in the ewe shows that KNDy neurons are activated during both surge and pulsatile modes of secretion and likely play a role in mediating both positive and negative feedback actions of estrogen on GnRH secretion in that species (Merkley et al., 2012). It is suggested that GnRH neurons transition from GABA to glutamate signaling network during puberty (Clarkson and Herbison, 2006a). Therefore, different neurotransmitters like glutamate, GABA, and dopamine may be involved in this complicated process (Oakley et al., 2009; Lehman et al., 2010; Cravo et al., 2011). Evidence from rats (Ciofi et al., 2006) and mice (Cravo et al., 2011) indicates that KNDy cells and their terminals colocalize the vesicular glutamate transporter-2 (vGLUT-2), suggesting that they are glutamatergic as well as peptidergic in signaling to GnRH neurons or other KNDy neurons that express N-methyl-D-aspartic acid receptors (NMDA) (Miller and Gore, 2002). Recent electrophysiological study shows that NMDA, leptin, and neurokinin B are potent activators of KNDy neuron electrical activity and GABA inhibits it. The firing pattern of Kiss1 neurons located in the AVPV/PeN fluctuates with the estrus cycle and is modulated by glutamate and GABA (Alreja, 2013).

Additionally, Kiss1 and GPR54 signaling is also found in ovaries (Castellano et al., 2006; Gaytan et al., 2009; Peng et al., 2013), hippocampal dentate gyrus (Arai et al., 2005; Arai and Orwig, 2008), vascular endothelial cells (Sawyer et al., 2011), adrenal
gland (Nakamura et al., 2007), and pancreas (Hauge-Evans et al., 2006; Bowe et al., 2009), indicating Kiss1 regulates a wide range of effects in the body besides its main function in reproduction. For example, it has been shown that GPR54 mRNA is expressed in smooth muscle of large vessels like aorta, coronary artery, umbilical vein and also in cutaneous microvasculature (Sawyer et al., 2011). Kisspeptin can cause vasoconstriction and edema (Mead et al., 2007; Sawyer et al., 2011). Hence, it has been suggested that Kiss1 signaling plays a role in the pathogenesis of atherosclerosis and preeclampsia (Oakley et al., 2009). Interestingly, kisspeptin has been found to inhibit insulin secretion at physiological concentrations of glucose from isolated mouse islets (Vikman and Ahren, 2009), although findings have not been consistent. Kisspeptin is capable of stimulating insulin release or potentiating glucose-induced insulin secretion in rat and rhesus monkeys in vivo (Bowe et al., 2009; Wahab et al., 2011a).

**Energy balance, puberty, and reproduction**

Puberty is a key developmental period when reproductive capacity develops and sexual maturation is completed (Ojeda et al., 2009). The onset of puberty occurs when GnRH neurons are released from the suppression of the prepubertal period (Sisk and Foster, 2004). GnRH triggers downstream gonadotropin release, which further stimulates sexual steroids secretion from gonads, activating the well-known hypothalamus-pituitary-gonad axis (HPG). Although much of the variance in timing of pubertal onset in humans is due to genetic factors (Sisk and Foster, 2004), an estimated 20%-50% of the variance is due to environmental and metabolic factors that modulate the reemergence of GnRH pulses. Reproduction is a metabolically demanding function that requires sufficient levels.
of energy stores (Fernandez-Fernandez et al., 2006). In humans and rodents, insufficient caloric intake or excessive energy expenditure can delay the pubertal transition (Bronson and Heideman, 1990; Klentrou and Plyley, 2003). In addition, disease states associated with metabolic disturbances, including malnutrition, chronic inflammatory states, thyroid disease, and growth hormone deficiency, are also associated with a disruption in the normal timing of puberty (Nathan and Palmert, 2005), suggesting that GnRH activation at puberty requires a positive energy balance.

As is discussed before, fertility is gated by nutrients and availability of stored energy reserves (Fernandez-Fernandez et al., 2006). Animals under adverse metabolic events must invest energy for survival first and reproduction second (Bronson and Heideman, 1990; Hill et al., 2008a). The HPG axis is closely linked to metabolic status. States of negative energy balance, such as starvation, excessive exercise or lactation inhibit the reproductive axis. However, the cellular and molecular mechanisms linking energy stores and reproduction are not well understood. There is compelling evidence to show that Kiss1 plays a crucial role in this process (Oakley et al., 2009; Navarro and Tena-Sempere, 2011).

The hypothalamic Kiss1 system undergoes a complex pattern of neuroanatomical maturation and functional activation during the course of puberty. Hypothalamic Kiss1 expression increases during puberty (Navarro et al., 2004a; Shahab et al., 2005), as does the number of AVPV Kiss1 neurons (particularly in the female) and their putative contacts with GnRH neurons (Clarkson and Herbison, 2006b). Pubertal maturation in humans and mice with inactivating mutations in the Kiss1 gene or the Kiss1r does not occur (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007;
Lapatto et al., 2007; Topaloglu et al., 2012). Furthermore, kisspeptin administration accelerates, while pharmacological blockade of kisspeptin activity disrupts, the normal progression of puberty in rats (Navarro et al., 2004b; Pineda et al., 2010). Thus, Kiss1 neurons have been hypothesized to be the central processor for relaying signals from periphery to GnRH neurons, essential in reproduction (Dungan et al., 2006) (Figure 1.4). Indeed, several studies have documented a clear impact of conditions of undernutrition or metabolic stress on Kiss1 expression in the hypothalamus. For example, short time fasting can decrease the expression of Kiss1, preceding the reduction of GnRH (Luque et al., 2007; Wahab et al., 2011b). Similarly, chronic subnutrition during puberty has been shown to reduce Kiss1 mRNA levels in the ARC in female rats (Roa et al., 2009). Food deprivation can induce a concomitant decrease in hypothalamic Kiss1 and increase in GPR54 mRNA levels in prepubertal rats (Castellano et al., 2005). Furthermore, chronic central kisspeptin administration can restore puberty in an undernutrition model (Castellano et al., 2005). Rats in lactation have lower levels of Kiss1 mRNA and kisspeptin protein in ARC compared with nonlactating controls (Yamada et al., 2007). Indeed, hyperprolactinemia-induced hypogonadotropic anovulation is reversed by kisspeptin administration (Sonigo et al., 2012). Thus we hypothesize that insulin and leptin, two of the most important metabolic factors, may also regulate puberty and reproduction through Kiss1 neurons, sensing and transmitting metabolic information to GnRH neurons.
Insulin

Peptide hormone insulin which is secreted from pancreatic \( \beta \) cells is central in regulating carbohydrate and fat metabolism. In humans the insulin gene is located on the short arm of chromosome 11. Insulin has a variety of roles in the regulation of metabolism, reproduction, and development. Insulin deficiency or insulin resistance is involved in the pathogenesis of diabetes, polycystic ovary syndrome and metabolic syndrome. In 1967, Steiner and his colleagues discovered that insulin is synthesized as the prohormone proinsulin, which is processed to insulin by site-specific protease dependent cleavage (Steiner et al., 1967). Specifically, expression of the insulin gene yields a precursor protein called preproinsulin, of 104 to 109 amino acids, depending on the species, including a 24 amino acid signal peptide. Proinsulin is produced after

Figure 1.4. *Kiss1* neurons may act as the central processors linking metabolism and reproduction. SCN, suprachiasmatic nucleus. Modified from (Dungan et al., 2006).
removal of the signal peptide. Then the C-peptide is removed and inter- and intra-chain disulphide bridges are generated. Finally, mature insulin with A- and B-chains linked by inter-chain disulphide bridges is produced and secreted together with C-peptide into bloodstream.

**Insulin receptor**

Insulin receptors (IR) are expressed in all tissues in mammals, including the classic insulin-responsive tissues skeletal muscle, fat, and liver and non-classical tissues such as brain, endothelial cells or gonadal cells. The insulin receptor is a typical receptor tyrosine kinase (RTK), which transmits a hormonal signal. The IR is a hetero-tetramer consisting of two \( \alpha \)- and two \( \beta \)-subunits linked by disulfide bonds (Figure 1.5). The \( \alpha \)-chain has a molecular weight of 135 kDa and is exclusively located extracellularly, whereas the \( \beta \)-chain (95 kDa) contains extracellular, transmembrane and cytosolic domains. The transmembrane and intracellular portions of the \( \beta \)-subunit contain the insulin-regulated tyrosine protein kinase that is critical for insulin activity. The extracellular domain of the \( \alpha \)-subunit contains the ligands binding site. There are some receptors sharing similarities with the IR. The most closely related receptors to the insulin receptor are the receptor for insulin-like growth factor-1 (IGF-1) and insulin receptor related receptor, both sharing 50 to 60% of the whole IR amino acid sequence, and being more than 80% identical in the kinase domain (Gautam, 2002).
Insulin signaling and mutations in insulin receptor

The insulin/insulin receptor interaction serves to amplify, diversify, and terminate insulin action. Amplification of insulin signaling is achieved through engagement of a number of intracellular substrates including insulin receptor substrate (IRS) (Figure 1.6), resulting in activation of a bunch of receptors can produce a full metabolic response. IRS proteins are a family of proteins comprised of IRS1-4 and the growth factor receptor binding protein 2 associated binder 1. IRS can activate phosphatidylinositol 3-kinase (PI3K), a heterodimer consisting of a catalytic subunit p110 and a regulatory subunit p85. PI3K binds to the PH domain of protein kinase B (PKB), also known as Akt, causing translocation of PKB to the plasma membrane and its colocalization with phosphoinositide-dependent protein kinase 1 (PDK1). The subsequent conformational change enables PDK1 to phosphorylate PKB on threonine 308 and serine 473, thereby activating the enzyme. PKB can phosphorylate downstream glycogen synthase kinase 3 (GSK3) and P70 S6 kinase (P70S6K) to activate glycogen synthesis and protein synthesis, respectively. Akt also phosphorylates forkhead box protein O1 (FOXO1), causing nuclear exclusion. The phosphorylated FOXO1 is then ubiquitinated and
degraded by the proteosome (Matsuzaki et al., 2003). Thus, transcription of glucose 6-phosphatase decreases, reducing the rates of gluconeogenesis and glycogenolysis.

Another major physiological role of insulin is the regulation of gene transcription through the mitogen-activated protein kinase (MAPK) cascade (Goodyear et al., 1996). IRs also participate in terminating insulin action by clearing insulin from the circulation through receptor-mediated endocytosis and through the action of tyrosine phosphatases or serine/threonine kinases (Figure 1.6).

Disruption of insulin signaling through deletion of IR leads to various phenotypes from early death to obesity. Table 1.1 lists the phenotype of tissue specific IR knockout mice (Kitamura et al., 2003).
Table 1.1. Phenotype of the tissue specific IR knockout mice.

<table>
<thead>
<tr>
<th>IR Knockout</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>Constitutive</td>
<td>Diabetic ketoacidosis</td>
</tr>
<tr>
<td>Muscle</td>
<td>Dyslipidemia</td>
</tr>
<tr>
<td>Muscle/adipose tissue</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>Adipocyte</td>
<td>Protection against obesity</td>
</tr>
<tr>
<td>Liver</td>
<td>Moderate insulin resistance, transient hyperglycemia</td>
</tr>
<tr>
<td>β-cell</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>Brown adipose tissue</td>
<td>β-cell failure</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Obesity, sub-fertility</td>
</tr>
</tbody>
</table>

Neuron-specific IR knockout mice have demonstrated a direct role of insulin signaling in different neuron populations in hypothalamus. For example, neuropeptide Y/agouti-related protein (NPY/AgRP) or proopiomelanocortin (POMC) neuron-specific IR knockout mice did not exhibit altered energy homeostasis. Thus, IRs in these neurons are not required for steady-state regulation of food intake and body weight. However, AgRP-IR knockout mice exhibited reduced insulin-stimulated hepatic interleukin-6 expression and increased hepatic expression of glucose-6-phosphatase (Konner et al., 2007). Catecholaminergic neuron-specific IR knockout mice showed increased body weight, increased fat mass, and hyperphagia, indicating a critical role of insulin signaling in catecholaminergic neurons for the control of food intake and energy homeostasis (Konner et al., 2011). Additionally, pituitary-specific knockout of IR can rescue the obesity-
induced infertility in female mice (Brothers et al., 2010). Recently, mice with IR knockout in steroidogenic factor-1 (SF-1) expressing-neurons in the ventral medial hypothalamus were shown to be protected from diet-induced leptin resistance, weight gain, adiposity and impaired glucose tolerance (Klockener et al., 2011).

**Insulin signaling and reproduction**

As mentioned, a sophisticated network of regulatory signals linking metabolism and reproduction has evolved to ensure appropriate regulation of GnRH secretion. One such metabolic signal is the pancreatic hormone insulin. Insulin levels circulate in proportion to adipose tissue stores in most mammals (Woods et al., 1979). IR and insulin signaling proteins are widely distributed throughout the hypothalamus (Schwartz et al., 1992). Hypothalamic insulin signaling plays a pivotal role in the regulation of reproduction. Indeed, insulin has been shown to activate GnRH and LH secretion *in vitro* (Burcelin et al., 2003; Salvi et al., 2006). Mice that lack insulin signaling in brain neurons (NIRKO mice) exhibit hypothalamic hypogonadism (Bruning et al., 2000) and a delay in puberty (Gautam, 2002). Moreover, diabetic rats display reproductive abnormalities, which can be ameliorated by central administration of insulin (Steger and Kienast, 1990; Steger et al., 1993; Kovacs P et al., 2003). In humans, type 1 diabetes also disrupts puberty and reproduction (Griffin et al., 1994; Elamin et al., 2006).

Although insulin was originally thought to be acting directly on GnRH neurons (Burcelin et al., 2003; Kim et al., 2005; Salvi et al., 2006; DiVall et al., 2007), a recent study suggests otherwise. Deletion of the receptor for a related growth factor, IGF-1, from GnRH neurons in mice resulted in a three day delay in pubertal onset, but deletion of the insulin receptor itself from these neurons had no effect on pubertal timing or adult
insulin signaling in presynaptic neurons is important for normal GnRH neuronal function.

**Leptin**

Leptin, discovered through positional cloning of ob/ob mice in 1994 (Zhang et al., 1994), is an adipocyte-secreted hormone with pleiotropic effects in the physiology and pathophysiology of energy homeostasis, endocrinology, and metabolism. Leptin has 167 amino acids, encoded by leptin gene in chromosome 7 (Paracchini et al., 2005). Studies in vitro and in animal models show that leptin regulates a number of physiological functions, including the control of energy balance and several neuroendocrine axes. Mice and humans lacking leptin (e.g. ob/ob mice) (Zhang et al., 1994) or the leptin receptor (e.g. db/db mice) (Chen et al., 1996) develop hyperphagia, obesity, and diabetes.

Leptin together with insulin act on central processors in the hypothalamus, repressing brain anabolic neural circuits that stimulate eating and inhibit energy expenditure, while simultaneously activating catabolic circuits that inhibit food intake and increase energy expenditure (Schwartz et al., 2000). Leptin inhibits anabolic NPY expression in the ARC (Schwartz et al., 1996) and genetic ablation of NPY reduces hyperphagia and obesity in ob/ob mice (Stephens et al., 1995). On the contrary, leptin activates catabolic POMC/CART in ARC expression (Schwartz et al., 1997; Kristensen et al., 1998), indicating leptin acts through hypothalamic neurons to regulation food intake and energy balance.

Similar to rodents, homozygous mutations of the leptin gene leading to complete leptin deficiency in humans have been found to cause morbid obesity (Bluher and Mantzoros, 2009). This finding led to the clinical use of leptin for various diseases.
Pharmaceutical effects of leptin depend on endogenous leptin levels. Available evidence from human studies indicates that leptin is effective in states of leptin deficiency, less effective in states of leptin adequacy, and largely ineffective in states of leptin excess (Kelesidis et al., 2010). In leptin deficiency state, for example in patients with congenital or HIV-related lipoatrophy, leptin treatment can improve insulin sensitivity and lipid profile, concomitant with reduced visceral and ectopic fat deposition (Shimomura et al., 1999; Oral et al., 2002). Leptin is used to treat hypothalamic amenorrhea in women (Welt et al., 2004). In a state of leptin excess, such as in obesity, however, leptin did not cause clinically significant body weight loss in several clinical trials (Heymsfield et al., 1999; Hukshorn et al., 2000; Hukshorn et al., 2003), probably due to leptin resistance. The mechanism of leptin resistance will be discussed in the following section.

**Leptin receptor and Leptin signaling**

Leptin mediates its effects by binding to specific leptin receptors (LepRs) expressed in the brain and in peripheral tissues. Alternative splicing generates several isoforms of LepRs. The LepRa isoform (the short isoform) is thought to play an important role in transporting leptin across the blood-brain barrier. The LepRb isoform (the long leptin receptor isoform) mediates signal transduction and is strongly expressed in the hypothalamus, an important site for the regulation of energy homeostasis and neuroendocrine function. As mentioned, LepRb isoform is expressed in POMC/CART and NPY/AgRP neurons in the ARC that are central to regulating food intake and energy expenditure. The binding of leptin to the LepRb receptor activates several signal transduction pathways, including Janus kinase signal transducer and activator of transcription 3 (JAK2-STAT3), which is important for regulating energy homeostasis.
(Bates et al., 2003), and PI3K, essential for regulation of both food intake and glucose homeostasis (Niswender et al., 2001). Other pathways MAPK, 5′-adenosine monophosphate–activated protein kinase (AMPK), and the mammalian target of rapamycin (mTOR), have been proposed to be the downstream signaling molecules of leptin and are under intense investigation (Kelesidis et al., 2010).

Leptin resistance was first thought to be due to mutations of the leptin receptor or downstream neural circuitry, e.g. mutation or single nucleotide polymorphisms of LepR, melanocortin 3 receptor (MC3R), melanocortin 4 receptor (MC4R) and POMC have been identified in obese humans (Mantzoros et al., 2011). However, recent studies have suggested that leptin resistance is much more complicated than expected. Leptin signaling negative regulators SOCS3 (Kievit et al., 2006), SHP2 (Carpenter et al., 1998), PTP1B (Bence et al., 2006) have been identified to be responsible for blunting the leptin sensitivity by dephosphorylation of JAK2/STAT3 pathway. Furthermore, impaired leptin transport, impaired LepRb trafficking and endoplasmic reticulum stress are also implicated in leptin resistance (Morris and Rui, 2009).

**Leptin signaling and reproduction**

Leptin plays a crucial role in regulating reproduction and the hypothalamic-pituitary-gonadal (HPG) axis. Leptin may serve as a signal to convey information to the reproductive system that the amount of energy stored in the body as fat is adequate for carrying a pregnancy to term. Because energy balance is closely linked to the onset of puberty and normal fertility (Schneider, 2004; Elias, 2012), leptin has been proposed to be a permissive signal that can activate the reproductive axis and maintain normal
reproductive function by conveying needed information on available energy reserves in
the adipose tissue (Bluher and Mantzoros, 2009).

It is well-established that leptin acts in the brain to increase the pulsatile rate of
GnRH/LH secretion in different species like mouse, rat, lamb, and primate (Ahima et al.,
1996; Yu et al., 1997; Finn et al., 1998; Parent et al., 2000). However, whether leptin
receptor is expressed in GnRH neurons has been controversial. Studies in immortalized
GnRH cell lines suggested that leptin receptor is expressed in GnRH neurons (Burcelin et
al., 2003). More recently, with advances in conditional knockout techniques, it was
demonstrated that leptin action in GnRH neurons is not required for pubertal
development. Mice engineered to lack leptin receptor selectively in GnRH neurons
showed normal progression through puberty and exhibit normal sexual maturation and
fertility (Quennell et al., 2009). In fact, with the development of mouse models
expressing leptin-receptor reporter genes it became clear that mouse GnRH neurons
express virtually no leptin receptor (Louis et al., 2011). Therefore, leptin’s action in
stimulating GnRH secretion is exerted via interneurons that project to GnRH neurons.

Studies have indicated that leptin effects on reproduction could be mediated by a
STAT3-independent alternative signaling pathway (Bates et al., 2003). In recent years a
series of important findings have suggested a role for cAMP response element-binding
protein regulated transcription coactivator-1 (Crtc1) and mTOR as potential signaling
pathways linking energy balance and reproduction (Altarejos et al., 2008; Roa et al.,
2009; Elias, 2012). Leptin can increase the dephosphorylated form of nuclear Crtc1,
stimulating \textit{Kiss1} expression by acting on the \textit{Kiss1} promoter (Altarejos et al., 2008).
These data obtained \textit{in vitro} suggests that leptin induces \textit{Kiss1} expression via a Crtc1-
dependent mechanism. Of note, mice with deletion of the Crtc1 gene are hyperphagic, obese and infertile (Altarejos et al., 2008). Subsequently, it was demonstrated that blockade of mTOR signaling in the brain disrupts the HPG axis at puberty and blunts the stimulatory effects of leptin on pubertal development in food-restricted female rats (Roa et al., 2009). However, the reproductive deficits reported in both studies may be secondary to a central metabolic imbalance or to the activation/inhibition of redundant signaling pathways that are not necessarily related to leptin signaling.

Based on evidence similar to that presented above, leptin effects on the reproductive neuroendocrine axis have previously been proposed to be mediated by Kiss1 neurons that in turn would regulate GnRH neuronal activity; a moderate-to-high degree of colocalization of Kiss1 neuron and leptin receptors was reported (Smith et al., 2006; Qiu et al., 2011). But other groups found only a very small percentage of colocalization between leptin receptors and Kiss1 neurons (Cravo et al., 2011; Louis et al., 2011). Deletion of LepR selectively from hypothalamic Kiss1 neurons in mice did not influence puberty or fertility, indicating that direct leptin signaling in Kiss1 neurons is not required for these processes.

Two populations of leptin receptor-expressing neurons in the ventral premammillary nucleus (PMV) and striohypothalamic nucleus directly project to GnRH neurons (Elias, 2012). Bilateral lesions of the PMV of ob/ob mice blunted the ability of exogenous leptin to induce sexual maturation. Moreover, unilateral reexpression of endogenous LepR in PMV neurons was sufficient to induce puberty and improve fertility in female LepR-null mice. In addition, it was recently reported that the acute effects of leptin on PMV neurons require the PI3K signaling pathway (Zhao et al., 2002; Williams
et al., 2011). These findings indicate that neurons in the PMV may be the interneurons that link leptin sensing and the HPG axis (Donato et al., 2011b). Additionally, pituitary cells also express LepR. The percentage of leptin-bearing cells within the pituitary varies in different reproductive states and depends on sex and menstrual cycle, suggesting a supportive role for leptin during key events involved with reproduction (Akhter et al., 2007).

**Mutations in leptin and other leptin signaling genes**

Deletion of LepR from POMC neurons does not reduce food intake, but is sufficient to normalize glucose and glucagon levels in mice otherwise lacking LepR (Berglund et al., 2012). Activation of POMC neurons is required for the chronic effects of leptin to raise mean arterial pressure and reduce insulin and glucose levels. Leptin receptors in other areas of the brain other than POMC neurons may play a key role in mediating food intake and oxygen consumption (do Carmo et al., 2011). Table 1.2 summarizes studies employing genetic or pharmacological manipulations to disclose site-specific or cell-specific effects of leptin.

Table 1.2. Examples of studies employing genetic or pharmacological manipulations to disclose site-specific or cell-specific effects of leptin.

<table>
<thead>
<tr>
<th>Reproductive phenotype</th>
</tr>
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<tbody>
<tr>
<td>Global deletion/blockade</td>
</tr>
<tr>
<td>LepR in the brain/neuron(Cohen et al., 2001; de Luca et al., 2005)</td>
</tr>
<tr>
<td>infertility, no pubertal development</td>
</tr>
<tr>
<td>LepR in the forebrain</td>
</tr>
<tr>
<td>infertility, no pubertal development</td>
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<tr>
<td>LepR in the hypothalamus(Ring and Zeltser, 2010)</td>
</tr>
<tr>
<td>infertility</td>
</tr>
<tr>
<td>Ubiquitous melanocortin antagonism (Ay mouse)</td>
</tr>
<tr>
<td>abnormal estrous cyclicity</td>
</tr>
<tr>
<td>Melanocortin antagonism (pharmacology)</td>
</tr>
<tr>
<td>decreased steroid-induced LH surge; normal leptin effect on reproduction (ob/ob mice)</td>
</tr>
<tr>
<td>MC4R deficiency</td>
</tr>
<tr>
<td>no deficits reported</td>
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<tr>
<td>Neuropeptide</td>
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<tr>
<td>-------------</td>
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<tr>
<td>NPY knockout</td>
</tr>
<tr>
<td>NPY knockout in <em>ob/ob</em> mouse</td>
</tr>
<tr>
<td>Cell-specific deletion</td>
</tr>
<tr>
<td>LepR from <em>Kiss1</em> neurons (Donato et al., 2011a)</td>
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<tr>
<td>LepR from GnRH neurons</td>
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<tr>
<td>LepR from POMC neurons</td>
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<tr>
<td>LepR from AgRP/NPY neurons</td>
</tr>
<tr>
<td>LepR from AgRP/NPY and POMC neurons</td>
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<tr>
<td>LepR and IGF from POMC neurons</td>
</tr>
<tr>
<td>LepR from SF-1 neurons</td>
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<tr>
<td>LepR from astrocyte (Jayaram et al., 2013)</td>
</tr>
<tr>
<td>LepR from somatotropes (Childs et al., 2011)</td>
</tr>
</tbody>
</table>

Site-specific reactivation

| Exogenous LepR in the NTS | no rescue of fertility | |
| Exogenous LepR in the ARC | improvement of cyclicity in Koletsky rats | |
| Endogenous LepR in the ARC | no rescue of fertility | |

The consequences of the manipulations for the animal reproductive function are summarized in the second column. Modified from (Donato et al., 2011a).

**Cre-loxP system**

Change of the mouse genome by conventional transgenic and gene-targeted approaches has greatly facilitated studies of gene function. However, a gene alteration expressed in the germ line may lead to an embryonic lethal phenotype impairing the study of gene function. Likewise, a gene alteration may exert its effect in multiple cell and tissue types, producing a mixed phenotype in which it is difficult to distinguish direct function in a particular tissue from secondary effects resulting from altered gene function in other tissues. Hence, methods have been developed to control conditions in terms of the timing, cell-type, and tissue specificity of gene activation or suppression (Kos, 2004). The Cre/loxP system is an important tool for these techniques. A loxP site is a 34 base
pair (bp) DNA sequence that is composed of an 8bp core (which determines directionality) flanked on each side by 13bp of palindromic (complementary) sequences (Figure 1.7A). Although loxP sites are prevalent in the genomes of bacteriophages, this exact 34bp of sequence is statistically unlikely to occur naturally in the mouse genome. Multiple loxP sites can be introduced into the mouse genome by targeted mutagenesis in embryonic stem cell lines. Sequences flanked by loxP sites are then said to be “floxed.”

The bacteriophage P1 encodes the 38kDa cyclization recombination recombinase enzyme known as Cre (creates recombination), which catalyzes recombination between two specific DNA repeats. Cre is a member of the integrase family of recombinases, recognizing a specific 34bp nucleotide sequence motif called a loxP site (“locus of crossover P1”). Cre can form a transient DNA-protein covalent linkage to bring the two loxP sites together and mediate site-specific recombination. Depending on the orientation of the paired loxP sites, the DNA segment between them will be either excised or inverted (Figure 1.7B-D). When the two loxP sites are in the same orientation, Cre excises the intervening DNA segment, leaving a single remaining loxP site. When the repeats are in an anti-parallel orientation (in opposite orientations), the DNA segment undergoes inversion and the two loxP sites remain (Kos, 2004).

**Aim of the study**

As mention before, two essential metabolic factors insulin and leptin are important in the regulation of reproduction, linking metabolism and fertility. We used Cre-loxP system to produce *Kiss1* neuron specific IR and IR/LepR knockout mice. Metabolic phenotype, pubertal and reproductive phenotype were carefully examined as described in Chapter 2 and Chapter 3.
Figure 1.7. Structure of a loxP site and Cre-mediated loxP-specific recombination events. (A) loxP sequence is schematically represented as a solid triangle with a (relative) orientation as defined by the nonpalindromic spacer sequence. Below, the canonical loxP sequence is given with arrows depicting inverted repeats and the spacer region underlined. (B) Cre-mediated excision between two directly repeated loxP sites, in an intramolecular reaction, results in the circularization of the loxP-flanked sequence and a linear molecule, each retaining a single loxP site. Cre/loxP-mediated integration is the reverse reaction that proceeds via an intermolecular reaction. (C) Cre-mediated recombination between two linear molecules, each harboring a single loxP site in the same orientation, is resolved through an intermolecular reaction. The reaction products have reciprocally exchanged flanking sequences. (D) Through an intramolecular reaction, Cre mediates an inversion of DNA sequence that is flanked by loxP sites in opposing orientation relative to each other. From (Kuhn and Torres, 2002).
Chapter 2

Delayed Puberty but Normal Fertility in Mice With Selective Deletion of Insulin Receptors From Kiss1 Cells
(Adapted from original paper published in Qiu et al. *Endocrinology*. 2013;154:1337-1348)

Introduction

The onset of puberty occurs when GnRH neurons are released from the suppression of the prepubertal period (Sisk and Foster, 2004). Although much of the variance in timing of pubertal onset in humans is due to genetic factors (Sisk and Foster, 2004), an estimated 20%–50% of the variance is due to environmental and metabolic factors that modulate the reemergence of GnRH pulses. Reproduction is a metabolically demanding function that requires sufficient levels of energy stores (Fernandez-Fernandez et al., 2006). In humans and rodents, insufficient caloric intake or excessive energy expenditure can delay the pubertal transition (Bronson and Heideman, 1990; Klentrou and Plyley, 2003). In addition, disease states associated with metabolic disturbances, including malnutrition, chronic inflammatory states, thyroid disease, and growth hormone deficiency, are also associated with a disruption in the normal timing of puberty (Nathan and Palmert, 2005), suggesting that GnRH activation at puberty requires a positive energy balance.
Accordingly, a sophisticated network of regulatory signals linking metabolism and reproduction has evolved to ensure appropriate regulation of GnRH secretion. One such metabolic signal is the pancreatic hormone insulin. Insulin levels circulate in proportion to adipose tissue stores in most mammals (Woods et al., 1979). Insulin receptors (IRs) and insulin signaling proteins are widely distributed throughout the hypothalamus (Schwartz et al., 1992). Hypothalamic insulin signaling plays a pivotal role in the regulation of reproduction. Indeed, insulin has been shown to activate GnRH and LH secretion in vitro (Burcelin et al., 2003; Salvi et al., 2006). Mice that lack insulin signaling in brain neurons (NIRKO mice) exhibit hypothalamic hypogonadism (Bruning et al., 2000) and a delay in puberty (Gautam, 2002). Moreover, diabetic rats display reproductive abnormalities, which can be ameliorated by central administration of insulin (Steger and Kienast, 1990; Steger et al., 1993; Kovacs P et al., 2003). In humans, type 1 diabetes also disrupts puberty and reproduction (Griffin et al., 1994; Elamin et al., 2006).

Although insulin was originally thought to be acting directly on GnRH neurons (Burcelin et al., 2003; Kim et al., 2005; Salvi et al., 2006; DiVall et al., 2007), a recent study suggests otherwise. Deletion of the receptor for a related growth factor, IGF-1, from GnRH neurons resulted in a three day delay in pubertal onset, but deletion of the insulin receptor itself from these neurons had no effect on pubertal timing or adult fertility (Divall et al., 2010). Together, these studies suggest that insulin signaling in presynaptic neurons is important for normal GnRH neuronal function.

Kisspeptin (a product of the Kiss1 gene) is a key hypothalamic neuropeptide involved in initiating puberty and maintaining reproductive function (Navarro et al., 2004a). The hypothalamic Kiss1 system undergoes a complex pattern of neuroanatomical
maturation and functional activation during the course of puberty. Hypothalamic Kiss1 expression increases during puberty (Navarro et al., 2004a; Shahab et al., 2005), as does the number of anteroventral periventricular nucleus (AVPV) Kiss1 neurons (particularly in the female) and their putative contacts with GnRH neurons (Clarkson and Herbison, 2006b). Pubertal maturation in humans and mice with inactivating mutations in the Kiss1 gene or the Kiss1r does not occur (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007; Topaloglu et al., 2012). Furthermore, kisspeptin administration accelerates, while pharmacological blockade of kisspeptin activity disrupts, the normal progression of puberty in rats (Navarro et al., 2004b; Pineda et al., 2010).

Kiss1 neurons have been hypothesized to serve as the primary transmitters of metabolic signals from the periphery to GnRH neurons (Dungan et al., 2006; Fernandez-Fernandez et al., 2006; Forbes et al., 2009). Several studies have documented a clear impact of conditions of undernutrition or metabolic stress on Kiss1 expression in the hypothalamus. Mice subjected to fasting display a significant reduction in hypothalamic Kiss1 mRNA levels, which precedes the decline in GnRH expression (Luque et al., 2007). Similarly, chronic subnutrition during puberty has been shown to reduce Kiss1 mRNA levels in the arcuate nucleus (ARC) in female rats (Roa et al., 2009). Furthermore, repeated central injections of kisspeptin to female rats with arrested puberty due to chronic subnutrition restored pubertal progression despite the persistent caloric restriction (Castellano et al., 2005). These findings suggest that Kiss1 neurons may transmit metabolic information provided by insulin levels to GnRH neurons.
Whether *Kiss1* neurons mediate the influence of metabolic factors on the timing of puberty is not clear. Leptin receptors colocalize with *Kiss1* neurons (Smith et al., 2006), yet we recently showed that leptin’s effect on puberty in mice do not require *Kiss1* neurons (Donato et al., 2011b). Using the Cre/loxP system to generate *Kiss1* neuron-specific insulin receptor knockout mice (IR\textsuperscript{ΔKiss}), we have ablated the insulin receptor in *Kiss1* cells to test whether insulin signaling plays a necessary role in the function of *Kiss1* neurons. Lack of insulin signaling in *Kiss1* neurons delayed puberty in both females and males in our model, but had no effect on adult fertility. Our results show that insulin sensing in *Kiss1* neurons modifies the timing of puberty, thus providing a mechanism that may advance puberty in well-nourished individuals.

**Materials and Methods**

Animals and genotyping. To generate mice with the IR specifically deleted in *Kiss1* neurons, *Kiss1*-Cre mice (Cravo et al., 2011) were crossed with insulin receptor floxed mice (Bruning et al., 2000) and bred to homozygosity for the floxed allele only. The IR\textsuperscript{floxflox} mice were designed with loxP sites flanking exon 4; excision of exon 4 in the presence of Cre recombinase results in a frameshift mutation and produces a premature stop codon. Littermates lacking Cre expression were used as controls (IR\textsuperscript{floxflox}). All mice were on a mixed C57BL/6J-129S6/SvEv background. Where specified, the mice also carried the Gt(ROSA)26Sor locus-inserted EGFP gene [B6.129-Gt(ROSA)26Sor\textsuperscript{tm2Sho}/J; Jackson labs], serving as a reporter under the control of Cre recombinase expression. Mice were housed in the University of Toledo College of Medicine animal facility at 22-24°C on a 12h light/12h dark cycle and were fed standard
rodent chow 2016 Teklad Global 16% Protein Rodent Diet (12% fat by calories, Harlan Laboratories). On postnatal day (PND) 22, mice were weaned if litter size was within 5 to 10 to prevent birth size effects on body weight. At the end of the study, all animals were sacrificed by CO₂ asphyxiation or by cardiac puncture under 2% isoflurane anesthesia to draw blood. All procedures were reviewed and approved by University of Toledo College of Medicine Animal Care and Use Committee. Mice were genotyped as previously described (Hill et al., 2010; Cravo et al., 2011). Additional genotyping was carried out by Transnetyx, Inc. using a real-time PCR-based approach.

**Western blotting.** Adult mice were sacrificed, and hypothalamus, liver, muscle, visceral adipose tissues and gonads were harvested. Tissues were snap frozen in liquid nitrogen and stored in -80°C until homogenized in radioimmunoprecipitation assay lysis buffer (Millipore) supplemented with protease inhibitor and phosphotase inhibitor (Thermo Scientific). After centrifugation, supernatant protein concentrations were determined by BCA protein assay (Thermo Scientific). 30μg denatured samples were subjected to SDS-PAGE electrophoresis and western blotting using insulin receptor β subunit (1:1000, Santa Cruz Biotechnology Inc.). β-actin (1:1000, Sigma) or α-tubulin (1:1000, Cell Signaling) was used as a loading control.

**Fluorescence activated cell sorting (FACS).** FACS and RNA extractions were done according to published protocols with minor modifications (Cravo et al., 2011). Briefly, 4 female Kiss1-Cre/EGFP mice and 4 female IRΔKiss mice (also carrying the EGFP reporter) were sacrificed via cervical dislocation and intact hypothalami were removed. The hypothalami were placed in cold Hank’s Balanced Salt Solution (HBSS, without calcium or magnesium, Thermo Scientific), cut into pieces, and digested by
papain (Worthington Biochemical Corporation) solution in HBSS (1mg/ml) in a 37 °C incubator with 5% CO₂ for 30 min with occasional mixing by pipetting. Cells were then centrifuged at 2000g for 5 minutes and the supernatant removed. FACS buffer (Bovine serum albumin 0.1%, 1% EDTA in 1×PBS) was used to stop digestion and resuspend cells. Cells were processed to isolate EGFP expressing cells via FACS Aria (BD Biosciences) according to EGFP wavelength. Total RNA was extracted by Arcturus PicoPure RNA isolation kit (Applied Biosystems) and reverse transcription PCR was performed for the IR cDNA in isolated EGFP expressing Kiss1 neurons (Applied Biosystems). IR primers were the same as previously published (Sense: TCATGGATGGAGGCTATCTGG; Antisense: CCTTGAGCAGGTTGACGATTT) (Luque et al., 2007).

Perfusion and immunohistochemistry. Adult male mice and female mice at diestrus at the age of 3-6 months were deeply anesthetized by ketamine and xylazine, and then a perfusion needle was inserted into the left ventricle. After briefly perfusing with a saline rinse, mice were perfused transcardially with 10% formalin for 10 minutes and the brain was removed. The brain was post-fixed in 10% formalin at 4°C overnight, and then immersed in 20% sucrose at 4°C for 48 hours. 25µm sections were cut by a sliding microtome into 5 equal serials. Sections were treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. After rinsing in phosphate-buffered solution (PBS), sections were blocked 2h in PBS-azide-T (PBS-azide; Triton X; 3% normal donkey serum). Then, samples were incubated for at least 48 h at 4°C in PBS-azide-T containing rabbit anti-kisspeptin IgG (1:1000, Millipore), which has been tested for specificity (Quennell et al., 2011). After several washes in PBS, sections were
incubated in PBS-T (Triton X, 3% donkey serum) containing biotinylated anti-rabbit IgG
(1:1000, Vector Laboratories), followed by incubation in ABC reagent (Vector
Laboratories) for 60 minutes at room temperature. Sections were washed and
immunoreactivity was visualized by 0.6mg/ml diaminobenzidine hydrochloride (Sigma)
in PBS with hydrogen peroxide. Finally, sections were washed, mounted on slides, dried
overnight, dehydrated, cleared and coverslipped. Kiss1-immunoreactive neurons in the
AVPV/PeN and ARC were quantified as previously described (Matsuwaki et al., 2010;
Quennell et al., 2011).

Dual label in situ hybridization/immunofluorescence. Females at the age of 4-5
months were perfused with DEPC treated saline and then formalin (n=3, Kiss1-Cre/EGFP
and IRΔKiss female mice on diestrus). Double-label ISH and immunofluorescence assays
were performed as previously described (Zigman et al., 2006; Cravo et al., 2011). Briefly,
free-floating hypothalamic sections were treated with 1% sodium borohydride for 15 min
followed by 0.25% acetic anhydride in 0.1 M triethanolamine (TEA, pH 8.0) for 10 min.
Sections were incubated at 50°C for 12-16 h in hybridization solution (50% formamide;
10 mM Tris-HCl, pH 8.0; 5 mg tRNA; 10 mM dithiotreitol/DTT; 10% dextran sulfate;
0.3 M NaCl; 1mM EDTA and 1× Denhardt’s solution) containing the 33P-labeled IR
riboprobe diluted to 10^6 cpm/mL. Sections were then washed in 2× SSC (sodium
citrate/sodium chloride) and treated with 0.002% RNase A (Roche) for 30 min, followed
by stringency washes in decreasing concentrations of SSC. Sections were incubated
overnight at room temperature in anti-GFP (made in chicken 1:5,000, Aves Labs)
primary antibody. The next day, sections were washed in PBS then incubated in
AlexaFluor 488-conjugated goat anti-chicken, 1:500 for 2h. Sections were mounted onto
SuperFrost plus slides, dehydrated in ethanol and placed in X-ray film cassettes with BMR-2 film (Kodak) for 4 days then dipped in NTB-2 photographic emulsion (Kodak), for 4 weeks. Slides were developed with D-19 developer (Kodak), dehydrated in ethanol, cleared in xylene, and coverslipped with Permaslip.

**Metabolic phenotype assessment.** Body weight was measured weekly in a single-occupant cage with ALPHA-dri bedding. Body composition in 4 months old mice was assessed by nuclear magnetic resonance (minispec mq7.5, Bruker Optics) to determine the percentage of fat mass, as previously described (Hill et al., 2009). Glucose Tolerance Tests (GTT) and Insulin Tolerance Tests (ITT) were done as previously described (Hill et al., 2010). Briefly, following a 6h fast, mice were injected with dextrose (2g/kg, i.p.). Tail blood glucose was measured using a mouse-specific glucometer (AlphaTRAK, Abbott) before and 15, 30, 45, 60, 90 and 120 min after injection. For ITT, following a 4 hour fast, mice were injected with recombinant insulin (0.75U/kg, i.p.). Tail blood glucose was measured again at specified timepoints. Food intake and indirect calorimetry were measured from females at the age of 2-3 months in a Calorimetry Module (CLAMS, Columbus Instruments) as previously described (Mesaros et al., 2008).

**Puberty and reproductive phenotype assessment.** Anogenital distance (AGD) was measured directly with a ruler from the middle of the anus to the middle of the penis. To minimize variation, AGD was measured by a single blinded observer. Balanopreputial separation was checked daily from weaning by manually retracting the prepuce with gentle pressure (Korenbrot et al., 1977). Singly housed female mice were checked daily for vaginal opening after weaning at 3 weeks of age. Vaginal lavages from female mice were collected from the day of vaginal opening for at least 3 weeks. Stages were assessed
based on vaginal cytology (Bingel and Schwartz, 1969; Nelson et al., 1982): predominant cornified epithelium indicated the estrus stage, predominant nucleated cells indicated the proestrus stage, and predominant leukocytes indicated the diestrus stage. At the age of 33 days, each male mouse was paired with one wildtype female of proven fertility for 4 weeks or until the female mouse was obviously pregnant. Then the paired mice were separated and the delivery date recorded. The age of sexual maturation was estimated from the birth of the first litter minus average pregnancy duration for mice (20 days). At 4-6 months old, animals were again paired with wildtype adult breeders to collect additional data on litter size and intervals between litters.

**Hormone assays.** Submandibular blood was collected at 10:00-11:00AM to detect basal LH and FSH levels using the rat pituitary panel (Millipore), performed by the University of Virginia Center for Research in Reproduction. This timepoint was chosen to avoid the LH surge in randomly cycling mice. The assay for LH had a detection sensitivity of 3.28 pg/ml. The intraassay and interassay coefficients of variance (CV) were 6.9% and 17.2 %, respectively. FSH was measured in females at diestrus and in males. For FSH the lower limit of detection was 7.62pg/ml, with intraassay and interassay CV 6.7% and 16.9 %, respectively. Serum estradiol was measured by ELISA (Calbiotech) with sensitivity <3pg/ml, and intraassey CV 3.1% and interassay CV 9.9%. Serum testosterone was measured by RIA at Oregon Health and Science University Endocrine Technology and Supporting Lab with a sensitivity range of 0.05-25ng/ml, and intrassay variation of <10% and interassay variation of <15%. Leptin was measured from random fed PND31 mice and adult mice. The leptin was measured by ELISA (Crystal Chem Inc.) with a sensitivity range of 0.2 to 12.8ng/ml, and intraassay and
interassay CV ≤10%. Serum collected after an overnight fast was used for measurement of insulin (Crystal Chem Inc.) and triglyceride (Point Scientific). The insulin ELISA kit had a sensitivity range of 50-3200pg/ml, with both intraassay and interassay CV ≤10%.

**Histology.** Ovaries and testes were collected from mice and fixed immediately in 10% formalin overnight. Then tissues were embedded in paraffin and cut into 5-8μm sections which were done by University of Toledo Pathology Department. Sections were stained by hematoxylin and eosin.

**Quantitative real-time PCR.** Mice were decapitated after isoflurane anesthesia and brains and other tissues were removed. The hypothalamus was divided into 2 blocks as previously described (Quennell et al., 2011). Specifically, 1 had RP3V containing AVPV and PeN and 1 had whole ARC containing medial basal hypothalamus (MBH). Total RNA were extracted from dissected tissues by RNeasy Lipid Tissue Mini Kit (Qiagen) and single-strand cDNA was synthesized by High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) using random hexamers as primers. 10μM cDNA template was used in a 25μl system in 96-well plates by SYBR green qPCR SuperMix/ROX (Smart Bioscience). Primers for amplification of Kiss1 mRNA were the same as previous publication (Sense: AGCTGCTGCTTCTCCTCTGT; Antisense GCATACCCGCATTCCCTTTT) (Luque et al., 2007). Each sample was analyzed in triplicate to measure Kiss1 expression level. The reactions were run in ABI PRISM 7000 sequence detection system (PE Applied Biosystems) and analyzed using the comparative Ct method (2^ΔΔCt) with GAPDH as the normalizer.

**Effects of ovariectomy (OVX) and acute estradiol benzoate (EB) treatment on LH.** Female mice 2-4 months of age were anesthetized by ketamine and xylazine
(9:00AM-12:00PM), and blood samples were collected submandibularly immediately before OVX. 7-14 days later, blood samples were collected again at the same time of day. Mice were then subcutaneously injected with 10µg/mouse EB (Sigma) in sesame oil. 2 hours after injection, blood samples were obtained for measurement of LH.

**High fat diet treatment.** One group of mice was on high fat diet with 60% kcal from fat with an energy density of 5.24kcal/g (Research Diets, Inc.; New Brunswick, NJ) from weaning day. Body weight was measured every 3 days until 8 weeks old. Vaginal opening and smear were checked every day until 8 weeks old. Date of vaginal opening and first estrus were recorded. The other group mice at the age of 8 weeks old were treated with high fat diet and continued for 5 months. During the feeding, the mice were measured for body weight every week. After 3 months of high fat diet treatment, NMR was performed to assess the body composition. Moreover, both fasting and nonfasting serum were obtained for glucose, insulin, leptin and lipid profile, LH, and FSH. At the age of 4-5 months, mice were paired with established breeders for at least 1 month. If mouse did not produce a new litter after 2 months breeding, it was considered to be sterile. Date of birth of new litters and litter size were recorded if a litter was produced.

**48h fasting experiment.** Adult mice were fasted for 48h beginning at 9:00-10:00AM. Submandibular blood was withdrawn to measure baseline and fasting LH levels.

**Data analysis and production of digital images:** Brain sections were visualized with a Zeiss Axioskop2 microscope. Photomicrographs were produced by capturing images with a Zeiss Axiocam HRc digital camera and AxioVision software. Only the sharpness, contrast, and brightness were adjusted. Quantification of dual labeled neurons
and percentage of colocalization were determined in the AVPV and in 2 rostro-to-caudal levels of the ARC. Cells were counted in one side of a determined level of each nucleus. We considered cells dual-labeled if the density of silver grains ($^{33}$P-labeled riboprobe) overlying a green fluorescent cytoplasm (GFP-ir) was at least $3 \times$ that observed in the background. For background determination, we used the density of silver grains overlying the superior cerebellar peduncle, where no cell bodies were detected.

 Statistical analysis. Data are presented as the mean±SEM. Two-tailed unpaired $t$ testing was used as the main statistical method. Mann-Whitney U test was used if the data did not assume a normal distribution. One way ANOVA was performed to compare 3 independent groups, followed by Bonferroni's multiple comparison test. Paired $t$ test was employed if the data is the same sample before and after an intervention, such as leptin level before and after fast. For body weight, GTT, ITT, and estrogen feedback data, repeated measures ANOVA was used to compare changes over time between two genotypes. For frequency comparison, $\chi^2$ test was used. $P<0.05$ was considered to be significant.

 Results

 Generation and confirmation of IR$^{\text{flox/flox}}$ mice

 To generate mice with the IR specifically deleted in Kiss1 neurons, we crossed IR$^{\text{flox/flox}}$ mice (Bruning et al., 1998) with Kiss1-Cre mice (Cravo et al., 2011) carrying the Cre recombinase gene driven by the Kiss1 promoter. To verify that the IR gene was excised in Kiss1 neurons, PCR was performed on DNA from different tissues. As expected, a 500bp band indicating gene deletion was produced from the hypothalamus.
and other tissues expressing \textit{Kiss1}, including cerebrum, ovary, testis, liver and pancreas (Figure 2.1A) (Ohtaki et al., 2001; Cravo et al., 2011). However, \textit{Kiss1} expression level in liver and pancreas were low by real-time PCR (Figure 2.1B). IR protein levels were similar between wildtype and targeted-knockout liver, muscle, visceral adipose tissue, testis and ovary (Figure 2.1C). Consistent with restriction of IR inactivation to a defined subpopulation of hypothalamic neurons, western blot analysis revealed no alteration of IR expression in hypothalamus (data not shown) (Konner et al., 2007). Thus, insulin sensing appears to be intact in the majority of cells in the ovary and other tissues.

\textbf{Kisspeptin neurons express insulin receptors}

To examine expression of IR specifically in \textit{Kiss1} neurons, these neurons were isolated by FACS sorting from EGFP reporter mice. Reverse transcription PCR of \textit{Kiss1}-EGFP neurons showed an IR band in wildtype but not IR$^{\Delta\text{Kiss}}$ mice, indicating that IR is present in \textit{Kiss1} neurons and is deleted successfully in IR$^{\Delta\text{Kiss}}$ mice (Figure 2.1D).

Furthermore, dual label \textit{in situ} hybridization/immunofluorescence showed that around 22\% \textit{Kiss1} neurons in the ARC were colocalized with IR in wildtype, while the percentage was sharply reduced to about 2\% in knockout mice (Figure 2.1E). Interestingly, in the AVPV, only 3-5\% \textit{Kiss1} colocalized with IR in wildtype mice. No colocalization was found in the AVPV of IR$^{\Delta\text{Kiss}}$ mice (Figure 2.1E).
Figure 2.1. Generation of IR$^{Δ\text{Kiss}}$ mice. (A) PCR of DNA from different tissues. The excised IR gene appears as a 500bp band and the un-excised IR gene sequence as a 2.2kb band. (B) Kiss1 expression in different tissues in a wildtype female by real-time PCR. (C) IRβ expression in different tissues and densitometry (n=3-5 each group). (D) FACS and reverse transcription PCR of IR from GFP positive cells isolated from 4 control female reporter mice (no IR$^{\text{flox/flox}}$) and 4 female reporter IR$^{Δ\text{Kiss}}$ mice. (E) Colocalization of Kiss1 neuron and IR and quantification of double staining percentages from female control and IR$^{Δ\text{Kiss}}$ mice in the AVPV and ARC (n=3). *P<0.05. (Qiu et al., 2013)
**IR\textsuperscript{ΔKiss} mice have a normal metabolic phenotype**

Due to the potential interaction between Kiss1 neurons and metabolic circuits in the hypothalamus, we examined the metabolic phenotype of IR\textsuperscript{ΔKiss} mice. The body weights of both females and males showed no significant difference between control and knockout mice (Figure 2.2A, B). 48h food intake was comparable between two groups in 2-3 months old females (Figure 2.2C). Fat mass, lean mass and fat percentage were comparable in both females and males (Figure 2.2D, E). Serum insulin was not statistically different between control and IR\textsuperscript{ΔKiss} mice in both sexes (Figure 2.2F, G), suggesting that β-cell insulin secretion is normal in these mice. Furthermore, Glucose tolerance was normal in both sexes of IR\textsuperscript{ΔKiss} mice (Figure 2.2H, I). No significant differences were seen in visceral fat weight, serum triglyceride and leptin levels at PND 31 and adulthood in males except the leptin level was different in females on both PND31 and adulthood (Table 2.1). Moreover, energy expenditure, oxygen consumption (Figure 2.3), or insulin tolerance did not differ between control and IR\textsuperscript{ΔKiss} adult mice (Supplemental figure 2.1A, B).

Table 2.1 Supplemental parameters in adults.

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Control</th>
<th>IR\textsuperscript{ΔKiss}</th>
</tr>
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<tbody>
<tr>
<td>Uterus mass (mg)</td>
<td></td>
<td>98.4 ± 7.5 n=7</td>
<td>117.4 ± 18.2 n=5</td>
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<td>Ovary mass (mg)</td>
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<td>10.5 ± 0.6 n=15</td>
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<tr>
<td>Testis mass (mg)</td>
<td></td>
<td>100.9 ± 3.285 n=13</td>
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<tr>
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<td>5.5 ± 0.8 n=6</td>
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<td>Visceral fat mass (mg)</td>
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<td>358.8 ± 41.7 n=5</td>
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<tr>
<td></td>
<td>Male</td>
<td>825.2 ± 119.2 n=13</td>
<td>537.2 ± 106.2 n=13</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td></td>
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<tr>
<td>--------------------------</td>
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<tr>
<td>Serum triglyceride (mg/dL)</td>
<td>53.4 ± 13.5 n=9</td>
<td>47.8 ± 9.6 n=9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27.7 ± 9.5 n=7</td>
<td>58.4 ± 16.5 n=7</td>
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<tr>
<td>Leptin PND31 (ng/ml)</td>
<td>3.2 ± 0.3 n=11</td>
<td>3.0 ± 0.6 n=6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.2 n=16*</td>
<td>2.4 ± 0.9 n=6</td>
<td></td>
</tr>
<tr>
<td>Leptin Adult (ng/ml)</td>
<td>2.2 ± 0.3 n=7</td>
<td>2.0 ± 0.2 n=16</td>
<td></td>
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<tr>
<td></td>
<td>1.3 ± 0.1 n=7*</td>
<td>1.9 ± 0.3 n=8</td>
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</table>

(*P<0.05)

Figure 2.2. Normal metabolic phenotype in adult IRΔKiss mice. (A) Weekly body weight of female mice (n=13 each group). (B) Weekly body weight of male mice (n=16 each group). (C) 48h food intake measured by calorimetric cages. (D) Female body composition at the age of 4 months in control (n=16) and IRΔKiss (n=14) mice. (E) Male body composition at the age of 4 months in control (n=10) and IRΔKiss (n=8) mice. (F) Female serum insulin at the age of 4-6 months in control (n=9) and IRΔKiss (n=10) mice.
(G) Male serum insulin at the age of 4-6 months in control (n=11) and IR\textsuperscript{ΔKiss} (n=10) mice. (H) 2-3 months old female GTT and AUC (inset) in control (n=9) and IR\textsuperscript{ΔKiss} (n=10) mice. (I) 2-3 months old male GTT and AUC (inset) in control (n=18) and IR\textsuperscript{ΔKiss} (n=13) mice. AUC, area under curve. (Qiu et al., 2013)

Figure 2.3. Indirect calorimetry from female mice. (A) O\textsubscript{2} consumption volume in daytime and nighttime. (B) CO\textsubscript{2} production volume in daytime and nighttime. (C) Respiratory expiratory ratio (n=6 each group).

Female IR\textsuperscript{ΔKiss} mice experience delayed puberty with normal fertility

To assess the progression of puberty in female mice, we measured vaginal opening and timing of the first entrance into estrus. In females, vaginal opening indicates the activation of the hypothalamic-pituitary-gonadal (HPG) axis at puberty (Safranski et al., 1993). Female IR\textsuperscript{ΔKiss} mice experienced vaginal opening approximately 3 days later than control mice (PND31.4±0.5 VS PND34.0±0.9 days, Figure 2.4A). The day of first estrus implies the establishment of the hormonal cyclicity necessary for female reproduction (Safranski et al., 1993). The age of first estrus was also significantly postponed in IR\textsuperscript{ΔKiss} mice (PND 39.6±1.2 VS PND 44.1±1.6 days, Figure 2.4B). LH levels in IR\textsuperscript{ΔKiss} mice were lower than controls on PND31 (Figure 2.4C), whereas estradiol was comparable between two groups on PND31 (Control, 5.2±0.6pg/ml, n=10 VS IR\textsuperscript{ΔKiss}, 5.2±0.6pg/ml, n=11). However, LH, FSH and estradiol were comparable between two groups in adulthood (Figure 2.5A-C). Female knockout mice also had
normal estrous cyclicity, characterized by normal estrous length and staging (Figure 2.5D). In addition, adult female IRΔKiss mice had similar ovary and uterus weights (Table 2.1). Ovarian morphology showed follicles at all stages of maturation and comparable number of corpora lutea between control and knockout mice (Figure 2.5E and Table 2.1). At 4-6 months old, mice were paired with established wildtype male breeders. Both the latency to birth and litter size were comparable between two groups (Figure 2.5F).

Figure 2.4. Delayed puberty in female IRΔKiss mice. (A) Vaginal opening was evaluated in control (n=12) and IRΔKiss (n=10) mice. (B) First estrous was evaluated in control (n=9) and IRΔKiss (n=10) mice. (C) LH on PND31 in control (n=11) and IRΔKiss (n=10) mice. *P<0.05. (Qiu et al., 2013)

Figure 2.5. Normal female reproduction in adult IRΔKiss mice. (A) Basal LH levels in adult females in control (n=16) and IRΔKiss (n=18) mice. (B) Basal FSH levels in adult females in control (n=5) and IRΔKiss (n=4) mice. (C) Estradiol in adult females in control (n=9) and IRΔKiss (n=10) mice. (D) Estrous cycle length (Control, n=9; IRΔKiss n=13) and estrous cycle analysis (Control, n=5; IRΔKiss, n=7) in 7-10 weeks old females. (E)
Representative light photomicrographs of adult ovaries in control and IR^{ΔKiss} mice (n=4). CL, corpora lutea. Scale bar, 100µm. (F) Fertility data from 4-6 months old females paired with established male breeders. Interval from mating to birth of a litter and litter size were compared between control (n=8) and IR^{ΔKiss} (n=12) mice. (Qiu et al., 2013)

**Male IR^{ΔKiss} have delayed puberty with normal fertility**

In males, AGD has been correlated with androgen exposure (Lapatto et al., 2007) and is therefore a useful measure of pubertal progression. On PND 28 and PND 43, AGD did not differ, yet on PND31 and PND 39, AGD in IR^{ΔKiss} mice was shorter than control mice (Supplemental figure 2.1C). Testis mass in IR^{ΔKiss} mice were lower than that of control, indicating a delay of gonadal development (Figure 2.6A). Balanopreputial separation is an indicator of activation of the reproductive axis in males (Korenbrot et al., 1977). However, this measure showed no difference between two groups (Figure 2.6B), nor did testosterone levels differ on PND25 (Control, 0.6±0.1ng/ml, n=10 VS IR^{ΔKiss}, 0.6±0.1ng/ml, n=7) or PND31 (Control, 1.1±0.3ng/ml, n=10 VS IR^{ΔKiss}, 1.6±0.5ng/ml, n=8). Nevertheless, LH levels in IR^{ΔKiss} mice were lower than controls on PND31 (Figure 3.6C). Finally, the age of sexual maturation, as measured by the age at which each male was able to impregnate a female of known fertility, was significantly delayed compared with controls (43.7±2.2 VS 49.4±1.1 days, Figure 2.6D).

Figure 2.6. Delayed male puberty in IR^{ΔKiss} mice. (A) Testis mass (Control, n=5; IR^{ΔKiss}, n=4). (B) Normal balanopreputial separation age (Control, n=12; IR^{ΔKiss}, n=10). (C) LH on PND31 in control (n=6) and IR^{ΔKiss} (n=5) mice. (D) Estimated age of sexual
maturation calculated by subtraction of 20 days from the time required for delivery of a litter (Control, n=8; IR\textsuperscript{ΔKiss}, n=10). *P<0.05. (Qiu et al., 2013)

In adults, LH, FSH and testosterone were not statistically different between two groups (Figure 2.7A-C). Furthermore, the testis weight was normal in adult IR\textsuperscript{ΔKiss} mice (Table 2.1). Testis histology showed all stages of spermatogenesis in seminiferous tubules and normal interstitial Leydig cells in IR\textsuperscript{ΔKiss} mice (Figure 2.7D). Fertility at 4-6 months old, characterized by litter size and days required to impregnate a female was not different between control and IR\textsuperscript{ΔKiss} males (Figure 2.7E).

We counted the Kiss\textit{l} neuron number in the AVPV/PeN and measured the Kiss\textit{l} immunoreactive areas in the ARC from PND31 mice (2.8A-D). In PND31 females, Kiss\textit{l}
neuron number in the AVPV/PeN in IR\(^{ΔKiss}\) mice was fewer than that in wildtype, while in the ARC Kiss1 neuron immunoreactive area was similar between two groups (Figure 3.8C). In PND31 males, Kiss1 neuron number in the AVPV/PeN did not differ, and Kiss1 immunoreactive area was similar in the ARC between two groups (Figure 2.8D). In adults, normal patterns of kisspeptin staining were seen in both AVPV/PeN and ARC (Figure 2.8E-H). In PND31 females, Kiss1 expression levels in RP3V and MBH were similar using real-time PCR. In PND31 males, Kiss1 expression in RP3V in wildtype was slightly lower than that in IR\(^{ΔKiss}\) mice, whereas Kiss1 expression in MBH was no difference (Supplemental figure 2.1D).

![Figure 2.8. Reduced Kiss1 cell number in the AVPV/PeN in juvenile IR\(^{ΔKiss}\) females. (A) and (B) Representative Kiss1 immunostaining micrograph in the AVPV and ARC in a female IR\(^{ΔKiss}\) mouse on PND31. (C) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in PND31 females (Control, n=3; IR\(^{ΔKiss}\), n=5). (D) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in PND31 males (Control, n=4; IR\(^{ΔKiss}\), n=3). (E) and (F) Representative immunostaining micrograph for kisspeptin in the AVPV and ARC in an adult female IR\(^{ΔKiss}\) mouse. (G) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in adult females (n=3). (H) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in adult...](image-url)
males (n=3). AVPV, anteroventral periventricular nucleus; PeN, periventricular nucleus; ARC, arcuate nucleus. *$P<0.05$. (Qiu et al., 2013)

**IR$^{AKiss}$ mice have normal response to estrogen feedback**

The secretion of GnRH in adulthood is regulated by feedback of gonadal sex steroids, and hypothalamic Kiss1 neurons may play a role in transmitting steroid feedback signals to the reproductive axis (Kauffman et al., 2007a). In addition, insulin and estrogens can activate shared intracellular signaling pathways (Malyala et al., 2008). We therefore investigated the possibility that insulin action in Kiss1 neurons participates in the negative feedback that estrogens exert on GnRH neurons. Mice were ovariectomized and then treated with EB. LH was significantly increased after ovariectomy and suppressed by administration of EB similarly in both IR$^{AKiss}$ mice and control animals. Contrary to the above hypothesis, the baseline, ovariectomy, and ovariectomy plus EB group did not differ between the two groups (Figure 2.9). 5 months after OVX, mice were subjected to NMR, fat percentage was comparable between the two groups (Supplemental figure 2.1E), indicating that insulin action in Kiss1 neurons does not mediate the rise in LH and body weight after OVX as previously proposed (Mittelman-Smith et al., 2012).
Figure 2.9. Normal response to estrogen feedback in IRΔKiss mice. Serum LH from intact, OVX, and OVX/EB injected (2h) females in the morning (Control, n=9; IRΔKiss, n=10). *P<0.05. (Qiu et al., 2013)

**IRΔKiss mice have normal response to negative energy balance**

In order to study whether insulin action in Kiss1 neurons is a required signal of energy balance to the reproductive axis in adulthood, we induced a negative energy balance by fasting mice for 48h. As expected, LH was dramatically decreased in control animals. IRΔKiss female mice showed a similar drop in LH (Figure 2.10), suggesting the HPG axis was still able to perceive the negative energy balance. Insulin may not be necessary to transmit negative energy balance to HPG axis through Kiss1 neurons in hypothalamus.
Figure 2.10. Normal response to negative energy balance. Serum LH from non-fast and 48h fast females with the age of 4 months at 9:00-10:00AM (Control, n=8; IR\textsuperscript{ΔKiss}, n=7).

**IR\textsuperscript{ΔKiss} mice have normal response to positive energy balance**

In order to study whether insulin action in Kiss\textsuperscript{l} neurons is a required signal of energy balance to the reproductive axis in puberty and adulthood, we induced a positive energy balance by high fat diet treatment in both peripubertal period and adulthood. Mice which were treated with HFD after weaning had no significant difference in body weight and puberty between control and IR\textsuperscript{ΔKiss} mice (Figure 2.11A and B), indicating insulin signaling in Kiss\textsuperscript{l} neurons does not advance pubertal timing in response to HFD treatment.

Figure 2.11. Comparable body weight and puberty after HFD treatment. (A) Body weight growth curve (n=4). (B) VO and 1st Estrus (Control, n=11; IR\textsuperscript{ΔKiss}, n=10).
For mice which were treated with HFD from 8th weeks old, no difference was detected in body weight and body composition (Figure 2.12A-D). This finding further shows that insulin signaling in \textit{Kiss1} neurons does not participate in the regulation of metabolism. After 3 months HFD treatment, more than half of female mice had an abnormal estrus cycle in both groups (Table 2.2). After 4 months HFD treatment, 60-70% female mice had stopped cycling regardless of their genotypes. However, in male mice, most mice in both groups retained their fertility (Table 2.3). 5 months after OVX, mice had similar percentage of fat (Supplemental figure 2.1E).

![Figure 2.12. Comparable body weight, body composition after HFD treatment. (A) Female body weight growth curve after HFD (n=8). (B) Male body weight growth curve](image-url)
after HFD (Control, n=7; IR\textsuperscript{ΔKiss}, n=5). (C) Female NMR 3 months after HFD (n=8). (D) Male NMR 3 months after HFD (Control, n=7; IR\textsuperscript{ΔKiss}, n=5).

Table 2.2. Estrus cycle after 3 months HFD treatment.

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<th>Control</th>
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</tr>
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<tbody>
<tr>
<td>Normal cycle</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>No cycle</td>
<td>4</td>
<td>5</td>
</tr>
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Table 2.3. Fertility after 4 months HFD treatment.

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<th>Control</th>
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<tbody>
<tr>
<td>Female Fertile</td>
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<td>4</td>
</tr>
<tr>
<td>Sterile</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Male Fertile</td>
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**Discussion**

Hyperinsulinemia is widely believed to advance pubertal maturation (Ahmed et al., 2009). Insulin rises in children around the time of adrenarche, in association with increasing adiposity, circulating IGF-1, and insulin resistance in peripheral tissues (Jeffery et al., 2012). Indeed, reduction of insulin levels by metformin administration in girls with precocious pubarche resulted in a delay in the clinical onset of puberty (Ibanez et al., 2006). Likewise, metformin blocked the ability of a high-fat diet to advance puberty in mice (Brill and Moenter, 2009). These effects may result in part from insulin inducing increased GnRH production. Increasing circulating insulin levels in adult male
mice with hyperinsulinemic clamp studies induces a significant rise in LH secretion (Burcelin et al., 2003). In women, hyperinsulinemic clamping similarly resulted in significant increases in the frequency of LH pulsatility (Moret et al., 2009), although this effect was not found in men (Pesant et al., 2012). Furthermore, mice that lack insulin signaling in the brain (NIRKO mice) have low luteinizing hormone (LH) levels that increase in response to GnRH, suggesting dysfunction at the hypothalamic level (Bruning et al., 2000). Male pubertal timing has not been reported in these mice, but NIRKO females exhibit a delay in vaginal opening of 3-4 days compared to their littermates (Gautam, 2002).

In this study, we have further defined the role of the insulin in the central control of reproduction, demonstrating that insulin signaling in the Kiss1 neurons is crucial for the normal timing of pubertal onset. The delayed pubertal onset was secondary to hypothalamic-pituitary dysfunction, as female IR^KissΔ^ mice had lower gonadotropin levels than control mice during the pubertal transition. Thus, elimination of insulin signaling by Kiss1 neurons completely recapitulated the effect of neuronal insulin deletion on puberty in females. For male mice, measures of maturation of the external genitalia were mixed. However, a functional test of when IR^KissΔ^ mice are able to impregnate a female showed a clear delay. IR^KissΔ^ mice eventually experienced pubertal onset, suggesting that insulin pathway activation in Kiss1 neurons is ultimately not required for GnRH activation. Clearly, GnRH activation at puberty is dependent upon a spectrum of converging genetic, developmental, and environmental signals. Such signals appear to overcome loss of IR activation to eventually induce GnRH pulse generator activity. Likewise, the normal reproductive parameters in adult IR^KissΔ^ mice suggest that other neuronal groups may
mediate insulin’s actions on fertility in mature mice. Alternatively, the infertility of adult NIRKO mice may be secondary to their metabolic derangements, including insulin and leptin resistance and hypertriglyceridemia, rather than the loss of IRs in neurons.

Insulin signaling in the central nervous system has been shown to play an important role in regulation of metabolism and energy balance (Bruning et al., 2000). NIRKO mice show increased food intake and diet-sensitive obesity with increases in body fat, hyperleptinemia, elevated plasma insulin levels, and hypertriglyceridemia. In addition, some evidence suggests that Kiss1 neurons can interact with hypothalamic circuitry controlling energy homeostasis (Hill et al., 2008a; Navarro and Tena-Sempere, 2012). In the current study, no abnormality was seen in body weight, visceral fat weight, and serum insulin levels in mice lacking insulin receptors in Kiss1 neurons. Other metabolic parameters, such as food intake, serum triglyceride, serum leptin, glucose tolerance and insulin tolerance, were also normal in IRΔKiss mice. These findings suggest that insulin’s role in the brain regulating energy disposal and fuel metabolism is not dependent on Kiss1 neurons. While we found no evidence of decreased insulin receptor expression in peripheral tissues, we did not analyze subpopulations of Kiss1 expressing peripheral cells (Ohtaki et al., 2001). Nevertheless, our findings also suggest that insulin signaling in these cells is not required for normal glucose homeostasis. These findings are in accord with previous studies in Kiss1 knockout mice, in which no abnormal metabolic phenotype was detected (Oakley et al., 2009).

Estrogens act on Kiss1 neurons to assist in the temporal coordination of juvenile GnRH restraint and subsequent pubertal activation (Mayer et al., 2010), although significant species differences exist in the kisspeptin system (Oakley et al., 2009). In
rodents, *Kiss1* neurons are direct targets for the action of sex steroids (Mayer et al., 2010), and *Kiss1* expression in the brain is strongly regulated by steroids (Han et al., 2005). Regulation of *Kiss1* neurons by an estrogen response element-independent nonclassical estrogen receptor pathway was found in the ARC of mice (Gottsch et al., 2009).

Intriguingly, both insulin signaling and the non-classical estrogen receptor pathway are coupled to PI3K intracellular signaling in the hypothalamus (Malyala et al., 2008; Park et al., 2011). We therefore considered the possibility that insulin signaling in *Kiss1* neurons can also influence sex steroids signaling in *Kiss1* neurons, and in such a way to modify the sex steroids feedback in *Kiss1* neurons. To identify whether insulin’s action in *Kiss1* neurons plays a role in estrogen negative feedback, we ovariectomized and EB treated adult female mice. Since EB fully suppressed LH production, it appears that insulin sensing by *Kiss1* neurons does not impact the negative feedback of estrogen on these neurons and the HPG axis.

A negative energy balance in adult animals impairs reproduction by down-regulating the HPG axis, which is associated with diminished expression of *Kiss1* mRNA expression (Castellano et al., 2005), as well as reduced circulating insulin levels (Ahima et al., 1996). However, we have shown that fasting similarly decreases LH in both control and IR<sup>ΔKiss</sup> mice. Thus, insulin signaling in *Kiss1* neurons is not required for suppression of the HPG axis due to a short term negative energy balance. Furthermore, while insulin signaling in the brain is required for normal adult fertility (Bruning et al., 2000), our results show that impaired insulin perception by *Kiss1* neurons does not alter
reproductive parameters in both adult males and females. Thus, other neuronal groups are likely to mediate insulin’s actions on fertility in mature mice.

In contrast, pubertal exposure to a positive energy balance in female animals leads to advanced puberty (Li et al., 2012). This treatment mimics the advance in puberty seen in obese humans (Davison et al., 2003; Herman-Giddens, 2006; Burt Solorzano and McCartney, 2010). Given the fact that childhood obesity has become a major health concern in recent decades, recent data also suggest that excess adiposity during childhood may influence pubertal development. In particular, excess adiposity during childhood may advance puberty in girls and delay puberty in boys (Burt Solorzano and McCartney, 2010). However, the exact mechanism underlying the obesity induced precocious puberty is not clear. Our data suggest that insulin itself does not act through Kiss1 neurons to regulate puberty. Therefore, other neurons, or other metabolic factors and hormones may be involved to control the onset of sexual maturation.

Substantial amounts of evidence support a role for kisspeptin neurons as gatekeepers of puberty (Tena-Sempere, 2012), but some unanswered questions remain. While ablation of Kiss1 neurons during the late infantile period disrupts the onset of puberty, congenital ablation of 97% of Kiss1 neurons does not prevent pubertal maturation (Mayer and Boehm, 2011). It therefore appears that a significant amount of developmental compensation is possible, allowing a small number of Kiss1 neurons or other neuronal circuits to drive pubertal maturation. Given the congenital nature of the targeted ablation we have described here, our results may underestimate the importance of insulin signaling in Kiss1 neurons in the juvenile or adult animal if a similar process of compensation occurred in our mice. Nevertheless, it should be noted that the overall
number of $Kiss1$ neurons in adult IR$^{AKiss}$ mice was not altered. Another question that remains unanswered is the role of ARC versus AVPV $Kiss1$ neurons in the control of puberty. Given that we found the majority of insulin receptors in the $Kiss1$ population in the ARC, our results support a role for this population in the modulation of pubertal timing.

In sum, insulin signaling in $Kiss1$ neurons is necessary for well-timed activation of the HPG axis during puberty. In contrast, insulin signaling in $Kiss1$ cells is unnecessary for normal adult reproductive or metabolic functions. This mechanism may play a role in promoting pubertal development in the presence of abundant energy stores and retarding puberty when the nutritional environment is unfavorable.
Supplemental figure 2.1. (A) Female ITT (n=10). (B) Male ITT (Control, n=11; IR\textsuperscript{\Delta Kiss}=7). (C) AGD in different timepoints (n=12). (D) PND31 Kiss1 mRNA expression in RP3V and ARC in females (n=8) and males (n=6). (E) 5 months after OVX body fat percentage measured by NMR (n=8).
Chapter 3

Normal Puberty and Fertility in Mice with Selective Deletion of Insulin Receptors and Leptin Receptors from Kiss1 Cells
(Unpublished manuscript)

Introduction

The onset of puberty is initiated when GnRH neurons are released from the suppression of the prepubertal period, causing increased secretion of luteinizing hormones (LH) and follicle stimulating hormone (FSH) from the anterior pituitary, followed by gametogenesis and production of gonadal steroids (Sisk and Foster, 2004). Hypothalamic reproductive circuits integrate numerous permissive signals that range from metabolic factors to environmental cues that modify the timing of puberty (Kennedy and Mitra, 1963; Frisch and McArthur, 1974). Indeed, reproduction is gated by nutrition and the availability of stored energy reserves (Crown et al., 2007; Hill et al., 2008a). However, the cellular and molecular mechanisms that link energy stores and reproductive maturation are not well understood.

Kisspeptin (a product of the Kiss1 gene) is a key hypothalamic neuropeptide involved in initiating puberty and maintaining reproductive function. Humans with loss-of-function mutation of kisspeptin and kisspeptin receptor (Kiss1r) lack pubertal development and are infertile due to hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). Both Kiss1 knockout and Kiss1r
knockout mice show abnormal sexual maturation and infertility (d’Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). So Kiss1 neurons have been hypothesized to serve as the central processor of signals from the periphery to GnRH neurons to initiate puberty and maintain normal reproduction (Dungan et al., 2006; George et al., 2010). Such signals including body weight, nutrition, metabolism and hormone levels may influence the activity of Kiss1 neurons (Fernandez-Fernandez et al., 2006; Forbes et al., 2009). However the mechanism underlying the metabolic regulation of Kiss1 neuron is not clear. Recent research shows that the adiposity signal insulin may play a permissive role in initiation of puberty through Kiss1 neurons whereas insulin sensing in Kiss1 neurons is not required in maintaining adulthood fertility (Qiu et al., 2013).

Another important adiposity signal is leptin, which circulates at levels proportional to body fat content (Considine et al., 1996). Similar to insulin, leptin signaling proteins are widely distributed throughout the hypothalamus and play a pivotal role in the regulation of food intake and reproduction (Baskin et al., 1988; Schwartz et al., 2000; Cohen et al., 2001). Mice and humans lacking leptin (e.g. ob/ob mice) or the leptin receptor (e.g. db/db mice) develop hyperphagia, obesity, diabetes and infertility (Zhang et al., 1994; Chen et al., 1996). However, the neuronal circuits underlying the role of leptin in reproduction remain to be elucidated. To test whether leptin works through Kiss1 neurons to regulate puberty and fertility, Donato and his colleagues used the Cre-loxP system to delete LepR from Kiss1 neurons. Yet, no abnormalities were seen in terms of puberty and reproduction (Donato et al., 2011a). Leptin and insulin signaling is suggested to be overlapped in different tissues (Niswender and Schwartz, 2003; Carvalheira et al., 2005). Hence, the compensatory signaling between leptin and insulin may be involved in
the network within the \textit{Kiss1} neuron. We hypothesized that ablating both leptin and insulin signaling in \textit{Kiss1} neurons would delay puberty and impair fertility. We used the Cre-loxP system to generate IR and LepR double knockout mice in \textit{Kiss1} neurons (IR/LepR^{ΔKiss}). Metabolic, pubertal and reproductive manifestations were characterized.

\textbf{Materials and Methods}

\textit{Animals and genotyping.} To generate mice with the IR specifically deleted in \textit{Kiss1} neurons, \textit{Kiss1}-Cre mice (Cravo et al., 2011) were crossed with insulin receptor floxed mice (Bruning et al., 2000) and bred to homozygosity for the floxed allele only. The IR^{flox/flox} mice were designed with loxP sites flanking exon 4. Excision of exon 4 in the presence of Cre recombinase results in a frameshift mutation and produces a premature stop codon. Exon 17 of LepR was flanked by loxP sites (LepR^{flox/flox}), containing the Box 1 motifs crucial for leptin signal transduction through JAK2 and STAT3 pathways (McMinn et al., 2004). Cre-mediated recombination produces a LepR that lacks exon 17 and has a truncated missense exon 18, which has previously been reported to recapitulate phenotypes found in the LepR^{db/db1J} mutation (Balthasar et al., 2004; McMinn et al., 2004; Ring and Zeltser, 2010). Two lines of mice were bred with each other to produce IR/LepR^{ΔKiss}. All mice were on a mixed C57BL/6J-129S6/SvEv background. Mice were housed in the University of Toledo College of Medicine animal facility at 22-24°C on a 12h light/12h dark cycle and were fed standard rodent chow 2016 Teklad Global 16% Protein Rodent Diet (12% fat by calories, Harlan Laboratories). On postnatal day (PND) 22, mice were weaned if litter size was within 5 to 10 to prevent birth size effects on body weight. At the end of the study, all animals were sacrificed by CO\textsubscript{2} asphyxiation or by cardiac puncture under 2\% isoflurane anesthesia to
draw blood. All procedures were reviewed and approved by University of Toledo College of Medicine Animal Care and Use Committee. Mice were genotyped as previously described (Hill et al., 2010; Cravo et al., 2011) (Table 3.1). Additional genotyping was carried out by Transnetyx, Inc. using a real-time PCR-based approach.

Table 3.1. Genotyping primer sequences.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>IR-loxP</td>
<td>gatgtgcaccccatgtctg</td>
<td>ctaaatagctgagaccacag</td>
</tr>
<tr>
<td>LepR-loxP</td>
<td>cagcttgagaaatcaacacacac</td>
<td>aatgaaaaagttttgacga</td>
</tr>
<tr>
<td>Kiss-Cre</td>
<td>tgcgaacctctactctgtgcat</td>
<td>gctctgtgaagtacgactctga</td>
</tr>
<tr>
<td>IR-delta</td>
<td>ggtagaaacaggtgg</td>
<td>ctaaatagctgagacgacag</td>
</tr>
<tr>
<td>LepR-delta</td>
<td>gcttgaccgaaggtagtgag</td>
<td>cagtaacgaaagagagatgatgtgg</td>
</tr>
</tbody>
</table>

Western blotting. Adult mice were sacrificed, and hypothalamus, liver, muscle, visceral adipose tissues and gonads were harvested. Tissues were snap frozen in liquid nitrogen and stored in -80°C until homogenized in radioimmunoprecipitation assay lysis buffer (Millipore) supplemented with protease inhibitor and phosphatase inhibitor (Thermo Scientific). After centrifugation, supernatant protein concentrations were determined by BCA protein assay (Thermo Scientific). 30μg or 50 μg denatured samples were subjected to SDS-PAGE electrophoresis and western blotting using insulin receptor β subunit (1:1000, Santa Cruz Biotechnology Inc.). β-actin (1:1000, Sigma) or α-tubulin (1:1000, Cell Signaling) was used as a loading control.

Perfusion and immunohistochemistry. Adult male mice and female mice at diestrus at the age of 3-6 months were deeply anesthetized by ketamine and xylazine, and
then a perfusion needle was inserted into the left ventricle. After briefly perfusing with a saline rinse, mice were perfused transcardially with 10% formalin for 10 minutes and the brain was removed. The brain was post-fixed in 10% formalin at 4°C overnight, and then immersed in 20% sucrose at 4°C for 48 hours. 25µm sections were cut by a sliding microtome into 5 equal serials. Sections were treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. After rinsing in phosphate-buffered solution (PBS), sections were blocked 2h in PBS-azide-T (PBS-azide; Triton X; 3% normal donkey serum). Then, samples were incubated for at least 48 h at 4°C in PBS-azide-T containing rabbit anti-kisspeptin IgG (1:1000, Millipore), which has been tested for specificity (Quennell et al., 2011). After several washes in PBS, sections were incubated in PBS-T (Triton X, 3% donkey serum) containing biotinylated anti-rabbit IgG (1:1000, Vector Laboratories), followed by incubation in ABC reagent (Vector Laboratories) for 60 minutes at room temperature. Sections were washed and immunoreactivity was visualized by 0.6mg/ml diaminobenzidine hydrochloride (Sigma) in PBS with hydrogen peroxide. Finally, sections were washed, mounted on slides, dried overnight, dehydrated, cleared and coverslipped. Kiss1-immunoreactive neurons in the AVPV/PeN and ARC were quantified as previously described (Matsuwaki et al., 2010; Quennell et al., 2011).

Metabolic phenotype assessment. Body weight was measured weekly in a single-occupant cage with ALPHA-dri bedding. Body composition in 4 months old mice was assessed by nuclear magnetic resonance (minispec mq7.5, Bruker Optics) to determine the percentage of fat mass, as previously described (Hill et al., 2009). Glucose Tolerance Tests (GTT) and Insulin Tolerance Tests (ITT) were done as previously described (Hill et
al., 2010). Briefly, following overnight fast, tail blood glucose was measured using a mouse-specific glucometer (AlphaTRAK, Abbott) before and 15, 30, 60, 90 and 120 min after dextrose (2g/kg, i.p.) injection. For ITT, following a 4 hour fast, mice were injected with recombinant insulin (0.75U/kg, i.p.). Tail blood glucose was measured again at specified timepoints. Food intake was measured every week until 4 months old.

**Puberty and reproductive phenotype assessment.** Anogenital distance (AGD) was measured directly with a ruler from the middle of the anus to the middle of the penis. To minimize variation, AGD was measured by a single blinded observer. Balanopreputial separation was checked daily from weaning by manually retracting the prepuce with gentle pressure (Korenbrot et al., 1977). Singly housed female mice were checked daily for vaginal opening after weaning at 3 weeks of age. Vaginal lavages from female mice were collected from the day of vaginal opening for at least 3 weeks. Stages were assessed based on vaginal cytology (Bingel and Schwartz, 1969; Nelson et al., 1982): predominant cornified epithelium indicated the estrus stage, predominant nucleated cells indicated the proestrus stage, and predominant leukocytes indicated the diestrus stage. At the age of 33 days, each male mouse was paired with one wildtype female of proven fertility for 4 weeks or until the female mouse was obviously pregnant. Then the paired mice were separated and the delivery date recorded. The age of sexual maturation was estimated from the birth of the first litter minus average pregnancy duration for mice (20 days). At 4-6 months old, animals were again paired with wildtype adult breeders to collect additional data on litter size and intervals between litters.

**Hormone assays.** Submandibular blood was collected at 10:00-11:00AM. Serum estradiol was measured by ELISA (Calbiotech) with sensitivity <3pg/ml, and intraassey CV 3.1% and interassay CV 9.9%. Serum testosterone was also measured by
ELISA (Calbiotech). Serum collected after an overnight fast was used for measurement of insulin and leptin (Crystal Chem Inc.). The leptin ELISA has a sensitivity range of 0.2 to 12.8ng/ml, with both intrassay and interassay CV≤10%. The insulin ELISA kit had a sensitivity range of 50-3200pg/ml, with both intraassay and interassay CV≤10%.

*Histology.* Ovaries and testes were collected from mice and fixed immediately in 10% formalin overnight. Then tissues were embedded in paraffin and cut into 5-8μm sections which were done by the University of Toledo Pathology Department. Sections were stained by hematoxylin and eosin.

*48h fasting experiment.* Adult mice were fasted for 48h beginning at 9:00-10:00AM. Submandibular blood was withdrawn to measure baseline and fasting LH levels. Mice were also i.p. injected 3mg/kg mouse recombinant leptin (AF Parlow of National Hormone and Pituitary Program, Torrance, CA) twice a day.

*Statistical analysis.* Data are presented as the mean±SEM. Two-tailed unpaired t testing was used as the main statistical method. Mann-Whitney U test was used if the data did not assume a normal distribution. One way ANOVA was performed to compare three independent groups, followed by Bonferroni's multiple comparison test. Paired t test was employed if the data is the same sample before and after an intervention, such as leptin level before and after fast. For body weight, GTT and ITT, repeated measures ANOVA was used to compare changes over time between two genotypes. \( P<0.05 \) was considered to be statistically significant.
**Results**

**Generation and confirmation of IR/LepR\(^{Δ}\text{Kiss}\) model**

To generate mice with the IR and LepR specifically deleted in *Kiss1* neurons, we crossed LepR\(^{\text{flox/flox}}\) mice with IR\(^{Δ}\text{Kiss}\) mice carrying the Cre recombinase gene driven by the *Kiss1* promoter (Figure 3.1A). Heterozygous mice were bred to produce homozygous IR/LepR\(^{Δ}\text{Kiss}\) mice. To verify that floxed IR and LepR gene was excised in *Kiss1* neurons, PCR was performed on DNA from different tissues. As expected, a 500bp band indicating IR and LepR gene deletion was produced from the hypothalamus and other tissues expressing *Kiss1*, including cerebrum, ovary and testis (Figure 3.1B) (Ohtaki et al., 2001; Cravo et al., 2011). IR protein levels were similar between wildtype and targeted-knockout liver, muscle, visceral fat tissue, testis and ovary (Figure 3.1C). Consistent with restriction of IR inactivation to a defined subpopulation of hypothalamic neurons, western blot analysis revealed no alteration of IR expression in hypothalamus (Figure 3.1C) (Konner et al., 2007). Thus, insulin sensing appears to be intact in the majority of cells in the ovary and other tissues.
Figure 3.1. Generation of IR/LepRΔKiss mice. (A) Construct in making IR/LepRΔKiss mice. Adapted from previous publications (Balthasar et al., 2004; Brothers et al., 2010; Cravo et al., 2011). (B) PCR of DNA from different tissues. The exercised LepR and IR both appear as a 500bp band, whereas unexercised LepR shown as 1kb band and unexercised IR as a 2.2kb band. (C) Representative IRβ expression in different tissues and densitometry.

IR/LepRΔKiss mice have normal metabolism

Due to the potential interaction between Kiss1 neurons and metabolic circuits in the hypothalamus, we examined the metabolic phenotype of IR/LepRΔKiss mice. The body
weight of both females and males showed no significant difference between control and knockout mice (Figure 3.2A, B). Average daily food intake was comparable between two groups in 2-3 months old in both sexes (Figure 3.2C, D). Fat mass, lean mass and fat percentages were comparable in both females and males (Figure 3.2E, F). Overnight fasting serum leptin was not statistically different between control and IR/LepR<sup>ΔKiss</sup> mice in both sexes, further proving that adipose tissue in knockout mice were comparable to control. Overnight fasting serum insulin was not statistically different between control and IR/LepR<sup>ΔKiss</sup> mice in both sexes (Table 3.2), suggesting that β-cell insulin secretion is normal in these mice. Furthermore, Glucose tolerance and insulin tolerance were normal in both sexes of IR<sup>ΔKiss</sup> mice (Figure 3.2G-J).

![Graphs and images showing data comparison between control and IR/LepR<sup>ΔKiss</sup> mice.](image-url)
weight of male mice (n=16 each group). (C) Female average daily food intake calculated from weekly measurement. (D) Male average daily food intake calculated from weekly measurement. (E) Female body composition at the age of 4 months in control (n=13) and IR/LepR\(\Delta\)Kiss (n=7) mice. (F) Male body composition at the age of 4 months in control (n=15) and IR/LepR\(\Delta\)Kiss (n=12) mice. (G) 4-5 months old female GTT and AUC (inset) in control (n=11) and IR/LepR\(\Delta\)Kiss (n=5) mice. (H) 4-5 months old male GTT and AUC (inset) in control (n=10) and IR/LepR\(\Delta\)Kiss (n=8) mice. (I) 5-6 months old female ITT and AUC (inset) in control and IR/LepR\(\Delta\)Kiss mice (n=7). (J) 5-6 months old male ITT and AUC (inset) in control (n=11) and IR/LepR\(\Delta\)Kiss (n=7) mice. AUC, area under curve.

Table 3.2. Overnight fast serum insulin and leptin.

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Control</th>
<th>IR/LepR(\Delta)Kiss</th>
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<tbody>
<tr>
<td>Serum insulin</td>
<td>Female</td>
<td>354.3 ± 66.6 n=14</td>
<td>287.8 ± 60.7 n=8</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td>Male</td>
<td>381.8 ± 92.2 n=18</td>
<td>600.0 ± 137.8 n=9</td>
</tr>
<tr>
<td>Serum leptin</td>
<td>Female</td>
<td>2.6 ± 1.1 n=9</td>
<td>1.4 ± 0.7 n=8</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>Male</td>
<td>1.4 ± 0.3 n=11</td>
<td>1.3 ± 0.2 n=9</td>
</tr>
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Normal puberty and fertility in females

Female IR/LepR\(\Delta\)Kiss mice did not show significant difference in the age of vaginal opening, first estrus and age of sexual maturation (Figure 3.3A-C). Estradiol was comparable between the two groups in adulthood (Figure 3.4A). Female IR/LepR\(\Delta\)Kiss mice had similar ovary weight (Figure 3.4B). Female knockout mice also had normal estrous cyclicity, characterized by normal estrous length and staging (Figure 3.4C). Ovarian morphology showed follicles at all stages of maturation and comparable number of corpora lutea between control and knockout mice (Figure 3.4D). At 5-6 months old, mice were paired with established wild-type male breeders. The latency to birth was comparable between the two groups, whereas litter size of IR/LepR\(\Delta\)Kiss females is slightly larger than that of controls (Figure 3.4E).
Figure 3.3. Normal puberty in female IR/LepRΔKiss mice. (A) Vaginal opening was evaluated in control (n=17) and IR/LepRΔKiss (n=8) mice. (B) First estrous was evaluated in control (n=17) and IR/LepRΔKiss (n=7) mice. (C) Estimated age of sexual maturation calculated by subtraction of 20 days from the time required for delivery of a litter (Control, n=5; IR/LepRΔKiss, n=4).

Figure 3.4. Normal female reproduction in adult IR/LepRΔKiss mice. (A) Estradiol in adult females in control (n=9) and IR/LepRΔKiss (n=6) mice. (B) Ovarian mass in adult females in control (n=9) and IR/LepRΔKiss (n=6) mice. (C) Estrous cycle analysis (Control, n=6; IR/LepRΔKiss, n=5) and estrous cycle length (Control, n=6; IR/LepRΔKiss, n=5) in 3 months old females. (D) Representative light photomicrographs of an adult IR/LepRΔKiss mouse ovary (n=4). CL, corpora lutea. Scale bar, 100μm. (E) Fertility data from 5-6 months old females paired with established male breeders. Interval from mating to birth of a litter and litter size were compared between control (n=12) and IR/LepRΔKiss (n=6) mice. * P<0.05.
Normal puberty and fertility in males

Male IR/LepRΔKiss mice show comparable anogenital distance growth curve, age of balanopreputial separation and age of sexual maturation (Figure 3.5A-C). In adults, testosterone was not statistically different between two groups (Figure 3.6A). Furthermore, the testis weight was normal in adult IR/LepRΔKiss mice (Figure 3.6B). Testis histology showed all stages of spermatogenesis in seminiferous tubules and normal interstitial Leydig cells in IR/LepRΔKiss mice (Figure 3.6C). Fertility at 5-6 months old, characterized by litter size and days required to impregnate a female, was not different between control and IR/LepRΔKiss males (Figure 3.6D).

Figure 3.5. Normal male puberty in IR/LepRΔKiss mice. (A) AGD growth curve (Control, n=10; IR/LepRΔKiss, n=8). (B) Normal balanopreputial separation age (Control, n=10; IR/LepRΔKiss, n=3). (C) Estimated age of sexual maturation calculated by subtraction of 20 days from the time required for delivery of a litter (n=4).
Figure 3.6. Normal male reproduction in adult IR/LepR\(^{\Delta \text{Kiss}}\) mice. (A) Testosterone in adult males in control (n=9) and IR/LepR\(^{\Delta \text{Kiss}}\) (n=8) mice. (B) Testis mass in control (n=9) and IR/LepR\(^{\Delta \text{Kiss}}\) (n=8) mice. (C) Representative sections of adult testis in IR/LepR\(^{\Delta \text{Kiss}}\) mice (n=4). Scale bar, 100\(\mu\text{m}\). (D) Fertility data from 5-6 months old males paired with established female breeders. Interval from mating to birth of a litter and litter size were compared between control (n=12) and IR/LepR\(^{\Delta \text{Kiss}}\) (n=11) mice.

**Normal Kiss1 expression in adults**

Normal patterns of kisspeptin staining were seen in both AVPV/PeN and ARC in adult mice in both sexes. (Figure 3.7A, B). We counted the Kiss1 neuron number in the AVPV/PeN and measured the Kiss1 immunoreactive areas in the ARC from adult mice. There was no difference in either of them after quantification (Figure 3.7C, D).
Figure 3.7. Normal pattern of Kiss1 cell number in adult IR/LepRΔKiss females. (A) and (B) Representative Kiss1 immunostaining micrograph in the PeN and ARC in a female IR/LepRΔKiss mouse. (C) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in adult females (Control, n=2; IR/LepRΔKiss, n=4). (D) Quantification of Kiss1 cell number in the AVPV/PeN and Kiss1 immunoreactive area in the ARC in adult males (Control, n=2; IR/LepRΔKiss, n=3). AVPV, anteroventral periventricular nucleus; PeN, periventricular nucleus; ARC, arcuate nucleus.

IR/LepRΔKiss mice have normal response to negative energy balance

In order to study whether both insulin and leptin action in Kiss1 neurons is a required signal of energy balance to the reproductive axis in adulthood, we induced a negative energy balance by fasting mice for 48h. Leptin was dramatically decreased in control animals even treated with leptin. IR/LepRΔKiss female mice showed a similar drop in leptin (Figure 3.8), suggesting the HPG axis was still able to perceive the negative
energy balance. Insulin and leptin may not be essential in transmission of negative energy balance to HPG axis through Kiss1 neurons in hypothalamus.

![Graph](image)

Figure 3.8. Normal response to 48h fast in IR/LepRΔKiss mice even treat with leptin. Serum leptin level was measured before and after 48h fast (Control, n=8; IR/LepRΔKiss, n=7). *P<0.05.

**Discussion**

Leptin and insulin signaling overlaps in many tissues (Niswender and Schwartz, 2003; Carvalheira et al., 2005). In the hepatocyte, an important site for insulin action, it was found that leptin can enhance the insulin induced association of IRS-1 or IRS2 with PI3K (Cohen et al., 1996; Szanto and Kahn, 2000; Anderwald et al., 2002). A similar situation was found in pancreatic β-cells. Leptin mimicked insulin to promote glucose uptake and glycogen synthesis via JAK activation of IRS-2 and PI3K (Kellerer et al., 1997). Using immortalized hypothalamic cell models, a recent study shows that insulin
resistance disrupts leptin-mediated control of neural signaling and transcription, highlighting the complex cellular cross-talk between insulin and leptin in development of neural insulin resistance and leptin resistance (Nazarians-Armavil et al., 2013). These in vitro studies show that leptin and insulin may cross-talk through IRS phosphorylation by JAK.

The same observation is seen with in vivo studies. Leptin was reported to be capable of activating IRS-PI3K pathways in adipose tissue and liver, albeit to a lesser extent than insulin. Likewise, intravenous insulin infusion in rodents was able to activate STAT molecules to differing degrees in several tissues (Kim et al., 2000). Thus, the ability of leptin to participate, perhaps synergistically, with insulin in the activation of classical insulin targets such as PI3K might explain the beneficial effects exerted by leptin on glucose homeostasis. Furthermore, overlapping effects of insulin and leptin on the CNS pathways that control glucose and energy homeostasis might explain the similarities in their actions in vivo (Niswender and Schwartz, 2003).

The cross-talk between leptin and insulin permits compensation to maintain normal homeostasis and exert anorexigenic actions in the hypothalamus. For example, leptin receptor knockout mice in POMC neurons show only mild obesity and mild effects on glucose homeostasis in males only (Balthasar et al., 2004; Shi et al., 2008). In mice lacking IR in POMC neurons, no discernable impact on body weight or glucose regulation was seen (Konner et al., 2007). However, POMC neuron-specific insulin receptor and leptin receptor double knockout mice show abnormal glucose metabolism and sub-fertility (Hill et al., 2010). Evidence from in vivo studies has further shown that cross-talk between insulin and leptin signaling at various levels of IRS-PI3K pathway
may be the underlying mechanisms of regulation of metabolism and reproduction (Duan et al., 2004; Morton et al., 2005; Hill et al., 2008b; Konner and Bruning, 2012). In leptin receptor deficient mice, disrupted leptin signaling caused hypothalamic insulin resistance (Koch et al., 2010).

Mice with deletion of LepR from Kiss1 neurons have normal puberty and fertility (Donato et al., 2011a). Our mice with IR and LepR double knockout from Kiss1 neurons also have normal puberty and fertility, which corrects the delay of puberty in IR knockout in Kiss1 neurons as previously described (Qiu et al., 2013). This finding raises the possibility that leptin and insulin overlapping signaling may be antagonistic. A previous study in white adipose tissue has shown that insulin signaling is blunted after in vivo treatment with leptin both directly and centrally (Perez et al., 2004). Perez and colleagues further characterized the downstream signaling of insulin to MAPK and GSK3β, which were decreased after leptin treatment. Additionally, an increase of suppressor of cytokine signaling-3 protein was also observed after leptin treatment, which was well recognized as a brake of insulin signaling. Therefore the authors concluded that leptin can modulate insulin signaling in an inhibitory manner in adipocytes by autocrine signal or through neuroendocrine pathways (Perez et al., 2004). In addition, chronic exposure to leptin centrally inhibits insulin signaling at the level of interaction of IR with IRS2 and activates PI3K by promoting JAK2-IRS2 association. Chronic central leptin treatment also increases SOCS3 association with IR (Burgos-Ramos et al., 2011). Therefore, it is possible that insulin and leptin in Kiss1 neurons act similarly.

Further studies are needed to address the antagonism between insulin and leptin. Using an electrophysiological approach to see how insulin and leptin act in the
electrophysiology of Kiss1 neurons may provide direct evidence for such antagonism. Electrophysiological recording of Kiss1 neurons has indicated that Kiss1 neurons in ARC have sex difference in their mean firing rates. 90% of Kiss1 neurons in males exhibited slow irregular firing whereas neurons from diestrus and ovariectomized mice were silent. By contrast, AVPV/PeN Kiss1 neurons were all spontaneously active, exhibiting tonic, irregular, and bursting firing patterns (de Croft et al., 2012). Furthermore, a recent study shows that Kiss1 neurons in both the AVPV/PeN and ARC have two neuronal populations defined as type I (irregular firing patterns) and type II (quiescent) (Frazao et al., 2013). AVPV/PeN exhibit a bimodal resting membrane potential influenced by K_{ATP} channels, whereas Kiss1 neurons in the ARC do not follow a bimodal distribution. However, this study showed that Kiss1 neuronal activity in the AVPV/PeN, but not in the ARC, is sexually dimorphic, which is different from a previous study (Frazao et al., 2013). These electrophysiological studies pave the way to define direct effects of insulin and leptin in the single Kiss1 neuron.

Another possibility is that the cross-talk between leptin and insulin occurs within a network of cells rather than within individual Kiss1 neurons, which is similar to POMC neurons (Williams et al., 2010). Acute leptin treatment results in a depolarization and simultaneous increase in the firing rate of a subpopulation of POMC neurons in the ARC. In contrast to leptin, insulin inhibits the activity of some POMC neurons in the ARC. Williams and his colleagues characterized the electrophysiological property of insulin and leptin responsive arcuate POMC neurons. They found that leptin-induced c-fos activity within arcuate POMC neurons did not overlap with POMC neurons that express the insulin receptor. Moreover, acute responses to leptin and insulin were segregated in
distinct subpopulations of POMC neurons. Hence, their data suggest that crosstalk between leptin and insulin occurs within a network of cells rather than within individual POMC neurons (Williams et al., 2010). Likewise, it is possible that crosstalk between leptin and insulin occurs within a network of Kiss1 neurons rather than a single neuron.

Additionally, the newly developed embryonic mouse hypothalamic immortalized cell line like CLU116 (N36-1) expressing kisspeptin, IR and LepR can be used as Kiss1 neuron model to further study the potential mechanisms underlying the antagonistic interacting signaling. Such immortalized mice or rat hypothalamic cell lines may prove a good model for studying the molecular signaling in neurons (Dalvi et al., 2011). Compared with primary cell culture and animal models, immortalized cell lines are easier to culture and maintain, and less expensive. Furthermore, the immortalized cell line offers a controlled physiochemical environment and allows for quick and simple experiments. For example, rHypoE-19 and mHypoA-2/10 have been used to study the overlapping signaling between insulin and leptin (Nazarians-Armavil et al., 2013). mHypoE-38 and mHypoE-42 are putative NPY-secreting cell lines (Kim et al., 2010). The N6 cell line has been used as a model of Kiss1 neurons, although expression level of Kiss1 is low (Luque et al., 2007). The immortalized rHypoE-8 cell line has been used as a model of Kiss1 neurons as well. Upon treatment with melatonin, they found that Kiss1 gene expression was decreased (Gingerich et al., 2009). We are employing the immortalized embryonic mouse hypothalamic cell line CLU116 (N-36/1) that expresses Kiss1 (although expression level is very low from preliminary experiments) and insulin receptor. Using this model, future work can examine the overlapping signaling pathways through which the insulin and leptin act upon Kiss1 neurons. However, the physiological
relevance of the above findings must be interpreted with caution, because CLU116 cells appear to jointly express *Kiss1*, GPR54, POMC and NPY genes. Moreover, as is the case with other immortalized cell lines used for the analysis of neuroendocrine mechanisms controlling reproductive function, some regulatory mechanisms may not be totally similar between CLU116 cell line and hypothalamic *Kiss1* neurons (Luque et al., 2007). It is possible during the immortalization process, the neuron physiology may change. Immortalized cells have inherent limitations due to the absence of the complex architecture and afferent cellular connections present in the intact brain (Dalvi et al., 2011).

Finally, additional *in vivo* experiments can be used to follow up on these findings. For instance, *in vivo* insulin and leptin can be centrally or peripherally injected to see how they influence *Kiss1* expression. In addition, animal models with insulin and leptin downstream signaling molecules knockout in *Kiss1* neurons will provide direct and compelling evidence to the overlapping signaling underlying the antagonistic interaction between insulin and leptin. For example, *Kiss1* neuron specific IRS2, p110 subunit of PI3K, or Akt knockout model which has not been done will further clarify the complicated overlapping signaling network.
Chapter 4

Discussion and summary

Pubertal onset only occurs in a favorable, anabolic hormonal environment. The neuropeptide kisspeptin, encoded by the *Kiss1* gene, modifies GnRH neuronal activity to initiate puberty and maintain fertility, but the factors that regulate *Kiss1* neurons and permit pubertal maturation remain to be clarified. The anabolic factor insulin may signal nutritional status to these neurons. To determine whether insulin sensing plays an important role in *Kiss1* neuron function, we generated IR$^{\Delta}$Kiss mice. IR$^{\Delta}$Kiss females showed a delay in vaginal opening and in first estrus, whereas IR$^{\Delta}$Kiss males also exhibited late sexual maturation. Adult reproductive capacity, body weight, fat composition, food intake, and glucose regulation were comparable between the two groups. These data suggest that impaired insulin sensing by *Kiss1* neurons delays the initiation of puberty but does not affect adult fertility. These studies provide insight into the mechanisms regulating pubertal timing in anabolic states (Qiu et al., 2013).

Mice with both insulin and leptin receptor knockout in *Kiss1* neurons showed normal puberty, metabolism and reproduction in both sexes. The correction of pubertal delay of insulin receptor knockout mice may be due to antagonistic interactions between insulin signaling and leptin signaling. This issue needs further clarification through cell study and electrophysiology.
Although one study demonstrated that up to 40% of Kiss1 neurons in the ARC in mice appear to have functional leptin receptors (Smith et al., 2006), recent studies challenged this finding by contending that leptin receptors were rarely colocalized with Kiss1 cell (Donato et al., 2011b; Louis et al., 2011). Cravo and his colleagues recently use the Cre-loxP system to re-express endogenous LepR selectively in kisspeptin cells of mice otherwise null for LepR. Kiss1-Cre LepR null mice showed no pubertal development and no improvement of the metabolic phenotype, remaining obese, diabetic and infertile. They further found that no coexpression of Kiss1 and LepR was observed in prepubertal mice (Cravo et al., 2013). Therefore, it is possible that LepR is expressed after sexual maturation under the regulation of other hormones or neurotransmitters. This mechanism could explain why leptin treatment can significantly raise Kiss1 expression via the Crtc1 or mTOR signaling pathway in adults.

Last but not least, Kiss1 neuron metabolic sensing may include other metabolic factors, like ghrelin, IGF-1, NPY, melanocortin, and melanin-concentrating hormone (MCH), etc. Ghrelin, an orexigenic hormone secreted by gastric mucosa, has recently been shown to reduce the Kiss1 mRNA expression in female rat hypothalamus (Forbes et al., 2009). Interestingly, ghrelin has effects that are opposite to those of leptin, thus functioning as signal for energy insufficiency (Tena-Sempere, 2008). Thus, ghrelin is a good candidate for a negative modifier of puberty onset and/or gonadotropin secretion in a variety of species (including rodents, sheep, monkey, and human), acting mainly at central levels (Fernandez-Fernandez et al., 2005; Tena-Sempere, 2008). IGF-1 was reported to activate Kiss1 gene expression in the brain of prepubertal female rat (Hiney et al., 2009), suggesting IGF-1 may be a positive modifier of puberty through its interaction
with Kiss1 neuron. Other potential metabolic regulators of the Kiss1 system are NPY and melanocortins. NPY, may function as stimulatory factor for Kiss1 expression, since Kiss1 mRNA levels are decreased in the hypothalamus of NPY KO mice, whereas NPY enhances Kiss1 mRNA expression in the hypothalamic cell line, N6 (Luque et al., 2007). While NPY expression is increased following conditions of negative energy balance, Kiss1 and the gonadal axis appear to be suppressed, indicating an inhibitory effects of NPY on GnRH secretion (Xu et al., 2009). So it is not clear how NPY regulates Kiss1 neurons in different conditions. In addition, melanocortins, which are anorectic neuropeptide products of POMC neurons in the ARC, have been suggested to stimulate hypothalamic Kiss1 gene expression at the preoptic area in the sheep (Backholer et al., 2009). In contrast, the orexigenic neuropeptide melanin-concentrating hormone (MCH), which is prominently expressed in the lateral hypothalamus, suppressed kisspeptin-induced stimulation of GnRH neurons (Wu et al., 2009). Yet, effects of MCH on Kiss1 expression have not been reported to date. Other metabolic factors like galanin, hypocretin, orexin, corticotrophin-releasing hormone, and glucagon-like peptide may also be involved in the Kiss1 neuron sensing, and await for further research.

In sum, insulin and leptin do not regulate metabolism through Kiss1 neurons. Insulin sensing by Kiss1 neurons plays a positive permissive role in puberty, but is not required for adult fertility. Leptin may counteract the effect of insulin in Kiss1 neuron. Further study will be required to characterize the mechanism underlying the antagonistic interaction between insulin and leptin.
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