Identification and Characterization of Leptin Responsive Neurons Involved in the Metabolic Control of Reproduction

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Abstract

Reproductive function is an adaptive system that is critical for the survival of all species. In vertebrates, fertility (the ability to reproduce) is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. This axis is centrally driven by the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. There are many endogenous and exogenous factors that can influence the HPG axis; here I focus on metabolic regulation of fertility.

The adipokine leptin, primarily produced by adipose tissue, circulates throughout the body in concentrations relative to the amount of energy stored in the adipose tissue. Leptin plays a critical role in the hypothalamic control of energy balance, but also has profound effects on central regulation of reproduction. The effect of leptin on the HPG axis is indirect, as GnRH neurons do not express leptin receptor (Quennell et al., 2009). Therefore it is assumed that intermediate leptin responsive neurons must exist that provides leptin-to-GnRH signaling. Trying to elucidate this neuronal pathway has been a challenge to many researchers in the field over the past years, as Lepr neurons exhibit a widespread and heterogynous distribution throughout the brain.

Here, I investigated different neuronal pathways and neurotransmitters that might relay the signal of leptin onto the HPG axis. I used transgenic mouse models to visualize Lepr neurons, and combined that with retrograde tract tracing to identify leptin responsive inputs to the region of GnRH neurons. Additionally, conditional deletion of the receptors for leptin from neuronal nitric oxide synthase (nNOS), glutamate, and γ-aminobutyric acid (GABA) neuron populations was used to assess the effects on reproductive function. With these experiments I investigated the role of these neurons and signaling molecules in mediating leptin-to-GnRH signaling.

From these studies I found that Lepr neurons, particularly from the arcuate nucleus of the hypothalamus (Arc), provide neuronal input to the region of
the GnRH neurons. Recent literature shows that leptin signaling in nNOS neurons might play a role in the metabolic regulation of fertility, however my attempt to conditionally delete Lepr from nNOS neurons was unsuccessful, presumably due to an incompatibility of the Cre and LoxP genes used. I found that leptin signaling in glutamate neurons is not critical for reproductive functioning. Most interestingly I found that GABA neuron specific Lepr knockout caused significant disruptions in reproductive functioning. Both females and males displayed delayed puberty onset. Observations of various adult fertility parameters revealed that these knockout animals have decreased fecundity and females had disordered estrous cycles. These experiments showed that leptin signaling in GABAergic neurons plays a critical role in the metabolic regulation of fertility. These exciting results were followed up by measuring changes in hypothalamic gene expression between control and GABA specific Lepr knockout females. The GABA specific Lepr knockout females showed a significant increase of Agrp gene expression in the Arc, and Npy gene expression in the LHA. These results will focus future research (including Lepr knockout or rescue experiments) to GABAergic leptin receptor-expressing AgRP neurons in Arc.

The experiments presented in this thesis form an important step in explaining the mechanisms by which central leptin signaling modulates the reproductive system. This work helps to narrow down the location and possible identity of mediators of leptin-to-GnRH signaling. Future research now, should be more focused on the GABAergic Lepr cells.
Publications

Peer-reviewed journal articles (see Appendix III for full articles)


Conference contributions

Zuure WA, Quennell JH, Anderson GM, A novel transgenic mouse model to study leptin responsive neurons in the regulation of fertility. Presented at the Medical Sciences Congress (MedSci) in Queenstown, New Zealand; August 2012

Zuure WA, Roberts AL, Quennell JH, Anderson GM, Leptin Action on γ-Aminobutyric Acid (GABA), not Glutamate Neurons is Required for Normal Reproductive Development. Presented orally at the Endocrine Society’s 95th annual meeting (ENDO, 2013) in San Francisco, USA. Won an outstanding abstract award and was selected for oral presentation.

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though it was a bit rough at the start; this part of our lives has turned out to be pretty amazing. I am so excited and ready for our next adventure together.
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<tbody>
<tr>
<td>18S</td>
<td>18S ribosomal RNA</td>
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<tr>
<td>3V</td>
<td>third ventricle</td>
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<tr>
<td>Abat</td>
<td>GABA transaminase</td>
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<tr>
<td>ABC</td>
<td>avidin-biotin peroxidase solution</td>
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<tr>
<td>Actb</td>
<td>beta-actin</td>
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<td>AgRP</td>
<td>agouti-related peptide</td>
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<td>AKT</td>
<td>protein kinase B</td>
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<td>Arc</td>
<td>arcuate nucleus of the hypothalamus</td>
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<td>AVPV</td>
<td>anteroventral periventricular nucleus</td>
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<tr>
<td>B2m</td>
<td>beta2 micro globulin</td>
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<tr>
<td>BIG</td>
<td>BL-IRES-GFP</td>
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<tr>
<td>BL</td>
<td>barley lectin</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CART</td>
<td>cocaine- and amphetamine regulated transcript</td>
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<tr>
<td>cDNA</td>
<td>copy DNA</td>
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<tr>
<td>ChT</td>
<td>chloramine T</td>
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<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
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<tr>
<td>ctx</td>
<td>cortex</td>
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<tr>
<td>D</td>
<td>diestrus</td>
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<tr>
<td>D3V</td>
<td>dorsal third ventricle</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td>db</td>
<td>diabetic gene</td>
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<td>DMN</td>
<td>dorsomedial nucleus of the hypothalamus</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>DSP</td>
<td>daily sperm production</td>
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<tr>
<td>E</td>
<td>estrus</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>eYFP</td>
<td>enhanced yellow fluorescent protein</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>L-glutamic acid decarboxylase</td>
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<td>GALP</td>
<td>galanin-like peptide</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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HDB  nucleus of the horizontal limb of the diagonal band
HPG axis  hypothalamic-pituitary-gonadal axis
HRP  horseradish peroxidase
hypo  hypothalamus
I-  Iodine
icv  intracerebroventricular
IHC  immunohistochemistry
iNOS  inducible nitric oxide synthase
ip  intraperitoneal
IRES  internal ribosomal entry site
IRS  insulin-receptor substrate
JAK-STAT  Janus kinase signal transducers and activators of transcription
Kiss1  kisspeptin
Lepr  leptin receptor
LH  luteinizing hormone
LHA  lateral hypothalamic area
LPO  lateral preoptic area
M  metestrus
MBH  mediobasal hypothalamus
MC  melanocortin
MCR4  melanocortin receptor 4
ME  median eminence
MePO  medial POA
MnPO  median preoptic area
mPOA  medial preoptic area
mRNA  messenger RNA
MS  medial septum
NBS  non specific binding
NDS  normal donkey serum
NGS  normal goat serum
NiDAB  nickel-enhanced DAB
nNOS  neuronal nitric oxide synthase
NO  nitric oxide
NOLEPKO  NO neuron specific Lepr knockout animals
NPY  neuropeptide Y
NTS  nucleus of the solitary tract
ob  obese gene
OPD  O-phenylenediamide
OVLT  vascular organ of the lamina terminalis
OVX  ovariectomized
ox  optic chiasm
P proestrus
PB phosphate buffer
PBS phosphate buffered saline
PCOS polycystic ovary syndrome
Pe periventricular hypothalamic nucleus
PEG polyethylene glycol
PFA paraformaldehyde
PH posterior hypothalamus
PI3K PI 3-kinase
PMV ventral premammillary nucleus
POA preoptic area
POMC proopiomelanocortin
PRV pseudo rabies virus
pSTAT3 phosphorylated STAT3
PVN paraventricular nucleus
PVpo periventricular nucleus, preoptic and anterior divisions
qPCR quantitative polymerase chain reaction
RFRP-3 RF-amide related peptide 3
RIA radioimmunoassay
RNA ribonucleic acid
RP3V rostral periventricular region of the third ventricle
RPII RNA polymerase II polypeptide A
rPOA rostral preoptic area
sc subcutaneous
SEM standard error of the mean
SOCS3 suppressor of cytokine signaling 3
SOX supra optic nucleus
STAT3 signal transducers and activator of transcription 3
Tac2 tachykinin 2
tamoxifen 4-hydroxytamoxifen
TBS tris buffered saline
TBS-TX TBS Triton X-100
TC total counts
VDB nucleus of the vertical limb of the diagonal band
Vgat vesicular GABA transporter
Vglut2 vesicular glutamate transporter
VMN ventromedial nucleus of the hypothalamus
WGA wheat germ agglutinin
αMSH alpha-melanocyte stimulating hormone
Chapter One
General Introduction and Literature Review
1.1 Introduction

The ability to adapt reproductive functioning to a constantly changing environment is an evolutionary capacity that all animals must be able to exploit for their survival. In vertebrates the central nervous system acts as an integrator of exogenous and endogenous stimuli and responds to changes in the environment, maintaining internal homeostasis. Reproductive success or fertility is sensitive to environmental challenges, for it would be unwise to spend valuable energy on creating offspring when circumstances governing survival are not optimal. Therefore, fertility is regulated by an adaptive system: the hypothalamic-pituitary-gonadal (HPG) axis. This axis can be attenuated by many different signals, for example; daylight length in different seasons, nutritionally regulated hormones, and physical or mental stress (Elias and Purohit, 2012; Evans and Anderson, 2012).

The research presented in this thesis aims to better understand the hypothalamic regulation of fertility by the adipocyte-derived hormone leptin. Different neuronal pathways and neurochemical signaling molecules that might allow this interaction are investigated. The experiments in this thesis make use of two different transgenic mouse models: the first model visualizes leptin responsive cells in the brain by labeling them with specific markers. The second model conditionally deletes the receptors for leptin from specific neuronal populations. These approaches complement each other by making it possible to study both anatomical and functional aspects of the molecular pathways linking fertility and leptin signaling within the hypothalamus of the murine brain.

1.2 Hypothalamic-pituitary-gonadal axis

Reproduction is the key to survival of all species, and therefore vital to sustain all life on earth even if it does not directly affect the survival of an individual. The hypothalamus acts as an important integrator of various signals from the internal and external environment, adapting where needed to maintain homeostasis. Some hormones from the periphery are able to
cross the blood brain barrier and bind to their receptors in the hypothalamus. Conversely, the hypothalamus communicates with the periphery by releasing neuropeptides into the portal vasculature or by release from the neuropituitary gland: i.e. the posterior pituitary gland (as described in chapter 27 of Marieb and Hoehn, 2007). Fertility is regulated by a neuroendocrine axis that has different compartments throughout the body; the hypothalamus in the ventral part of the brain, the pituitary gland found just below the brain, and the distal gonads. These organs and organ parts collectively make up the hypothalamic-pituitary-gonadal (HPG) axis (Fig 1.1). Situated within the hypothalamus are the gonadotropin releasing hormone (GnRH) neurons, which project to the vascular contact zone in the median eminence. The position of GnRH cell bodies varies amongst mammals (King and Anthony, 1984; Silverman et al., 1994). Steroids and peptides from the periphery form a modulating signal for GnRH release at the GnRH neuron terminals in the median eminence, at the base of the hypothalamus. Pulsatile release of GnRH into the hypophyseal portal system induces the pulsatile secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary. These gonadotropins, LH and FSH, in turn promote fertility and sex steroid production in the gonads (testes and ovaries). Steroids, including estradiol and testosterone, form a negative feedback loop with the hypothalamus and pituitary (review by Genazzani et al., 1992). Regulation of fertility can take place at all three levels of the HPG axis: in the GnRH neurons in the rPOA, in the gonadotropin producing cells of the anterior pituitary, and/or in the steroid producing cells of the gonads (Freeman, 2006).

1.2.1 Hypothalamus: Gonadotropin releasing hormone

The GnRH cell bodies can be found in a scattered pattern extending from the olfactory bulbs to the medial septal nucleus, diagonal band of Broca and medial preoptic area (mPOA) through to the mediobasal hypothalamus (MBH). In the rodent brain the distribution is often referred to as an inverted Y, in which GnRH soma are restricted to a continuum extending from the
medial septum through to bilateral ‘arms’ in the ventrolateral hypothalamus (Herbison, 2006). Of these GnRH neurons, about 60% project to the median eminence, whereupon GnRH is released into the portal blood system. Cytoplasmic GnRH peptide concentration is kept at a very high level in the neurons, indicating a regulatory system at the level of release rather than GnRH biosynthesis (Maurer, 1999). A network of steroid (and other hormone) sensitive neurons, provide input to the GnRH neurons to regulate release and therefore fertility (Herbison, 2006; Wintermantel et al., 2006). In females, the output of these neuroendocrine neurons changes according to the circulating estradiol concentration during different phases of the estrous cycle. The GnRH neurons can switch from a regular pulsatile pattern to a continuous release of high GnRH output (the preovulatory GnRH surge) at the middle of the female cycle. This event triggers the LH surge released from the pituitary and ovulation of the oocyte from the ovaries (reviewed by Herbison, 2008).

1.2.2 Pituitary: Gonadotropins

After GnRH is released into the portal circulation it is transported to the anterior pituitary gonadotroph cells via the pituitary portal blood vessels. These cells produce both FSH and LH and express the G-protein coupled receptor for GnRH. Binding of GnRH to the GnRH receptor causes phosphorylation of intracellular signaling molecules and upregulation of the gonadotropin subunits (i.e. LHβ, FSHβ and the common α subunits) biosynthesis and secretion (Knobil and Neill, 2006). Sex steroids (testosterone, estrogens and progesterone) exert direct feedback to the gonadotroph cells, which express estrogen, progesterone, and androgen receptors (Stefaneanu, 1997). Both LH and FSH stimulate spermatogenesis or oogenesis in the gonads. Androgens and estrogens are produced as a result of gametogenesis and form an inhibitory input to the gonadotrophes (Fig 1.1).
Figure 1.1  An overview of the hypothalamic pituitary gonadal axis (adapted from: Saner-Amigh and Halvorson, 2010). The hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the gonadotrophes in the pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). The anterior pituitary released LH and FSH travels down throughout the body to effect the gonad's steroid and gamete production and maturation. The resulting sex steroids, testosterone in males, and estrogens and progesterone in females feed back to the upstream components of the axis.

1.2.3 Gonads: Steroid hormone production and feedback

In males the main steroid hormone that influences hypothalamic and pituitary function is testosterone, produced by the Leydig cells of the testes. Luteinizing hormone stimulates testosterone production, which in turn provides negative feedback to both the hypothalamus and the pituitary,
lowering GnRH and LH release. Follicle stimulation hormone stimulates inhibin production by the Sertoli cells in the testes. Inhibin negatively regulates the release of FSH from the pituitary gonadotrophes (Childs, 2006).

In females there is a combined effect from estrogens and progesterone, which form a feedback loop within the HPG axis. Progesterone (produced by theca cells of the developing follicle and by the corpus luteum after ovulation) and estradiol (produced by granulosa cells) provide negative feedback, which lowers GnRH and LH pulse frequency (Sullivan and Moenter, 2005). Feedback from granulosa cells of the developing follicles on FSH release occurs via inhibin, as in males. After ovulation, high levels of progesterone and moderate levels of estradiol produced by the corpus luteum potently inhibit GnRH and LH/FSH release. When the corpus luteum undergoes luteolysis (in the late luteal phase) progesterone and estrogen production is lowered and LH/FSH levels rise, permitting the maturation of new follicles. The granulosa cells surrounding the growing follicle increase the release of estrogens, which in the absence of a high progesterone concentration only mildly inhibits LH and FSH release. In later stages of the follicular phase, estradiol levels reach a certain threshold and cause a shift in the feedback effects. Firstly, a marked suppression of pulsatile GnRH and LH secretion occurs, this is then followed by a switch to a robust high amplitude preovulatory GnRH surge accompanied by the LH surge (Christian and Moenter, 2010). The exact mechanism on how estradiol changes from suppressive to highly stimulatory is still relatively unclear, however recent studies show that kisspeptin plays a major role (Smith et al., 2006b). Kisspeptin produced by neurons within the hypothalamus is the most potent stimulator of GnRH release, and mice without kisspeptin or its receptor are completely infertile (Messager et al., 2005; Clarkson et al., 2008). Kisspeptin neurons reside in two distinct hypothalamic populations, the rostral periventricular region of the third ventricle (RP3V) and arcuate nucleus (Arc). Experiments have shown that the RP3V kisspeptin population is involved in the positive feedback of estradiol at the time of the preovulatory
GnRH/LH surge (Smith et al., 2005; Smith et al., 2006b; Herbison, 2008), whereas the Arc population is thought to be involved in the regulation of negative feedback by estradiol (Smith et al., 2005; Glidewell-Kenney et al., 2007; Navarro et al., 2009).

1.3 Energy homeostasis

To maintain energy homeostasis caloric intake must be matched to energy output. Caloric intake solely comes from food intake and energy output is the sum of resting metabolic rate and other energetic activities (e.g. exercise or foraging). Energy homeostasis is primarily regulated by the hypothalamus and is fundamental for the survival of the organism. An early study by Heterington and Ranson (1940) showed that the hypothalamus is key in energy homeostasis of rats, because when lesioned they rapidly became obese. This indicates that the hypothalamus must contain centers that regulate body weight, and under normal conditions these suppress caloric intake and/or stimulate energy output by the organism.

The hypothalamus monitors energy stores in the body via hormonal messengers that are produced in the periphery yet signal centrally. Important examples of energy storage hormones include insulin and leptin. Insulin, produced by the pancreas in response to food consumption, can act in the brain to acutely reduce energy intake. Obese individuals produce higher amounts of insulin, often leading to insulin resistance (i.e. type II diabetes). The hormone leptin, produced by the adipocytes, seems to have a more chronic effect on caloric intake and energy expenditure, and is discussed in more detail below.

Leptin’s effects on energy homeostasis were first noticed in 1950 in the ob/ob mouse (Ingalls et al., 1950; Runner and Gates, 1954). A naturally occurring homozygous mutation in the obese gene of the mouse caused extreme obesity, infertility and other complications (reviewed by Houseknecht et al., 1998). It was not until 1994 that the obese gene that
codes for the hormone leptin was sequenced and linked to the phenotype of these mice (Zhang et al., 1994). Soon after this discovery, exogenous administration of leptin was shown to overcome the effects of the ob/ob mutation (Muzzin et al., 1996; Mounzih et al., 1997). The receptor for leptin was discovered in another mutated mouse line, the db/db mouse (Hummel et al., 1966), where a mutation in the leptin receptor gene (diabetic, now called Lepr) causes disruptive leptin receptor signaling (Lee et al., 1996). The db/db animals show similar pathophysiology to the ob/ob mice, giving further evidence for leptin’s role in energy balance. However, the effects of the db/db mutation are not reversible by leptin treatment as shown in an elegant parabiosis experiment by Coleman (1978).

1.3.1 Leptin signaling

Leptin is mainly produced by white adipose tissue (Ahima, 2006), and in smaller amounts by the stomach (Bado et al., 1998), placenta (Masuzaki et al., 1997; Mercer et al., 2000) and muscle (Wang et al., 1998). It circulates in the peripheral blood in amounts proportional to the energy storage in the body.

The obese gene codes for a 4.5 kb messenger RNA (mRNA) that is translated into a 167 amino-acid polypeptide (Zhang et al., 1994). It was long thought that this gene was mammal specific, but in recent years orthologues have been found in amphibians and fish (Denver et al., 2011). Leptin is secreted from adipose tissue cells as a 146-residue protein weighing 16 kDa. It has been difficult to identify the three dimensional structure of leptin because it has no strong sequence similarities with other proteins (Zhang et al., 1994). However, its receptor is similar to those of many cytokine receptors, and because of this the leptin protein is thought to be folded in a manner similar to that of many cytokines (Houseknecht et al., 1998).

Discovery of the leptin receptor in mice and humans by Tartaglia et al. (1995) opened up ways to identify the targets and actions of leptin more
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specifically. Leptin protein circulates through the body, either in a free form or bound to a soluble receptor (Lepr-e, Sinha et al., 1996). Next to the soluble form, there are four different splice variants of the membrane bound receptor (Fig 1.2). These four receptors all have a single transmembrane domain and are members of the GP130 family of cytokine kinase receptors (Tartaglia, 1997). These isoforms all have the same extra cellular peptide-binding site, but they differ at C-terminal ending (Fig 1.2). The shorter forms (Lepr-a, -c, -d) do not activate an intracellular signaling response and are likely to mediate the transport over the cell membrane and the clearance of the leptin from the periphery (Bjørbaek et al., 1997; Bifulco et al., 2003; Merino et al., 2006). The membrane bound receptor with the long C-terminal ending (further referred to as Lepr), contains the two protein motifs (Box 1 and Box 2; Fig 1.2) that together are capable of activating the Janus kinase signal transducers and activators of transcription (JAK-STAT) pathway of gene activation.

**Figure 1.2** Schematic drawing of the leptin receptor gene and its splice variants formed by alternative splicing of the mRNA. There are 5 different leptin receptor molecules, four membrane bound (Ob-Ra-d) and one soluble (Ob-Re). All membrane spanning leptin receptors show an identical structure until Lys889. Only the long form leptin receptor (Ob-Rb) expressed both protein motifs (box 1 and box 2) that are necessary for activation of the JAK-STAT pathway. Ob-Re is identical to the others until His796, after which only 9 amino acids are added, lacking the transmembrane part of the protein (Caprio et al., 2001). aa: amino acid, EC: extracellular, IC: intracellular, TM: transmembrane
When leptin binds to the long-form leptin receptor (Lepr), a homo-dimer is formed with another Lepr molecule. The proline-rich box 1, and the less-conserved sequence of box 2 recruit the cytoplasmic kinase JAK2, as depicted in Figure 1.3. When leptin associates with the receptor, conformational changes cause phosphorylation of JAK2 and tyrosine residues in box 2 of the intracellular compartment. These phosphorylated sites predominantly provide binding sites for signal transducer and activator of transcription 3 (STAT3) proteins, which are activated by phosphorylation (pSTAT3) and then translocated to the nucleus where they stimulate transcription of target genes (Fruhbeck, 2006). One of the target gene promoters of these activated STAT molecules is the suppressor of cytokine signaling 3 (SOCS3, Bjørbaek et al., 1997). Upregulation of SOCS3 targets can inhibit Lepr-STAT3 signaling, forming an intracellular negative feedback loop.

Figure 1.3 A model of intracellular leptin signaling (adapted from: Gao and Horvath, 2008). At least 3 signal pathways, JAK-STAT, PI3K, and MAPK, are activated upon leptin binding to Lepr. Binding of leptin to its receptor causes phosphorylation of JAK2, which in turn phosphorylates STAT3. Two pSTAT3 molecules form a dimer and this is translocated to the nucleus to promote gene
transcription (e.g. SOCS3). Phosphorylated JAK2 can also activate the PI3K-AKT pathway via insulin-receptor substrate (IRS), and box 2 can activate MAPK signaling via SHP2 and GRB2. Although the JAK-STAT3 pathway is dominant, both PI3K and MAPK are modestly involved in energy homeostasis and may interact with STAT3 to regulate homeostasis.

1.3.2 Regulation of energy homeostasis by leptin

The hypothalamus is known to be a major site for integration of different signals contributing to the regulation of energy homeostasis (Sahu, 2004). The membrane bound leptin receptors are found in different nuclei throughout the hypothalamus (Obici, 2009). In particular, the Arc plays an important role in the regulation of energy balance. Two important types of leptin responsive cells are found in this area, the proopiomelanocortin (POMC) and the agouti-related peptide (AgRP) neurons. The POMC neurons co-express cocaine- and amphetamine regulated transcript (CART). Binding of leptin to these neurons causes up regulation of both POMC and CART (Cowley et al., 2001). Both melanocortin signaling (via αMSH, a POMC derived peptide) and CART suppress food intake, and are therefore anorexigenic. The AgRP neurons co-express neuropeptide Y (NPY), which are orexigenic proteins, leptin inhibits the expression of these substances (Fig 1.4). High circulating leptin levels will normally cause a net decrease of energetic intake through these neuropeptide pathways (Baskin et al., 1999; Elias et al., 1999; Balthasar et al., 2004; Sahu, 2004). Leptin is able to specifically regulate and modulate this system because it circulates in amounts proportional to the amount of adipose tissue of an individual at a given time (Zhang et al., 1994).
Control of energy homeostasis by arcuate nucleus neurons (Seeley and Woods, 2003). Two sets of neurons, AgRP/NPY and POMC/CART neurons, are regulated by circulating metabolic hormones. AgRP (agouti-related protein) and NPY (neuropeptide Y) are neuropeptides that stimulate food intake and decrease energy expenditure, whereas α-melanocyte stimulating hormone (αMSH, a post-translational derivative of proopiomelanocortin, POMC) and CART (cocaine- and amphetamine-regulated transcript) are neuropeptides that inhibit food intake and increase energy expenditure. Insulin and leptin are hormones that circulate in proportion to body adipose stores; they inhibit AgRP/NPY neurons and stimulate adjacent POMC/CART neurons. Lower insulin and leptin levels are therefore predicted to activate AgRP/NPY neurons, while inhibiting POMC/CART neurons. MC, melanocortin.

1.4 Metabolic regulation of fertility

Reproduction is an energy demanding function; it is therefore not surprising that reproductive function is dependent on metabolic status (review by Elias and Purohit, 2013). The maturation (puberty) and adult functioning of the HPG axis are sensitive to the amount and availability of energy reserves within the body (Tena-Sempere, 2013). This relationship is most evident in
mammalian females due to the high energetic investment made for the successful completion of gestation and lactation. The HPG axis can be regulated at different levels, but it has become evident that the most substantial part of the metabolic regulation takes place within the hypothalamus. There are multiple metabolic hormones that act on the reproductive axis: insulin from the pancreas (Brüning et al., 2000; Evans et al., 2014), ghrelin from the stomach (Garcia et al., 2007) and leptin produced by adipose tissue (Barash et al., 1996).

Ghrelin production by the stomach under the control of mechanical sensors is increased when the stomach is empty. Acting as a potent orexigenic signal in the hypothalamus, it will increase food intake (Fernandez-Fernandez et al., 2006). Contrary to its effects on metabolism, ghrelin has predominantly suppressive effects on circulating gonadotropin levels and inhibitory effects on puberty onset (Tena-Sempere, 2013). For example, in vivo administration of ghrelin systemically or centrally causes a significant inhibitory response especially in terms of circulating LH (Furuta et al., 2001; Tena-Sempere, 2013). Additionally, treatment of leptin deficient (ob/ob) mice with a ghrelin receptor antagonist improved the reproductive phenotype (Zhu et al., 2013).

For the maintenance of glucose homeostasis, insulin promotes glucose uptake by peripheral tissues. Insulin is produced by the pancreas, and receptors are widely distributed throughout the body, including the brain. Elevated levels of circulating insulin are associated with an increase in GnRH and LH release (reviewed by Comninos et al., 2014). In a transgenic study where insulin receptors were selectively deleted from neuronal progenitor cells, female mice showed low LH levels and diet-induced obesity (Brüning et al., 2000).

It is thought that insulin and leptin might work together to regulate reproduction in POMC neurons of the Arc. Deletion of insulin or leptin receptors individually from this population does not impair fertility (Konner et al., 2007; van de Wall et al., 2008). However, female mice that lack both of
these receptors from POMC neurons show elevated testosterone and ovarian abnormalities (Hill et al., 2010).

1.4.1 Metabolic regulation of fertility by leptin

Leptin is an important regulator of reproduction; it forms a link between energy availability and fertility control centers (Barash et al., 1996; Chehab et al., 1996). Peripheral administration of leptin can overcome fasting induced suppression of LH pulsatility in rats (Nagatani et al., 1998), and critical levels of leptin are needed for normal puberty onset in humans and rats (van der Spuy, 1985; Cheung et al., 1997b). Additionally, early high circulating leptin can advance puberty onset in mice (Ahima et al., 1997). Leptin signaling in the brain is necessary for normal reproductive functioning as selective deletion of functional leptin receptors from all forebrain neurons prevents onset of puberty and results in infertility in both female and male mice; a similar reproductive phenotype to that seen in db/db animals (Quennell et al., 2009). Additionally, restoration of Lepr solely in neuronal tissues, is sufficient to regain reproductive function in a db/db mouse (de Luca et al., 2005). Overall, low levels of circulating leptin inhibit HPG axis functioning, however, this is also true when leptin levels are too high or continuously elevated causing insensitivity to leptin or leptin resistance (Munzberg and Myers, 2005). There seems to be a critical ‘leptin window’ in which it is optimal for fertility to occur. Although leptin has many targets throughout the brain, Lepr signaling in the hypothalamus is crucial to the control of fertility as intra-hypothalamic infusions of leptin are sufficient to overcome the suppressive effects of low circulating leptin (Watanobe, 2002).

1.4.2 Dysregulation of energy homeostasis and fertility

Human obesity is a severely growing health problem in the western societies, and this is why the role of leptin in metabolism and appetite regulation has become a point of interest to many researchers (Houseknecht
et al., 1998; Friedman, 2000; Biro et al., 2006). In healthy people and animals, a rise in peripheral leptin will cause an increase in energy expenditure and a decrease in food intake, maintaining body weight equilibrium. When this process is disrupted, by incorrect functioning of the leptin system (i.e. leptin resistance), the body cannot regulate body weight appropriately and becomes obese. Studying leptin’s actions in the body in order to understand this problem may uncover new therapeutic strategies (Morris and Rui, 2009). The discovery of leptin generated a short-lived hope that it would provide a cure for the growing obesity problem. Subsequent studies now show that leptin signaling deficiency via a gene mutation in leptin is very rare, and that obesity is actually associated with hyperleptinemia and leptin resistance (Ostlund et al., 1996; Montague et al., 1997).

The relationship between hyperleptinemia in humans and reproductive capacity is not well understood. Research has shown that paternal body weight correlates with lower sperm quality in males (Chavarro et al., 2010), and that infertility in women is often associated with obesity-associated disorders such as type 2 diabetes and polycystic ovary syndrome (PCOS, reviewed by Seli et al., 2014). It remains uncertain what role leptin plays in these pathologies because there are a myriad of factors that contribute to the dysfunctioning. On the other side of the spectrum, the effects of a lack of leptin are more straightforward. It is clear that hypoleptinemia caused by extreme exercise or eating disorders like anorexia nervosa, is associated with low gonadotropin levels (Laughlin and Yen, 1996, 1997); these levels can be raised with exogenous leptin administration (Welt et al., 2004).

1.4.3 Hypothalamic leptin receptor neurons that regulate fertility

Leptin receptor expression is found in many discrete regions throughout the brain (Scott et al., 2009; Patterson et al., 2011), therefore it has been challenging to identify the exact mechanism and population of Lepr neurons responsible for the regulation of reproduction. The rPOA GnRH neurons
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would be the most obvious target for leptin as they form the final output from the hypothalamus and drive the HPG axis. Although in vitro experiments in immortalized GnRH secreting cell lines show Lepr expression (Magni et al., 1999), there is little evidence for Lepr mRNA or protein expression by GnRH neurons in vivo (Finn et al., 1998; Håkansson et al., 1998). A more recent study confirms these neurons do not express Lepr and shows that selective deletion of Lepr from GnRH neurons does not have any effect on fertility parameters (Quennell et al., 2009). This key paper suggests that regulation of fertility by leptin is indirect from GnRH neurons but centrally linked to the HPG axis, since the authors also showed that leptin actions on forebrain neurons are critical for normal fertility.

1.5 Possible leptin to GnRH intermediate neurons

Comparing Lepr distribution with GnRH retrograde tracing has identified possible pathways of leptin to GnRH. Scott et al. (2009) and Patterson et al., (2011) both used a transgenic mouse model (Fig 1.5) in which the expression of enhanced yellow fluorescent protein (eYFP) was under the control of the Lepr promoter, to identify leptin responsive cells. Yoon et al. (2005) and Wintermantel et al. (2006) use a Cre dependent attenuated strain of pseudo rabies virus (PRV) to retrogradely infect GnRH connected neurons in GnRH Cre animals. Retrograde tracing of GnRH afferents is made visible by green fluorescent protein (GFP) expressed by the activated PRV strain (Yoon et al., 2005; Wintermantel et al., 2006). Hypothalamic regions that are both leptin sensitive and afferent to GnRH neurons are discussed below.
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Figure 1.5 Transgenic mouse models using the Cre-LoxP system. The Cre-Lox recombinase system is a widely used genetic tool to cause deletion, insertion or inversions at specific sites in the DNA of specific cell types. Cre recombinase is an enzyme derived from a bacteriophage not normally expressed in mammalian cells. Deletions of target genes can be cell type specific depending on the promoter used to drive Cre expression. For example, GnRH specific Cre expression can be established by using the GnRH promoter to drive Cre transcription. Only cells that use this promoter will express Cre recombinase. The target gene is the gene to be recombined or deleted. Part or all of the coding region of the target gene is flanked by LoxP sites (=floxed), which are 34 bp recognition sequences also derived from bacteriophages. When Cre recombinase is expressed in specific cell type it will recognize these LoxP sites and cause recombination resulting in deletion of the floxed sequence and an inactive target gene. There are multiple variations on this system, for example a ‘stop-floxed’ sequence in front of the target gene. Where a stop sequence in front of the target gene inhibits transcription in cells that do not express Cre recombinase, but when Cre recombinase is present the stop sequence is excised and the target gene will be expressed. This system is used commonly in fluorescent reporter mice; labeling Cre cells with a fluorophore (i.e., green fluorescent protein -GFP).

1.5.1 The rostral hypothalamus

The RP3V is a continuum that includes the anteroventral periventricular nucleus (AVPV), the preoptic and anterior hypothalamic divisions of the periventricular nucleus (PVpo) and the median preoptic area (MnPO, Herbison, 2008; Quennell et al., 2009; Scott et al., 2009). The RP3V is the
location of an estrogen-sensitive population of kisspeptin neurons that project to GnRH cell bodies (identified by neuroanatomical tracing studies). Experiments that point to the importance of these neurons include demonstrations that they express the early immediate gene cFos during the preovulatory GnRH/LH surge, and loss of female fertility in lesion studies (Herbison, 2008). Additionally, neurons in this region are leptin-responsive (Quennell et al., 2009; Scott et al., 2009). The RP3V kisspeptin neurons have been proposed as one of the possible mediators of leptin to GnRH signaling (Messager et al., 2005; Luque et al., 2007; Backholer et al., 2010). Kisspeptin expression in the RP3V is reduced by leptin deficiency (ob/ob mice) as well as high fat diet-induced obesity (Quennell et al., 2011). Only recently it has been shown that the kisspeptin neurons in the RP3V are not Lepr positive; there is no colocalization of the leptin-induced second messengers (pSTAT3 and others) and kisspeptin immunoreactivity, nor co-expression of kisspeptin and Lepr mRNA shown by quantitative polymerase chain reaction (qPCR) performed on a kisspeptin-enriched cell population (Cravo et al., 2011; Quennell et al., 2011). It has been suggested that the effect of leptin on kisspeptin neurons is achieved via an indirect pathway, making the relation of leptin and GnRH even more complex and possibly mediated via two interneuron projections (i.e. Lepr neuron → kisspeptin neuron → GnRH neuron).

The GnRH neurons located in the POA, rostral of the RP3V, do not express Lepr (Quennell et al., 2009). However, moderate expression of Lepr is found within the surrounding medial POA (MePO) and MnPO (Scott et al., 2009; Patterson et al., 2011). Systemic treatment with leptin shows activation of the Lepr neurons in these areas by pSTAT3 expression (Cravo et al., 2011). The Lepr neurons in these POA populations co-express α-adrenergic receptors for either noradrenaline or dopamine. Nerve terminals projecting from ascending noradrenergic populations are thought to be involved in thermoregulation, arousal and sleep/wake rhythm but not reproduction (Frontini and Giordano, 2010). In this region, leptin induced pSTAT3 has also been shown in nitric oxide (NO) producing neurons (Donato et al., 2010).
Additionally, stimulation of GnRH secretion and the preovulatory LH surge by leptin is blocked by NO inhibitors (Yu et al., 1997a; Watanobe and Schiöth, 2001). It is not known whether these leptin responsive neurons are in direct contact with GnRH neurons of the rPOA.

1.5.2 Arcuate nucleus

The Arc is a heterogeneous region with the highest Lepr protein and mRNA levels found in the hypothalamus (Scott et al., 2009; Patterson et al., 2011) this being very important in the regulation of energy homeostasis as discussed previously (Section 1.3). This hypothalamic nucleus contains many types of neurons including POMC/CART and NPY/AgRP neurons which both express Lepr. These might also be involved in conveying metabolic status to GnRH neurons. The POMC/CART neurons project to rPOA (Rondini et al., 2004) and POMC positive appositions are shown on GnRH neurons (Leranth et al., 1988).

Neuropeptide Y lowers GnRH pulse frequency in adult rodents via NPY itself or by γ-Aminobutyric acid (GABA) produced in the same neurons (Lebrethon et al., 2000). The pre-pubertal stimulatory effects on fertility of both leptin and NPY are mediated via two distinct mechanisms: (1) NPY increases the GnRH firing frequency via the Y5-receptor, and (2) leptin effects are determined by CART signaling (Lebrethon et al., 2000). However, genetic removal of Lepr from POMC neurons or from both AgRP and POMC populations does not result in any disturbance to the reproductive phenotype (Balthasar et al., 2004; van de Wall et al., 2008). AgRP is known as a potent antagonist of melanocortin signaling and melanocortin receptor (MCR4) signaling is thought to be involved in LH stimulation by leptin (Ollmann, 1997; Watanobe et al., 1999). Interestingly, modulation of melanocortin signaling by ablation of AgRP neurons restores puberty onset and fertility in db/db animals (Israel et al., 2012). This suggests AgRP and melanocortin signaling might play a role in mediating leptin's effects on the HPG axis.
A small proportion (5-20%) of the population of kisspeptin neurons found in the Arc appears to be leptin responsive (Smith et al., 2006a; Cravo et al., 2011). Additionally, Kiss1 mRNA levels in the Arc are reduced in leptin deficient animals (Quennell et al., 2011). These neurons form a likely candidate for leptin-to-GnRH intermediates because kisspeptin is known to stimulate GnRH (Messager et al., 2005). The kisspeptin population in the Arc exhibits no colocalization with POMC or NPY (Cravo et al., 2011). It is now clear however, that Lepr signaling through this pathway is neither necessary nor sufficient for the regulation of HPG axis. Selective deletion or re-expression of Lepr in kisspeptin neurons does not impair or restore fertility (Cravo et al., 2013). This suggests that, as is probably the case with RP3V kisspeptin neurons, there may be intermediates linking leptin signaling to kisspeptin neurons in the Arc.

Another neuropeptide produced by a different population of leptin-responsive neurons in the Arc is galanin-like peptide (GALP, Ohtaki et al., 1999). The GALP neurons project to the anterior paraventricular nucleus (PVN) and also make connections with GnRH neurons in the rPOA (Gundlach, 2002). Shiba et al. (2010) suggest two distinct GALP pathways with GALP neurons projecting from the Arc to the PVN, and from the Arc to the rPOA. The GALP neurons express Lepr and respond to leptin by increasing Galp mRNA expression (Juréus et al., 2001). Specifically, central (intracerebroventricular) administration of GALP activates GnRH neurons in rPOA and increases plasma LH levels (Gundlach, 2002). Absence of leptin, for example in the ob/ob mice, might therefore reduce GALP levels in the Arc to the extent that GnRH release is inhibited (Kageyama et al., 2005).

Lastly, there are Lepr-expressing neurons in the Arc that use the neurotransmitters GABA or glutamate (Vong et al., 2011). These populations overlap with the populations of Arc neurons discussed above. It is known that AgRP/NPY neurons are GABAergic and POMC/CART use glutamate as a neurotransmitter (Cowley et al., 2001; Vong et al., 2011), but less is known
about the other populations. In a study where leptin receptors were selectively deleted from either all glutamate or all GABA neurons it was shown that the majority of metabolism related effects of leptin are mediated by GABAergic Lepr neurons rather then glutamatergic neurons (Vong et al., 2011). It needs to be noted that these deletions were not confined to the Arc, but rather a knockout of leptin receptors on GABA or glutamate neurons throughout the brain.

1.5.3 Ventral premammillary nucleus

Leptin signaling in the ventral premammillary nucleus (PMV) seems to be exclusively linked to reproduction rather then energy metabolism. Lesion of the PMV in rats caused no changes in metabolic status but did impair the ability to activate the HPG axis during the afternoon of proestrus (Donato et al., 2009). The PMV strongly innervates areas related to reproductive control such as the RP3V and mPOA (Canteras et al., 1992), and trans-synaptic retrograde tracing with barley lectin from the GnRH neurons, shows a direct connection between leptin responsive cells in the PMV and GnRH neurons (Leshan et al., 2009). In addition to leptin receptors, PMV neurons express large amounts of estrogen and androgen receptors (Simerly et al., 1990), which adds to the potential role of the PMV as a fertility regulating nucleus (Hahn and Coen, 2006). Interestingly however, PMV lesion studies showed no change in Kiss1 mRNA expression within the AVPV neurons at the time of proestrus (Donato et al., 2009).

Intermediate signaling molecules might include glutamate, NO, and CART which are all highly expressed in the PMV. Glutamate neurons from the PMV project to the mPOA, and might form crucial GnRH afferents (Kocsis, 2003; Leshan et al., 2009). Leptin responsive CART neurons in the PMV project to GnRH expressing regions (Elias et al., 2001; Rondini et al., 2004), however, it is not clear whether the CART and Lepr expressing neurons are the same neurons that project to GnRH-expressing cells. Another possible mediator of leptin signaling to GnRH neurons in the PMV is the gaseous neurotransmitter
NO. High levels of colocalization of leptin induced pSTAT3 and NAPDH (indicating NO synthesis) are found in the PMV (Donato et al., 2010). Additionally, a negative energy balance (i.e. fasting/low leptin) reduces whole hypothalamic neuronal nitric oxide synthase (nNOS) mRNA expression; but this reduction in nNOS was not restored by acute leptin treatment (Donato et al., 2010). Inhibitors of NOS can block a leptin induced rise in circulating LH (Watanobe and Schiöth, 2001), showing a relationship between leptin, NO and GnRH. However, it is not known if these effects are relayed by leptin responsive nNOS neurons in the PMV.

1.5.4 Ventromedial nucleus of the hypothalamus

The ventromedial nucleus of the hypothalamus (VMN) was one of the first areas to be associated with energy homeostasis and is mainly known for its function as a satiety center (reviewed by Elmquist et al., 1999). The VMN Lepr neurons are predominantly glutamatergic. Deletion of Lepr specifically from the VMN or from glutamate neurons leads to mild obesity, showing the critical role of the VMN in metabolic regulation (Dhillon et al., 2006; Vong et al., 2011). Part of the VMN is thought to play a role in sexual behavior in females, but there is no other apparent role for the VMN in fertility regulation (Blaustein, 2008; Chee et al., 2010).

1.5.5 Lateral hypothalamic area

Stimulation of the lateral hypothalamic area (LHA) causes a desire to eat; an opposite function to the VMN (Schwartz et al., 2000). Therefore leptin has an inhibitory effect on the LHA, since high leptin levels cause satiety. The LHA contains a mixture of neurons that are GABAergic and glutamatergic. GABAergic neurons are known to inhibit food intake via the LHA (Stanley et al., 2011). Some LHA neurons express nesfatin-1, a peptide involved in food intake that also acts as a controller in puberty onset in female rats (Garcia-Galiano et al., 2010). However, nesfatin-1 acts independently from leptin signaling, at least for regulation of food intake, so an intermediate role for
nesfatin-1 is unlikely in regards to leptin-modulated puberty onset (Goebel-Stengel et al., 2011). A different population of LHA neurons express the neuropeptide orexin; these orexin neurons have been found to provide direct input to GnRH neurons in sheep (Iqbal et al., 2001; Gaskins and Moenter, 2012). Orexin plays a role in appetite regulation, but is not a direct target of leptin (Louis et al., 2010; Leinninger et al., 2011). Taken together, there is little evidence for the LHA as a region important for regulation of fertility, despite it being labeled as a GnRH afferent in retrograde tracing studies (Iqbal et al., 2001; Yoon et al., 2005; Wintermantel et al., 2006).

1.5.6 Dorsomedial nucleus of the hypothalamus

High levels of Lepr expression are seen in the dorsomedial nucleus of the hypothalamus (DMN, Scott et al., 2009; Patterson et al., 2011), and the DMN is involved in mediating reproductive behavior (Yoon et al., 2005). The DMN seems a likely location for neurons which act as leptin to GnRH intermediates. Tracing studies from the DMN show projections to a number of other hypothalamic nuclei including the preoptic regions (Thompson and Swanson, 1998), and some of these efferent projections come from leptin-responsive DMN neurons (Gautron et al., 2010).

The DMN is the exclusive location of RF-amide related peptide 3 (RFRP-3) cell bodies in mice, rats, and hamsters (Kriegsfeld et al., 2006; Qi et al., 2009; Rizwan et al., 2009). Projecting fibers and terminals of RFRP-3 neurons are shown in midline brain regions that have a high concentration of GnRH neurons (MS, diagonal band of broca, rPOA and anterior hypothalamus, Rizwan et al., 2009), and these make direct contact with GnRH neurons (Kriegsfeld et al., 2006; Rizwan et al., 2012). RFRP-3 is involved in the control of stress responses, food intake, and reproductive functioning and behavior (Kriegsfeld et al., 2010; Takayanagi and Onaka, 2010). The RFRP-3 neurons express estrogen receptor α, and RFRP-3 mRNA expression is lowered by estradiol (reviewed by Khan and Kauffman, 2012). These neurons are the counterpart of the highly stimulatory effects of kisspeptin
and thus form a likely metabolic intermediate; however recently it has been shown that RFRP-3 neurons are not leptin responsive (Rizwan et al., 2014).

The DMN has a population of GABAergic Lepr neurons (Vong et al., 2011), which are distinct from the RFRP-3 population since RFRP-3 neurons are not GABAergic (Rizwan et al., 2014). It is currently unknown if these GABAergic neurons project to GnRH cells in the rPOA.

In addition to the Arc CART is expressed in the DMN, and these neurons also express Lepr and project to the region of the GnRH cell bodies (Elias et al., 2001; Rondini et al., 2004). The CART neurons in the DMN therefore provide a possible pathway by which leptin could modulate GnRH release.

1.5.7 Paraventricular nucleus

The PVN plays a central role in the mediation of reproductive behavior. It connects with the nucleus of the solitary tract (NTS) and is upstream to the Arc (Simerly, 2002; Yoon et al., 2005). In the PVN there are neurons which secrete oxytocin and vasopressin at their nerve terminals in the posterior pituitary gland (Landgraf and Neumann, 2004). Oxytocin fibers have been shown in close proximity to GnRH cell bodies, and a small population of GnRH neurons (10%) express oxytocin receptors (Caligioni et al., 2007).

There is evidence that PVN oxytocin neurons link hypothalamic leptin action to caudal brain stem nuclei controlling food intake (Kutlu et al., 2010; Perello and Raingo, 2013). Leptin is able to inhibit oxytocin secretion by lowering noradrenaline levels in the PVN of rats; this effect might be related to alterations in feeding behavior but not to reproductive regulation (Landgraf and Neumann, 2004; Kutlu et al., 2010).

Corticotrophin-releasing hormone (CRH) from the PVN drives the stress induced reaction of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis, and is able to suppress the HPG axis (Petraglia et al., 1987). This action might be mediated by direct innervation of the GnRH cells by
CRH/vasopressin neurons (Rivalland et al., 2006), or via enkephalin-producing neurons also found in the PVN, since enkephalin has also been shown to be able to modulate the HPG axis (Bruni et al., 1977). Low leptin (fasting) activates the HPA axis by increasing CRH expression (Heiman et al., 1997), although there is no convincing evidence for direct actions of leptin onto CRH neurons in the PVN.

1.5.8 Summary

The review of overlapping brain nuclei in studies concerning Lepr expression and GnRH input provides a literature-based selection of neuronal populations that could accommodate the regulation of HPG axis functioning by leptin. It must be noted that most initial anatomical studies were performed in rats, with transgenic manipulation becoming increasingly available the field shifted to mainly use (transgenic) mice. In the preoptic area NO-Lepr neurons might form an intermediate pathway to the reproductive axis (Fig 1.6). From the Arc the likely candidates are the NPY/AgRP-, POMC/CART-, GALP-, GABA-, or glutamate-Lepr neurons, from the PMV NO-, CART-, or glutamate-Lepr neurons, and from the DMN CART and GABAergic Lepr neurons might provide this role. In this thesis these different hypotheses will be addressed. One exciting technique that has greatly advanced this field over the last decade is conditional deletion of Lepr specifically from target neuronal populations (as explained in Fig 1.5 and e.g. studies by Balthasar et al., 2004; Quennell et al., 2009; Mayer et al., 2011). While this approach has yet to reveal a single population of neurons that is critical for fertility, the increasing availability of Cre-expressing mouse lines for targeting the Lepr knockout will enable more widespread screening in neuronal pathways linking nutritional cues to the HPG-axis. A recent development also allows researchers to knock Lepr in to specific cell populations on a Lepr-null mouse, which has the same phenotype as db/db animals but has the compatibility with the Cre-Lox system (Berglund et al., 2012; Cravo et al., 2013).
Figure 1.6 An overview of possible pathways mediating leptin responses to GnRH neurons. Nitric oxide (NO) Lepr neurons from the preoptic area, CART and GABA neurons from the DMN, GALP, POMC/CART, NPY/AgRP, glutamate, and GABA Lepr neurons from the Arc, and glutamate, NO, and CART Lepr neurons form the PMV might form intermediates between GnRH cell bodies (solid red arrows) or GnRH terminals (opaque red arrows). ac, anterior commissure; AgRP, agouti related peptide; Arc, arcuate nucleus; CART, cocaine- and amphetamine regulated transcript; DMN, dorsomedial nucleus of the hypothalamus; f, fornix; GABA, γ-aminobutyric acid; GALP, galanin-like peptide; GnRH, gonadotropin releasing hormone; MS, medial septum; NPY, neuropeptide Y; ox, optic chiasm; PH, posterior hypothalamus; PMV, premammillary nucleus; POA, preoptic area; POMC, pro-opiomelanocortin; SOX, supra optic nucleus; VDB, nucleus of the vertical limb of the diagonal band; VMN, ventromedial nucleus of the hypothalamus.
1.6 Aims

The aim of this work is to study where and how leptin regulates HPG axis functioning, specifically targeting the potential intermediary neuronal candidates mentioned above. Trying to identify or narrow down the possible leptin to GnRH intermediate candidates areas and linking neurotransmitters, will do this. A combination of immunohistochemistry, gene expression measurements, tract tracing and mouse transgenics combined with assessments of reproductive competence will be used to try and reach these goals. Functionally, Cre-lox transgenics (Fig 1.5) will be used to remove Lepr signaling from specific neuronal populations so that the effects on metabolic and reproductive parameters can be assessed. Anatomically, Lepr-GFP transgenic animals in combination with retrograde tracing will be used to identify the location of the neurons that convey the leptin signal to the GnRH neurons. Combining this with immunohistochemical and molecular biology techniques, the neurochemical nature of these neurons can be further explored. These methods will give insight to both location, and the neuronal mechanisms underlying HPG axis regulation by leptin.

Specific aims:

1. To determine which hypothalamic regions send leptin-responsive neuronal projections to the region where GnRH cell bodies are located.

2. To test whether nNOS, glutamate, or GABA neurons are critical conduits for leptin’s effects on the reproductive axis.

3. To measure changes in expression of various genes in response to GABA neuron specific Lepr deficiency.

1.7 Outline of this thesis

In this thesis I will explore the hypothalamic regions and neurotransmitters that mediate the regulation of fertility by leptin. Chapter 2 will contain specific background information about methods and techniques that are
used throughout the experimental **Chapters 3-6**. Using a retrograde tracer, the anatomical location of potential leptin responsive inputs to the rPOA is investigated in **Chapter 3**. The possibility of NO being a mediator of leptin to the HPG axis signaling is explored in **Chapter 4**, using an inducible transgenic model to specifically delete Lepr from nNOS expressing cells. To investigate the major stimulatory and inhibitory pathways in a number of hypothalamic nuclei, leptin receptors are deleted from these two large heterogeneous neuronal populations in **Chapter 5** using Cre-Lox transgenics. Using GABA (major inhibitory neurotransmitter) and glutamate (major excitatory neurotransmitter) specific Lepr knockout animals, different hypothalamic populations are targeted, particularly the Arc, LHA, and DMN in GABAergic Lepr knockout mice, and the Arc, VMN, and PMV in the glutamatergic knockout of Lepr. In **Chapter 6** the underlying mechanism of the reduced fertility in the GABA specific Lepr knockout is further explored using quantification of candidate gene expression levels. In the last chapter, **Chapter 7**, the results of all the experimental chapters are discussed from the perspective of the general aims of this thesis. Additionally, this chapter contains suggestions for future research into the metabolic control of the HPG axis functioning by leptin.
Chapter Two
General Methods
In this chapter the experimental procedures that were used in Chapters 3-6 are described. In Appendix I the recipes for the commonly used solutions can be found.

2.1 Animals

All mice were obtained from the University of Otago animal breeding facility. Animals were group housed (unless stated otherwise) and maintained on a 12 hour light-dark cycle (lights on between 0600 and 1800 h) at a constant temperature (22 ± 1°C). Mice had *ad libitum* access to water and rodent chow, (< 5% fat by weight; Specialty feeds, Glenn Forrest, WA, Australia) unless stated otherwise. To assess growth and development of obesity, the weight of the animals were measured on a regular basis. The University of Otago Animal Ethics Committee approved all animal experimental protocols.

2.2 Leptin treatment

2.2.1 Intracerebroventricular leptin treatment

For intracerebroventricular (icv) leptin treatment animals were fasted overnight and anaesthetized using isoflurane (1-2.5%) and 70% oxygen. The head was secured in stereotaxic device using the ear bars (Kopf Instruments; 943-45). A 10 mm mid-sagittal skin incision was made to expose the skull and bregma point, which was calibrated as zero. From there, the tip of a 0.5 ml Hamilton syringe (Hamilton Company; #7105) was moved 0.8 mm lateral and 0.1 mm rostral. At this point a small hole was drilled using a ball shaped tip (Leica; 39416023). One microliter of recombinant mouse leptin solution (0.1 mg/ml; National Hormone and Peptide Program) or vehicle (0.01 M phosphate buffered saline (PBS), pH 7.4) was drawn up and the Hamilton syringe was then lowered into the lateral ventricle (2.4 mm dorsal to the skull surface). Leptin or vehicle was slowly injected over 3 minutes, the needle was left in place for a further 2 minutes, and was then pulled up. The skin incision was closed using three sutures (4-0 Vicryl, Softsilk sutures;
Chapter Two

GS-831) and as an analgesic animals were given 5 mg/kg carprofen (5 mg/ml; Carprieve) subcutaneously before removing them from stereotaxic device and placing back into their home cage. Recovery from surgery (becoming ambulatory) usually occurred between 5-10 min later. Thirty minutes after icv injection animals were given an overdose of sodium pentobarbital (240 mg/kg, Pentobarb 300) and perfused as described below (Section 2.3.1).

2.2.2 Subcutaneous leptin treatment

Animals were fasted overnight (starting at ~1700 h) and the next morning (between 0900-1100 h) treated with a subcutaneous (sc) injection into the fold of the hind limb, with vehicle (200 μl/20 g body weight; 0.01 M PBS, pH 7.8) or recombinant mouse leptin (1 mg/kg, National Hormone and Peptide Program, Anderson et al., 2003). Two hours after the injection animals were overdosed with sodium pentobarbital (240 mg/kg, Pentobarb 300), and perfused transcardially.

2.3 Tissue collection and preparation

2.3.1 Transcardial perfusion; fixed brain tissue for immunohistochemistry

After deep anesthesia with a 240 mg/kg pentobarbital injection, and once pedal withdrawal reflex was absent, the chest cavity of the mouse was opened to expose the heart. Then, 20 ml of 4% paraformaldehyde (PFA in 0.1 M phosphate buffer (PB), pH 7.3) was slowly injected into the left ventricle. Afterwards, the brain was removed, and post-fixed overnight at 4 °C in the same 4% PFA in PB (pH 7.3) solution. The following day, the brain was transferred to 30% sucrose in 0.01 M PBS (pH 7.4) for cryoprotection. When brains had sunk to the bottom of the vial (48-72h later) they were cut into four series of 30 μm sections, at -20 °C on a sliding microtome (Leica SM2400, Leica Microsystems). Sections were transferred to a 12 well plate filled with cryoprotectant (0.1 M PB with 30% sucrose, 1% polyvinyl-
pyrrolidone, and 30% ethylene glycol; pH 7.2) to be stored at -20 °C until later use.

2.3.2 Blood collection

When blood plasma collection was required, a 1 ml heparinized syringe (0.1 ml of 5000 units/ml heparin drawn up, then expelled) with a 27-gauge needle was prepared in advance. Once mice were anaesthetized and the heart was exposed 0.4-1 ml of whole blood was collected from the right atrium. Blood was transferred to a 1.7 ml Eppendorf tube and animals were further perfused as described above. To separate plasma from red blood cells, blood samples were spun at 5000 x g for 10 min and plasma was transferred to a clean 0.6 ml Eppendorf tube. Plasma samples were kept frozen at -20 °C until further use. Repetitive freeze/thaw cycles were avoided.

2.3.3 Reproductive organ collection

Uteri or testes were collected by dissection after perfusion. In females, the cervix was located at the base of the abdomen, and a cut was made directly below it. The uterus with attached ovaries was removed from the pelvic cavity; the fatty tissue, cervix and ovaries were carefully removed before collecting wet weights. In males, the testes were exposed by gently pulling on the gonadal fat pads. Each testis was then separated from the surrounding tunica vaginalis and epididymis; testes wet weight was recorded and they were stored at -20 °C until daily sperm production assessment.

2.3.4 Decapitation; fresh brain tissue

For the collection of fresh brain tissue and serum blood samples, animals were decapitated and trunk blood samples were collected using a funnel and 1.7 ml Eppendorf tubes. Brain tissue was quickly dissected from the head,
and was directly frozen on foil on dry ice before storage at −80 °C. Blood samples were put on wet ice (~30 min) until they were able to be spun down at 5000 x g for 10 min. The serum was collected and transferred to a clean 0.6 ml Eppendorf tube and the samples were stored at -20 °C until further use.

2.4 Immunohistochemistry

2.4.1 Chromogen (DAB) staining

For single label chromogen immunohistochemistry: 30 μm coronal sections were transferred to net wells (15 mm diameter, 500 μm mesh; Corning; COR3478) for all wash steps. A minimum of three 5-10 minute washes was performed between all subsequent steps. After washing in TBS-TX (Tris buffered saline (TBS) with 1% Triton X-100, pH 7.6), endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ in dH₂O for 10 mins. Tissues were then incubated for one hour in blocking solution: 1% bovine serum albumin (BSA; Sigma-Aldrich; 05470) and 3% normal goat serum (NGS) in TBS-TX. Primary antibody (Table 2.1) was added to make up the final concentration and this tissue was incubated at 4°C for 24-72 hours. Sections were then incubated for 60 mins in secondary antibody that was either biotinylated or conjugated to horseradish peroxidase (HRP; Table 2.2). For biotinylated secondary antibodies only: the signal was amplified using avidin-biotin peroxidase solution (ABC; Elite Vectorstain, Vector Laboratories) for another hour. Sections were transferred to filtered diaminobenzidine solution (0.05% DAB, Sigma-Aldrich; D5905) containing urea as a source of hydrogen peroxide to visualize brown staining. Nickel-enhanced DAB (NiDAB) was used for dark purple/black staining. After careful checking of tissue sections for sufficient levels of staining they were washed and mounted on gelatin coated microscope slides. They were allowed to dry overnight, and then dehydrated by brief (2 min) consecutive immersions in graded ethanol solutions (50, 70, 90 and 100%), followed by two consecutive 5 min immersions in 100% xylene. Microscope slides were
then cover-slipped using DPX mounting medium (Scharlau; SCARDP00500500).

For immunohistochemical staining of leptin-induced pSTAT3, sections were submitted to an antigen retrieval step after the initial wash. This consisted of 15 min incubation in 1 mM EDTA at 90 °C.

Double label chromogen immunohistochemistry was performed by repeating the above (from the primary antibody step), using different primary and secondary antibodies (Tables 2.1 and 2.2). Note that the primary antibodies are ideally raised in different host species to eliminate cross reactivity at the secondary antibody stage. Furthermore, two different secondary antibodies were always used (one biotinylated (plus ABC amplification; usually used for the least abundant target) and one HRP conjugated), and DAB staining was nickel enhanced for one substrate and not for the other.

### Table 2.1  Primary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Concentration</th>
<th>Label</th>
<th>Cat. #</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-GnRH precursor</td>
<td>Guinea pig</td>
<td>1:2000</td>
<td>fluoro</td>
<td>In-house</td>
<td>G. Anderson</td>
</tr>
<tr>
<td>anti-nNOS</td>
<td>Sheep</td>
<td>1:5000</td>
<td>DAB or fluoro</td>
<td>K 205</td>
<td>Herbison (1996)</td>
</tr>
<tr>
<td>anti-pSTAT3</td>
<td>Rabbit</td>
<td>1:3000</td>
<td>DAB or fluoro</td>
<td>9145</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-Vgat</td>
<td>Rabbit</td>
<td>1:750</td>
<td>fluor</td>
<td>5062P</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

### Table 2.2  Secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Concentration</th>
<th>Cat. #</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-rabbit(HRP)</td>
<td>Goat</td>
<td>1:500</td>
<td>PO488</td>
<td>Dako</td>
</tr>
<tr>
<td>anti-rabbit(biotinylated)</td>
<td>Goat</td>
<td>1:1000</td>
<td>BA1000</td>
<td>Vector</td>
</tr>
<tr>
<td>anti-sheep(biotinylated)</td>
<td>Donkey</td>
<td>1:1000</td>
<td>7131659003</td>
<td>Jackson(ImmunoResearch</td>
</tr>
<tr>
<td>anti-rabbit Alexa568</td>
<td>Goat</td>
<td>1:500</td>
<td>A11011</td>
<td>Molecular</td>
</tr>
<tr>
<td>anti-chicken Alexa488</td>
<td>Goat</td>
<td>1:500</td>
<td>A110039</td>
<td>Molecular</td>
</tr>
<tr>
<td>anti-guineapig Alexa488</td>
<td>Goat</td>
<td>1:500</td>
<td>A110073</td>
<td>Molecular</td>
</tr>
<tr>
<td>strepdavadin Alexa568</td>
<td>n/a</td>
<td>1:500</td>
<td>S11226</td>
<td>Molecular</td>
</tr>
</tbody>
</table>
2.4.2 Immunofluorescence

Single label fluorescent immunohistochemistry was used in a similar way as described for chromogen staining. After the first TBS-TX wash step sections were blocked in for one hour in blocking solution (1% BSA and 3% NGS or 3% NDS (for nNOS staining) in TBS-TX, pH 7.6). Primary antibody (Table 2.1) was added to make up the specific final antibody concentration and incubation was at 4 °C over 24-72 hours. Tissues were removed from primary incubation solution, washed and incubated for 60 mins in fluorescent secondary antibody (Table 2.2) when a direct label was used. Alternatively, sections were washed and incubated with a biotinylated secondary antibody (Table 2.2) followed by 60 mins of strepavidin-568 (Molecular Probes; P36930) fluorescent label (indirect labeling). Tissue sections were mounted onto glass microscope slides and cover slipped using Vectashield fluorescent mounting medium (Vector Laboratories; H-1000).

After adding fluorescent secondary antibodies all incubation steps were performed under low-light conditions.

Double immunofluorescent labeling was done by co-incubation of primary antibodies (Table 2.1). As for dual-label chromogen staining, both primary antibodies had to be raised in different hosts.

In each immunohistochemistry experiment primary antibody omission was used as an internal measure of non-specific binding of the secondary antibody. No staining was observed when this was done. Other measures of antibody validation are described in the appropriate chapters.
2.5 Measuring mRNA levels

2.5.1 Micro punching

Frozen fresh brains were removed from −80 °C storage and sectioned into 300 µm coronal slices at −9 °C on a cryostat (Slee, London, UK). Sections were collected onto glass microscope slides, and specific hypothalamic regions were identified under a dissecting microscope and punched out using RNase free blunted 21 gauge needles (inner diameter 0.8 mm). Micro punches were collected in 300 µl of lysis buffer (buffer RLT, RNeasy mini kit, QIAGEN; 74104) containing 3 µl of β-mercaptoethanol (Sigma-Aldrich; M3148-25ML). Samples were frozen on dry ice and stored at −80 °C. Next, samples were sonicated for 180 sec at 100 Hz to disrupt cells within the tissue (3 sec interval; Vibra Cell, Sonics & Materials Inc.), then vortexed, frozen on dry ice, and stored at −80 °C until RNA extraction.

2.5.2 RNA extraction

Total RNA was extracted following the manufacturers protocol of the RNeasy mini kit (QIAGEN; 74104). Briefly, an equal volume (300 µl) of 70% ethanol was added to the lysate and mixed. The whole mixture (600 µl) was transferred to an RNeasy spin column, placed in a 2 ml waste collection tube and centrifuged for 15 sec at 8000 x g, and the flow-through was discarded. Spin column membranes with total RNA bound to them were washed once with 700 µl Buffer RW1 followed by another two washes with 500 µl Buffer RPE. Columns were then placed into clean 2 ml waste collection tubes and centrifuged for 1 min for an extra cleaning step. Then columns were transferred to a clean 1.5 ml collection tube. To elute total RNA, 30 µl of RNase-free H₂O was directly pipetted into the center of the membrane and centrifuged for 1 min. The last step was repeated by pipetting the eluent onto the membrane again. Directly after elution total RNA concentrations were measured in 1 µl using a Nanodrop spectrophotometer (Thermo Fisher
Scientific Inc.;ND-1000). Then samples were stored at −80 °C until required for further processing.

2.5.3 DNase treatment

Since it could not be guaranteed that all of the proprietary quantitative polymerase chain reaction (qPCR) primers were designed to span intro-exon boundaries, the RNA was DNase treated. The same amount of RNA was added to each DNase reaction. The amount of RNA was calculated from Nanodrop readings. Equal final concentrations were achieved by adding different amounts of RNA eluent and RNase-free H2O to make up a final volume of 27 µl. For DNase treatment, samples were mixed with 3 µl of 10x RQ1 RNase-free DNase buffer (10x; Promega; PMM6101) and treated with 1 µl each of RQ1 RNase-free DNase (Promega) and RNase out (40 U/µl; Invitrogen; 10777-019). Then samples were incubated at 37 °C for 30 mins. To terminate the reaction, 1 µl of RQ1 DNase STOP solution was added (Promega) and samples were incubated at 65 °C for 10 mins.

2.5.4 Reverse transcription

DNase treatment was directly followed by cDNA synthesis. For this, 3 µl of dNTPs (deoxyribonucleotide triphosphate, 10 mM, Invitrogen; 18427-013) and 3 µl of random hexamers (50 µM, Invitrogen; N8080127) were added to the DNase treated samples, and the mixture was incubated at 65 °C for 5 mins. After cooling down on ice (for at least 1 min), 12 µl of first strand RT buffer (5x; 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3; Invitrogen), 3 µl of DTT (dithiothreitol, 100 mM, Invitrogen), 3 µl of RNase out (40 U/µl; Invitrogen; 10777-019), and 3 µl of Superscript III RT (200 U/µl, Invitrogen; 18080-044) were added to each sample. Samples were incubated for 5 mins at 25 °C, 50 mins of 50 °C and 15 mins at 70 °C in a thermo cycler (Biometra TProfessional Basic Thermo cycler, Biomedizinishe Analytic GmBH). The cDNA was stored at −20 °C until use in qPCR analysis.
2.5.5 Preparing the plate for real-time quantitative PCR using probe/primer sets

Reactions (20 µl) were prepared for analysis in a 96-well plate (Roche, #04729692001) with adhesive optical covers. For each reaction, 1 µl of cDNA was added to 10 µl of 2x Lightcycler 480 probes master (Roche, #04887301001), 1 µl of each primer and the probe, and 6 µl of H2O. Using a Roche Lightcycler 480 qPCR thermo cycler (Roche), samples were heated to 95 °C for 10 min, before 45 cycles of 95 °C for 10 sec and then 60 °C for 30 sec. Amplification curves were assessed using the Lightcycler 480 software (Roche). Any obviously poor replicates were discarded.

2.5.6 Preparing real-time quantitative PCR TaqMan array cards

These cards were purchased pre-loaded with proprietary primer/probe sets, as shown in Table 6.2. Loading and preparing of the samples to the array cards was done as described by the manufacturer¹. Briefly, 55 µl of cDNA was mixed with 55 µl of gene expression master mix (TaqMan; #4369016). Of this mixture, 100 µl was added to each loading port, where each port gives access to 48 wells in the array card. Array cards were inserted into the custom cardholders and buckets, and then centrifuged (331 x g) twice for 1 minute each to evenly distribute the sample (1 µl per well). After centrifuging the samples, the fill ports of the array cards were checked to see if there was any blockage in the system. Once clear, the cards were sealed using the TaqMan array micro fluidic card sealer, and fill ports were removed. Using a ViiA 7 Real-Time PCR machine (Applied Biosystems), samples were heated to 50 °C for 2 min then 95 °C for 10 min, before 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Amplification curves were assessed using the ViiA 7 software (Roche). Any poor replicates were discarded.

2.6  Hormone assays

2.6.1 Estradiol radioimmunoassay

Levels of serum estradiol were quantified by radioimmunoassay (RIA) using a commercial kit (Diagnostic Systems Laboratories; DSL-4800) according to manufacturer’s protocol. Two modifications were made: reactions were performed with 50 µl aliquots of sample in duplicate (instead of 200 µl), and an overnight incubation (instead of one hour) was used after addition of the radioactive tracer $^{[125]}$Iestradiol. Sample concentrations were calculated according to the standard curve.

2.6.2 LH radioimmunoassay

For measurement of plasma LH in Chapter 5 a RIA was used. Iodination of rat luteinizing hormone (rLH, NIDDK-rLH-I-10, batch number AFP11536B) was carried out with iodine$^{125}$ (I$^{125}$I, Perkin-Elmer; NEZ033A005MC), using the chloramine T (ChT) method. This was done by adding 5 µl of I$^{125}$I to the vial containing rLH (4 µg of LH in 20 µl of 0.01 M NaHCO$_3$ buffer), in the presence of 20 µl ChT (2 mg/ml in 0.05 M PB, pH 7.4). Chloramine T is an oxidizing agent that converts Na$I^{125}$ to the more reactive form, free iodide (I$^-$). The reaction was terminated after 60 sec with 20 µl sodium metabisulphate (5 mg/ml in 0.05 M PB, pH 7.4) followed by addition of 100 µl of RIA buffer (0.1 M PB containing 50 mM EDTA, 137 mM NaCl, 1 mM sodium azide and 5% BSA; pH 7.4).

The iodinated solution was run through a Sephadex G50 column to separate out bound I$^{125}$-rLH molecules. First, 50 µl of dextran-blue dye was added to the column and allowed to drain into the Sephadex. Immediately after, the LH iodination mixture was added to the column and allowed to drain. The hormone was eluted with 0.05 M PB (pH 7.4) in 6-drop fractions (≈0.3 ml). Collection was started when the dye approached the bottom of the column. Each fraction was counted in a gamma counter (Wizard 1470 Automatic
Gamma Counter, Perkin-Elmer), and the best fraction of first peak containing the most radioactivity was kept. The second peak contained free iodine and was discarded. To these fractions 200 µl 5% BSA buffer (0.05 M PB, 1% NaN₃, 5% BSA) was added and stored at 4 °C.

On the first day of the RIA, 25 µl of standards (0.049-25 ng/ml; NIDDK-rat LH-RP-3), quality control samples (Low, Med, High), and samples were added to appropriate polystyrene tubes (5 ml, 75 x 12 mm; Sarstedt; D-51588) in triplicate. To non-specific binding (NSB) and blank (Bo) tubes 25 µl RIA Assay Buffer was added. Then 100 µl of primary antibody (1:300,000; NIDDK rabbit anti-rat LH-S11) in RIA Assay Buffer was added to all tubes except total counts (TC) and NSB (NSB tubes got 100 µl of RIA assay buffer instead). Tubes were vortexed briefly and incubated overnight at 4 °C.

On day two; 50 µl of ¹²⁵I-labelled hormone (diluted to ~5500 cpm/50 µl in RIA assay buffer containing 1:400 normal rabbit serum) was added to all tubes, shaken gently and incubated for at least 20h at 4 °C.

On the third day; 50 µl of 1:50 sheep anti-rabbit serum (a gift from AgResearch Invermay, SAR 10 AB2) in RIA assay buffer was added to all tubes except TC. Tubes were shaken gently and incubated for at least 15h at 4 °C.

On day four, TC tubes were set aside and 1 ml 5% PEG buffer (5% polyethylene glycol in 0.05 M PB) was added to all other tubes and centrifuged for 30 min (1200 x g, 4°C; Hereaus Cryofuge 6000i). Tubes were decanted and blotted dry on paper towels. Pellets were counted for 2 min/tube on the gamma counter (Wizard 1470 Automatic Gamma Counter, PerkinElmer). Any single measures that were not within the range of the other two were removed before analysis.
2.6.3 Luteinizing hormone enzyme-linked immunosorbent assay

Serum LH concentrations were measured in Chapter 6 by an in-house sandwich enzyme-linked immunosorbent assay (ELISA), as described by Steyn et al. (Steyn et al., 2013). This method of measuring LH in smaller samples became available to our lab partway throughout the experiments of my PhD, therefore both methods are used in this thesis.

Briefly, on the first day plates were coated (overnight, 4 °C) with 50 μl monoclonal primary antibody (1:1000; bovine anti-LHβ 518B7, obtained from L. E Sibley at University of California, Davis) in 0.1 M PBS. Washes were done between all steps, three times for 3 minutes, using 200 μl PBS-T (0.1 M PBS with 0.05% Tween 20; pH 7.4). On the second day plates were decanted and 200 μl blocking buffer (5% skim milk powder in PBS-T) was added and incubated for two hours. Plates were then washed and loaded with 50 μl of either standards or samples in duplicate, and left to incubate for two hours at room temperature. Next, 50 μl of polyclonal detection antibody in blocking buffer was added (1:10000, rabbit-anti LH AFP240580Rb, from AF Parlow mouse RIA kit), and plate was incubated for 90 minutes at room temperature and then 50 μl of HRP-conjugated secondary antibody (1:1000, goat anti-rabbit IgG-HRP; DAKO; P0448) in 50% blocking buffer and 50% PBS was added and incubated for 90 min. For the colimetric reaction, 100 μl of O-phenylenediamide (OPD; Invitrogen; 00-2003) in citrate buffer (0.1 M; pH 5.0) with 0.01% H₂O₂ was added, and the plate was protected from light and incubated for 30 mins. Then 50 μl of HCl (3 M) was added to stop the reaction, and the absorbance of the wells was read at 490 nm (Victor X3, PerkinElmer Inc.). Sample concentrations were calculated according to the standard curve.

2.6.4 Leptin ELISA

Measuring plasma leptin concentration by ELISA was done using antibody-coated micro plates from a commercial kit (Mouse Leptin ELISA kit; Crystal
The micro plate was washed using wash buffer provided. Next, 45 µl sample diluent, 50 µl guinea pig anti-leptin serum, and 5 µl of standard or sample were added to the appropriate wells in duplicate. The plate was incubated overnight and calorimetric experiment was performed. At the end the absorbance at wavelengths of A450 and A630 values were measured using a plate reader (Victor X3, PerkinElmer Inc.). Sample concentrations were calculated according to the standard curve.

2.6.5 Testosterone ELISA

Serum total testosterone concentration was measured using a commercial rat/mouse ELISA kit (Labor Diagnostika Nord; AR E-8000). Briefly, standards and blood serum samples (10 µl/well) were incubated in duplicate with enzyme conjugate (50 µl in 100 µl of the provided incubation buffer) for 60 mins. After washing, wells were incubated with 200 µl substrate solution for 30 mins, before adding 50 µl of the stop solution. The absorption reading was measured within 15 mins for 1 sec per well at 450 nm on a plate reader (Victor X3, PerkinElmer Inc.). Sample concentrations were calculated according to the standard curve.

2.7 Generation of transgenic animals

2.7.1 Breeding of transgenic animals

All transgenic animals were generated using the Cre-Lox genetic recombination system, which was explained in Figure 1.5. Cre and floxed mouse lines (Table 2.3) were obtained from colonies maintained by Assoc. Prof. Anderson within the University of Otago animal breeding facilities. Pairing homozygous Cre and floxed animals started the first round of breeding. Offspring (f1) were heterozygous for both Cre and flox and were used in the second round of breeding. Heterozygous f1 males and females were paired with animals homozygous for the floxed gene to create the f2 generation. F2 animals that were positive for Cre and homozygous for flox,
as determined by genotyping (Section 2.7.2), were used as the experimental group, while animals homozygous for flox but negative for Cre were the littermate controls. All genotypes were checked using standard genotyping protocols outlined below.

Table 2.3 Transgenic animals used in this thesis

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cre promoter</th>
<th>Floxed sequence</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepr reporter</td>
<td>Lepr-Cre</td>
<td>stop-eGFP</td>
<td>GFP expression in Lepr cells</td>
</tr>
<tr>
<td>nNOS reporter</td>
<td>nNOS-ER-Cre</td>
<td>stop-eGFP</td>
<td>inducible eGFP expression in nNOS neurons</td>
</tr>
<tr>
<td>Vgat reporter</td>
<td>Vgat-Cre</td>
<td>stop-ttdtomato</td>
<td>ttdtomato expression in GABAergic neurons</td>
</tr>
<tr>
<td>NOLEPKO</td>
<td>nNOS-ER-Cre</td>
<td>Lepr</td>
<td>inducible Lepr knockout in nNOS neurons</td>
</tr>
<tr>
<td>GABA specific LEPR knockout</td>
<td>Vgat-Cre</td>
<td>Lepr</td>
<td>knockout of Lepr in GABAergic neurons</td>
</tr>
<tr>
<td>Glutamate specific LEPR knockout</td>
<td>Vglut2-Cre</td>
<td>Lepr</td>
<td>knockout of Lepr in Glutamatergic neurons</td>
</tr>
</tbody>
</table>

2.7.2 Genotyping

DNA extraction. Approximately 2 mm of the tip of the tail was cut off and collected in 0.6 ml of lysis buffer (pH 8.5) containing 5 µl proteinase K (20 mg/ml; Roche; 03115887001) and left to digest at 55 °C overnight. To remove all protein and lipid membrane residues, samples were centrifuged for 10 min at maximum speed (16000 x g). Supernatant containing genomic DNA was decanted into a new tube containing 0.6 ml isopropyl alcohol. After mixing by inverting the tubes, samples were centrifuged again for 5 min, causing the DNA to form a pellet at the bottom of the tube. Pellets were resuspended in 200 µl Tris-EDTA buffer (TE buffer; pH 8.0), and subsequently stored at -20 °C until used in polymerase chain reaction (PCR).

PCR. For every genotyping experiment, a negative water control and wild type or homozygous mouse DNA controls were included. For every reaction, premixed PCR master mix was made containing 12.5 µl 2x Reddymix (Thermo Fisher Scientific; AB-0575/DC/LD/B), 0.5 µl of each of the primers (Table 2.4) and this was made up to 23 µl per reaction with H2O. Two µl of DNA sample was added to each reaction. The PCR reaction was performed in a thermocycling machine (Biometra TPProfessional Basic Thermocycler,
Biomedizinische Analytische GmBH) as follows: initially 95 °C for 3 min to activate Taq polymerase and denature DNA strands, then 35 rounds of: 95 °C for 30 sec to denature, 60 °C for 1 min to anneal primers to DNA, and 72 °C for 45 sec to elongate (XX is primer pair specific; Table 2.4). Final elongation was done over 5 min and then samples were cooled to 10 °C. Products were visualized by 1.5% or 3% agarose gel electrophoresis.

Table 2.4  Primer pairs used for genotyping.

<table>
<thead>
<tr>
<th>Transgenic gene</th>
<th>Primers (5'-3')</th>
<th>Primer concentration</th>
<th>MgCl2 concentration</th>
<th>Annealing temperature</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic Cre</td>
<td>1: CTT GGA AAA TGC TTC TGT CCG</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>55 °C</td>
<td>wt: none mutant: 400 bp</td>
</tr>
<tr>
<td></td>
<td>2: CAG GGT GTC ATA AGC ATG CCC</td>
<td>1 μM</td>
<td>1.25 mM</td>
<td>52 °C</td>
<td>wt+DNA control: 324 bp mutant: 100 bp</td>
</tr>
<tr>
<td>LepR Cre</td>
<td>1: GCG GTG TGG CAG TAA AAA CTA TC</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>2: GTG AAA CAG CAT TGC TGT CAT TT</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>3: CTA GGG CAC AGA ATT GAA AGA TCT</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>4: GTA GGT GGA AAT TCT AGC ATG CCC</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td>Lepr-floxed</td>
<td>1: AAT GAA AAA GTT TTG GGA CGA</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>2: CAG GTG TGA GAA CAT GAA CAC AAC AAC</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>3: CTG ATG TGA TAG ATG GTC TGG AG</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>59 °C</td>
<td>wt: ~200 bp mutant: 250 bp</td>
</tr>
<tr>
<td>nNOS-cre</td>
<td>1: CTT GGC TTC GAG TTC TTC TTG</td>
<td>1+2: 50 μM</td>
<td>1.25 mM</td>
<td>62 °C</td>
<td>wt: 313 bp mutant: 168 bp</td>
</tr>
<tr>
<td></td>
<td>3: CAG GTT CTT GCG AAC CTC AT</td>
<td>3: 83.5 μM</td>
<td>1.25 mM</td>
<td>62 °C</td>
<td>wt: 313 bp mutant: 168 bp</td>
</tr>
<tr>
<td>Tau eGFP</td>
<td>1: CGA AGT CGC TCT GAG TTA TC</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>60 °C</td>
<td>wt: 600 bp mutant: 400 bp</td>
</tr>
<tr>
<td></td>
<td>2: GCA GAT GGA GCG GGA GAA AT</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>50 °C</td>
<td>wt: 600 bp mutant: 400 bp</td>
</tr>
<tr>
<td></td>
<td>3: GCT CTT ATG GGC GTT ACT ATG</td>
<td>10 μM</td>
<td>0.75 mM</td>
<td>50 °C</td>
<td>wt: 600 bp mutant: 400 bp</td>
</tr>
</tbody>
</table>

Gel electrophoresis. Agarose (Merck, 1.5%, or 3% gel) was added to the appropriate volume of TBE buffer (pH 8.3). The solution was then mixed and heated until dissolved, then cooled to about 60 °C when 0.03 μl/ml ethidium bromide (10 mg/ml, Invitrogen; 15585-011) was added. The gel was poured into an appropriate tray with combs in place and allowed to set. Combs were removed and gel was submerged in TBE buffer in an electrophoresis tank (Bio-Rad Laboratories Inc.). Ten μl of PCR samples or 5 μl of DNA ladder (TrackIt ladders, Invitrogen; 10488-043) were added to each well. Gels were electrophoresed at 70-90 V for 30-45 min. Products were visualized under UV light and images were captured using BioDocAnalyze software (BioDocAnalyze, Biometra GmbH).
2.8 Reproductive physiology

2.8.1 Puberty onset

In female experimental and control mice, puberty onset was measured by the age of vaginal opening and first estrus, which are the first signs of transition to sexual maturity. Animals were monitored daily from 26 days of age. Vaginal opening was assessed by eye and noted when a clear opening was visible. Starting on the day following vaginal opening, vaginal cytology (Section 2.8.2) was studied daily to assess the signs of first estrus. First estrus is indicative of the first female cycle, and thus the true sign of entering reproductive adulthood.

Male puberty onset was assessed by preputial separation, which happens at the start of the transition into adulthood. Preputial separation was assessed by manually trying to separate the prepuce from the penis by applying gentle pressure on either side of the prepuce. When this was clearly established the age of the animal was noted. Male animals were monitored daily starting from 28 days of age. Another way to assess onset of reproductive competence in male animals is by pairing them with C57BL/6J (wild type) females of reproductive age. Experimental animals were paired from 34 ± 1 days of age. Attainment of male fertility was calculated by subtracting 21 days (gestation period) from the date when their first litter was born.

2.8.2 Vaginal cytology assessment

Vaginal smears were collected between 0900 and 1000h by inserting a small wire loop into the vagina of the mouse using water as a lubricant. Collected cells were spread on glass microscope slides, and histochemically stained with a drop of 0.05% toluidine blue. Smears were assessed under a light microscope using 4x, 10x and 20x objectives. A proestrus stage (P) was noted when cells were predominantly nucleated, estrus (E) when the smear contained mainly cornified cells and met/diestrus (M/D) when many
leukocytes were present or the smear was very sparse (Caligioni, 2009; Byers et al., 2012).

To determine adult female estrous cyclicity, vaginal smears were taken for a minimum of 10 consecutive days starting at least 14 days after the occurrence of first estrus. Average estrous cycle duration was calculated as the time between two proestrus phases.

2.8.3 Adult fertility assessment

Female and male animals were paired with C57BL/6J (wild type) mice of the opposite sex of reproductive age (minimum 8 weeks old) to further assess adult fertility. At least three times a week breeding pairs were checked for litters (starting 18 days after pairing); date of birth and litter size was noted and any litters were culled. Breeding studies were performed for at least a 100 days; from the data collected average number of litters, inter-litter interval, and litter size could be calculated.

2.8.4 Estimation of daily sperm production

Daily sperm production (DSP) was determined from testes stored at -20 °C, as described previously with some modifications (Robb et al., 1978; Singireddy et al., 2013). Briefly, fragments of testes (~25 mg) were placed in 600 µl of solution containing 0.9% NaCl, 0.01% sodium azide, and 0.05% Triton X-100 and homogenized in a tissue lyser (TissueLyser II, Qiagen) for 10 min. Spermatids in stages 14-16 of spermatogenesis (Leblond and Clermont, 1952) are resistant to this homogenization and their nuclei were counted in 10 µl aliquots of homogenate using a hemocytometer. The total number of counted nuclei was divided by 4.84, because developing spermatids spend 4.84 days in stages 14-16 during spermatogenesis in mice. This value is the DSP.
2.9 Surgical manipulations

All mouse surgeries were performed under 1-2.5% isoflurane and 70% oxygen anesthesia. Animals were placed on a heated pad, and the skin was cleaned using a hibitane solution (Sumitomo Pharmaceuticals C. Ltd.). Standard aseptic techniques were used throughout all surgical procedures. All animals were given a sc bolus of 5 mg/kg carprofen (Carprieve) during the surgery as a long lasting analgesic. At the end of each surgery mice were left to recover in clean, warmed cages, before returning to their normal animal housing rooms. On the five days following surgery the animals’ appearance, body weight, and fluid intake was monitored closely (further referred to as post-surgery monitoring).

2.9.1 Ovariectomy

Female mice were anaesthetized, the hair on the dorsal surface of the animal was removed and skin was cleaned. A 10-20 mm incision was made in the skin about 2/3 down the back of the animal. The ovarian fat pad was visualized through the abdominal wall before making a small (2-4 mm) incision through the muscle wall. The ovary in its fat pad was pulled through the opening using forceps, then clamped and removed. Once the clamp was removed, the uterine horn was checked for bleeding and the abdominal wall incision was closed with a single suture (4-0 Vicryl, Softsilk sutures; GS-831). A drop of 2 mg/ml Lopaine (lignocaine hydrochloride) was applied to the incision site for local analgesia. This was repeated on the contralateral side of the animal, and the skin incision was closed using wound clips (Harvard Autoclips; 340557/340555).

2.9.2 Estradiol implant

Estradiol implants were used to assess estradiol negative feedback. To make the implants, 1 mg/ml 17β-estradiol (Sigma-Aldrich; E8875-1G) dissolved in absolute ethanol was mixed in a 1:10 ratio with Silastic Medical grade A
silicone adhesive (Dow Corning; 01019821). This viscous 0.1 mg/ml solution was then put into Silastic tubing (inner diameter 1.02 mm, outer diameter 2.16 mm; Dow Corning; 508-005), resulting in approximately 1 μg estradiol per 10 mm of tubing. To insert the implant, female ovariectomized animals were anaesthetized and a 10 mm incision was made into the skin of the dorsal surface (below the neck). Using blunt dissection methods a subcutaneous pocket was created and the implant (measured by length to contain 1 μg estradiol per 20 g body weight) was inserted. Skin was closed using wound clips (Harvard Autoclips) and animals were allowed to recover.

2.9.3 Multiple time point blood sampling

Tail tip blood samples were collected when animals were under isoflurane anesthesia. The tip (2-4 mm from the end) of the tail was cut using a scalpel blade and tail was massaged to release drops of blood that were collected into heparinized capillary tubes (Chase Scientific; CSE2501). Three tubes were collected per animal (150 μl of whole blood) and plasma was separated from the red blood cells in a centrifuge (5000 x g) at room temperature with a hematocrit rotor. Capillary tubes were snapped at the separation level of plasma from cells and plasma was combined into a 0.6 ml Eppendorf tube and immediately frozen in dry ice. Samples were stored at -20 °C until further use.

2.9.4 Stereotaxic injection of RetroBeads

Animals were anesthetized, and secured via ear bars (Kopf Instruments; 943-45) into a stereotaxic device. A rostral to caudal 10 mm skin incision was made to expose the skull and bregma point. The bregma point was calibrated as zero and from there the tip of a 0.5 μl Hamilton syringe
(Hamilton Company; #7000.5) was moved 0.9-1.1 mm rostral (according to the body weight of the animal <19 g: 0.9 mm; 20-22 g: 1.0 mm; >23 g: 1.1 mm). At this point on the skull a small hole was made using a ball shaped drill tip (Leica; 39416023), being very careful not to disturb underlying tissue. Then 50 nl of red or green RetroBeads (Lumafluor; red: R165, green: G165), was drawn up into the Hamilton syringe. The sagittal sinus was pulled aside using a small surgical hook, and the tip of the Hamilton syringe was lowered into the rPOA (4.2-4.4 mm ventral; according to body weight <19 g: 4.2 mm; >21 g: 4.4 mm). After waiting for 5 minutes the tracer was injected slowly over the course of another 5 minutes. The syringe was left in place for 10 minutes, and then pulled up slowly. Skin was closed with 3 sutures (4-0 Vicryl, Softsilk sutures; GS-831) and animals received 5 mg/kg carprofen (Carprieve) for pain relief and were removed from stereotaxic device. After recovery animals were subjected to post-surgery monitoring.

### 2.10 Data analysis

#### 2.10.1 Outliers

Removal of outliers was applied to results of hormone assays and qPCR experiments. Two rules were applied to filter out the outliers. First, when samples were measured in triplicate if a single replicate was out of line with the other two it was removed from the analysis. Secondly, if a sample from an animal was more than two standard deviations removed from the mean of the group (with a minimum of 50% of the samples to calculate the mean) the sample was removed. Removal of outliers was in all cases done manually.

#### 2.10.2 Statistical analysis

All numerical data are presented as mean ± standard error of the mean (SEM), and a value of p < 0.05 was considered to be significant. GraphPad Prism software (versions 5 and 6) was used for all analyses except for bodyweight analysis in **Chapter 5** this was done using Sigma stat.
The two main parametric (assuming Gaussian distributions) statistical methods used within this thesis are Student’s t-tests and two-way ANOVA’s. Two-tailed unpaired Student’s t-testing was used to compare significant differences between two groups. If there were more than two groups to compare with each other a one-way ANOVA was used to show significant differences between groups. Repeated measures two-way ANOVA was used to compare changes over time between two groups, and if a main effect of group was found, Bonferroni’s multiple comparison *post hoc* testing was used to determine the time points at which the significant differences occurred.

When the data was not equally distributed or the sample size was less than 4 (like in Chapter 3), a more conservative non-parametric equivalent test the Mann-Whitney U test instead of the Student’s t-test was used.
Chapter Three
Leptin Responsive Projections to the Rostral Preoptic Area

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Appendix III

Additional data to be prepared for publication
3.1 Abstract

The adipose tissue derived hormone leptin plays a critical role in the control of reproduction via signaling in the brain. The GnRH neurons do not have the receptors for leptin; therefore intermediate leptin responsive neurons that provide leptin-to-GnRH signaling must exist. In this chapter, I investigated the populations of the leptin responsive neurons that provide input to the rPOA, where GnRH cell bodies reside. Fluorescent retrograde tracer beads (RetroBeads, Lumafluor) were injected into the rPOA of transgenic Lepr reporter mice (Lepr-eGFP). Uptake of the tracer by Lepr-eGFP neurons was assessed throughout the hypothalamus, with emphasis on regions that are leptin responsive and GABAergic (as these neurons play a critical role in leptin's regulation of fertility). Tracer uptake was most evident in the medial Arc, the DMN, and the PMV. The uptake of the tracer by Lepr-eGFP neurons was the highest in the medial Arc. To address whether GABAergic Lepr neurons in the Arc and DMN project to the rPOA, the above experiment was repeated in GABA reporter mice (Vgat-tdTomato). Interestingly, 24.1 ± 7.6% of tracer labeled neurons in the Arc were GABAergic, and uptake of tracer by GABAergic neurons in the DMN was very low (5.2 ± 5.2%). Together these results suggest that both leptin responsive and GABAergic neurons from the Arc project to the region of the GnRH cell bodies. This work helps to narrow down the location and possible identity of mediators of leptin-to-GnRH signaling.
Chapter Three

3.2 Introduction

Leptin signaling in the brain plays a critical role in the control of reproduction. Reproductive capacity is driven by GnRH release into the portal bloodstream (Knobil and Neill, 2006). However, GnRH neurons in the rPOA do not have the receptors for leptin (Quennell et al., 2009); therefore there must be intermediate neurons that provide leptin-to-GnRH signaling in the brain. This chapter investigates the populations of the leptin responsive neurons that project to and provide input to the location of GnRH cell bodies in the rPOA.

Neurons sensitive to leptin, i.e. Lepr expressing neurons, are found throughout the extent of the hypothalamus and other regions of the mouse brain (Scott et al., 2009; Patterson et al., 2011). Leptin receptor neurons in different hypothalamic centers are involved in different leptin responsive functions. For example, many of the Lepr neurons of the Arc regulate energy homeostasis (Håkansson et al., 1996; Mercer et al., 1996; Cheung et al., 1997a; Baskin et al., 1999) and Lepr neurons in the PVN are involved in mediating leptin’s effects on the stress axis (Heiman et al., 1997). A single specific region that is involved in the control of reproduction by leptin has not been identified yet.

One hypothalamic region that is thought to be involved in the regulation of fertility rather than energy expenditure by leptin is the PMV (Donato et al., 2011b). Lesion of the PMV impairs reproductive functioning, but does not alter metabolic properties in rats (Donato et al., 2009). Additionally, selective re-expression of Lepr only within the PMV in female Lepr-null mice causes puberty onset, and about 50% of those animals were also able to get pregnant (Donato et al., 2011b). Thus, leptin signaling only in the PMV is sufficient for fertility to occur. However, experiments trying to show if leptin signaling is necessary in the PMV for fertility (i.e. deletion of functional Lepr only from PMV neurons) have been unsuccessful thus far (Elias, 2014). Glutamate and CART neurons from the PMV project to the mPOA, and might form crucial GnRH afferents (Kocsis, 2003; Rondini et al., 2004; Leshan et al., 2009). However, it is not known if these glutamate and CART neurons are also leptin responsive.
Previous neuronal tracing experiments have revealed hypothalamic regions that provide input to the GnRH neurons (Yoon et al., 2005; Wintermantel et al., 2006). However, these experiments mainly focus on olfactory and estrogenic feedback to GnRH neurons, and do not provide information about leptin responsive cells. In this chapter I will look at leptin responsive inputs to GnRH neurons.

It is known that both POMC neurons and NPY neurons found in the Arc express Lepr and project to the rPOA and make contact with GnRH and kisspeptin neurons (Leranth et al., 1988; Baskin et al., 1999; Cowley et al., 2001; Turi et al., 2003). However, when Lepr are deleted from POMC and AgRP neurons there is no reduction in reproductive function (van de Wall et al., 2008; Israel et al., 2012). This interaction is therefore not crucial for metabolic feedback to the HPG axis. In 2011 Louis et al. (2011) showed that GnRH neurons are innervated by Lepr neurons using the trans-synaptic tracer wheat germ agglutinin (WGA); however this technique does not show where these projections are coming from. Louis et al. (2011) used the BIG transgenic mouse model to evaluate the location of Lepr neuronal inputs to GnRH neurons. This mouse expresses barley lectin (BL), an internal ribosomal entry site (IRES), and GFP (BL-IRES-GFP; BIG) under the control of the GnRH promoter; which results in GFP expression in GnRH neurons and labeling of anterograde (postsynaptic) and retrograde (presynaptic) neurons with BL. In this study they found very low numbers of leptin responsive inputs to GnRH neurons in the PMV and preoptic area. It should be noted that labeled neurons in BIG mice will presumably include those that contact GnRH neurons during development, when GnRH neurons migrate from the olfactory placode.

For leptin to have an indirect effect on GnRH neurons, leptin responsive cells have to be linked to the GnRH neuronal system. I hypothesize that ‘first order’ leptin responsive innervations originate from the Arc, LHA, DMN, PMV or from within the preoptic area itself. In the current chapter this hypothesis is explored using a retrograde tracer. The RetroBeads are injected into the region of GnRH
neurons and retrograde uptake of this tracer by Lepr neurons is assessed throughout the hypothalamus. This will disclose the residence of first order leptin sensing neurons in the GnRH system.

3.3 Materials and methods

3.3.1 Generation of Lepr and Vgat reporter mice

Leptin receptor eGFP reporter mice were generated using four breeding pairs in which leptin receptor Cre (Lepr-Cre, JAX mice stock number 008320, DeFalco et al., 2001) mice were crossed with enhanced green fluorescent protein (eGFP) stop-flox mice (stopflox-Tau-eGFP, ROSA26-CAGS-τGFP, Rodrigues et al., 1999), as described in Section 2.7. The eGFP is connected to the Tau protein that stabilizes neuronal microtubules, which shows the cytoplasmic structure of the cells expressing it. The Vgat-tdTomato reporter mice were generated by crossing vesicular GABA transporter Cre (Vgat-ires-Cre, Vong et al., 2011) with red fluorescent protein (tdTomato) stop-flox animals (stopflox-tdTomato, Ai9, JAX mice stock number 007905/007909, Madisen et al., 2010; Rizwan et al., 2014). The resultant offspring from two breeding pairs have red fluorescent labeling expressed by Vgat neurons. Mice were identified using DNA isolated from tail tips and primers for generic Cre, Tau-eGFP and tdTomato, as described in Section 2.7.2. Animals heterozygous for both the Cre and floxed genes were used in this experiment.

3.3.2 Validation of eGFP expression in leptin receptor neurons

To validate eGFP expression in the Lepr reporter mice, animals were treated with leptin and immunohistochemical staining for pSTAT3 was employed (a widely used marker of leptin signaling, e.g. Anderson et al., 2003). Animals were treated with two different leptin treatment regimes: 1 mg/kg leptin was injected sc, or 0.1 μg was injected icv (Section 2.2). Fixed brain tissue was collected and sectioned, as described in Section 2.3.1. Tissue of all reporter
animals was kept in the dark to retain fluorescence. For icv leptin treatment female animals were subjected to leptin (n = 3) or vehicle (n = 2) treatment as described in Section 2.2.1. Animals were treated with sc leptin as described in Section 2.2.2: female animals had leptin (n = 3) or vehicle (n = 2) treatment.

3.3.3 Fluorescent immunohistochemistry for pSTAT3

To verify that eGFP expressing cells were leptin responsive, brain sections of leptin and vehicle treated animals were subjected to fluorescent immunohistochemical staining for leptin-induced pSTAT3 (Section 2.4.2). There was one major modification: antigen retrieval was performed for 15 minutes at 75°C instead of 90°C, as it was found that fluorescent eGFP signal was lost at higher temperatures. Antigen retrieval at temperatures below 75°C were found to be ineffective for pSTAT3 labeling. Positive leptin responsive cells were counted when a clear circular and brightly red stained nucleus was present. Leptin induced pSTAT3 reactivity was virtually absent in fasted vehicle-treated animals.

3.3.4 Fluorescent immunohistochemistry for GnRH

To verify the placing of tracer injections, rPOA sections of Lepr-eGFP mice (n = 4) were subjected to green fluorescent immunohistochemical staining for GnRH as described in Section 2.4.2. Validation of the primary antibody for GnRH was described by Rizwan et al. (2012), and shown by colocalization with a well validated GnRH antibody (Hu4H) and no staining was observed following preabsorption with GnRH. Immunofluorescent labeling of GnRH showed up as cytoplasmic green labeling of neuronal cell bodies with some labeling of proximal dendrites, and was confined to the previously described region of GnRH expression (i.e. medial septum, rPOA and anterior hypothalamic area for soma and primarily the OVLT and median eminence for fibers).
3.3.5 Stereotaxic injection of RetroBeads

Twelve female Lepr-eGFP animals and four female Vgat-tdTomato animals (20-32 g) received intra-cerebral rPOA injections of red (in Lepr-eGFP mice) or green (in Vgat-tdTomato mice) RetroBeads, as described in Section 2.9.4. As a control, red RetroBeads were injected into the striatum (0.9 mm rostral, 3.0 mm lateral and, 2.5 mm caudal of bregma) of one Lepr-eGFP animal.

Seven days after the injection of the RetroBeads animals were fasted overnight, treated with sc leptin and perfused with 4% PFA (Section 2.3.1). Rostral brain sections were checked for accuracy of injection site based on the placement of the fluorescent tracer; only animals with the right injection site (midline injection, placed between 0.74-0.38 mm rostral to bregma) were used for further analysis. Brains were cut into four equivalent series of 30 µm sections on a sliding microtome, so that within each series every fourth section (120 µm apart) was collected from the rostral extent of the preoptic area until the caudal extent of the 3rd ventricle.

3.3.6 Analysis

Brain sections from Lepr-eGFP mice with leptin induced pSTAT3 staining photomicrographs were taken at 20x magnification in different hypothalamic areas using an Olympus BX51 fluorescent microscope with a Diagnostic instruments SPOT RT KE camera. An overlay of the green and red images was made using ImageJ software (National Institutes of Health; NIH). Using the count cells plugin within ImageJ, single label red and green neurons, as well as double labeled cells (red nuclear staining surrounded by green cytoplasmic eGFP) were counted and percentages of colocalization were calculated.

For the retrograde tracer experiment, sections were viewed and photographed under the fluorescent microscope (as above). The number of retrogradely labeled cells, defined as a cluster of fluorescent beads in the shape of a cell, was counted for the specific hypothalamic regions directly from viewing at 10x or
20x under the microscope. By switching fluorescent filters, tracer-labeled cells were checked for colocalization with eGFP or tdTomato. From these data, the percent of colocalization was calculated. With the use of the confocal microscope (Zeiss LSM 710) and ZEN 2009 software, Z-stack images were taken for representative images of areas shown in Figures 3.2 and 3.5.

All results are presented as mean ± SEM. Mann-Whitney U tests were used to identify statistically significant differences (p < 0.05) between icv and sc leptin treatment regimes.

### 3.4 Results

#### 3.4.1 Validation of eGFP expression in Lepr neurons

The Lepr reporter mouse has eGFP expression in Lepr neurons caused by Cre mediated recombination of the floxed stop sequence in front of the eGFP gene (stop-flox-eGFP). These animals were validated using colocalization of the eGFP signal with the leptin induced second messenger pSTAT3: an established way to assess leptin responsive cells in the brain (Anderson et al., 2003). Additionally, two different leptin administration regimes (icv and sc) were compared, to see if there were any different results in the areas of interest due to the different leptin availabilities from these two approaches. The levels of colocalization of leptin induced pSTAT3 immunohistochemistry with eGFP in the Lepr reporter animals are shown in Table 3.1 and 3.2. Tissue of vehicle treated animals showed absence of pSTAT3 IHC in all regions except the Arc, where it was significantly lower than in leptin treated tissue (p < 0.05).

In the Arc approximately 70% of eGFP expressing neurons co-expressed pSTAT3 in both treatments. In the PMV ~61% of eGFP neurons in icv and ~84% in sc treated mice colocalized with leptin induced pSTAT3. This shows that most of the eGFP neurons in the reporter animals were responsive to leptin, and these animals were appropriate to use in further experiments at least as far
as these regions are concerned. The percentage of leptin-responsive eGFP cells was lower in the OVLT and AVPV (Table 3.1). Also, a smaller percentage (36-64%) of pSTAT3-positive neurons co-expressed eGFP, indicating that the eGFP label may only reveal about half of the actual leptin-responsive neurons in these regions. The two different routes of injection showed no significant differences when analyzed with Mann-Whitney U tests. Thus, for practical reasons, sc leptin was used in subsequent experiments. These data for sc leptin treatment, together with data of J. Kim (male animals) have been published in *Endocrinology* (Singireddy *et al.*, 2013,' Appendix II).

**Table 3.1** The average number of positively labeled cells by icv leptin injection (n = 3). The counts are averaged per section, and percentage of double-labeled cells is displayed.

<table>
<thead>
<tr>
<th>Hypothalamic Nucleus</th>
<th>Number of pSTAT3 positive cells</th>
<th>Number of eGFP cells</th>
<th>Number of double labeled cells</th>
<th>pSTAT3 cells expressing eGFP (%)</th>
<th>eGFP cells expressing pSTAT3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVLT</td>
<td>25.9 ± 3.2</td>
<td>29.0 ± 6.1</td>
<td>9.7 ± 2.5</td>
<td>37.9 ± 4.7</td>
<td>32.2 ± 1.3</td>
</tr>
<tr>
<td>AVPV</td>
<td>56.0 ± 2.2</td>
<td>47.1 ± 3.8</td>
<td>22.7 ± 2.0</td>
<td>39.9 ± 3.2</td>
<td>49.4 ± 1.8</td>
</tr>
<tr>
<td>rArc</td>
<td>69.0 ± 20.7</td>
<td>37.2 ± 6.4</td>
<td>26.3 ± 7.9</td>
<td>36.0 ± 4.0</td>
<td>76.6 ± 1.6</td>
</tr>
<tr>
<td>mArc</td>
<td>103.2 ± 14.6</td>
<td>78.1 ± 7.7</td>
<td>56.8 ± 6.0</td>
<td>55.9 ± 2.2</td>
<td>73.3 ± 4.2</td>
</tr>
<tr>
<td>cArc</td>
<td>53.0 ± 9.3</td>
<td>44.6 ± 6.6</td>
<td>29.8 ± 5.8</td>
<td>55.3 ± 8.5</td>
<td>65.2 ± 3.9</td>
</tr>
<tr>
<td>PMV</td>
<td>164.7 ± 63.2</td>
<td>163.0 ± 28.4</td>
<td>109.0 ± 44.8</td>
<td>64.5 ± 2.5</td>
<td>60.9 ± 17.8</td>
</tr>
</tbody>
</table>

**Table 3.2** The average number of positively labeled cells by sc leptin injection (n = 3). The counts are averaged per section, and percentage of double-labeled cells is displayed.

<table>
<thead>
<tr>
<th>Hypothalamic Nucleus</th>
<th>Number of pSTAT3 positive cells</th>
<th>Number of eGFP cells</th>
<th>Number of double labeled cells</th>
<th>pSTAT3 cells expressing eGFP (%)</th>
<th>eGFP cells expressing pSTAT3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVLT</td>
<td>18.8 ± 1.7</td>
<td>23.2 ± 4.1</td>
<td>6.5 ± 1.0</td>
<td>41.1 ± 9.9</td>
<td>29.4 ± 6.7</td>
</tr>
<tr>
<td>AVPV</td>
<td>38.1 ± 7.6</td>
<td>55.4 ± 7.0</td>
<td>15.8 ± 3.6</td>
<td>40.2 ± 2.3</td>
<td>28.5 ± 5.2</td>
</tr>
<tr>
<td>rArc</td>
<td>46.6 ± 7.3</td>
<td>30.3 ± 8.4</td>
<td>20.7 ± 2.3</td>
<td>38.3 ± 3.1</td>
<td>69.9 ± 10.8</td>
</tr>
<tr>
<td>mArc</td>
<td>83.3 ± 14.1</td>
<td>88.8 ± 15.8</td>
<td>63.1 ± 13.8</td>
<td>74.7 ± 4.0</td>
<td>69.2 ± 5.3</td>
</tr>
<tr>
<td>cArc</td>
<td>71.5 ± 12.6</td>
<td>80.4 ± 7.8</td>
<td>55.9 ± 12.2</td>
<td>77.7 ± 3.7</td>
<td>68.5 ± 8.6</td>
</tr>
<tr>
<td>PMV</td>
<td>118.2 ± 17.1</td>
<td>112.7 ± 10.6</td>
<td>91.6 ± 14.8</td>
<td>76.7 ± 1.5</td>
<td>80.3 ± 6.5</td>
</tr>
</tbody>
</table>

3.4.2 Injection of RetroBeads into the rostral preoptic area

To find out where leptin responsive inputs to the region of the GnRH neurons (rPOA) are, the Lepr-eGFP animals were used in a retrograde tracer
Lepr projections to rPOA

experiment. Retrograde tracing is a technique in which a tracing dye, in this case fluorescent latex microbeads (RetroBeads), is taken up by neuronal terminals exposed to it. When taken up the properties of the substance cause it to be transported retrogradely along the axon to the neuronal cell body. Here, the tracer was injected in close proximity to the GnRH cell bodies in the midline of the rPOA (Fig 3.1A-C). Stereotaxic injection of the beads varied per animal; therefore the exact site of tracer injection was assessed for each animal after sectioning of the brain. Animals where the injection was on the midline and extending at least between 0.74 and 0.38 mm rostral to bregma (Fig 3.1D-G), were used subsequently.

Prior to conducting experiments presented here, RetroBead tracer was injected into the more rostral medial septal (MS) region. This was done to try and include inputs of Lepr-eGFP neurons in the rPOA as one of the regions to be analyzed. However, extensive trials (n = 22) with the mid-point of the injection site around 0.9 mm rostral to bregma did not reveal any labeled cells in the regions investigated. This suggested that the GnRH neurons in these more rostral regions receive less input from the hypothalamus, or the number of GnRH neurons targeted at this site is insufficient to show labeling. In a different control experiment where the tracer was injected into the striatum, also no retrogradely labeled neurons were found in any of the hypothalamic nuclei investigated. Both of these ‘misplaced’ injections add support to the idea that the tracing observed in rPOA injections is specific to GnRH neurons.

To show the distribution of GnRH neurons at the level of the injection site, sections containing the rPOA were labeled with GnRH immunohistochemistry (Fig 3.1B). Injection of RetroBeads was in close proximity to GnRH cell bodies within the rPOA (Fig 3.1A-C). When the injection site is compared with the GnRH neuron distribution, it can be appreciated that injections were done at or just above the site where GnRH neurons are most concentrated (Herbison, 2006).
Figure 3.1 Evaluation of retrograde tracer injection site. A, GnRH immunofluorescence in the rPOA of a representative animal injected with tracer. The intense green fluorescence at the injection site is an artifact of the highly concentrated microbeads in this place. B, Photomicrograph of a brain section at the level of the rPOA showing RetroBead tracer auto-fluorescence. C, A merged image showing GnRH immunofluorescence in close proximity to the site of injection. D-G, Schematic diagrams showing the extent of the correctly-placed retrograde tracer injections into the rPOA (grey shaded area) in Paxinos mouse brain atlas diagrams at 0.74 mm, 0.62 mm, 0.50 mm, and 0.38 mm rostral to bregma (Paxinos and Franklin, 2001). 3V, third ventricle; AVPe, anteroventral periventricular nucleus; HDB, nucleus of the horizontal limb of the diagonal band; LPO, lateral preoptic area; MnPO, median preoptic nucleus; MPA, medial preoptic area; MS, medial septum; VDB, nucleus of the vertical limb of the diagonal band; VOLT, vascular organ of the lamina terminalis. Scale bar: 50 μm
3.4.3 Leptin responsive neurons that project to the rPOA

To assess retrograde uptake of the fluorescent beads, every section in one series (i.e. 120 µm spacing) was viewed under the microscope using fluorescent filters. Labeled cells were counted when there was a clear group of tracer beads in the shape of a cell. High power examples can be seen in Figure 3.2B and D. Neurons which were labeled with RetroBeads were seen in different areas throughout the extent of the hypothalamus. Analysis of coronal sections was started at the rostral anatomical landmark of anterior commissure fusion (0.14 mm rostral of bregma) and was carried through until the end of the hypothalamic 3rd ventricle (-2.80 mm caudal of bregma). In Figure 3.3, the location of labeled neurons in the hypothalamus is presented for one animal; one circle represents one cell.

Quantification of the number of labeled neurons was only carried out in regions known to contain high levels of leptin responsive neurons (Table 3.1 and 3.2). On average there were 6 ± 1.63 labeled neurons per section in the AVPV, none of which were eGFP positive. The Arc was divided into three different regions: the rostral Arc (bregma -0.94 to -1.34 mm), medial Arc (bregma -1.46 to -1.94 mm), and caudal Arc (bregma -2.06 to -2.54 mm). In the rostral and caudal Arc there were 4.2 ± 1.39 and 5.3 ± 2.70 labeled neurons respectively, of which 9.5% and 4.6% were eGFP positive. In the medial Arc there were more tracer labeled neurons (10.0 ± 4.75), of which 18.7% were eGFP positive. In the DMN numbers of labeled neurons averaged 9.8 ± 3.04, but the percentage of colocalization was low (1.1%). Tracer labeled neurons were mainly found in the more ventral parts of the DMN bordering the dorsal part of the VMN, as can be seen in Figure 3.2F. In the LHA the number of RetroBead labeled neurons was relatively low (5.2 ± 1.32 per section), with only 3.5% of these colocalizing with eGFP. The PMV had 9.1 ± 2.72 labeled neurons per section, but only 3.5% were eGFP positive (Fig 3.4).
Figure 3.2  Retrograde tracer uptake in Lepr-eGFP neurons. A, Lepr-eGFP epifluorescence in a neuron of the lateral hypothalamic area (LHA). B, RetroBead fluorescence in a neuron of the LHA. C, Colocalization of Lepr-eGFP and RetroBead fluorescence in a LHA neuron. D, Three RetroBead labeled neurons in the dorsomedial nucleus of the hypothalamus (DMN). E, Double label fluorescent image of the arcuate nucleus (Arc) showing green Lepr-eGFP neurons and red RetroBeads. Arrowhead indicates colocalization of the latter. F, Overview of Lepr-eGFP and RetroBead labeling in the Arc and DMN. 3V, third ventricle. Scale bar C: 10 μm; Scale bar F: 50 μm.
Figure 3.3  A schematic overview of retrograde tracer labeled neurons and Lepr-eGFP double labeled neurons in one RetroBead-treated animal. A-H, Coronal diagrams adapted from the Paxinos mouse brain atlas at regular intervals between 0.26 and −2.46 mm from bregma (Paxinos and Franklin, 2001), showing the cell bodies of RetroBead-labeled neurons that project to the rPOA that did not co-express Lepr-eGFP (red circles) and Lepr-eGFP and RetroBead-colabeled neurons projecting to the rPOA (blue circles). 3V, third ventricle; ArcC, arcuate hypothalamic nucleus, caudal part; ArcM, arcuate hypothalamic nucleus, medial part; ArcR, arcuate hypothalamic nucleus, rostral part; AVPV, anteroventral periventricular nucleus; D3V, dorsal third ventricle;
DMN, dorsomedial hypothalamic nucleus; LHA, lateral hypothalamic area; ME, median eminence; MnPO, median preoptic nucleus; MPA, medial preoptic area; MS, medial septum; ox, optic chiasm; Pe, periventricular hypothalamic nucleus; PH, posterior hypothalamic area; PMV, ventral premammillary nucleus; VMN, ventromedial hypothalamic nucleus.

**Figure 3.4**  Analysis of retrograde labeling in Lepr-eGFP animals (n = 6). A, The number of RetroBead-labeled neurons per section that project to the rPOA averaged per section in each area (black bars) and the average number of Lepr-eGFP neurons labeled with retrograde tracer in the same hypothalamic areas (grey bars; at least three sections per animal). B, Percentage of neurons projecting to the rPOA that are also Lepr-eGFP positive. AVPV, anterior periventricular nucleus; ArcR, arcuate hypothalamic nucleus, rostral part; ArcM, arcuate hypothalamic nucleus, medial part; ArcC, arcuate hypothalamic nucleus, caudal part; DMN, dorsomedial hypothalamic nucleus; LHA, lateral hypothalamic area; PMV, premammillary nucleus, ventral part.
3.4.4 GABAergic neurons that project to the rPOA

The injection of green RetroBeads into the rPOA of Vgat-tdTomato animals was tested due to leptin signaling in GABA neurons now being recognized as a critical factor for normal reproductive function (Chapter 5 and Zuure et al., 2013). A total of four animals were injected with RetroBead tracer, and 50% of the injections ended up in the same region as discussed previously (Fig 3.1). The Vgat-tdTomato reporter animals express the tdTomato fluorophore under the control of the vesicular GABA transporter gene (i.e., Vgat-tdTomato mice label GABAergic neurons specifically). While cellular colocalization of Vgat-tdTomato and GABA cell markers has not yet been demonstrated, tdTomato expression is high in the Arc (Fig 3.5A), absent in the VMN, and moderate in the DMN (Fig 3.5B), a pattern that mirrors that of GABAergic neurons in the hypothalamus (Vong et al., 2011).

Figure 3.5 Retrograde tracer in Vgat-tdTomato neurons. A, Vgat-tdTomato epifluorescence and green RetroBead tracer fluorescence in the arcuate nucleus of the hypothalamus. B, Vgat-tdTomato epifluorescence and green RetroBead tracer fluorescence in the dorsomedial hypothalamic nucleus. Insets show magnification of double-labeled cells, arrowheads show colocalization. 3V, third ventricle. Scale bar 50 μm

Green RetroBead uptake exhibited the same distribution as the red RetroBead tracer, i.e. there was no marked difference between the pattern of red and green tracer uptake in the areas assessed (Fig 3.4 and 3.6). The only noticeable exception to this was the AVPV, in which the number of green RetroBead-
labeled neurons was lower than the number of red RetroBead-labeled neurons. This variability probably reflects the close proximity of this region to the injection site. There were similar numbers of tracer labeled neurons throughout the Arc, and again relatively high numbers were seen in the DMN and PMV with lower numbers in the LHA (Fig 3.6A).

The highest percentage of colocalization between green RetroBead tracer and Vgat-tdTomato was seen in the caudal Arc (Fig 3.6B), even though the Arc contains a mixture of GABAergic and glutamatergic neurons (Vong et al., 2011). Colocalization of tracer and tdTomato was low in the DMN although this nucleus is considered to be largely GABAergic. Not surprisingly given the almost exclusively glutamatergic nature of the PMV (Vong et al., 2011), few (4.1%) of the RetroBead-labeled neurons in this region colocalized with Vgat-tdTomato.
Figure 3.6  Analysis of retrograde labeling in Vgat-tdTomato animals (n = 2). A, the average number of RetroBead-labeled neurons that project to the rPOA averaged per section in each area (black bars) and the average number of Vgat-tdTomato neurons labeled with retrograde tracer in the same hypothalamic areas (grey bars; at least three sections per animal). B, percentage of neurons projecting to the rPOA that are also Vgat-tdTomato positive. AVPV, anterior periventricular nucleus; Arc R, arcuate hypothalamic nucleus, rostral part; Arc M, arcuate hypothalamic nucleus, medial part; Arc C, arcuate hypothalamic nucleus, caudal part; DMN, dorsomedial hypothalamic nucleus; LHA, lateral hypothalamic area; PMV, premammillary nucleus, ventral part.

3.5 Discussion

In this chapter leptin inputs to the area of GnRH cell bodies were investigated. The use of a Lepr reporter (Lepr-eGFP) animal was validated by showing that the eGFP neurons are responsive to leptin. Then, a retrograde tracer was
injected into the rPOA of Lepr-eGFP and GABAergic reporter (Vgat-tdTomato) mice, and colocalization of tracer and reporter fluorophores were evaluated. This experiment highlighted Lepr and GABAergic neurons in the arcuate nucleus as key projections to the rPOA - the area where GnRH cell bodies reside. Lower numbers of Lepr projecting cells were found in the LHA and PMV, and GABAergic projections in the LHA. This work helps to narrow down the location and possible identity of mediators of leptin-to-GnRH signaling.

Validation of the Lepr reporter animals was necessary because eGFP expression is turned on by Cre recombinase in all cells that have ever used the Lepr promoter (including throughout embryonic development). To show if these eGFP neurons were actually leptin responsive at an adult age (>3 months) the leptin induced pSTAT3 was used; a technique widely used in the leptin neurobiology field (Anderson et al., 2003; Ladyman and Grattan, 2004; Quennell et al., 2011). Overall, there was a high percentage of eGFP neurons co-expressing leptin induced pSTAT3 in all regions except the OVLT and AVPV, showing that the Lepr reporter mouse is a good model to investigate leptin responsive neurons in these regions. Lepr reporter mice were treated with two different leptin administration routes; icv and sc. The main difference between pSTAT3 labeled neurons in these treatments was that the more rostral areas (regions of the OVLT and AVPV) had higher pSTAT3 numbers and the most caudal area (PMV) was lower in icv treated tissue compared to sc treatment. Differences in the rostral areas are probably due to timing of perfusion after the leptin treatment (30 min for icv vs. 120 min for sc). It has been shown previously that a shorter time (45 min vs. 120 min) between leptin treatment and sacrifice of the animal significantly alters pSTAT3 immunoreactivity in these areas (Cravo et al., 2011). Also, a shorter timeframe does not allow for leptin to penetrate deep into the hypothalamic areas further from the blood brain barrier; therefore lower numbers of pSTAT3 labeled cells are seen in the more lateral areas (for example in the PMV).

The tracing experiments used fluorescent latex microbeads because they have a minimal diffusion area around the site of injection and are not taken up by
passing fibers (Katz and Iarovici, 1990; Vercelli et al., 2000; Schofield, 2008). This makes the target area more concentrated compared to, fluorogold for example (Rizwan et al., 2009; Yeo and Herbison, 2011), which is an advantage because innervations can be observed closer to the injection site. One concern when injecting dye around GnRH neurons is the scattered nature of the GnRH neurons, so that only a subset of GnRH cell bodies will be contacted. Since non-GnRH neurons in the rPOA would also have taken up the tracer, it is impossible to be certain that the tracer labeled neurons observed at the various distal hypothalamic sites examined specifically innervate GnRH cell bodies. Nevertheless, injecting the tracer in the area where GnRH cell bodies are most concentrated gives the highest chance of tracing the inputs to these neurons. Additionally, the injected tracer forms an elongated injection site along the needle tract similar to that of the distribution of GnRH neurons at this rostral level (Spergel et al., 1999; Suter et al., 2000; Campbell, 2007). In my hands accurate placement of the RetroBead tracer was achieved in about 50% of the injections.

As GnRH neuron cell bodies are distributed in an elongated scattered pattern, it is impossible to target all of them using this technique. Injection of RetroBeads in the current location (the center of the injection being above and strictly anterior to the rostral tip of the 3rd ventricle) was chosen because this results in the largest number of cell bodies targeted with a single injection. Prior to these experiments trials were done with injections placed more rostrally, however these were not successful in labeling any neurons in the hypothalamic areas assessed. If the site and size of current injections are put next to the general GnRH expression pattern, it can be estimated that 20-30% of the GnRH cell bodies were within the injected region. Therefore the results are an underestimation of the total of inputs to the GnRH neurons, excluding cell bodies located in the more rostral medial septal region and the lateral ‘arms’ of the inverted Y pattern (Herbison, 2006). An alternative approach that was considered would be to use viral tract tracing in GnRH-Cre animals (Yoon et al., 2005; Wintermantel et al., 2006; Campbell, 2007), in which a virally delivered tracer would only be expressed in GnRH neurons themselves. However, this
technique would not be compatible with Lepr-eGFP or Vgat-tdTomato expression in the reporter animals as they use the same Cre-Lox transgenics.

The results of these experiments show that rPOA GnRH neurons receive input from the AVPV, Arc, DMN, LHA and PMV in all animals investigated, confirming regions identified by others (Yoon et al., 2005; Wintermantel et al., 2006; Louis et al., 2011). In this study none of the AVPV inputs to the rPOA were from Lepr neurons, which is consistent with experiments from our lab (Quennell et al., 2011) and others (Cravo et al., 2011) showing that few cells in this region, including kisspeptin neurons, express Lepr. It is more likely that they are the estrogen responsive kisspeptin inputs to the GnRH neurons et al. (Clarkson and Herbison, 2006; Wintermantel et al., 2006; Clarkson and Herbison, 2011; Yeo and Herbison, 2011). The most interesting finding is that many Arc Lepr neurons project to the rPOA. It is known that Arc kisspeptin neurons innervate the rPOA (Yeo and Herbison, 2011), but only a small percent of these kisspeptin neurons also respond to leptin (Smith et al., 2006a; Cravo et al., 2011). It is more likely that these leptin responsive innervations from the Arc to the rPOA come from POMC/CART, AgRP/NPY, or GALP neurons (Baskin et al., 1999; Ohtaki et al., 1999). All of these cell types are difficult to identify by immunohistochemistry. To help narrow down the type of neurons providing input to the rPOA, the tracer experiment was repeated in Vgat reporter mice, in which there was a similar percentage of colocalization of retrograde tracer and the Vgat-tdTomato signal in the Arc as was observed for Lepr cells. Together, these results may indicate that the leptin responsive neurons projecting to the rPOA are AgRP/NPY neurons, as these are GABAergic whereas POMC/CART neurons are not (Vong et al., 2011), and NPY neurons are known to innervate GnRH cells (Turi et al., 2003). It should be noted that the GABAergic nature of the GALP neurons is presently unknown.

Neuronal inputs to the rPOA originating from the DMN exhibited little colocalization with either Lepr-eGFP or Vgat-tdTomato. A well defined population that projects to the GnRH neurons in this area are the RFRP-3 neurons (Rizwan et al., 2009), so it is very likely that these were filled with the
tracer in this experiment. It has recently been shown that RFRP-3 neurons in the DMN are neither leptin sensitive or GABAergic (Rizwan et al., 2014). Only a small percentage (4%) of neurons projecting from the LHA to the rPOA were Lepr positive. However, around 20% of these inputs were GABAergic. A small number of PMV Lepr neurons were shown to project to the rPOA as has also been shown by Louis et al. (2011), although the proportion of cells from this important region that innervate the rPOA has not been quantified until now. As expected, colocalization of Vgat-tdTomato and retrograde tracer in the PMV was very low as the PMV is mainly glutamatergic (Chapter 5, and Vong et al., 2011; Zuure et al., 2013).

Leptin receptor neurons close to the injection site in the region of the OVLT and rPOA could not be studied using this technique. This was due to variability in injection site between individual mice, and the intense fluorescence of the tracer around the site of injection. Although roughly in the same area, this technique undoubtedly comes with slight variations in depth and spread of the tracer at the site of injection, making it impossible to gather reliable data close to this region. Therefore there may still be undiscovered leptin responsive neurons in close proximity to the GnRH neuron cell bodies that are not picked up using this technique.

Recently, it has been shown that GnRH neuronal projections have both axonal and dendritic properties (Herde et al., 2013). This new idea changes the view on the site of leptin synaptic contacts with GnRH neurons (as well as other types of inputs to GnRH neurons), because the whole surface of the ‘dendron’ rather than just the cell body could be important for receiving neuronal inputs and regulation of GnRH release. Therefore, there is a strong possibility that Lepr neurons do not regulate the release of GnRH solely at the level of the cell bodies in the rPOA, but also along the dendron and even at the terminals in the median eminence, making the results in this study limited to a potentially small proportion of the GnRH neuron network.
From the results of this study I can conclude that leptin responsive inputs to the region of the GnRH cell bodies arise primarily from the medial Arc, with a few inputs also arising from the caudal Arc and PMV. The projections coming from the Arc are likely to be GABAergic. In the following chapters these possibilities will be further investigated. More specifically, nitric oxide or glutamate expressing Lepr neurons in the PMV (Chapter 4 and 5) and GABAergic and glutamatergic Lepr neurons in the Arc (Chapter 5) will be studied.
Lepr projections to rPOA
Chapter Four

Metabolic Control of Fertility via Leptin Receptor Nitric Oxide Neurons
4.1 Abstract

One of the possible intermediate signaling molecules for leptin-GnRH communication is the signaling molecule NO, produced in nNOS neurons. NO acts by volume transmission rather than across synaptic junctions. Leptin responsive nNOS neurons are found in the preoptic area and the PMV, regions that are implicated to have interactions with GnRH neurons in the rPOA. Here Lepr-nNOS neurons are studied as a possible intermediates of leptin-to-GnRH signaling. Firstly, inducible nNOS-ER-Cre animals were crossed with stop-flox-eGFP mice to form nNOS reporter animals. These animals were used to confirm that the tamoxifen treatment regimen was effective: i.e. able to induce eGFP in nNOS neurons. Next, inducible nNOS-Lepr knockout animals were made by crossing nNOS-ER-Cre with Lepr flox lines resulting in NO neuron specific Lepr knockout animals (NOLEPKO) and controls. NOLEPKO animals were treated with tamoxifen to induce the knockout. Before and after knockout induction the metabolic phenotype was assessed by body weight measurements. No significant differences were observed at both time points. Adult fertility of both male and female NOLEPKO and control animals was assessed. Surprisingly, there were no differences between the groups. Leptin signaling by leptin-induced pSTAT3 was assessed at the end of the experiments. This showed that knockout of Lepr from nNOS neurons was unsuccessful. It was concluded that, for at least some floxed genes, nNOS-ER-Cre expression in these mice is insufficiently activated by tamoxifen to cause full recombination and therefore knockout of functional Lepr.
4.2 Introduction

Leptin signaling in the brain is a major regulator of metabolism and reproduction (Chehab et al., 1996; Quennell et al., 2009). One of the possible signaling molecules used as an intermediate by leptin responsive neurons to signal to the HPG-axis is nitric oxide (Sections 1.5.1 and 1.5.3, Yu et al., 1997a; Watanobe and Schiöth, 2001). Nitric oxide is a gaseous signaling molecule. When synthesized by nitric oxide synthase (NOS) it can diffuse out of the cell to stimulate receptors located nearby. The NOS enzyme family consists of inducible (iNOS), endothelial (eNOS) and the neuronal (nNOS) form (Bredt and Snyder, 1990; Förstermann et al., 1991). The focus of this chapter is on nNOS that is expressed in nervous tissue and in skeletal muscles. Neuronal NOS activity is dependent on cellular calcium (Ca²⁺) concentration, location with in the cell and phosphorylation. For example, when glutamate binds to the NMDA receptors, Ca²⁺ channels open to allow an influx of Ca²⁺, and the rise in Ca²⁺ triggers nNOS phosphorylation as reviewed by Bellefontaine et al. (2011).

For leptin to be able to directly change NO synthesis or secretion, leptin receptors and nNOS would need to be expressed within the same cells. It has been shown that the number of neurons expressing the activated (i.e. phosphorylated) nNOS was significantly reduced in fasted or in leptin deficient ob/ob animals (Donato et al., 2010). This reduction was able to be lifted by administration of leptin. This shows that there are hypothalamic nNOS neurons that respond to circulating leptin, possibly directly via their co-expressed leptin receptors. Additionally, there is colocalization of leptin induced pSTAT3 and NAPDH activity in the hypothalamus. NO is produced from l-arginine and uses oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent. Histochemical staining of NADPH is therefore an indicator of nNOS (Dawson et al., 1991; Hope et al., 1991). Colocalization of pSTAT3 and NADPH is seen in moderate levels in the lateral mPOA, posterior hypothalamus, Arc, and ventral DMN, while high levels of colocalization are found in the PMV (Donato et al., 2010). Additionally,
Donato et al. (2010) showed that a negative energy balance (i.e., fasting) reduces whole hypothalamic nNOS mRNA expression; but a reduction in nNOS was not restored by acute leptin treatment. So, there is evidence for leptin signaling in nNOS neurons and leptin’s ability to modulate NO synthesis, but it is not known if/how this relates to reproduction.

On the other hand, it is known that GnRH perikarya in the mPOA are surrounded by nNOS neurons but do not express nNOS themselves (Herbison et al., 1996; Bellefontaine et al., 2011). If nNOS levels are directly modulated by leptin, then this may in turn affect the NO levels available to GnRH neurons. In vitro experiments with explants that include the mPOA show that NO stimulates GnRH release (Bonavera et al., 1993). However, in an in vitro GnRH patch-clamp setup, endogenous and exogenous NO is able to inhibit GnRH neuron firing (Clasadonte et al., 2008). Interestingly, synaptic uncoupling does not ablate this effect because NO is a gaseous signal. These findings suggest that NO directly acts on the GnRH membrane, by diffusion, rather than synaptic connection. Whether NO excites or inhibits GnRH neurons is not clear, and probably depends on many other factors such as changes in circulation estrogens during different stages of the estrous cycle. It was shown that NO production is key to the occurrence of preovulatory surge of GnRH, and it is thought that NO might set GnRH neurons to a phasic bursting pattern needed this surge (Clasadonte et al., 2008). To support this hypothesis it has been shown that NOS inhibitors can block a leptin induced LH surge (Watanobe and Schiöth, 2001), showing a relationship between leptin, NO and GnRH.

Taking the above mentioned findings in regard, it is not surprising that a global knockout experiment where exon 6 of the Nnos gene was deleted, caused a reduction in fertility (Gyurko et al., 2002). Male nNOS knockout mice were completely infertile, and two-thirds of female nNOS knockouts were infertile when mated to wild type mates (for a 21-day period). In both sexes body and gonadal weights were lower than controls, and ovaries showed a lower number of corpora lutea. Male knockout animals showed
significantly lower levels of hypothalamic GnRH and circulating FSH concentrations. Interestingly, female mice showed higher hypothalamic GnRH levels and higher circulating LH concentrations.

To investigate if leptin acts upon nNOS neurons to indirectly regulate GnRH release, and thus fertility, an inducible form of Cre-Lox transgenics (Section 1.5, Fig 1.5) was used to knock out Lepr from nNOS neurons. With this model the time of knockout induction can be specified and any potential ‘developmental compensatory’ effects of knockout during embryonic development are avoided (Inui, 2000). Here I examined metabolic and reproductive phenotypes before and after knock out induction.

4.3 Materials and methods

4.3.1 Generation of nNOS specific reporter mice

Transgenic mice that express estrogen receptor (ER) bound Cre under the control of the nNOS promoter (nNOS-CreER-KI, JAX mice stock number 014541, Taniguchi et al., 2011) bred on a C57BL/6J background were crossed with Tau-eGFP-stop-flox mice (stopflox-Tau-eGFP, ROSA26-CAGS-τGFP Rodrigues et al., 1999) to generate nNOS-eGFP reporter animals, using two different breeding pairs as described in Section 2.7. To check if the ER-dependent Cre expression system worked in our hands, we produced these nNOS-eGFP reporter animals to quantify the ability of tamoxifen treatment to induce Cre recombinase activation. Animals that were heterozygous for Cre and floxed genes were used in the experiment.

4.3.2 Tamoxifen treatment regimen

To induce eGFP expression in the reporter animals, the Cre transgene linked to the ER has to be activated by the injection of tamoxifen. The ligand dependent Cre-recombinase is bound to a mutated human ER. This mutation
causes the receptor to have a high affinity for the synthetic estrogen receptor-modulator 4-hydroxytamoxifen (tamoxifen) and not for endogenous 17β-estradiol (Feil et al., 1997). From this trial study, it was determined that the best treatment regimen was as follows: twice daily (0900 and 1600) subcutaneous injections of tamoxifen (50 mg/kg; Sigma-Aldrich, #T5648) in 0.1 ml sunflower seed oil were given on five consecutive days.

To establish the optimal tamoxifen treatment regimen in the inducible reporter animals, different treatment regimens were tested based on protocols described in the literature (Feil et al., 1997; Erdmann et al., 2007; Campbell et al., 2011; Taniguchi et al., 2011). I chose to try five different regimens as listed in Table 4.1. After tamoxifen treatment, animals were left to recover for two weeks before brain tissue was collected (Section 2.3.1). After sectioning, brain tissue was checked for tamoxifen induced eGFP expression. This was done in the rPOA and the PMV, which are hypothalamic sites with high nNOS expression (Donato et al., 2010).

Table 4.1 Different treatment regimens (n = 2-4 for each) tested for the effectiveness of activating Cre driven deletion of the LoxP flanked stop sequence, allowing expression of eGFP in nNOS neurons. The vehicle for all treatments was sunflower seed oil.

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4.3.3 Generation of nNOS specific Lepr knockout mice

To remove Lepr from nNOS neurons, nNOS-CreER-KI mice were crossed with Lepr-floxed mice (Lepr-flox bred on a C57BL/6J background, McMinn et al., 2004) and the heterozygous offspring backcrossed to homozygous Lepr-flox mice (in eight founding breeding pairs), as described in Section 2.7.
Animals homozygous for Lepr-flox and expressing nNOS-ER-Cre were used as the experimental animals (NOLEPKO), and homozygous Lepr-flox mice lacking Cre expression were used as littermate controls (control). Animals were weighed every fortnight throughout the study to monitor growth and the occurrence of any metabolic phenotype. All mice were housed according to the standard conditions described in Section 2.1.

After Lepr knockout induction, male and female animals were divided into three different groups: vehicle treated nNOS-Cre animals (NOLEPKO-V), tamoxifen treated Lepr-flox controls (Control-T), and tamoxifen treated nNOS-Cre animals (NOLEPKO-T). Tamoxifen or vehicle treatment was performed at 9-10 weeks of age in both female and male animals using the optimal treatment regimen determined in the pilot study described in Section 4.3.2 (i.e. twice daily 50 mg/kg injections for five days).

4.3.4 Fertility assessment before and after knock out induction

Puberty onset was measured in all female and male animals before knockout induction with tamoxifen, as described in Section 2.8.1. Female NOLEPKO (n = 8) and control (n = 8) mice were checked for vaginal opening and first estrus, and male NOLEPKO (n = 8) and control (n = 11) mice were assessed for first fertile mating from day 34 ± 1 until day 54 ± 1.

Five days after the last tamoxifen injection, male animals were re-mated with non-pregnant wild type females and their fertility was assessed for a 120 day period, as described in Section 2.8.3. Female animals of all three groups were left to recover from the tamoxifen treatment for five weeks, then vaginal smears were taken for 14 consecutive days to assess estrous cycles (Section 2.8.2). After vaginal cytology assessment females were mated with a wild type male for 120 days to assess fertility.
4.3.5 Tissue collection and preparation

At the end of the fertility study, animals were fasted overnight and treated with a sc injection of recombinant leptin (1 mg/kg) or vehicle and perfused as described in Sections 2.2.2 and 2.3.1. Blood and reproductive organs were collected at the time of perfusion (Section 2.3.2 and 2.3.3).

4.3.6 Immunohistochemistry for leptin induced pSTAT3

Brain sections were stained for leptin-induced pSTAT3 using anti-pSTAT3 antibodies and chromogen staining, as described in Section 2.4.1. This was done in a subset of the female animals: one NOLEPKO-V, four Control-T, and four NOLEPKO-T mice. Leptin induced pSTAT3 staining was virtually absent in vehicle treated animals.

Representative photomicrographs of pSTAT3 single label were taken on an Olympus BX45 microscope (Olympus C5060 camera). Quantification of leptin-induced pSTAT3 was done in the mPOA, Arc, VMN, DMN and PMV of Control-T, NOLEPKO-V and NOLEPKO-T animals. Positive leptin responsive cells were counted when a clear circular and darkly stained nucleus was present. Using ImageJ software (NIH), images were converted to gray scale for analysis and positively stained nuclei were then counted in different hypothalamic nuclei using a macro program (size > 50 pixels, circularity = 0.5-1.0, and adjacent nuclei were separated using ‘watershed’ feature).

4.3.7 Fluorescent immunohistochemical labeling of nNOS

Brain sections of one nNOS-eGFP reporter animal were labeled with primary sheep anti-nNOS antibody and staining was visualized by incubating with strepdavidin-Alexa568 (Section 2.4.2). Validation of the nNOS antibody was done previously using western blotting, which yielded a single band at the position of 155 kDa corresponding to neuronal nNOS (Herbison et al., 1996).
Omission of the primary nNOS antibody resulted in absence of fluorescent staining.

Fluorescent photomicrographs of reporter animal tissue were taken on Olympus BX51 with Diagnostic instruments SPOT RT KE camera.

4.3.8 Detection of Lepr excision in brain tissue

Lepr gene excision was assessed using PCR analysis of genomic DNA using the primers 2 and 3 for the Lepr-floxed gene (2: CAG GCT TGA GAA CAT GAA CAC AAC AAC; 3: CTG ATT TGA TAG ATG GTC TTG AG; Table 2.4). For each animal two coronal 30 µm brain sections containing the rPOA, and two containing the caudal Arc and PMV were selected. For the caudal Arc and PMV regions the sections were separated into two parts: the hypothalamic regions, and the cortex. Genomic DNA was extracted from the brain tissue as described in Section 2.7.2. PCR reactions were performed as described in the same section, with two modifications: only 0.25 µl of each primer was used and 40 cycles were performed. Products were visualized using gel electrophoresis, in which the un-excised flox gene showed up at ~600 bp and the excised at ~220 bp.

4.3.9 Analysis

All results are presented as mean ± SEM. Student’s t-tests were used to identify significant statistical differences (p < 0.05) between control and knockout mice in measures of puberty onset. To analyze the body weights of the different groups at different time points, a repeated measures two-way ANOVA and a Bonferroni multiple-comparison post hoc test were used. Fertility and pSTAT3 counts were analyzed using one-way ANOVAs to show significant (p < 0.05) differences between Control-T, NOLEPKO-V and NOLEPKO-T groups.
4.4 Results

4.4.1 Establishing tamoxifen treatment regimen

To assess the induction of eGFP by the different tamoxifen treatments, three different individuals were asked to assign an intensity number (0-2) blindly to matched photos taken from the mPOA, PVN, Arc, and PMV of the different treatment animals. It was found that treating the mice twice daily for five days with subcutaneous injections of 1 mg tamoxifen (treatment 5, Table 4.1) gave the most widespread and intense eGFP expression in the reporter animals (Fig 4.1A-E). Treatment regimen 5 had the highest score and was used for subsequent experiments. Therefore this regimen was used to knock out leptin receptors in nNOS expressing cells, in the NOLEPKO mice.
Figure 4.1  Example of tamoxifen activated eGFP expression in nNOS neurons in the rPOA (A,C) and the PMV (B,D). A and B, Treatment of the animal with tamoxifen twice daily for 5 days caused the activation of Cre recombinase in nNOS neurons to allow eGFP synthesis. C and D, Vehicle treated animals had no induction of eGFP expression. E, The relative amount of eGFP expression after Cre activation by tamoxifen (n = 2-4 for each). Scale bar: 100 µM

4.4.2 Metabolic effects before and after tamoxifen treatment

Before tamoxifen treatment, control and NOLEPKO animals had similar body weights in both males and females (Fig 4.2A and B). The treatment had no effect on male body weights (Fig 4.2C). However, female Control-T mice had significantly increased bodyweight at three weeks after treatment (two-way repeated-measures ANOVA: $F_{(2,20)} = 21.78$, $p < 0.01$) when compared to NOLEPKO-V mice; this effect became normalized seven weeks post-treatment (Fig 4.2D). This temporary increase in female body weight suggests an orexigenic effect of the tamoxifen. Tamoxifen is an antagonist to the ER, therefore endogenous estrogen is not able to bind. It has been shown that estrogen signaling in the brain plays an important role in bodyweight regulation, and with tamoxifen occupying the ER endogenous estrogens are unable to exert their anorexigenic effects (as reviewed by Clegg, 2012).
Figure 4.2  Body weights of animals in the inducible knockout of leptin receptor from nNOS neurons. **A**, Body weights of control (n = 12) and NOLEPKO (n = 15) male groups before treatment were the same. **B**, Female body weight of control (n = 11) and NOLEPKO (n = 17) animals were not different before knockout induction. **C**, Average male body weight in weeks after treatment for the control group treated with tamoxifen (n = 7), and the knockout groups treated with vehicle (n = 8) or tamoxifen (n = 7). **D**, Average female body weight in weeks after treatment for the control group treated with tamoxifen (n = 9), and the knockout groups treated with vehicle (n = 7) or tamoxifen (n = 8). No significant differences were seen between the groups.

4.4.3 Fertility assessment before and after tamoxifen treatment

As expected, puberty onset in both male and female animals was normal when comparing NOLEPKO and controls before tamoxifen treatment (**Fig 4.3A**). After the induction of the knockout, the estrous cycles and fecundity of the females were assessed as measures of fertility. No differences were found between Control-T or NOLEPKO-V and the NOLEPKO-T groups in the time spent in each estrous cycle stage; the average time spend in each stage (in a 12 day period) was the same for all groups (**Fig 4.3B**). When these animals were mated with wild type males all mice
remained fertile; there was no difference in the time between litters, nor the number of pups per litter (Fig 4.3D). As an index of circulating estradiol levels I collected the uteri from the females in the three different groups after the fertility study and recorded their weights. Here, I saw a significant decrease in the uterine weight of NOLEPKO-T treated animals compared to those who were vehicle treated (one-way ANOVA: $F_{(2,12)} = 6.45$, $p < 0.05$; Fig 4.3C). As these tissues were collected at a single time point animals were in different stages of the estrus cycle, although stages were evenly distributed through all groups. Even 7 months after tamoxifen treatment, this estrogen antagonist seems to reduce uterine weight.

Male animals were separated from their wild type mates during treatment with tamoxifen, so that the tamoxifen (a potent estrogen) would not influence the mates’ fertility. One week after tamoxifen treatment the males were reunited with wild type females to further assess fertility. No differences were evident in time between litters or number of pups per litter when Control-T or NOLEPKO-V were compared with NOLEPKO-T experimental animals (Fig 4.3D).
**Figure 4.3** Puberty and adult fertility assessment in female and male NOLEPKO and control mice.  
**A,** Puberty onset before treatment with tamoxifen for females (control: n = 8; NOLEPKO: n = 8) and males (control: n = 6; NOLEPKO: n = 5).  
**B,** Percent of time spent in estrous cycle stages during a 14 day period, after treatment with tamoxifen (Control: n = 9; NOLEPKO: n = 8) or vehicle (NOLEPKO: n = 7).  
**C,** Uterine wet weights were not different between groups (Control-T, n = 5; NOLEPKO-V, n = 6; NOLEPKO-T, n = 4).  
**D,** Average interlitter interval and number of pups per litter for NOLEPKO males and females (Control-T, n = 6-7; NOLEPKO-V, n = 6-8; NOLEPKO-T, n = 6-7). No significant differences were seen between any of the fertility measurements.
4.4.4 Verifying the knockout using leptin induced pSTAT3

Verification of the tamoxifen-induced knockout could not be performed until the end of all experiments. To show the removal of Lepr from nNOS producing neurons after tamoxifen or vehicle treatment, the number of pSTAT3 positive nuclei in different hypothalamic areas were counted. The areas counted (rPOA, Arc, DMN and PMV) were chosen because they have high colocalization of NADPH and pSTAT3 as shown by (Donato et al., 2010). The VMN of the hypothalamus was added as an internal control where no changes were expected. As can be seen in Figure 4.4, there were no differences between groups for the number of pSTAT3 labeled cells in the VMN after tamoxifen treatment. However, there were also no evident differences in the regions where knockout of leptin signaling was expected. This indicates that the knock out of leptin receptors from nNOS neurons was not complete.

4.4.5 Verifying the knockout using excision of Lepr-flox

To see if the tamoxifen treatment caused activation and translocation of Cre and excision of Lepr from the genomic DNA, different hypothalamic regions were checked for the occurrence of excision. The results show that tamoxifen treatment did not induce any detectable excision of the floxed part of the Lepr gene. This was tested across all animals (n = 40; representative image shown in Fig 4.5).
Figure 4.4 Average number of pSTAT3 positive neurons in specific hypothalamic nuclei, in Control-T treated animals (n = 4), NOLEPKO-V (n = 1), and NOLEPKO-T (n = 4) animals. There were no significant differences between any of the groups. Rostral preoptic area (rPOA), arcuate nucleus (Arc), ventromedial nucleus of the hypothalamus (VMN), dorsomedial nucleus of the hypothalamus (DMN), and ventral premammillary nucleus (PMV).

Figure 4.5 Excision of Lepr-flox did not occur in tamoxifen treated animals. Photomicrograph of electrophoresis gel showing amplified DNA bands for non-excised Lepr-flox ~600 bp and excised Lepr-flox at ~220 bp. Gaba specific Lepr knockout tissue was used as a positive control for excision of Lepr. Tissue came from animals generated for Chapter 5.Ctx, cortex; hypo, hypothalamus, rPOA, rostral preoptic area.
4.4.6 Verifying the induction of eGFP using immunohistochemistry

There was no reduction of pSTAT3 labeling or evidence of excision in the NOLEPKO animals that received tamoxifen compared with those that received vehicle, indicating that the knockout of Lepr did not work as expected (Fig 4.4 and 4.5). To better understand why the eGFP reporter mouse did indicate tamoxifen induced Cre activation and the Lepr knockout did not, fluorescent immunohistochemical labeling for nNOS was done on the reporter tissue. Looking at the merged images of the tamoxifen induced eGFP (green) and nNOS immunohistochemistry in red it is clear that there was a high colocalization (yellow) in the mPOA, the medial septum, the PVN, and this colocalization is present but lower in the PMV (Fig 4.6). In general there were more nNOS immunoreactive (red) neurons than the number of eGFP positive neurons. Due to low n numbers (n = 2) this was not quantified. This limited colocalization study suggests that eGFP expression was specific to most nNOS neurons in the nNOS-eGFP reporter animal in the medial and rostral hypothalamus, but not in the PMV.
Figure 4.6 Colocalization of nNOS immunohistochemistry and tamoxifen induced reporter eGFP expression. Examples of the rostral preoptic area (rPOA), medial septum (MS), paraventricular nucleus (PVN) and the ventral premammillary nucleus (PMV) are shown. Scale bars: 50 µm
4.5 Discussion

The experiment described in this chapter was aimed to investigate whether leptin acts on nNOS neurons in the hypothalamus to regulate fertility. It is known that NO signaling plays a role in fertility regulation, and that leptin signaling and nNOS activity are colocalized in parts of the hypothalamus. To investigate this I used Cre-Lox transgenics, with an inducible Cre system to selectively delete Lepr from nNOS neurons in adult mice.

To check if leptin receptors were removed from nNOS neurons, labeling of leptin induced pSTAT3 was done. A decrease of leptin responsiveness in specific hypothalamic regions was expected in the NOLEPKO-T animals because tamoxifen treatment should remove the LoxP flanked part of the Lepr gene. Unfortunately, in the knockout animals the number of leptin responsive neurons was not reduced when compared with the controls. This result was inconsistent with my validation experiment, where nNOS-eGFP reporter expression was successfully established with the tamoxifen treatment. In this situation, the modified ER was activated by tamoxifen; causing the translocation of Cre recombinase. Here, Cre was able to recombine the floxed STOP sequence preceding the GFP encoding gene. In the experimental NOLEPKO model the ER-Cre duplex should have translocated to the nucleus to recombine the floxed sequence of the exon 17 of the Lepr gene (McMinn et al., 2004). This region of the gene codes for the Janus kinase docking site required for STAT3 signaling; recombination ultimately results in disabling JAK-STAT signaling. In both transgenic animal lines the tamoxifen would need to bind to the membrane bound ER-Cre and translocate to the nucleus to recombine a LoxP flanked sequence.

It is interesting that this process worked well in removing a stop sequence to allow eGFP expression, but did not work to inactivate the Lepr gene. Despite homozygous Lepr-flox expression, there may have been deletion of the Lepr in only one allele (heterozygous recombination), leading to one functional allele still remaining (in contrast, eGFP expression in the reporter animals
Lepr nNOS neurons

requires only one recombined floxed sequence). In other words, maybe the dose and regimen used was only sufficient to target the floxed gene hetrozygously, therefore keeping one of the floxed Lepr alleles intact. However, this is unlikely because similar treatment regimens have been used successfully to delete connexin 36 or induce lacZ expression (Feil et al., 1997; Campbell et al., 2011). Removal of the floxed Lepr sequence from the genome by activated Cre recombinase was checked using excision primers for Lepr (Balthasar et al., 2004; Quennell et al., 2009). This showed that no excision on either allele occurred in current experiment.

Another possibility as to why the recombination was unsuccessful is that Cre could not access the LoxP sites. This option is again unlikely because tamoxifen was clearly able to translocate the ER-Cre duplex to the nucleus in the nNOS-eGFP reporter mice, and the Lepr-floxed transgenic animal has been used successfully in many studies before in our and in other labs (Chapter 5 and McMinn et al., 2004; Quennell et al., 2009; Donato et al., 2011b; Leinninger et al., 2011; Leshan et al., 2012; Zuure et al., 2013).

Finally, Lepr protein or mRNA might still be stable many weeks after gene manipulation. Again this is unlikely because Campbell et al. (2011) show a complete ablation of the floxed connexin 36 gene at five weeks after tamoxifen treatment. The animals in the current experiment were kept alive for over ten weeks after treatment. Because I was unable to validate the knockout, the fertility results probably do not reflect a Lepr-deficient situation and so it remains unclear from this experiment whether NO is a critical linking neurotransmitter between leptin and GnRH neurons.

At the time I was doing this experiment, a paper by Leshan et al. was published (2012). In this article a constitutively expressing Cre model was used to mediate deletion of Lepr from nNOS cells. Phenotypes of these animals were compared with both Lepr-null and Cre negative Lepr-flox control animals. Leshan et al. (2012) show a major loss of leptin induced pSTAT3 in the nNOS specific Lepr knockout animals, in the PMV and to lesser
extent in the Arc and DMN. The animals with Lepr ablation from nNOS neurons showed both an increase in body weight and in food intake. This indicates a role for leptin signaling in nNOS neurons in energy homeostasis, although this is not directly via anorexigenic POMC neurons in the Arc as there is no colocalization of POMC and nNOS in this region (Leshan et al., 2012). More pertinent was their data on puberty onset; the nNOS specific Lepr knockout females showed a significant delay in puberty onset (measured by first estrus). They also assessed adult fertility, but showed no difference in the females ability to deliver pups after mating with a male (Leshan et al., 2012).

From the experiment presented here it remains inconclusive if leptin's effects on reproduction are mediated via nNOS producing neurons. From the data presented by Leshan et al. (2012) it can be concluded that leptin signaling in nNOS neurons seems to be at least partially required for puberty onset, but not for adult reproductive functioning. However, a recent publication by Bellefontaine et al. (2014) investigated the NO neurons surrounding the GnRH cell bodies in the preoptic region. They showed that NO signaling originating from the preoptic region is needed for leptin-induced LH release, as leptin is unable to stimulate LH release in mice lacking the Nos1 gene or when NOS was inhibited pharmacologically. Even though nNOS neurons in the preoptic regions express receptors for kisspeptin (Hanchate et al., 2012), the stimulation of LH release by leptin was not dependent on kisspeptin signaling because the results were similar between wild type and kisspeptin receptor knockout animals (Bellefontaine et al., 2014). This paper provides compelling data to support a role for leptin sensitive nNOS neurons in the preoptic area in providing a link between energy stores and GnRH release.

A possible future approach of these experiments would be to cross the inducible nNOS-CreER-KI animals with stop-flox Lepr mice (Lepr-null mice, JAX mice stock number 018989, Berglund et al., 2012) to test if leptin receptors in nNOS neurons are sufficient for fertility, as opposed to being
required for fertility as tested in current experiment. The tamoxifen-activated nNOS-Cre may be able to cause recombination of the stop sequence (as occurred in the reporter animal) and hence permit Lepr expression, even though it could not cause noticeable recombination in the Lepr-flox mice in the current experiment.
Chapter Five
Leptin Signaling in GABA Neurons, but Not Glutamate Neurons, is Required for Reproductive Function

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Appendix III
5.1 Abstract

Here it is tested whether GABAergic or glutamatergic neurons provide the intermediate pathway between the site of leptin action and the GnRH neurons. Leptin receptors were deleted from GABA and glutamate neurons using Cre-Lox transgenics and the downstream effects on puberty onset and reproduction were examined. Both mouse lines displayed the expected increase in body weight and region-specific loss of leptin signaling within the hypothalamus. The GABA neuron specific Lepr knockout females and males both showed significantly delayed puberty onset. Observations of various adult fertility parameters revealed that these knockout animals have decreased fecundity and disordered female estrous cycles. In contrast, glutamate neuron specific Lepr knockout mice displayed normal fertility. Assessment of estrogenic HPG axis regulation in females showed that leptin action on GABA neurons is not necessary for estrogen mediated suppression of tonic LH secretion (an indirect measure of estrogenic negative feedback), but is required for regulation of a full preovulatory-like LH surge (an indirect measure of estrogenic positive feedback). In conclusion, leptin signaling in GABAergic (but not glutamatergic neurons) plays a critical role in the timing of puberty onset and is involved in fertility regulation throughout adulthood in both sexes. These results form an important step in explaining the role of central leptin signaling in the reproductive system. Narrowing down the possible mediators of leptin signaling to the HPG axis will direct future research to be more focused on the GABAergic Lepr cells.
5.2 Introduction
Circulating leptin is an important metabolic signal regulating food intake and energy expenditure. Leptin also forms a permissive modulator of fertility (Ahima et al., 1997; Nagatani et al., 1998). Reproduction is centrally regulated by the drivers of the HPG axis: the GnRH neurons. A significant body of research has been devoted to trying to understand how leptin's permissive effects are passed to the reproductive axis. Leptin acts directly on the brain, as transgenic removal of Lepr from forebrain neurons results in the same infertile and obese phenotype as does global mutation in the leptin gene itself (Ingalls et al., 1950; Swerdloff et al., 1975; Quennell et al., 2009). However, leptin affects GnRH neurons indirectly as they do not express leptin receptors (Quennell et al., 2009). To elucidate the identity of the neuronal network that must exist between leptin responsive neurons and GnRH neurons, leptin signaling was investigated in the major inhibitory and excitatory neuronal cell populations: GABA and glutamate neurons respectively.

The GnRH neurons express both GABA_A and GABA_B receptor isoforms. Although GABA is generally an inhibitory neurotransmitter, the effect of GABA_A receptor activation in GnRH neurons can be excitatory or inhibitory depending on the chloride concentration within the neuron (Krnjevic, 1974; Herbison and Moenter, 2011). Binding of GABA to the GABA_B receptor causes an inhibition of pulsatile GnRH release (Todman et al., 2005; Zhang et al., 2009). Hypothalamic locations where GABAergic Lepr neurons are concentrated include the Arc, DMN, and LHA (Ovesjo et al., 2001; Vong et al., 2011). Functionally, acute fasting (i.e., reducing leptin levels) alters GABAergic transmission to GnRH neurons, and decreases GnRH neuronal activity (Sullivan et al., 2003; Sullivan and Moenter, 2004), suggesting that leptin sensing GABAergic afferents integrate metabolic cues to modulate GnRH release.
Glutamate is the main excitatory neurotransmitter of the central nervous system. The cell bodies of GnRH neurons are innervated by glutamatergic terminals that stimulate GnRH release (Bourguignon et al., 1995; Kiss et al., 2003). Hypothalamic glutamatergic Lepr neurons can be found in the Arc, the VMN, and the PMV (Kocsis, 2003; Vong et al., 2011). Lesion experiments indicate that PMV Lepr neurons are involved in leptin’s effects on fertility (Donato et al., 2009; Leshan et al., 2009). Combining these data suggests that glutamatergic Lepr neurons form a likely leptin-to-GnRH intermediate pathway.

In neurons both GABA and glutamate are transported by vesicular transporter proteins: vesicular GABA transporter (Vgat) and vesicular glutamate transporter 2 (Vglut2). The expression of these transporters can be utilized to specifically target Lepr deletion to either GABA or glutamate neurons, using the Cre-Lox transgenic system (as described in Section 1.5). The effects of these deletions on fertility were studied in female and male animals. Specifically, puberty onset, adult fertility and estradiol feedback on the HPG axis were assessed to determine which of these two major neuronal populations is primarily responsible for leptin’s effects on reproductive function.

5.3 Materials and methods

5.3.1 Generation of GABA and glutamate specific Lepr knockout mice

Female and male transgenic mice with Vglut2 (Vglut2-ires-Cre) or Vgat (Vgat-ires-Cre) specific Lepr deletions were bred from transgenic lines as described by Vong et al. (2011) and in Section 2.7 of this thesis (on a mixed FVB and C57BL/6J background). These animals are hereafter referred to as GABA and glutamate specific Lepr knockouts. Homozygous floxed littermates without Vglut2- or Vgat-Cre of each line were used as controls. All experimental animals came from a mix of offspring from the founding
breeding pairs, 10 and 12 pairs for glutamate and GABA specific Lepr knockout specifically.

5.3.2 Immunohistochemistry and image analysis

All animal groups were treated with leptin or saline and brain tissue collected for immunohistochemistry as described in Sections 2.2.2 and 2.3.1. Immunohistochemical labeling of leptin-induced pSTAT3 was performed to determine loss of hypothalamic leptin responsiveness in the knockout mice. This was done using chromogen staining with a monoclonal anti-pSTAT3 antibody, as described in Section 2.4.1. To analyze the effects of Lepr knockout on the number of pSTAT3 positive neurons, stained cells were counted in hypothalamic nuclei (median preoptic area (MnPO), rPOA, mPOA, rostral Arc (bregma -0.94 to -1.34 mm), medial Arc (bregma -1.46 to -1.94 mm), caudal Arc (bregma -2.06 to -2.54 mm), VMN, LHA, DMN, PMV) and the nucleus of the tractus solitarus (NTS). Positive leptin responsive cells were counted when a clear circular and darkly stained nucleus was present. Counts were performed in photomicrographs of at least three tissue sections per area from each animal, except for the Arc where nine tissue sections (three for each subregion) were counted.

Fluorescent double label immunohistochemistry of GnRH and Vgat was performed in control and GABA specific Lepr knockout male animals. Brain sections containing GnRH neurons were incubated in anti-GnRH and anti-Vgat primary antibodies and visualized using fluorescent secondary antibodies (as described in Section 2.4.2). Omission of the primary Vgat antibody resulted in an absence of stained punctate terminals. Additionally, the antibody was thoroughly characterized by western blotting yielding a single band of the predicted molecular weight, cellular morphology and distribution (Wang and Sun, 2012). Validation of the primary antibody for GnRH was described by Rizwan et al. (2012). Immunofluorescent labeling of GnRH showed up as cytoplasmic green labeling of neuronal cell bodies with
labeling of proximal dendrites, and labeling was confined to the region of GnRH expression. Confocal microscopy was performed on a Zeiss LSM 710 microscope, using an argon laser exciting at 488 nm (GnRH) and a helium neon laser exciting at 543 nm (Vgat). With a ×20 PlanApochromat objective lens, Z-stacks (0.5 µm optical slices) were made through each individual GnRH soma and proximal projections. Twenty GnRH neurons per animal were photographed and used for analysis (10 within the medial septum population and 10 in the rPOA). Zeiss LSM image browser software was used to count the number of appositions (where red pixels touched the green, for at least two consecutive optical slices) on the GnRH soma and in three 10 µm segments progressing distally along the projections. This tool was also used to measure the circumference of the soma. Where data could be obtained from two projections per GnRH neuron, these were averaged.

5.3.3 Body weight measurements

To assess the metabolic phenotype of these animals, body weights were recorded on a weekly basis, starting at weaning (3 weeks of age). Body weights for females could not be recorded during the fertility study as animals were in different stages of pregnancy. A final body weight measurement was taken at least 10 days after the last litter was born and males were removed from breeding pairs.

5.3.4 Plasma leptin concentrations

Plasma samples were collected at the end of the fertility studies (as described in Section 2.3.2), males were 18 weeks of age and females 25 weeks of age. The plasma leptin levels of control and knockout animals were measured by ELISA, as described in Section 2.6.3. The average coefficient of variation was 4.1%, and the sensitivity of the assay was 0.04 ng/ml. All samples were repeated in duplicate on one single ELISA plate.
5.3.5 Glucose measurements

Fasting (18h) and non-fasting blood glucose concentrations were measured from whole venous blood (~2 μl) collected from the tail vein of the animals. Blood glucose values were determined using glucose test strips (Accu-Chek Performa; 05967270020, Roche) in an automatic glucose monitor (Roche; Accu-Chek Performa). These experiments were performed on 4 month-old female glutamate specific Lepr knockouts and controls and on 5 month-old female GABA specific Lepr knockouts.

5.3.6 Food intake

In a cohort of female GABA specific Lepr knockout and control animals (10 mice per group, housed with 5 mice per cage), daily food intake was measured at four weeks of age. This was done by weighing the cage top with food hub every 24 hours for 3 consecutive days. Daily food intake was calculated by dividing the total amount eaten by the number of animals in the cage. Assessment of food intake was not done for glutamate specific Lepr knockout females, or for males of either group.

5.3.7 Fertility experiments

In female animals, puberty onset was measured by vaginal opening along with the age at first estrus. Male mice were mated with adult wild type females to assess first fertile mating (Section 2.8.1).

To determine adult female estrous cyclicity, vaginal smears were taken for 10 consecutive days (glutamate specific knockouts and controls) or 28 consecutive days (GABA specific knockouts and controls) starting at least 14 days after first estrus (Section 2.8.2). Estrous cycle duration was calculated as the average time between two proestrus phases. Adult fertility was assessed by evaluating breeding success over a certain period, for both sexes in knockout and control animals of both experimental groups, as
described in Section 2.8.3. For female and male glutamate specific knockouts and controls, breeding studies were performed for 120 consecutive days. Male GABA specific Lepr knockout and control animals were with wild type females for a period of 100 days. Whereas female GABA specific knockouts and controls were with wild type males for 40 days, breeding pairs were separated due to cases of dystocia in the GABA specific Lepr knockout females.

In males of GABA and glutamate specific Lepr knockouts and controls daily sperm production was measured at 4 months of age (Section 2.8.4).

5.3.8 Assessment of HPG axis regulation by estradiol

This assessment was only conducted in female GABA specific Lepr knockouts and controls, since no fertility phenotype was observed in the glutamate specific Lepr knockouts. A separate cohort of animals from those used for the fertility experiment was used. Negative feedback assessment of estradiol was based on the experiments described by Moore et al. (2012). The GABA specific Lepr knockout and control mice were anesthetized with isoflurane and tail blood samples (Section 2.9.3) taken at baseline (day 0; intact). Ovariectomy was performed (Section 2.9.1), and 14 days later another blood sample was taken (OVX). Animals were subsequently implanted with a chronic slow-release 17-β-estradiol sc implant, as described in Section 2.9.2; eight days later another blood sample was taken (OVX+implant).

Estradiol positive feedback was measured in an established model for preovulatory-like LH surge induction, as described in Quennell et al. (2009). On day 22, all mice received a bolus injection of estradiol benzoate (50 μg/kg, sc) nine hours before lights out. At lights out on day 23, final trunk blood samples were taken as described in Section 2.3.4.
The plasma LH concentration was measured by radioimmunoassay (Section 2.6.1). The sensitivity of the assay (95% confidence interval at 0 ng/ml) was 0.14 ng/ml and the intra-assay coefficient of variation was 5.5%. All samples were analyzed in triplicate within the same assay.

5.3.9 Analysis

All results in this chapter are presented as mean ± SEM. Student’s t-tests were used to identify significant statistical differences (p < 0.05) between control and knockout mice in the pSTAT3 cell counts, plasma leptin concentrations, daily food intake, and all measures of fertility. The body weight and negative feedback data were compared using a two-way ANOVA with repeated measures and a Bonferroni multiple comparisons post hoc test. The control Lepr-flox/flox animals were pooled into one group for immunohistochemical, plasma leptin concentration and body weight comparisons since the two experiments were run concurrently and controls were identically produced.

5.4 Results

5.4.1 Loss of leptin signaling in GABA and glutamate specific Lepr knockout neurons

To validate the transgenic knockout models I identified the loss of leptin responsive cells using pSTAT3 immunohistochemistry. The Cre-Lox animal models used in this study disrupt leptin signaling by deletion of exon 17 of the Lepr gene. This exon encodes for part of the receptor responsible for JAK-STAT docking and downstream signaling (McMinn et al., 2004); loss of leptin-induced phosphorylated STAT3 therefore provides an accurate representation of where Lepr was deleted. The transgenic disruption of Lepr from either GABA or glutamate neurons resulted in a marked reduction of leptin induced pSTAT3 immunoreactivity in the hypothalamic nuclei where these neurotransmitters are known to be strongly expressed (Vong et al.,
Removing leptin receptors from GABA neurons caused a 42% reduction of leptin-induced pSTAT3 in the Arc (particularly in the medial and caudal Arc sections; the effect was not seen in the most rostral sections; **Fig 5.1E, H and 5.2**). Similar reductions were seen in the LHA (49%) and the DMN (31%; **Fig 5.1E and 5.2**).

Deletion of leptin receptors from glutamate neurons caused a different pattern of pSTAT3 staining. Strikingly, pSTAT3 immunoreactivity was 80 and 97% reduced in the PMV and VMN respectively (**Fig 5.1F, I and 5.2**). Significant decreases were also evident in the ventral rPOA (55%; **Fig 5.1C and 5.2**) and mPOA (47%; **Fig 5.2**) as well as the LHA (30%; **Fig 5.1F and 5.2**) when glutamate specific Lepr knockouts were compared with controls.

Extra-hypothalamic Lepr deletion was addressed by counting pSTAT3 positive nuclei in the NTS. No significant differences were found between the GABA specific knockouts and controls (**Fig 5.2**); this was expected because several studies have shown that the NTS Lepr neurons are not GABAergic (Vong et al., 2011; Garfield et al., 2012). The number of animals in the glutamate specific Lepr knockout group was low; nevertheless a trend towards a reduced pSTAT3 response (albeit non-significant; $t_{8} = 1.261$, $p = 0.24$) was observed similar to what has been reported (Vong et al., 2011).
Figure 5.1  Characterization of the knockout models by labeling leptin-induced phosphorylated STAT3. A-I, Representative photomicrographs of staining in different hypothalamic areas counted. A-C, MnPO and rPOA, D-F, Arc, VMN, LHA, and DMN, and G-I, Arc and PMV. The first column shows leptin induced (5 mg/kg) pSTAT3 labeling in control animals (n = 11). Second and third columns show, pSTAT3 labeling in GABA and glutamate specific Lepr knockout animals respectively (n = 10 and n = 8). MnPO, median preoptic nucleus (including the region of the organum vasculosum of the lamina terminalis: OVLT); rPOA, rostral preoptic area (ventral part); mPOA, medial preoptic area; Arc, arcuate nucleus; VMN, ventromedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; DMN, dorsomedial nucleus of the hypothalamus; PMV, ventral premammillary nucleus, Scale bar: 100 μm
Lepr GABA and glutamate neurons

Figure 5.2  Quantification of immunohistochemical staining for leptin-induced pSTAT3. Number of positive labeled cells per section in control animals (n = 11), GABA specific (n = 10) and, glutamate specific (n = 8; n = 2 for NTS) knockouts. Note: There was no difference between the pSTAT3 counts of the two control groups (n=6 and n=5) that are derived from the two different Cre lines, and therefore they have been pooled in this graph. In all regions experimental groups are compared to the control animals to show significant differences. Arc, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; MnPO, median preoptic nucleus (including the region of the organum vasculosum of the lamina terminalis: OVLT); MPA, medial preoptic area; NTS, nucleus of the solitary tract; PMV, ventral premammillary nucleus; rPOA, rostral preoptic area (ventral part); VMN, ventromedial nucleus of the hypothalamus. * p < 0.05.

In all groups a marked increase in pSTAT3 labeling throughout the hypothalamus and in the NTS was seen when fasted animals were treated with leptin rather than vehicle (Fig 5.1-5.3). However, in the absence of exogenous leptin the overnight fast was unable to reduce pSTAT3 immunoreactivity to the same extent in the GABA specific Lepr knockouts when compared to the controls (Fig 5.3). This phenomenon is likely due to the high circulating leptin concentrations found in these very obese animals (Fig 5.54).

The above-mentioned findings agree with previously described distributions of Vgat and Vglut2 mRNA in the hypothalamus (Vong et al., 2011),
confirming that the Lepr deletions were indeed targeted to neurons expressing Vgat and Vglut2. There appeared to be very little overlap between the regions affected by GABA and glutamate Lepr knockout. The LHA was an exception; both GABA and glutamate specific Lepr deletion reduced the numbers of neurons able to respond to leptin here.

Figure 5.3 Characterization of vehicle induced levels of pSTAT3 immunohistochemistry. A-C, Representative photomicrographs of minimal pSTAT3 staining in different hypothalamic areas counted in a vehicle treated control animal. D, Quantification of immunohistochemical staining (number of positive labeled cells per section) in control animals, GABA specific and, glutamate specific knockouts (n = 4 in all groups). In all regions experimental groups are compared to the control animals to show significant differences. Arc, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; MnPO, median preoptic nucleus (including the region of the organum vasculosum of the lamina terminalis: OVLT); MPA, medial preoptic area; PMV, ventral premammillary nucleus; rPOA, rostral preoptic area (ventral part); VMN, ventromedial nucleus of the hypothalamus. *p < 0.05, Scale bar: 100 μm.
5.4.2 Body weight regulation in GABA and glutamate specific Lepr knockout animals

Body weights of all knockout and control groups were measured fortnightly. The genotype of the knockouts had a significant effect on body weight in females (two-way repeated measures ANOVA: $F_{2,32} = 247.06, p < 0.001$) and in males (two-way repeated measures ANOVA: $F_{2,26} = 69.50, p < 0.001$) when compared to controls. As previously described, Lepr knockout from GABA neurons caused an obese phenotype (Vong et al., 2011). Post hoc testing revealed that female GABA specific Lepr knockout animals were significantly heavier than littermate controls from the age of four weeks onwards (Fig 5.4A), whereas for males a significantly greater body weight was reached at six weeks of age (Fig 5.4B). Glutamate specific Lepr knockout caused a milder metabolic phenotype. Females were significantly heavier than their littermate controls by nine weeks of age (Fig 5.4A). The males showed a significant weight difference from 12 weeks of age when compared to control animals (Fig 5.4B). These results are similar to the body weights described by Vong et al. (2011); with the GABA specific knockouts being notably heavier than the glutamate specific knockout animals.
Figure 5.4   Effects of Lepr knockouts on body weight in female and male GABA and glutamate specific Lepr knockout and control animals. A, Female GABA specific knockout animals ($n = 10$) were significantly heavier than controls ($n = 19$) from 4 weeks of age. Glutamate specific Lepr knockout animals ($n = 9$) were significantly heavier than controls ($n = 19$) from 9 weeks of age. B, Male GABA specific Lepr knockout animals ($n = 9$) had an increased body weight from 6 weeks onwards and glutamate specific knockout males ($n = 6$) from 12 weeks onwards compared to controls ($n = 15$). Bodyweight data was not collected for the female groups throughout the breeding study. * $p < 0.05$. 
5.4.3 *Metabolic phenotype in GABA and glutamate specific Lepr knockout animals*

Blood collected at the end of the breeding study showed that plasma leptin concentrations were significantly elevated in both female and male GABA specific Lepr knockouts (female: $t_{14} = 16.46, p < 0.0001$ and male: $t_3 = 9.85, p = 0.0022$; Fig 5.5A). In glutamate specific knockouts a significant increase in leptin concentration was only evident in male animals ($t_3 = 4.56, p = 0.02$; Fig 5.5A).

No differences in blood glucose were observed between female GABA specific Lepr knockout and control mice, nor was there a decrease in blood glucose when the animals were fasted (Fig 5.5C). This was unexpected as the supplementary data of Vong *et al.* (2011) show a significant decrease of glucose levels after fasting, the only differences being the sex and age of the animals measured (male animals, 10-12 weeks vs. female animals, 5 months of age). Glutamate specific Lepr knockout females did not show any differences when compared directly to control animals. Both glutamate control and knockout animals showed a significant decrease in blood glucose when fasted (glutamate controls: $t_{16} = 6.26, p < 0.0001$; glutamate specific Lepr knockouts: $t_{16} = 5.83, p < 0.0001$; Fig 5.5B).

To see if differences in body weight in the GABA specific Lepr knockout females were due to higher food intake, daily food intake was measured for three consecutive days. Figure 5.5D shows that the daily food intake of the knockout animals was significantly higher than in control animals ($t_6 = 4.66, p = 0.0035$). Results were similar to the phenotype described by Vong *et al.* (2011). When leptin signaling was removed from GABAergic neurons, leptin was unable to inhibit food intake via NPY/AgRP neurons as these are GABAergic.
Figure 5.5  Effects of Lepr knockouts on plasma leptin, glucose concentration, and daily food intake. A, Plasma leptin concentrations of GABA specific knockout animals (female n = 8, male n = 2) were significantly higher than those of control animals (female n = 8, male n = 3). In glutamate specific Lepr knockouts only male animals showed a significantly higher plasma leptin concentration than the controls (female n = 4, male n = 2). B, Fasting blood glucose was significantly lower than fed blood glucose in both female glutamate specific knockouts (n = 9) and controls (n = 9). C, Fed and fasting glucose measurements were not different between female GABA specific knockouts (n = 7) and controls (n = 9). D, Daily food intake of female control animals was significantly lower than in GABA specific knockout animals (n = 2 cages of 5 animals for both groups). * p < 0.05, ** p < 0.01, *** p < 0.001.

5.4.4 Delayed puberty onset in GABA specific Lepr knockout animals

To assess puberty onset, date of vaginal opening and first estrus in females were measured, while for males the age of first fertile mating was determined. Female mice with Lepr deleted from GABAergic neurons showed a significant, seven day delay in vaginal opening ($t_{18} = 2.40$, $p = 0.027$), coupled with a 20 day delay in the appearance of first estrus ($t_{16} = 10.31$, $p < 0.0001$; Fig 5.6A and C). When male GABA specific Lepr knockout animals and controls were mated with wild type C57BL/6J females
of reproductive age, they showed a significant delay in puberty onset. When the age at puberty was calculated by backdating the length of gestation, it was revealed that the knockout animals went through puberty around 13 days later than the controls ($t_{12} = 3.40, p = 0.005$; Fig 5.6A and D).

Puberty onset of glutamate specific Lepr knockout females and males did not differ from controls (Fig 5.6B). Female knockout and control animals showed exactly the same age at vaginal opening ($30.2 \pm 0.7$ days). In both these animal groups, first estrus was evident about three to four days later. Male glutamate specific Lepr knockout animals did not show any significant difference in the timing of puberty onset (Fig 5.6B).

**Figure 5.6** Puberty onset in GABA and glutamate specific Lepr knockout animals. **A,** In GABA specific Lepr knockout animals ($n = 7-10$) vaginal opening, first estrus and male puberty onset were all significantly delayed, when compared to their control littermates ($n = 7-10$). **B,** Vaginal opening, first estrus and male puberty onset all occurred at the same time in glutamate specific knockout animals ($n = 6-9$) compared to their control littermates ($n = 8-9$). **C,** Survival profiles showing puberty onset of female GABA specific Lepr knockout (grey lines) and control animals (black lines). The percentage of mice showing vaginal opening (intermittent lines) and first estrus (continuous lines) is plotted for each time point. **D,** Puberty onset profiles of male GABA specific knockouts (grey line) and controls.
(black line), percentage of mice successfully reproducing over time are plotted. VO: vaginal opening; * p < 0.05, ** p < 0.01, *** p < 0.001.

5.4.5 Reduced adult fertility in GABA specific Lepr knockout animals

To assess adult fertility, female estrous cycles and fecundity in both sexes was investigated. Vaginal cytology was determined for 28 consecutive days beginning at day 60 in female GABA specific Lepr knockout animals; significantly less time was spent in proestrus ($t_{17} = 7.32$, $p < 0.0001$; **Fig 5.7A**) and more time in estrus compared to controls ($t_{17} = 2.28$, $p = 0.036$). The time spent in met- or diestrus was not different between groups ($t_{17} = 0.63$, $p = 0.54$; **Fig 5.7A**). Estrous cycle duration averaged $13.6 \pm 1.8$ days for knockouts compared with $4.9 \pm 0.2$ days for controls, showing that GABA specific Lepr knockout resulted in significantly prolonged cycles ($t_{17} = 5.02$, $p = 0.0001$). For glutamate specific Lepr knockout females, there was no difference in the frequency of cycle stage compared to controls. On average, one day was spent in each the pro- and estrus phases and two days in met- and diestrus per cycle (**Fig 5.7C**).

Fecundity of adult mice was assessed by litter size and frequency following pairing with wild type mates. In the GABA specific Lepr knockout females, three animals had to be euthanized due to dystocia complications, and due to these ethical considerations the experiment had to be terminated prematurely (after 38 days). Only thirty percent (3/10) of GABA specific Lepr knockout females produced two litters within this timeframe. A further four of the knockout females delivered successfully but only had one litter. In contrast, all control females produced two litters within 38 days of mating. The inter-litter interval for the multiparous three knockouts was not significantly different compared to controls ($t_{11} = 1.12$, $p = 0.29$; **Fig 5.7B**). However, there was a significant delay in the time from pairing to first delivery for knockout females ($t_{18} = 4.21$, $p = 0.0005$; **Fig 5.7B**). The average number of pups in the litters that were born did not differ between groups (knockout: $6.2 \pm 0.4$ and control $7.6 \pm 0.6$ pups per litter; $t_{17} = 1.79$, $p = 0.09$). Uterine weights of the different female groups were collected as a proxy
indicator of circulating estradiol concentration. No differences were found between the knockout and control groups (Fig 5.8A). Male knockout and control animals were kept in their breeding pairs for 100 days after their first litter was born. The time between litters over this period was significantly longer in the knockout group compared to the control animals ($t_{12} = 2.74, p = 0.0178$; Fig 5.7B). The average number of pups per litter was not different between groups (knockout: $7.8 \pm 0.7$ and control $7.8 \pm 0.5$ pups per litter; $t_{12} = 0.11, p = 0.91$). To assess if this reduced fertility in the male animals was due to lower sperm production, testes were weighed and daily sperm production was measured in GABA specific Lepr knockout males and controls. Paired testes weights were similar between control and knockout groups (Fig 5.8B), and also daily sperm production assessment also revealed no significant differences between the groups (control: $1.2 \times 10^6 \pm 0.3 \times 10^6$; knockout: $1.4 \times 10^6 \pm 0.5 \times 10^6$ spermatocytes per testis per day; $t_{8} = 0.32, p = 0.76$).

![Figure 5.7](image_url)

**Figure 5.7** Adult fertility in knockout and control groups. A, Frequency of estrous cycle stage was monitored for 28 days in GABA specific Lepr knockout females ($n = 9$) and controls ($n = 10$). A significant decrease in time spend in
proestrus was seen in the knockout females. **B**, The time to first litter in GABA knockout females (n = 10) was longer than in littermate controls (n = 10). Number of days between litters was significantly increased in male GABA specific knockout animals (n = 7 in both groups). The same trend was seen in surviving females but low animal number limited statistical comparison (n = 3). **C**, Frequency of estrous cycle stage was monitored for 10 days in glutamate specific knockout mice (n = 9) and control littermates (n = 9). No significant differences were seen between the groups. **D**, Time to first litter and number of days between litters was the same for all glutamate specific Lepr knockout females and males (n = 6 and 9) when compared to littermate controls (n = 8-9). P: proestrus, E: estrus, M/D: met/diestrus; * p < 0.05, *** p < 0.001.

Glutamate specific Lepr knockouts and their controls were also paired with a wild type mate and assessed for litter frequency and time to first litter. Both female and male knockouts and controls were proven to be equally fertile (**Fig 5.7D**).

**Figure 5.8**  Reproductive organ weights of knockout and control groups. 

**A**, Uterine and testes weights of GABA specific Lepr knockouts (females n = 7; males n = 5) and controls (females n = 9; males n = 5), no significant differences were found. **B**, No significant differences were seen between paired testes and uterine weights of glutamate specific Lepr knockouts (females n = 9; males n = 3) and their controls (females n = 5 and males n = 3 respectively).

**5.4.6 Estradiol negative and positive feedback in GABA specific Lepr knockout animals**

The experiments described above have demonstrated a significant delay in puberty onset and a reduction in fertility when Lepr signaling is disrupted in GABA neurons. To explore the underlying mechanism of this decrease in fertility, hypothalamic responses to estradiol was assessed. Measuring the
circulating concentration of LH in different estrogenic states provides a direct index of GnRH neuronal activity since GnRH is a potent stimulator of LH release (Knobil and Neill, 2006). Estrogens inhibit GnRH/LH secretion at most times during the female cycle (negative feedback), however on the day prior to ovulation a rising estradiol concentration triggers a massive preovulatory GnRH/LH surge (positive feedback), eventually causing ovulation (Herbison, 1998).

Intact control and GABA specific Lepr knockout mice had similar levels of plasma LH that increased significantly after OVX (controls $t_{16} = 6.12$; knockouts $t_{16} = 5.70$; $p < 0.0001$ for both; Fig 5.9A). The ability of the estradiol implant to suppress LH levels was undiminished in GABA specific Lepr knockout animals compared to control animals (controls $t_{16} = 5.01$; knockouts $t_{16} = 5.27$; $p < 0.0001$ for both; Fig 5.9A). This indicates that estrogenic negative feedback remains functional in GABA specific Lepr knockout mice despite their fertility defects and high circulating leptin concentration. To test if leptin signaling in GABA neurons is required for positive estradiol feedback, an ovariectomy (OVX) plus high estradiol dose model was used. In this model, a bolus injection of estradiol benzoate results in an artificially induced preovulatory-like LH surge the following day at the time of lights out (Winternantel et al., 2006). When GABA specific Lepr knockout animals were subjected to this treatment, the knockout animals had a 46% lower plasma LH concentration at the time of the preovulatory-like surge when compared to controls ($t_{16} = 2.49$, $p = 0.025$; Fig 5.9B). These data suggest that an impaired positive feedback mechanism may underlie the subfertile phenotype seen in the GABA specific Lepr knockout mice.
Figure 5.9  The effects of estradiol on LH levels in ovariectomized GABA specific Lepr knockouts and controls. A, Estradiol negative feedback was assessed by measuring plasma LH concentration in serial blood samples. Intact plasma samples were taken on day 0, 14 (OVX), and 22 (OVX+implant). The negative feedback actions of estradiol remained intact in GABA specific Lepr knockout animals compared to littermate controls. B, Estradiol positive feedback was assessed based on plasma LH concentration in trunk bloods taken at the time of an estradiol induced preovulatory-like surge. There was a significant suppression of LH concentration in GABA specific knockout female animals compared to controls. n = 9 for all groups; * p < 0.05.

5.4.7 GABAergic input to GnRH neurons in GABA specific Lepr knockout animals

The reduced fertility in the GABA specific Lepr knockouts could be a result of a difference in GABAergic wiring onto GnRH neurons in these animals. To investigate this I counted GABAergic (Vgat positive) appositions on GnRH soma and the proximal projections in both groups. Double label immunohistochemistry and confocal imaging was used to visualize GnRH
neurons and surrounding Vgat-positive terminals. I found that the circumference (knockout: 31.5 ± 0.78 μm and controls: 32.3 ± 1.83 μm; t\_8 = 0.85, p = 0.42) and number (knockout: 5.9 ± 1.32 GnRH neurons per section and controls: 5.4 ± 0.95 GnRH neurons per section; t\_8 = 0.35, p = 0.74) of GnRH soma did not differ between knockout and control animals. When the number of GABAergic appositions onto the GnRH soma and proximal dendrites were counted there were no differences between the groups (Fig 5.10). This indicates no detectable deficits in GABAergic GnRH inputs in the GABA specific Lepr knockout mice.

**Figure 5.10** GABAergic (Vgat positive) appositions on GnRH neurons in male GABA specific knockouts and controls. Images are single optical sections from confocal z-stacks. **A**, Representative image of a GnRH neuron (green) with Vgat (red) appositions on the soma and proximal projection (white arrowheads). White lines indicate 10 μm segments of the neuronal projection. **B**, Representative image of GnRH labeled neuron with omission of Vgat primary antibody. **C**, Number of Vgat positive appositions.
positive appositions per 10 μm of GnRH soma membrane and first three 10 μm segments of the projection, in control (n = 5) and GABA specific Lepr knockout animals (n = 5). No significant differences were found between groups. Scale bar: 10 μm.

5.5 Discussion

Neuronal pathways that communicate leptin and other metabolic signals to the HPG axis are poorly understood. Here, leptin receptors were selectively deleted from the principal inhibitory (GABA) and excitatory (glutamate) neuronal populations to identify whether these neurons mediate interactions between leptin and the reproductive axis. Using these validated mouse models, I conclude that leptin signaling in GABAergic neurons is critical for HPG axis functioning in both sexes, and surprisingly leptin signaling in glutamatergic neurons is not.

Leptin signaling in the PMV is thought to play a major role in regulating fertility rather than metabolism. Lesion studies of this nucleus caused a disruption of estrous cyclicity in rats (Donato et al., 2009), and selective PMV Lepr re-expression in Lepr-null mice induces female puberty onset and limited fertility (Donato et al., 2011b). Additionally, PMV Lepr neurons (of which >80% are glutamatergic) are known to project to GnRH cell bodies, AVPV kisspeptin neurons, and GnRH terminals in the median eminence (Rondini et al., 2004; Leshan et al., 2009; Donato et al., 2011b; Louis et al., 2011). While the present study showed a reduction of ~80% in PMV leptin signaling in the glutamate specific Lepr knockouts, it was unexpected that the glutamate specific Lepr knockout showed no fertility phenotype. These glutamatergic Lepr neurons in the PMV may well participate in this role under normal conditions and even be sufficient to permit fertility in the absence of other brain leptin receptors (Donato et al., 2011b), but with Lepr neurons in other regions intact the loss of PMV Lepr signaling can presumably be compensated. Alternatively, the remaining ~20% of Lepr
neurons in the PMV that are not glutamatergic may fulfill the leptin-to-GnRH role.

Another major population of Lepr neurons within the PMV are the nNOS neurons. Leshan et al. (2012) removed Lepr from nNOS neurons causing a delay in puberty onset. Thus, PMV nitric oxide neurons might mediate leptin’s effects to the HPG-axis. Interestingly, these nNOS Lepr knockout mice were considerably more obese than our glutamate specific Lepr knockouts. This is probably due to the fact that the nNOS Lepr knockout targets Arc neurons in addition to those in the PMV (Leshan et al., 2012). From this work it is unclear that leptin signaling in PMV glutamate neurons is not critical for the metabolic regulation of fertility, but there still might be a role for nNOS-Lepr neurons in this region, as discussed in Chapter 4.

In contrast to the fully fertile glutamate specific Lepr knockouts, male and female GABA specific Lepr knockouts exhibited markedly reduced fertility and the females displayed impaired estrous cycles. To try to identify the cause of subfertility in the GABA specific Lepr knockout mice, I assessed estradiol feedback in females and daily sperm production in males. The results of the estrogenic feedback experiment indicate that knockout mice exhibit normal negative feedback regulation of LH, but are impaired in their ability to mount a full preovulatory-like LH surge in the positive feedback model. This finding is consistent with the reduced occurrence of proestrus in these mice. Similar findings in regard to the LH surge have been reported in Lepr deficient (in all forebrain neurons) and high fat diet-fed mice (Quennell et al., 2009; Sharma et al., 2013). It is not clear why there is a reduced response to positive estradiol feedback, and not to negative feedback in these animals. Perhaps the ‘switch’ from negative to positive feedback requires coordination by leptin-responsive GABA neurons. Deletion of Lepr from certain neuronal populations or metabolic disturbances, such as diet-induced leptin resistance, might therefore only affect the more orchestrated hypothalamic function of mounting a full pre-ovulatory LH surge. As with the fertility data in intact animals, the concurrence of severe obesity with this
effect means that caution must be exercised in attributing the disrupted preovulatory-like LH surge specifically to deficient leptin signaling in GABA neurons. For example, male ob/ob mice completely lacking leptin produced relatively few mature spermatocytes, but this deficit appears to be due to apoptotic effects of leptin deficiency at the gonadal level (Bhat et al., 2006; Zhu et al., 2013). Daily sperm production in our animals, which were not leptin deficient, did not differ between GABA specific Lepr knockout mice and controls, suggesting that our male mice had no such apoptotic effects.

The neurotransmitter GABA is well known to be an important modulator of GnRH neurons, and the present results are consistent with the idea that leptin may communicate with GnRH through GABA. It is known that fasting alters GABA receptor-mediated transmission to GnRH neurons, and leptin is able to modulate this change (Sullivan et al., 2003; Sullivan and Moenter, 2004). Similarly, GABA specific Lepr knockout mice in a study by Vong et al. (2011) were characterized by an increased inhibitory GABAergic tone upon Arc POMC neurons. Although this study did not assess fertility, the increase of inhibitory tone (upon POMC neurons) might also be present upon GnRH neurons in these animals, either directly or via suppression of POMC afferents to GnRH. GABAergic input upon GnRH neurons may therefore be compromised in our GABA specific Lepr knockout mice. In this regard, I have shown that there is no change in GABAergic appositions upon GnRH neurons in the knockout animals, but this does not eliminate the possibility of a change in GABAergic tone causing the subfertile phenotype.

There are a few other candidates for providing the leptin-to-GnRH intermediate signal, in the form of neuropeptides that are potentially co-released from GABAergic Lepr neurons. Firstly, the neuropeptide kisspeptin is the most potent known stimulator of GnRH release and regulates the timing of puberty onset (Han et al., 2005; Clarkson and Herbison, 2006). Additionally, leptin is able to regulate kisspeptin expression (Quennell et al., 2011). Disruption of the function of kisspeptin-producing cells would provide a convenient explanation for the inability of GABA specific Lepr
knockout mice to mount a full LH surge in this study. However, conditional Lepr deletion in kisspeptin neurons or even re-expression in Lepr-null mice has revealed that a direct action is neither required nor sufficient for fertility (Donato et al., 2011b; Cravo et al., 2013). Indeed, few kisspeptin neurons (and none of the AVPV population known to drive the GnRH/LH surge) have leptin receptors (Smith et al., 2006a; Cravo et al., 2011; Quennell et al., 2011). Therefore, if kisspeptin function is reduced in these mice, it probably does not occur as a direct response of Lepr deficiency. Secondly, AgRP/NPY neurons in the Arc are affected by the knockout of Lepr because they are GABAergic (Horvath et al., 1997). Both the AgRP and NPY neuropeptides have been previously linked to the metabolic control of reproduction by direct actions onto GnRH neurons (Klenke et al., 2010; Roa and Herbison, 2012), and indirectly by inhibiting the stimulatory α-melanocyte-stimulating hormone (αMSH) produced from the Pomc gene (Israel et al., 2012). In both leptin deficient ob/ob and Lepr deficient db/db animals, ablation of AgRP neurons restores normal puberty onset and fertility (Israel et al., 2012; Wu et al., 2012). These experiments suggest that leptin responsive AgRP/NPY/GABA neurons are likely to act as intermediates between metabolism and GnRH neurons, and thus suppress the HPG axis in the absence of leptin or leptin signaling. I suggest that these Lepr/AgRP/NPY/GABA neurons are at least partly responsible for the subfertile phenotype observed in our GABA specific Lepr knockout mice. Lastly, there are GALP neurons in the Arc and CART neurons in the DMN that express Lepr and project to the area of the GnRH neurons (Lebrethon et al., 2000; Takatsu, 2001; Rondini et al., 2004). Whether GALP and CART neurons co-release GABA has not been identified, therefore it is unclear if they would have been targeted in our knockout model.

If leptin’s permissive actions on the HPG axis occur via GABA/NPY/AgRP inhibitory inputs to the GnRH neurons, deletion of leptin receptors from GABA neurons would be predicted to cause upregulation of GABA/NPY/AgRP function leading to an increased inhibitory tone upon GnRH neurons. Alternatively, if leptin’s signals are relayed by stimulatory
neuropeptides (i.e. GALP and CART), and knowing that GABA has the ability to stimulate GnRH neuronal activity in some circumstances (Herbison and Moenter, 2011), then a reduction in the tone of these pathways upon GnRH neurons would be predicted in our knockout model. It would be interesting to determine the nature of such changes in our GABA specific Lepr knockout mice. This is addressed using gene expression analysis in Chapter 6.

The knockout animals in which a delay in puberty onset was observed were also obese and had high circulating leptin concentrations. Transgenic removal of exon 17 from the Lepr gene happens at early embryonic stages since Vgat expression is detectable from mid-gestation (Oh et al., 2005). Therefore, leptin receptor deletion in GABA neurons would have encompassed the second half of gestational development in all GABA specific Lepr knockout animals. The reproductive phenotype that was observed in these animals could be an effect of defective wiring or the resulting obesity. However, early obesity or high circulating leptin is known to cause an advance in puberty onset rather than a delay (Ahima et al., 1997; Li et al., 2012). Additionally, Lepr expression and leptin binding in hypothalamic neurons is observed only in late gestation and early postnatal life (Carlo et al., 2007). Also, HPG axis functioning can be successfully stimulated by exogenous administration of gonadotropins and progesterone, or of leptin in ob/ob animals (Smithberg and Runner, 1957; Chehab et al., 1996). These studies indicate that neurotropic deficits due to deficient leptin signaling throughout development are relatively minor, at least as far as fertility is concerned. In support of this, no deficits in GABAergic GnRH inputs were apparent in the GABA specific Lepr knockout mice, since no detectable differences were noted in the number of Vgat positive appositions on the GnRH neurons. Collectively these results suggest that the obesity or any differences in neuronal wiring in our GABA specific Lepr knockouts are unlikely to be primarily responsible for their reduced fertility.

Here, we have limited the first order leptin-to-GnRH mediators to GABAergic neurons. These experiments show that there is a pivotal role for leptin
Lepr GABA and glutamate neurons

receptors in GABA neurons, but notably not in glutamate neurons, in metabolic regulation of male and female fertility. This serves to focus attention on GABA or a few known peptide co-transmitters, specifically NPY, AgRP, or possibly GALP and CART as critical neurotransmitters for the control of reproduction. Future studies should determine which neurons are GABAergic, leptin responsive, and send inputs to GnRH neurons in order to control fertility and then delete leptin receptors specifically from these populations to determine their role as critical Lepr-GnRH conduits.
Chapter Six

Regulation of Reproductive Function by Leptin Signaling in GABA Neurons: A Molecular Biology Approach
6.1 Abstract

In Chapter 5 I have shown that GABAergic neurons provide an intermediate pathway between leptin action and GnRH neurons. In the current chapter, leptin receptors were deleted from GABA neurons using Cre-Lox transgenics. First, the downstream effects on puberty onset and reproductive hormones were confirmed. Both female and male GABA specific Lepr knockout mice showed the expected delay in the onset of puberty. Adult male GABA specific Lepr knockout animals showed a significant decrease in serum LH and testosterone concentrations, whereas there were no differences in the reproductive hormone levels of diestrus female groups. Secondly, hypothalamic gene expression in diestrus females was measured using RT-qPCR for a custom-selected panel of 13 genes. The expression of Gnrh and Kiss1 genes remained unchanged in the knockout animals compared to controls, possibly suggesting a role for GABAergic Lepr neurons acting at the level of GnRH release rather than transcriptional regulation. No expression differences were found in any genes of GABA synthesis or GABA turnover. The GABA specific Lepr knockout females showed a significant increase of Agrp gene expression in the Arc, and Npy gene expression in the LHA. These results will focus future research (including Lepr knockout or rescue experiments) to GABAergic leptin receptor-expressing AgRP and NPY neurons in Arc and LHA.
6.2 Introduction

Leptin signaling in the hypothalamus forms a permissive modulatory signal to the central drivers of the HPG axis: the GnRH neurons (Ahima et al., 1997; Nagatani et al., 1998; Quennell et al., 2009). A significant body of research has been devoted to trying to understand how leptin’s effects are relayed to the HPG axis. As the GnRH neurons in the rPOA do not have the receptors for leptin (Quennell et al., 2009); there must be intermediate neurons that provide leptin-to-GnRH signaling in the brain.

Experiments in the previous chapter show that leptin signaling in GABAergic neurons is required for normal fertility (Chapter 5). There was a significant delay in puberty onset in both sexes when leptin receptors were deleted from Vgat neurons. Additionally, female animals displayed estrous cycle abnormalities, an inability to mount a full pre-ovulatory like LH surge, and an increase in time to produce their first litter. These effects probably originated centrally because the deletion was GABA neuron specific, but because GABA is produced by many types of neurons the specific pathways responsible for the subfertile phenotype in these animals is unknown. Investigating changes in the gene expression of various potential interneuronal pathways in GABA specific Lepr knockouts and controls might uncover a specific neuronal pathway affected by this broad-spectrum deletion.

One interesting feature to look at in the GABA specific Lepr knockout animals is whether levels of GABA are altered. The direct effects of GABA signaling upon GnRH neurons are complicated by the fact that the chloride concentration within the neuron can alter the effect of GABA (which is has until recently been considered to be an inhibitory neurotransmitter, Herbison and Moenter, 2011). It is known that leptin can change GABAergic effects upon GnRH neurons in brain slice electrophysiology studies (Sullivan et al., 2003; Sullivan and Moenter, 2004). Thus, Lepr deletion from GABA neurons might induce a change in GABA signaling in our in vivo knockout
model, which could in turn form the origin of the sub fertility. Measuring GABA levels directly by gene expression techniques is not possible as GABA is a modified form of the amino acid glutamate rather than a peptide or protein. Instead, expression of the enzymes responsible for GABA synthesis, L-glutamic acid decarboxylase (Gad), can be investigated. There are two isoforms of the Gad enzyme that are expressed in the brain: Gad 1 and Gad 2. These enzymes decarboxylate glutamate to form GABA and CO\(_2\) (see review by Martin and Rimvall, 1993). Additionally, the expression of GABA transaminase (Abat) can be measured as an indicator of GABA catabolism (Bernasconi et al., 1982). Together, the expression of these genes in the GABA specific Lepr knockout animals will provide an indication on how much GABA is being made and broken down.

From Chapter 5, there were three hypothalamic regions where a reduction of leptin induced pSTAT3 was seen in the GABA specific Lepr knockout animals: the Arc, DMN and LHA. Therefore, GABAergic Lepr neurons in these regions form possible mediators of leptin-to-GnRH signaling.

In the Arc there are AgRP/NPY neurons that are also GABAergic and express leptin receptor. These neurons are very important in the regulation of food intake and energy metabolism, and they might also play a role in the metabolic regulation of fertility. Israel et al. (2012) showed that ablation of AgRP in the infertile db/db mouse restored reproductive functioning, suggesting a suppressive role of AgRP on fertility. There are also additional GABAergic Lepr neurons in the Arc that might provide a change in GABAergic, or other neuropeptide input to the GnRH neurons following deletion of Lepr (Vong et al., 2011). The Arc contains GALP neurons that are good candidates to convey actions of leptin to GnRH neurons; leptin is known to regulate Galp expression as it is down regulated in ob/ob animals and this can be reversed by leptin treatment (Juréus et al., 2001). Also, GALP neurons provide input to GnRH cell bodies (Takenoya et al., 2006), and icv administration of GALP activates GnRH neurons in the rPOA and increases plasma LH levels (Matsumoto et al., 2001; Gundlach, 2002). AgRP/NPY,
GABA and GALP form likely candidates for Arc leptin-to-GnRH signaling. The GABAergic nature of GALP neurons remains to be investigated, however.

The DMN might contain GABAergic leptin-to-GnRH signaling neuronal pathways, as within this region there are Lepr neurons that are GABAergic and colocalize with CART (Elias et al., 2001) or NO (Donato et al., 2010). Retrograde tracer studies have shown that DMN CART neurons project to areas containing GnRH and kisspeptin neurons, but their role in the regulation of fertility has not been well explored (Rondini et al., 2004).

The LHA contains a mixture of neurons that are GABAergic and glutamatergic, and the GABA neurons found here are known to inhibit food intake (Stanley et al., 2011). There is not much known about leptin signaling in LHA GABA neurons in relation to fertility regulation, but my previous experiment in the GABA specific Lepr knockout animals highlights this population as a potential GABAergic mediator of leptin's effects.

As mentioned in Chapter 5, it has been shown that leptin signaling in GABAergic neurons is required for normal reproductive function. In the current chapter, these results are further explored in regards to effects on gene transcription in the same transgenic mouse model. The underlying mechanism of the subfertile phenotype in the GABA specific Lepr knockouts was investigated using steroid hormones immunoassays before and after puberty onset to show changes throughout development. In adult diestrus female control and GABA specific Lepr knockout mice RT-qPCR was used to look at the expression of several metabolic, reproductive, and GABA related genes.
6.3 Materials and Methods

6.3.1 Generation of GABA specific Lepr knockout mice

Female and male transgenic mice with Vgat (Vgat-ires-Cre) specific Lepr deletions were bred in five pairs from transgenic lines as described in Sections 2.7 and 5.3.1. These animals are hereafter referred to as GABA specific Lepr knockouts. Homozygous Lepr floxed littermates were used as negative controls.

6.3.2 Body weight and puberty onset assessment

Starting one week after weaning, weekly body weights were taken from male and female GABA specific Lepr knockout animals and controls. Female puberty onset was assessed by age at vaginal opening and male puberty onset by age at preputial separation, as described in Section 2.8.1.

6.3.3 Tissue collection and preparation

Prepubertal female and male brain and blood samples were collected at 24 days of age. Adult brain and blood samples were collected at 49-56 days of age (seven weeks; as described in Section 2.3.4). Fresh brain tissue of female animals was collected by decapitation on the day of diestrus (assessed by vaginal cytology, Section 2.8.2). At the time of writing, only the female adult brains have been analyzed for changes in gene expression due to the cost of the array cards used for this.

6.3.4 Reproductive hormone assessment

Serum LH concentrations were measured by an in-house sandwich ELISA, as described in Section 2.6.3 and by Steyn et al. (2013). The average inter-assay coefficient of variation was 1.8% and the average sensitivity was 0.1 ng/ml for both plates. Two plates were run concurrently, one for all
female and one for all male serum samples. Prepubertal and adult serum samples were run in duplicate on the same plate.

Total serum testosterone concentration was measured using a commercial rat/mouse ELISA kit (Section 2.6.5), in samples collected from prepubertal and adult males. The average coefficient of variation was 2.7%, and the sensitivity of the assay was 0.03 ng/ml. All samples were repeated in duplicate on a single plate.

Levels of serum estradiol were quantified using a commercial RIA, as described in Section 2.6.1, in samples of prepubertal and adult diestrus females. The average coefficient of variation was 24%, and the sensitivity of the assay was 7.5 pg/ml. All samples were repeated in duplicate in a single run.

6.3.5 Micro dissection of hypothalamic regions

Micro punches of five different hypothalamic regions were collected from 300 µm coronal sections, as described in Section 2.5.1. Specific hypothalamic regions were identified under a dissecting microscope and punched out using blunted 21 gauge needles. Regions and number of punches per area are specified in Table 6.1 and Figure 6.1.
Figure 6.1  Map of micro punched hypothalamic regions. Representative coronal mouse brain atlas pictures at 0.62 mm, −1.82 mm, and −2.46 mm from bregma (Paxinos and Franklin, 2001). Arc, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PMV, ventral premammillary nucleus; rPOA, rostral preoptic area.

Table 6.1 Micro-dissected hypothalamic regions

<table>
<thead>
<tr>
<th>Hypothalamic region</th>
<th>Atlas coordinates (from bregma in mm)</th>
<th>Number of sections</th>
<th>Punches per section</th>
<th>Average RNA concentration (ng/μl)</th>
<th>Amount of RNA added to cDNA reaction (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPOA</td>
<td>0.86 to 0.38</td>
<td>3</td>
<td>2</td>
<td>20.67 ± 8.18</td>
<td>297</td>
</tr>
<tr>
<td>Arc</td>
<td>-1.34 to -2.54</td>
<td>4</td>
<td>1</td>
<td>15.54 ± 7.08</td>
<td>297</td>
</tr>
<tr>
<td>DMN</td>
<td>-1.46 to -2.18</td>
<td>3</td>
<td>2</td>
<td>17.99 ± 3.61</td>
<td>378</td>
</tr>
<tr>
<td>LHA</td>
<td>-1.46 to -2.19</td>
<td>3</td>
<td>2</td>
<td>13.20 ± 2.52</td>
<td>292</td>
</tr>
<tr>
<td>PMV</td>
<td>-2.30 to -2.70</td>
<td>2</td>
<td>2</td>
<td>16.59 ± 5.32</td>
<td>324</td>
</tr>
</tbody>
</table>
6.3.6 RNA extraction, DNase treatment and cDNA synthesis

Total RNA extractions, DNase treatment, and cDNA synthesis were performed as described in Sections 2.5.2-2.5.4. At the start of the DNase treatment step, final RNA concentrations were made equal across regions (as specified in Table 6.1) by adding different amounts of total RNA to water to reach a final volume of 27 µl.

Real-Time Quantitative PCR for leptin receptor long-form using probes

As a positive control for the GABA Lepr knockout animals, Lepr mRNA was measured by RT-qPCR using primers and probes specifically targeted to the excised region of the Lepr gene (i.e. exon 17 of the long Lepr-b form). Efficiency for the Lepr primer/probe set was calculated from a 10 fold dilution series of whole hypothalamus cDNA and found to be 0.903. Duplicate uniplex reactions for measurement of Lepr mRNA were carried out on cDNA samples using the primers and probe listed in Table 6.2. Primers and probe specific for RNA polymerase II polypeptide A (RpII, Table 6.2) were used as a reference gene. Quantitative PCR 96-well plates were prepared as described in Section 2.5.5.

Table 6.2 RpII and Lepr long-form primer probe sets.

<table>
<thead>
<tr>
<th>Primer/Probe Type</th>
<th>Sequence (5'-3')</th>
<th>Primer concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RpII forward</td>
<td>GCA CCA CGT CCA ATG ATA T</td>
<td>600 µM</td>
</tr>
<tr>
<td>RpII reverse</td>
<td>GTG CTG CTG CTT CCA TAA</td>
<td>600 µM</td>
</tr>
<tr>
<td>RpII probe</td>
<td>FAM/ATT ATC GGC/ZEN/ATT TGG CGC</td>
<td>250 µM</td>
</tr>
<tr>
<td>Lepr forward</td>
<td>ATT TCC TCT TGT GTC CTA CTG</td>
<td>900 µM</td>
</tr>
<tr>
<td>Lepr-long reverse</td>
<td>AAG ATG CTC AAA TGT TTC AGG</td>
<td>900 µM</td>
</tr>
<tr>
<td>Lepr probe</td>
<td>FAM/CGA TGT TCC/ZEN/AAA CCC CAA</td>
<td>250 µM</td>
</tr>
</tbody>
</table>
6.3.7 Real-Time Quantitative PCR experiment using TaqMan array cards

Custom 384 well TaqMan microfluidic array cards were designed using the Life Technologies array configuration tool\(^2\). Primer-probe sets for β-actin, β2 micro globulin, and 18s ribosomal RNA were added as reference genes (Table 6.3). Gene of interest primer-probe sets added to the array are listed in Table 6.3 below. The supplier did not make the sequences available.

Table 6.3 Primer-probe sets used in the TaqMan microfluidic array cards.

<table>
<thead>
<tr>
<th>Primer-probe set</th>
<th>Abbreviation</th>
<th>Reference code</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Actb</td>
<td>Mm00607939_s1</td>
</tr>
<tr>
<td>β2 micro globulin</td>
<td>B2m</td>
<td>Mm00437762_m1</td>
</tr>
<tr>
<td>18s ribosomal RNA</td>
<td>18S</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Leptin receptor</td>
<td>Lepr</td>
<td>Mm00440181_m1</td>
</tr>
<tr>
<td>Glutamic acid decarboxylase 1/67</td>
<td>Gad1</td>
<td>Mm00725661_s1</td>
</tr>
<tr>
<td>Glutamic acid decarboxylase 2/65</td>
<td>Gad2</td>
<td>Mm00484623_m1</td>
</tr>
<tr>
<td>GABA transaminase</td>
<td>Abat</td>
<td>Mm00556951_m1</td>
</tr>
<tr>
<td>Neuronal nitric oxide synthase</td>
<td>Nos1</td>
<td>Mm00435175_m1</td>
</tr>
<tr>
<td>Agouti related peptide</td>
<td>Agrp</td>
<td>Mm00475829_g1</td>
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<tr>
<td>Neuropeptide Y</td>
<td>Npy</td>
<td>Mm03048253_m1</td>
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<td>Pro-opiomelanocortin</td>
<td>Pomc</td>
<td>Mm00435874_m1</td>
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<tr>
<td>Galanin-like peptide</td>
<td>Gapa</td>
<td>Mm00626135_m1</td>
</tr>
<tr>
<td>Cocaine and amphetamine related peptide</td>
<td>Cart</td>
<td>Mm04210469_m1</td>
</tr>
<tr>
<td>Tachykinin 2</td>
<td>Tac2</td>
<td>Mm00436885_m1</td>
</tr>
<tr>
<td>Kisspeptin</td>
<td>Kiss1</td>
<td>Mm03058560_m1</td>
</tr>
<tr>
<td>Gonadotrophin releasing hormone</td>
<td>Gnrh</td>
<td>Mm01315605_m1</td>
</tr>
</tbody>
</table>

The array cards were prepared and loaded as described in Section 2.5.6. All genes were assessed in triplicate for each sample, as shown in Appendix II; samples of the same hypothalamic areas were loaded on to two cards and

run on the same day in the ViiA7 Real-Time PCR machine with a TaqMan Array block (Applied Biosystems; #4453537). Raw RT-qPCR Ct values (the cycle number at which the fluorescence reading is first recorded above threshold levels) were analyzed using the ViiA 7 software package. Replicates were checked thoroughly and outliers were omitted where appropriate.

6.3.8 Analysis

Quantitative PCR data were analyzed using the comparative Ct method. Subtracting the average Ct value for the gene of interest from the average Ct value for the reference gene gives the ΔCt. The comparative Ct method is a relative measure of mRNA expression; thus a fixed arbitrary number (100% in this case) can be used as a calibrator. Subtracting the calibrator from the ΔCt for all samples gave the relative change (ΔΔCt). Finally the arithmetic formula $2^{-\Delta \Delta C_t}$ was used to achieve relative quantitation (Bustin, 2000).

All graphs are presented as mean ± SEM, and Student’s t-test was used to identify significant statistical differences between GABA specific Lepr knockout animals and controls. The body weight data were compared using a two-way ANOVA with repeated measures (treatment group and time as factors) and a Bonferroni multiple comparisons post hoc test.
6.4 Results

6.4.1 Body weights and puberty onset in GABA specific Lepr knockout animals

Animals were bred as described in Chapter 5 and as this was a new cohort of animals, bodyweight and puberty onset were again monitored to confirm phenotypes. In this experiment assessment of preputial separation was added as an anatomical indicator of male pubertal onset. Both knockout females and males had significantly increased body weight from four weeks of age onwards when compared to same sex controls (females: $F_{3,42} = 21.55$, $p < 0.0001$, males: $F_{4,52} = 20.88$, $p < 0.0001$; Fig 6.2A). The first signs of puberty onset were assessed by vaginal opening in females and by preputial separation in males. In both female and male animals this was significantly delayed in the GABA specific Lepr knockout animals (vaginal opening: $t_{14} = 2.23$, $p = 0.0425$, preputial separation: $t_{12} = 3.89$, $p = 0.002$; Fig 6.2B).

Figure 6.2 Body weight phenotype and puberty onset of GABA specific Lepr knockout used in RT-qPCR experiment. A, body weights of control females (n = 8) and GABA specific Lepr knockout females (n = 8). Male body weights of control animals (n = 7) and male GABA specific Lepr knockouts (n = 8). Knockout animals had increased bodyweight when compared with same sex littermate controls using a two-way ANOVA. B, puberty onset as measured by vaginal opening (n = 8 for both groups) and preputial separation (n = 7 for both groups) was significantly delayed in GABA specific Lepr knockout animals compared to controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$
6.4.2 Reproductive endocrinology

To further assess the reproductive physiology of the GABA specific Lepr knockout animals described in Chapter 5, reproductive hormones (LH, estradiol and testosterone) were measured in adult (7-8 weeks old) and prepubertal animals (24 days old). There was no significant difference in LH or estradiol concentration in prepubertal females or adult females, perhaps because all adult female blood samples were collected in the diestrus stage of the cycle and because of a large amount of samples being below the detection limit of the assay in the prepubertal levels (n = 3-4 out of 8; Fig 6.3A and B).

In the male animals, serum LH might have been higher in adult control animals compared with prepubertal animals (t9 = 1.32, p = 0.22; Fig 6.3A), but this increase in LH was not evident in adult knockout animals. When adult control and GABA specific Lepr knockout males were compared levels of serum LH were significantly lower in knockouts (t13 = 2.19, p = 0.047; Fig 6.3A). A similar pattern was seen when serum testosterone was measured in the male groups. The increase of testosterone in control animals over time (prepubertal vs. adult) showed a non-significant increase (t10 = 2.06, p = 0.07; Fig 6.3C), while the GABA specific Lepr knockout males did not show this phenomenon. Within the adult male group control animals showed significantly higher levels in serum testosterone than knockouts (t12 = 2.56, p = 0.025; Fig 6.3C).
Figure 6.3  Characterization of reproductive endocrinology in GABA specific Lepr knockout animals and controls. A, Serum LH concentration in prepubertal (n = 3 for females; n = 4 for males) and adult female (n = 8 for both sexes) and male animals. B, Serum estradiol concentration in prepubertal (controls: n = 6, knockouts: n = 5) and adult female controls (n = 8) and knockouts (n = 8). No significant differences were found. C, Total testosterone in serum of prepubertal (controls: n = 5, knockouts: n = 8) and adult male animals (n = 7 for both groups). * p < 0.05
6.4.3 Loss of Lepr expression in hypothalamic regions

Unfortunately, for approximately one third (33.0 ± 1.92%) of the samples no C_t could be determined due to poor or insufficient cDNA amplification. Therefore, the numbers remaining for analysis are shown on the graph bars in the following sections.

It was previously shown in Chapter 5 that GABA specific Lepr knockout caused a reduction in leptin induced pSTAT3 immunohistochemistry. In this knockout model, LoxP sites flanked exon 17 of the Lepr, and Cre mediated recombination caused the removal of this exon (McMinn et al., 2004). To confirm this recombination occurred, RT-qPCR specifically for the Lepr gene was performed using a primer/probe set on cDNA extracted from hypothalamic micro punches (rPOA, Arc, DMN, LHA and PMV). In this experiment the reverse primer was designed to be complementary to part of exon 17, specifically targeting Lepr-b (long-form). However when the Cre-mediated recombination has occurred the reverse primer will not have any cDNA that is complementary to this primer. This makes the assay suitable for indicating a decrease in the expression of Lepr in hypothalamic regions of the GABA specific Lepr knockouts. (Note that this is not the case for the Lepr primer set on the array cards; as disclosed by the supplier in response to my query about this after having run the assay)

Figure 6.4 shows the relative expression of Lepr using RpII as a reference gene. No differences were found between RpII expression and the different regions when controls and GABA specific Lepr knockouts were compared. As expected from pSTAT3 data there were no differences in Lepr expression in rPOA and PMV when GABA specific Lepr knockouts are compared to controls. The expected decrease of Lepr was seen in the LHA ($t_8 = 2.58$, $p = 0.032$), and there was a decreasing trend in the DMN even though it was not significant ($t_7 = 1.94$, $p = 0.093$). Surprisingly, Lepr expression in the Arc did not reflect the decrease seen in leptin induced pSTAT3.
Figure 6.4 Relative expression of Lepr in diestrus female GABA specific knockout and control animals using RNA polymerase II (RPII) as a reference gene. A significant decrease in Lepr expression was seen in the lateral hypothalamic area (LHA), but not in other regions. Numbers at the bottom of the bars indicate number of samples in each group. Arc, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; PMV, ventral premammillary nucleus; rPOA, rostral preoptic area.

* \( p < 0.05 \)

6.4.4 Changes in gene expression in the rPOA

To investigate a wide range of different genes in the small micro punch samples custom 384 well TaqMan microfluidic array cards were used. The small 1 \( \mu l \) reactions make it possible to assay up to 16 different genes in triplicate within a 50 \( \mu l \) cDNA sample. In the current experiment every fill port contained 300-400 ng total RNA converted to cDNA (Table 6.1). Relative expression was calculated using the expression of the 18S ribosomal RNA gene (18S) as a reference gene, \( C_t \) values of the reference gene did not differ between groups.

In the rPOA of diestrus female GABA specific Lepr knockouts and controls there were no differences in Gnrh expression (Fig 6.5). As another indicator of reproductive capacity kisspeptin expression was investigated, since the rPOA micro punched region includes the AVPV kisspeptin population known to be crucial for estradiol positive feedback (Wintermantel et al., 2006).
Kisspeptin expression in the rPOA had a trend towards being downregulated, however only 3 knockout mice were included and this was not significant ($t_7 = 0.74, p = 0.48$, Fig 6.5).

Expression levels of the two different Gads (1 and 2) as a proxy indicator of GABA biosynthesis, and Abat indicating GABA catabolism were measured in this experiment. No differences were seen in the relative expression of these genes in GABA specific Lepr knockouts compared to controls (Fig 6.5).

The rPOA is also known to be a region where leptin responsive nitric oxide (NO) neurons reside (Donato et al., 2010; Bellefontaine et al., 2014). Therefore the expression of nNOS was investigated. There was no difference in Nnos gene expression between the groups (Fig 6.6). There is also a population of CART expressing neurons called the preoptic and periventricular CART neurons in this region. This neuropeptide does not colocalize with GnRH (Larsen et al., 2003; Vrang, 2006), and it is not known if these neurons are GABAergic. Here, there was no difference in the expression of Cart in the rPOA of the GABA specific Lepr knockout animals (Fig 6.5).

![Figure 6.5](image_url)  
**Figure 6.5** Relative expression of different genes in the rostral preoptic area (rPOA) of diestrus female GABA specific knockout and control animals. Relative expression was calculated using the expression of the 18S ribosomal RNA gene as a reference gene. Numbers at the bottom of the graph indicate number of samples in each group. Abat, 4-aminobutyrate transaminase; Cart, cocaine and amphetamine regulated transcript; Gad1 and 2, glutamic acid decarboxylase 1 and 2; Gnrh,
gonadotropin releasing hormone; Kiss1, kisspeptin; Nnos, neuronal nitric oxide synthase.

6.4.5 Changes in gene expression in the Arc

The Arc showed some interesting results, however some expected changes were not detected. In contrast to the trend towards lower expression in the rPOA, kisspeptin expression was unchanged in the Arc (Fig 6.6). The expression levels of GABA turnover genes remained unchanged here, even though Lepr were removed from the GABAergic neurons in this region. Additionally, Nnos expression did not change in the Arc, which was expected as colocalization of nNOS and Lepr is low in the Arc (Donato et al., 2010).

It was presumed that Lepr deletion made in GABAergic neurons included AgRP/NPY neurons in the Arc. The gene expression results show a significant up-regulation of Agrp expression in the knockout females ($t_9 = 2.78, p = 0.021$; Fig 6.6). A similar trend was seen for Npy, however this was not significant between the groups ($t_9 = 1.53, p = 0.16$; Fig 6.6). The increase in circulating leptin concentration seen in the Lepr knockout animals (see Section 5.4.3), or the increased levels of Agrp expression did not suppress the expression of Cart and Pomc. There were no differences between control and knockout Cart and Pomc expression levels. This is surprising since high circulating leptin concentrations normally stimulate Cart and Pomc expression (Cowley et al., 2001; Sahu, 2008). Additionally, the AgRP/NPY and CART/POMC neurons form a close contact network and high Agrp or Npy can inhibit Cart and Pomc expression (Horvath et al., 1992; Cowley et al., 2001). The relative expression of Galp in the Arc was non-significantly higher in the GABA specific Lepr knockout animals ($t_8 = 1.91, p = 0.09$; Fig 6.6).
Figure 6.6  Relative expression of different genes in the arcuate nucleus (Arc) of diestrus female GABA specific knockout and control animals. *Agrp expression was significantly increased in GABA specific Lepr knockout females (grey bars) when compared to controls (black bars). Numbers at the bottom of the graph indicate number of samples in each group. Abat, 4-aminobutyrate transaminase; Agrp, agouti related protein; Cart, cocaine and amphetamine regulated transcript; Gad1 and 2, glutamic acid decarboxylase 1 and 2; Galp, galanin-like peptide; Kiss1, kisspeptin; Nnos, neuronal nitric oxide synthase; Npy, neuropeptide Y; Pomc, proopiomelanocortin.* p < 0.05

Relative expression (in Fig 6.6) was calculated using the expression of 18S gene as a reference gene. However it must be noted that for the Arc samples, C\textsubscript{t} values of the reference gene were significantly different between groups (t = 2.35, p = 0.044). To check if the results that were found are truly significantly different, relative expression levels were calculated with a different reference gene from the same array that did not differ in mean C\textsubscript{t} value between different groups (Lepr). The Lepr gene expression from the array card was used because in hindsight the primer-probe set used to detect this gene was not detecting the floxed part. This resulted in no changes in Lepr gene expression in any of the regions, because it was measuring an upstream region. Using Lepr C\textsubscript{t} values as a reference gene confirmed that Agrp expression is upregulated by threefold in the Arc.

6.4.6 Changes in gene expression in the DMN

Relative expression was calculated using the expression of the 18S ribosomal RNA gene as a reference gene, C\textsubscript{t} values of the reference gene did not differ between groups. In the DMN of the GABA specific Lepr knockout females
there were no differences in the expression of GABA synthesis genes (Gad1,2 for GABA) or the catabolic enzyme Abat gene (Fig 6.7). Neuronal Nos expression was evident in DMN, which is consistent with the literature (Donato et al., 2010; Leshan et al., 2012). There was no difference between the expression of Nnos, Cart, and Pomc in the DMN of GABA specific Lepr knockout females when compared to controls (Fig 6.7).

**Figure 6.7** Relative expression of different genes in the DMN of diestrus female GABA specific knockout and control animals. Relative expression was calculated using the expression of the 18S ribosomal RNA gene as a reference gene. Numbers at the bottom of the graph indicate number of samples in each group. Abat, 4-aminobutyrate transaminase; Cart, cocaine and amphetamine regulated transcript; DMN, dorsomedial nucleus of the hypothalamus; Gad1 and 2, glutamic acid decarboxylase 1 and 2; Nnos, neuronal nitric oxide synthase; Pomc, proopiomelanocortin.

### 6.4.7 Changes in gene expression in the LHA

No differences in the expression of GABA synthesis genes (Gad1 and 2 for GABA) or the catabolic enzyme Abat gene, or Pomc expression were seen in the LHA of the GABA specific Lepr knockout females. The LHA Npy expression was significantly increased in GABA specific Lepr knockout females when compared to controls (t5 = 3.07, p = 0.028; Fig 6.8). Levels of Cart expression showed a non-significant trend towards being down regulated in the LHA of the GABA specific Lepr knockout animals (t9 = 1.45, p = 0.18; Fig 6.8).
Figure 6.8  Relative expression of different genes in the LHA of diestrus female GABA specific knockout and control animals. Relative expression was calculated using the expression of the 18S as a reference gene, $C_t$ values of the reference gene did not differ between groups. In the LHA $Npy$ expression was significantly increased in GABA specific Lepr knockout females (grey bars) when compared to controls (black bars). Numbers at the bottom of the graph indicate number of samples in each group. Abat, 4-aminobutyrate transaminase; Cart, cocaine and amphetamine regulated transcript; Gad1 and 2, glutamic acid decarboxylase 1 and 2; LHA, lateral hypothalamic area; Npy, neuropeptide Y; Pomc, proopiomelanocortin. * $p < 0.05$

6.4.8 Changes in gene expression in the PMV

In PMV of the GABA specific Lepr knockout females there were no differences in the expression of GABA synthesis genes ($Gad1$ and $2$) or the catabolic enzyme $Abat$ gene (Fig 6.9). Expression of $Nnos$, $Cart$, and $Pomc$ in PMV also did not differ, although the statistical power was limited by the low number of animals in the knockout group ($Nnos$: $t_{10} = 0.83$, $p = 0.42$; $Cart$: $t_{10} = 0.73$, $p = 0.48$; $Pomc$: $t_{10} = 0.56$, $p = 0.59$; Fig 6.9). No difference in $Npy$ expression was seen in the PMV.
Figure 6.9  Relative expression of different genes in the PMV of diestrus female GABA specific knockout and control animals. Relative expression was calculated using the expression of the 18S as a reference gene, $C_t$ values of the reference gene did not differ between groups. Numbers at the bottom of the graph indicate number of samples in each group. Abat, 4-aminobutyrate transaminase; Cart, cocaine and amphetamine regulated transcript; Gad1 and 2, glutamic acid decarboxylase 1 and 2; Nnos, neuronal nitric oxide synthase; Npy, neuropeptide Y; Pomc, proopiomelanocortin; PMV, ventral premammillary nucleus.

6.5 Discussion

In Chapter 5 the existence of a critical GABAergic link that communicates leptin signals to the HPG axis is described. In this chapter, the effects on reproductive hormones and gene expression in the hypothalamus were studied in the GABA specific Lepr knockout mouse model. Overall, this work shows that leptin signaling is required to maintain normal gene expression in the GABAergic arcuate nucleus AgRP/NPY neurons. Therefore, it is possible that these particular GABAergic neurons are critical for HPG axis functioning.

The experiments in this chapter were performed on a new cohort of GABA specific Lepr knockout animals. First, I confirmed puberty onset delay and strengthened findings presented in Chapter 5 with the addition of preputial separation data. Then to further investigate HPG axis functioning in the GABA specific knockout animals the circulating levels of different
reproductive hormones were examined. The results show that adult GABA specific Lepr knockout males have significantly reduced circulating LH and testosterone concentrations. In fact, these remained at prepubertal levels in the adult mice. Because of the central modifications in leptin signaling in the knockout animals, this is likely the result of a reduction in hypothalamic GnRH output. In female GABA specific knockout animals and controls blood samples were collected at the diestrus stage of the cycle in order to standardize ovarian influences across the groups. There were no differences between control and knockout groups in circulating estradiol or LH concentrations in prepubertal and diestrus adult females. Results of the estradiol assay must be interpreted with caution as the variation within the assay was high. The is likely due to the changes that were made to the commercial kit to be able to add a lower volume of blood. Serum LH seems to be lower in prepubertal females, this was not significant because a lot of the samples were below the detection range of the assay, however samples in a similar range of what has been reported before for animals at this age (Risma et al., 1997).

Diestrus female brains were chosen to be examined because circulating hormones were even across the groups, and thus would not confound with the expression of the various genes. Perhaps collecting the brain tissue at the time of the preovulatory surge (afternoon of proestrus) would have painted a different picture since preovulatory-like surge LH levels were reduced in the GABA specific Lepr knockout animals (Section 5.4.6). The main objective here was analysis of central gene expression changes under similar steroid levels. In the gene expression part of this work I choose to look at five different hypothalamic regions: the rPOA, Arc, DMN, LHA, and PMV. Differences in gene expression in the rPOA were investigated because GnRH and some kisspeptin cell bodies reside in this region. These neuronal populations were not targeted by the knockout as neither of them express Lepr (Quennell et al., 2009; Cravo et al., 2013). Examining the gene expression in the rPOA provides a view of the downstream effects of the knockout, as GnRH is the final output of the reproductive axis and kisspeptin
is the most potent known modulator of GnRH release. The Arc, DMN, and LHA were investigated, because there was a reduction in leptin-induced pSTAT3 staining in these GABA neuron-rich regions (Chapter 5). The PMV was examined because this region has been shown to be important for the regulation of fertility by leptin (Donato et al., 2009; Donato et al., 2011b). A lack of changes in the PMV might support the specificity of our effects to GABAergic neurons, as the PMV is largely glutamatergic.

When looking at a reduced reproductive phenotype the first thing one might think about is a reduction in the hypothalamic GnRH output. Therefore, Gnrh mRNA expression in the rPOA was investigated. Despite the known subfertility of these mice, there were no differences between the groups. This is consistent with the fact that GnRH neuron numbers are similar in both groups, as shown in Section 5.4.6. This suggests that the GnRH neuron population is intact in these animals and that reproductive suppression is probably due to a reduced GnRH release, rather then GnRH protein synthesis (as reviewed by Christian and Moenter, 2010). A reduction in GnRH release in these animals might be assessed by measuring hypothalamic GnRH protein content rather then gene expression (for example by RIA as described by d’Anglemont de Tassigny et al., 2007).

Kisspeptin is the most potent known stimulator of GnRH release and is probably the main conveyor of estradiol positive and negative feedback (Gottsch et al., 2004; Messager et al., 2005; Smith et al., 2006b). However, only a small proportion of kisspeptin-expressing neurons express Lepr (Cravo et al., 2011). In Chapter 5 an impaired estradiol positive feedback was noted in the female GABA specific Lepr knockout animals. This impairment could be explained by a dysregulation of kisspeptin signaling. Kisspeptin expression in the rPOA was insignificantly down regulated in the GABA specific Lepr knockout females. This trend could in part explain why the GABA specific Lepr knockout females failed to exhibit a full pre-ovulatory LH surge (Section 5.4.6). The lack of statistical significance of this result may be due to low endogenous estradiol levels in the diestrus stage of both
groups. When similar animals were treated with an estrogen, a significant decrease in AVPV kisspeptin gene expression has been shown recently using \textit{in situ} hybridization (Martin \textit{et al.}, 2014). This paper also showed a reduction in kisspeptin mRNA expression in the Arc when estradiol was removed by ovariectomy. However, the punches that were made for the rPOA samples only included a variable part of the AVPV kisspeptin population. In the current experiment we saw no change in Arc kisspeptin expression, but due to variability in the sampling technique it is not comparable with results of Martin \textit{et al.} (2014).

As leptin signaling in GABAergic neurons was specifically targeted, changes in GABA release (rather than changes in a co-expressed neuropeptide) might cause the subfertile phenotype. In the previous chapter, I showed that the number of Vgat positive appositions on GnRH neurons is the same in both groups (Section 5.4.6). This shows that GABAergic neuronal wiring to GnRH cell bodies (and to the first 30 µm of the dendrites) appears to be intact in the GABA specific Lepr knockout mice. Although the anatomy is intact it remains important to check whether there are any differences in the enzymes that make and break down GABA (as a proxy indicator of GABA turnover). There was no difference in the expression of the three GABA turnover genes investigated in any of the different hypothalamic areas, suggesting that removing Lepr from GABA neurons does not change GABAergic transmission within the hypothalamus. This is surprising because Vong \textit{et al.} (2011) showed changes in inhibitory GABAergic tone upon POMC neurons in the Arc, measured by electrophysiology. A lack of gene expression changes in the GABA system does not mean that GABAergic signaling from Lepr neurons does not play a role. Additionally, in the Arc samples there is a mixture of GABAergic and non-GABAergic Lepr neurons therefore gene expression changes might be diluted out in these samples. Transgenic models in which Vgat is deleted from either Lepr or AgRP neurons, inhibiting the transport and release of GABA from these neurons, show that GABA release from Lepr neurons is important for bodyweight regulation (Tong \textit{et al.}, 2008; Xu \textit{et al.}, 2012). This results in an inversion of what has been done
in this thesis, removing leptin signaling from Vgat neurons. The effects of removing GABA transport from Lepr or AgRP neurons on reproduction are not investigated in the papers mentioned above and would be interesting to explore.

The colocalization of nNOS and leptin receptors has been reported in all hypothalamic areas investigated here, except the LHA (Donato et al., 2010). However there were no differences detected in Nnos expression in any of these regions. This is likely due to the fact that hypothalamic nNOS neurons are not GABAergic (Leshan et al., 2012). A recent study showed that leptin signaling in the rostral POA nNOS neurons plays an important role in facilitating fertility, including the preovulatory LH surge (Bellefontaine et al., 2014). Although Lepr in nNOS neurons maybe important for fertility, it seems that this role is not mediated via GABA neurons.

Even though there was a significant decrease of leptin signaling (pSTAT3) in the Arc of the knockout animals, no difference in expression of Lepr in this region was evident. One of the possible explanations for not seeing this change at the mRNA level is that the loss of Lepr expression is diluted down and therefore lost in the mixture of cells collected in the micro punch. As visualizing phosphorylation of STAT3 is generally an on-off signal of cellular communication it does not tell us anything about protein or mRNA content within the activated cells. Thus it is possible that the total number of leptin responsive cells has decreased in the various regions, but not the level of Lepr expression. Or it could be due to the low fidelity of the micro punch technique especially in diffuse populations like the Arc.

A concern regarding the Arc samples is that the mean Ct values of the reference genes were different between the GABA specific Lepr knockout and control group. In the original setup of the TaqMan array cards, three different reference genes were added (Actb, B2m, and 18S). The levels of 18S were least different between the groups (p = 0.044) and therefore chosen to produce **Figure 6.6**. The results in the Arc show a significant increase in
Agrp expression when checked with a gene that did not change between the groups.

The increase in Arc Agrp is consistent with the suppressive effect leptin signaling has on AgRP expression (as reviewed by Schwartz et al., 2000). When leptin receptors are removed from the GABAergic AgRP neurons, Agrp will no longer be suppressed by leptin and this will therefore lead to an elevation of Agrp mRNA in our knockout model. It is then tempting to hypothesize that the increase in AgRP might in turn act to suppress fertility at the level of GnRH release into the portal blood system, as both AgRP and NPY are known to suppress LH release in rats (Catzeflis et al., 1993; Schioth et al., 2000). This is consistent with studies that show deletion of functional AgRP in Lepr deficient db/db mice (Israel et al., 2012; Sheffer-Babila et al., 2013), or ablation of AgRP neurons in leptin deficient ob/ob animals restores fertility (Wu et al., 2012). These papers suggest that the fertility suppression observed may be due to an increase in the expression of inhibitory AgRP. It remains unclear if AgRP neurons project to the GnRH cell bodies in the rPOA, or if the effect of increased AgRP has a local effect on the release of GnRH at the median eminence. There has been one study in which Lepr were specifically deleted from AgRP neurons (van de Wall et al., 2008), however this paper focused on the metabolic functions of AgRP neurons and did not investigate the resulting reproductive phenotype. It would be very interesting to further investigate this specific deletion; an AgRP-Cre transgenic mouse line (JAX mice stock number 012899) has recently become available.

Vong et al (2011) previously showed that GABAergic Lepr neurons in the Arc decrease the inhibitory tone upon CART/POMC neurons. This suggests the removal of Lepr from these neurons increases the inhibitory tone upon CART/POMC neurons. However in this chapter there was no change in the expression of Cart and Pomp in the Arc, showing that the increased inhibitory tone does not affect gene expression levels. As a distinct population from the AgRP/NPY and CART/POMC neurons there are GALP
Lepr neurons in the Arc. In this experiment an increase in \textit{Galp} expression was predicted (if these neurons are GABAergic, which remains to be explored), because fasting (which is accompanied by reduced circulatory leptin levels) is known to lower \textit{Galp} expression (reviewed by Shiba \textit{et al.}, 2010). Deletion of leptin receptors mimics a low leptin a situation (fasting) because the affected neurons cannot receive input from circulating leptin. However, there were no differences in \textit{Galp} mRNA expression between the two groups. This may suggest that the GALP Lepr neurons in the Arc are not GABAergic. The current difficulty in labeling GALP neurons by immunohistochemistry makes this difficult to confirm. A GALP-Cre mouse is available which could make visualizing of GALP neurons easier using a reporter animal (Eberhard \textit{et al.}, 2012), however for good visualization of GABAergic neurons this is also needed and also uses this approach. Maybe only dual label in situ will be able to answer whether GALP neurons are GABAergic.

In this chapter, I have followed the initial findings of sub fertility in GABA specific Lepr knockout animals up with a gene expression study. The key result shows that Arc AgRP/NPY/GABA neurons are upregulated by this knockout. And therefore might provide the intermediate pathway that results in the metabolic regulation of fertility by leptin.
Chapter Seven
General Discussion
7.1 Main findings

Reproductive function in females is energetically demanding due to the high-energy costs of pregnancy and lactation (Hill et al., 2008; Roa et al., 2010). As a consequence, the central HPG axis is in constant dialog with metabolically informative hormones that circulate in the periphery and pass into the central nervous system. These hormones include leptin, insulin, and ghrelin that provide short and long term information about energy stores and satiety. This thesis focused on the adipocyte derived hormone leptin, and how it interacts with the HPG axis. Studies using genetically modified mice have shown that these effects are primarily relayed through forebrain neurons (Cohen et al., 2001; de Luca et al., 2005; Quennell et al., 2009). Leptin receptors are densely expressed in parts of the hypothalamus, suggesting that leptin might act directly on hypothalamic components of the HPG axis such as: GnRH and kisspeptin neurons. However, it is now clear that GnRH and kisspeptin neurons are not the direct anatomical link between leptin signaling and reproductive function (Quennell et al., 2009; Donato et al., 2011a; Donato et al., 2011b; Louis et al., 2011; Cravo et al., 2013). Subsequent research is now trying to identify the intermediate connections that enable this leptin-to-GnRH signaling.

The experiments in this thesis worked towards acquiring knowledge about the hypothalamic regulation of fertility by the leptin. Two main questions were asked:

1. Where are the hypothalamic Lepr-expressing neurons that provide leptin-to-GnRH signaling located?
2. What is the intermediate signaling molecule for HPG axis regulation by leptin?

With solving these anatomical and functional questions I have tried to narrow down and identify the possible leptin to GnRH intermediate pathways. After an extensive literature review (which is summarized in Chapter 1), a selection of potential intermediary neuronal populations was
noted. These populations are both known to have anatomical connection with GnRH neurons and express leptin receptors (although whether the same individual neurons fulfill both of these criteria is difficult to assess). To more specifically link the anatomical location of leptin responsive neurons that provide input to GnRH neurons, neuronal retrograde tract tracing was used in Chapter 3. This experiment highlighted the arcuate nucleus as a key area that contains Lepr positive neurons that project to the rPOA - the area where GnRH cell bodies reside. Additionally, lower numbers of leptin responsive projecting cells were found in the LHA and PMV.

To try and identify the type of neurons, and hence the intermediate signaling molecule that provides leptin-to-GnRH signaling, three different hypothalamic leptin receptor populations were studied: nitric oxide, glutamate, and GABA neurons. These large neuronal populations, known to colocalize with a range of neuropeptides, were chosen based on published evidence suggesting their involvement, and because transgenic deletion of Lepr from smaller neuronal populations has been unsuccessful in identifying any likely candidates in leptin’s control of the HPG axis in the past (i.e., kisspeptin or POMC and AgRP, van de Wall et al., 2008; Donato et al., 2011b). First, I investigated whether leptin acts on NO producing neurons to regulate fertility. Unfortunately, the knockout of Lepr from nNOS neurons was unsuccessful (Chapter 4), emphasizing that not every Cre driver can be relied on to recombine every floxed gene. However, recent transgenic and functional studies within this field have shown a role for NO signaling in the control of puberty onset and leptin induced LH release (Leshan et al., 2012; Bellefontaine et al., 2014). Secondly, leptin receptors were selectively deleted from the glutamate population to identify whether these neurons mediate interactions between leptin and the reproductive axis. Surprisingly, this revealed that leptin signaling in glutamatergic neurons are not needed for normal HPG axis function (Chapter 5). Lastly, leptin signaling in GABAergic neurons was investigated and found to be critical for normal pubertal development and adult reproductive functioning. When this pathway was investigated further it was found that leptin sensitive
GABAergic arcuate nucleus AgRP/NPY neurons are significantly altered (in terms of AgRP gene expression) in GABA specific Lepr knockout mice, raising the possibility that these cells may be critical for normal HPG axis functioning (Chapters 5 and 6).

Four different hypothalamic regions are highlighted by these studies and the current literature. These regions form the current ‘hotspots’ for focus in the reproductive field, together with likely signaling molecules expressed in these regions (as shown Table 7.1). The following sections will give a brief overview of the likely role and importance of these interactions between Lepr, intermediary neurons and GnRH neurons.

Table 7.1 Likely mediators of leptin-to-GnRH signaling

<table>
<thead>
<tr>
<th>Neurotransmitters</th>
<th>NO</th>
<th>GABA</th>
<th>Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPOA</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Arc</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>PMV</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>LHA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropeptides</td>
<td>AgRP/NPY</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GALP</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

7.1.1 Nitric oxide in the rostral preoptic area

Neurons synthesizing the gaseous neurotransmitter NO may represent a key pathway in leptin signaling to the HPG axis. Selective deletion of Lepr from nNOS neurons results in obesity and a delay in puberty onset (Leshan et al., 2012). This publication achieved much of what I hoped to demonstrate in Chapter 4, although the nNOS-Cre mouse I used had the potential advantage
of being inducible to bypass the caveats of developmental compensation. Additionally, global genetic deletion or pharmacological blockade of nNOS in adulthood renders leptin unable to stimulate LH release (Bellefontaine et al., 2014). This provides evidence for leptin signaling in nNOS neurons, and that the functioning of nNOS is important in leptin-to-GnRH signaling. The studies of Bellefontaine et al. (2014) pinpoint the effects of NO signaling in the rPOA: infusions of a nNOS inhibitor into this region effectively diminished the leptin induced increase in circulating LH concentration. It is important to note that NO transmission is accomplished by simple diffusion rather than via synaptic junctions, and the Lepr NO neurons in this region are in close proximity to the GnRH neurons. However, it is unclear if the two types of neurons are related as deletion of Lepr from nNOS neurons does not necessarily impair NO production and only about 34% of nNOS neurons have leptin induced pSTAT3 in the rPOA (Donato et al., 2010). Investigation of the rPOA Lepr neurons in the retrograde tracer experiment was not practical because these were found to be too close to the tracer injection site, and thus were masked by the intense fluorescence of the injection site (despite the limited diffusion properties of the Retrobeads compared to many other tracers).

7.1.2 GABA, galanin-like peptide, and agouti-related peptide in the arcuate nucleus

The literature suggests many possible signaling molecules that might provide an intermediate link between leptin and GnRH: the neurotransmitters NO, glutamate, and GABA as well as the neuropeptides NPY, AgRP, POMC, CART, and GALP. A large number of these are located in the Arc, an important region for leptin’s effects of metabolism. From the neuronal tracing studies (Chapter 3), I found the highest absolute number and percentage of Lepr and GABAergic neurons that project to the rPOA come from the Arc. This connection to GnRH soma has also been described for Arc kisspeptin neurons (Yeo and Herbison, 2011), however colocalization
of leptin signaling and kisspeptin is very low (Cravo et al., 2011; Quennell et al., 2011) to moderate (Smith et al., 2006a) in the Arc.

Functional studies in this thesis found GABA-Lepr neurons are critical for normal reproductive function, but this was not the case for glutamate neurons. The effects on fertility cannot for certain be accredited to the GABA neurotransmitter itself, because transgenic deletion of Lepr from GABA neurons does not appear to directly impair GABA turnover gene expression (Chapter 6) or GANA synapses on GnRH soma (Chapter 5) in these neurons. Thus, the signaling molecules that are responsible for the subfertile phenotype seen in the GABA specific Lepr knockout animals remains uncertain. There is a good case for increased AgRP coming from Lepr/GABA/AgRP/NPY neurons in the Arc as the causative factor, as AgRP was upregulated in our model (Chapter 6). Additionally, previous research has shown that high levels of AgRP are suppressive to the HPG axis (Israel et al., 2012; Wu et al., 2012). Future research could knockout Lepr specifically from AgRP neurons and assess fertility. I can conclude from the GABA specific Lepr knockout studies that leptin signaling in GABA neurons (but not nNOS and glutamate neurons) is critical for normal reproductive functioning, because when leptin signaling in GABA neurons is intact adult fertility is intact (as is seen in nNOS and glutamate specific Lepr knockout(Chapter 5 and Leshan et al., 2012). However, it should be emphasized that these experiments have little bearing of whether leptin receptors in GABA neurons are sufficient for, or even involved in, reproductive function. To assess this, a GABA specific Lepr ‘rescue’ approach would be required.

Expression of nNOS neurons in the Arc is anatomically distinct from AgRP and POMC expression (Leshan et al., 2012), and co-expression with Lepr in this region has shown to be very low (Donato et al., 2010). Reproductive changes seen in nNOS-Lepr knockout are therefore unlikely to be mediated via Arc nNOS-Lepr neurons, but rather related to leptin signaling in rPOA and PMV (Leshan et al., 2012). In the Arc, glutamate is co-expressed with POMC/CART neurons (about 10% of POMC neurons are Vglut2 positive,
Vong et al., 2011), and because glutamate specific Lepr knockout animals did not show a phenotype these are unlikely to mediate leptin’s effects on fertility.

One major concern with using transgenic knockout mouse models is developmental compensation that might mask the predicted phenotype. This is especially pertinent as metabolism and reproduction are highly protected functions. Removal of the transgene happens early in development (in the case of the GABA Lepr knockout mice, Vgat is detectable from mid-gestation; (Oh et al., 2005)), and thus changes in neuronal wiring and neurochemical signaling may be able to compensate for the loss of Lepr in certain neuronal populations. For example, this protective effect could potentially result in a situation where glutamate specific Lepr knockout drives GABA and nNOS Lepr neurons to compensate. To overcome this phenomenon, inducible knockout mouse models could be employed to study reproductive phenotypes. Inducing the knockout during adulthood would avoid developmental compensation effects on reproductive phenotype. This was the aim of the inducible model of nNOS-Lepr knockout, but unfortunately this experiment failed for unknown reasons. At this point in time glutamate and GABA inducible knockout mouse models are unavailable.

There are some limitations to the techniques used to identify the Arc by neuronal tracing (Chapter 3). The retrograde tracer injections were centered on the region where GnRH cell bodies are most concentrated; however, this is not a GnRH neuron specific technique. When the regions mapped by my retrograde tracing approach are compared with regions found in GnRH neuron specific tracing (Yoon et al., 2005; Wintemantel et al., 2006), similar hypothalamic tracing patterns are seen. To investigate leptin responsive inputs it was important to be able to identify endogenous Lepr neurons and thus a transgenic reporter animal model was used. Innervations from the regions mentioned above have been previously described, but it is only known that there are Lepr neurons within these regions and not whether these exact same neurons are the ones that project to the rPOA.
From the work presented in this thesis, it can now be concluded that there are leptin responsive neurons from the Arc, LHA and PMV that project to the rPOA, and it is plausible that these may interact directly (or indirectly) with GnRH neurons. I have also demonstrated that there are GABAergic neurons projecting to the rPOA, whether these are leptin responsive needs to be further investigated. Attempts to colocalize leptin-induced pSTAT3 in the Vgat-tdTomato reporter animals have been unsuccessful thus far. This caveat could be overcome by breeding the Vgat-Cre line onto the eGFP reporter line, as staining for leptin-induced pSTAT3 in that fluorophore reporter line has been successful (Chapter 3).

It is important to note that the possibility of Arc (or other regions) Lepr neurons regulating the release of GnRH at their terminals in the median eminence should also be considered. There is evidence that GnRH stimulation can happen along remarkably distal regions of the neuron, and that the very long GnRH projections are found to have both axonal and dendritic properties (Herde et al., 2013).

7.1.3 Nitric oxide and glutamate in the PMV

The PMV is characterized by a concentrated expression of leptin receptors (Yoon et al., 2005; Leshan et al., 2009; Patterson et al., 2011). Exogenous re-expression of Lepr in the PMV of Lepr-null mice induces puberty onset and improves fertility in females. Additionally, bilateral lesion of the PMV impairs leptin to induce reproductive function in ob/ob mice (Donato et al., 2011b). These studies form compelling evidence for an important role of the PMV in the regulation of the HPG axis by leptin. It is however unknown what intermediate signaling molecules are responsible. Two abundant messengers in this area that form possible intermediates are glutamate and NO. From the data presented in Chapters 4 and 5, it appears more likely that NO, rather than glutamate, might be a critical intermediate signaling molecule. However, glutamate cannot be ruled out as effects might be ‘masked’ by
developmental changes (as discussed in Section 7.1.2), or it might play a facilitating/sufficient role but not a critical one.

7.1.4 GABA in the lateral hypothalamic area

Surprisingly, in both the anatomical and functional studies, the LHA remains a potential mediator of leptin-to-GnRH signaling. There are Lepr inputs projecting from the LHA to the rPOA (Chapter 3), and there is a significant decrease in leptin-induced pSTAT3 in the GABA specific Lepr knockout animals (Chapter 5). Additionally, in Chapter 6 I found that there is an increase in NPY expression in the LHA. It is known that GABAergic LHA neurons inhibit food intake (Stanley et al., 2011), but the results presented in this thesis show that they might also play a role in leptin’s effects on fertility. This agrees with previous findings that LHA neurons project to GnRH cells in the rPOA (Yoon et al., 2005; Wintermantel et al., 2006). This potential pathway could be further explored by region specific deletion of Lepr here, or even GABA neuron specific regional deletion of Lepr (as described in Section 7.2.3).

7.2 Future research

7.2.1 Tackling redundancy

As reproduction is such a critical and energy-consuming process, redundancies in the metabolic pathway controlling reproduction would ensure survival of the species in case one pathway failed. This has been thought to be the case for many of the experiments done in this field using genetic knockout of specific genes where no pronounced phenotype was observed (reviewed in Elias, 2014).

In Chapter 5, no differences in fertility in the glutamate specific Lepr knockout were discovered, even-though the animals in this study showed a
very large decrease in PMV Lepr signaling. This was a surprising result because when leptin receptors are re-expressed unilaterally in this mostly glutamatergic region of female Lepr-null mice, puberty is induced and fertility improved (Donato et al., 2011b). In the case of the glutamate specific Lepr knockout experiment presented here, there may be redundant pathways making up for the large loss of leptin signaling in the PMV. It would be interesting to investigate reproductive phenotypes in an inducible model of this knockout to assess the contribution of developmental compensation. Additionally, it could be tested if leptin signaling in glutamatergic neurons is sufficient to allow fertility (vs. required as tested in Chapter 5) in a rescue experiment where leptin receptors are only expressed in Vglut2 expressing neurons.

Although transgenic deletion of Lepr from nNOS neurons results in a delay in puberty onset, adult animals have normal fertility (Leshan et al., 2012). However, selective deletion of leptin receptors from nNOS neurons does not necessarily impair NO signaling from these neurons. To ensure this, nNOS would have to be deleted from Lepr neurons (the opposite to what has been done by Leshan et al.). This would be an interesting experiment because a recent study shows that functional nNOS is needed for the leptin induced rise in LH in female adult animals (Bellefontaine et al., 2014). Another future approach would be to selectively re-express Lepr in only nNOS neurons. This can be established by crossing the inducible nNOS-ER-Cre animals with stop-flox Lepr mice (Lepr-null mice). This will test if leptin receptors in nNOS neurons are sufficient for fertility (as opposed to being required for fertility as I attempted to test). Alternatively, pharmacological leptin stimulation experiments could be performed on nNOS-Lepr knockout animals to show that the effects on leptin stimulated LH secretion are coming from direct leptin actions on these neurons and not via secondary pathways.

In this thesis I have shown that leptin signaling in GABA neurons is critical for normal reproductive functioning. However, even these very obese animals were all able to go through puberty and produce offspring. This
suggested that leptin receptors in GABAergic neurons are not an absolute requirement for fertility, and possibly that redundant pathways are in place to ensure reproductive function is not lost even if Lepr signaling in GABAergic neurons is absent. It would be interesting to investigate whether leptin signaling solely in GABA neurons (using Lepr-null mice with GABA Lepr rescue) would be sufficient for normal reproductive functioning. This has been done for a small number of animals using the Flp-Neo recombinase system (as described in, Elias, 2014), where knock-in of Lepr in GABAergic neurons resulted in lean and fertile animals (C. Elias personal communication). Additionally, further investigation of Lepr signaling in AgRP neurons would be worthwhile, even though this is a small population so there might not be any phenotype due to redundancy. It is known that simultaneous deletion of Lepr from AgRP and POMC neurons does not impact fertility (van de Wall et al., 2008). Repeating this study might be worthwhile as the researchers mainly focus on metabolism and ingestion rather than reproductive function. Furthermore, it would be interesting to investigate if Lepr signaling in AgRP neurons is sufficient and/or necessary for reproductive functioning, because deletion of AgRP in db/db mice or ablation of AgRP neurons in leptin deficient ob/ob animals restores fertility (Israel et al., 2012; Wu et al., 2012; Sheffer-Babila et al., 2013).

7.2.2 Leptin acting at the level of the median eminence to regulate GnRH release

A high density of Lepr positive fibers are seen in the preoptic regions (Patterson et al., 2011). While many of these Lepr positive fibers surrounding the GnRH neurons may originate from Lepr neurons within the rPOA, at least a portion for these comes from the PMV (Leshan et al., 2009). On the other hand, there is evidence for Lepr neurons acting at the level of the GnRH terminals in the median eminence. The density of Lepr fibers in the Arc and median eminence regions is high (Patterson et al., 2011), and in vitro treatment of median eminence-Arc explants (without GnRH cell bodies) with low doses of leptin induces release of GnRH (Yu et al., 1997b). The same
group showed that this GnRH release was mediated by the gaseous signaling molecule NO, as inhibition of NOS caused the leptin induced release of GnRH to be completely blocked (Yu et al., 1997a). This suggests that the leptin induced GnRH release is mediated by release of NO. However, this is probably not a direct effect of leptin on Lepr-NO neurons, as leptin signaling in NO neurons is low in this region (Donato et al., 2010). Therefore it is suggested that there are NO producing intermediates between the leptin responsive neurons and GnRH terminals (as depicted in Fig 7.1 and reviewed by Bellefontaine et al., 2011).

Other reproductive signals that may work in a similar manner at the median eminence are kisspeptin and estrogens. Exogenous kisspeptin can stimulate GnRH release in mediobasal hypothalamic explants of mice, even in the presence of tetrodotoxin (TTX) which blocks possible action potential firing (d'Anglemont de Tassigny et al., 2008). This indicates that kisspeptin could directly stimulate GnRH release from its terminals. Similar results were shown in a large mammal model (the ovine hypothalamus) where kisspeptin stimulated GnRH release from median eminence explants (Smith et al., 2011). Estradiol can manipulate GnRH release indirectly by promoting NO production at the GnRH terminals in the ME (reviewed by Bellefontaine et al., 2011). Perhaps this is true for leptin, possibly via GABAergic AgRP/NPY neurons or Lepr neurons projecting directly to the median eminence from LHA or PMV.

These are complex ideas to investigate further. In vitro electrophysiology might be able to elucidate the mechanism. After patching on to a GnRH dendrite in the median eminence, then applying a localized “puff” of leptin to either the median eminence or Arc nucleus, the reaction of the GnRH terminal could be measured. Another way to examine this possibility would be to infuse NPY or AgRP or GABA (or their antagonists) into this region and examine cFos activation in GnRH neurons, or to measure leptin induced LH secretion. If effects are relayed via NO signaling in the median eminence, experiments with pharmacological inhibition of NO production could be
performed in this region (similar to those described in Bellefontaine et al., 2014).

7.2.3 Other experiments

There are several additional options to further explore the questions raised by this thesis. For instance, with the viral delivery of Cre, Lepr knockout models can be produced that have site specific deletions or rescue. A Cre recombinase expressing adeno-associated virus (Kaspar et al., 2002; Donato et al., 2011b; Guo et al., 2013), or TAT-Cre protein (Peitz et al., 2002), could be injected into the rPOA, Arc, PMV, or LHA of Lepr-flox animals. This would result in a specific hypothalamic regional Lepr knockout. This technique would also avoid the potential problems of developmental compensation, as the deletion will only be induced in adulthood. A major downside of this method is that the effect on puberty onset will not be evaluated because the animals are too small to easily inject before puberty begins. This method can be expanded by using a Cre virus that is under the control of a neurochemical or neuropeptide specific promoter, for example nNOS or Vgat/GAD67 (Tolu et al., 2010). This could provide a means to delete Lepr in a region and neuron specific manner. Experiments I would suggest include using an nNOS specific Cre-virus to delete Lepr in the rPOA and PMV, or a Vgat specific Cre-virus to delete Lepr in Arc and LHA.

Pharmacological stimulation or inhibition could be exerted via insertion of designer receptors into Lepr neurons of specific hypothalamic regions. The Cre-lox system can be used to express receptors for pharmacogenetic activity silencer (hM4D) or stimulator (hM3D) in a neuron and region specific manner. The agonist for both of these receptors (clozapine-N-oxide) is then injected peripherally and causes chronic suppression or activation of the transfected neurons (Atasoy et al., 2012; Zhan et al., 2013). To investigate the effects on reproduction this could be coupled with serial blood sampling to measure the effects on pulsatile LH release or the preovulatory LH surge (Steyn et al., 2013).
To specifically trace afferents of GnRH neurons, GnRH-Cre animals could be used in combination with a Cre-activated viral tracer (Wintermantel et al., 2006; Card et al., 2011). This method can be combined with leptin induced pSTAT3 immunohistochemistry to identify leptin responsive neuronal inputs to GnRH neurons. However, this will not tell us where on the extensive GnRH neuronal network these Lepr neurons make contact.

7.3 Conclusion

From the experiments presented in this thesis I can conclude that the regulation of fertility by leptin is complex. The results show that there are still a number of potential but unproven leptin sensitive populations that could mediate leptin-to-GnRH functions. However, this work has narrowed down the number of possibilities for critical intermediary neurons significantly. I conducted anatomical tracing (which highlighted Lepr and GABAergic neurons in the Arc as potential leptin-to-GnRH conduits), gene knockout experiments (which showed that leptin must act via GABA neurons for normal fertility) and measurements of gene expression (which showed that Lepr deletion from GABA neurons upregulates Agrp expression in the Arc). Together, these results point to the AgRP neurons as being a likely pathway for control of reproduction by leptin. However, future studies are needed to address this hypothesis, and others raised by the experiments in this thesis. Even though the numbers of options are smaller, the research presented here supports the concept that there is not a single leptin responsive neuronal population that provides leptin-to-GnRH signaling. Instead, there seem to be many redundant pathways in place to preserve reproductive function.
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Appendix I

Commonly used solutions

**Citrate buffer** (0.1 M, pH 5.0)
To make 1 l:
- Add 10.3 g of citric acid monohydrate and 18.16 g of sodium phosphate (Na₂HPO₄·2 H₂O)
- Make up to 1 l
- Adjust pH to 5.0

**DAB** (diaminobenzidine 0.05%)
To make 20 ml:
- Add 1 tablet DAB and 1 tablet urea to 20 ml distilled water and dissolve
  (- For NiDAB, dissolve 160 mg in water first)
- Keep in the dark and use immediately for immunohistochemical staining

**EDTA** (0.5 M, pH 8.0)
To make 250 ml:
- Add 46.525 g of EDTA to 150 ml milli-Q water
- Adjust pH with NaOH to 8.0
- Make up to 250 ml with more water

**Gelatin coated slides**
- Dissolve 1.25 g granular gelatin in 500 ml milli-Q water, while heating (do not bring to boil)
- Add 125 mg chromalum (CrK(SO₄)₂·12 H₂O)
- Dip cleaned slides into solution and drain and dry for 45-60 minutes
- Dip slides into solution a second time and allow them to dry completely

**Lysis buffer**
- Add 12.11 g Tris base (100 mM), 1.86 g EDTA (5 mM), 2 g SDS (0.2 %), and 11.68 g NaCl (200 mM) 500 ml distilled water and dissolve
- Make to 1 l with water; adjust pH to 8.5 with HCl
Appendix

Proteinase K – store as 20 mg/ml stock aliquots at -20°C. Dilute 1:200 in lysis buffer just before use (final concentration of 100 μg/ml)

**PFA** (paraformaldehyde, 4% in 0.1 M PB, pH 7.3)
- Make up half of the required end volume of 0.2 M PB (pH 7.3)
- Measure the other half of the volume of milli-Q water and heat to ~60 °C on a plate stirrer
- Weigh out PFA (40 g/L) and add to heated water
- Add 6-7 drops of 10 M NaOH, leave stirring until clear
- Cool down and filter (using a pre-wet filter) into the 0.2 M PB
- Check pH and adjust if necessary

**PB** (phosphate buffer, 0.2 M stock, pH 7.3-7.4)
To make final 0.2 M PB, combine dibasic and monobasic solutions in a ratio 4:1 and add milli-Q water (60% of the final volume).
(Example: to make 100 ml: 32 ml dibasic, 8 ml monobasic and 60 ml milli-Q water)

Dibasic (disodium hydrogen phosphate, 1 l, 0.5 M):
- Weigh out 89 g disodium phosphate (Na₂HPO₄·2H₂O; Mw 177.99) or 71 g of anhydrous disodium phosphate (Na₂HPO₄; Mw 141.96), and make up to 1 l with milli-Q water

Monobasic (sodium dihydrogen phosphate, 500 ml, 0.5 M):
- Weigh out 39 g sodium dihydrogen phosphate (NaH₂PO₄·2H₂O; Mw 156.01), and make up to 500 ml with milli-Q water

**PBS** (phosphate buffered saline, 10x, pH 7.4)
To make 2 l of 10x PBS:
- Add 160 g of NaCl, 4 g of KCl, 28.8 g of Na₂HPO₄ and 4.8 g of KH₂PO₄ to 1 l of distilled water
- Adjust pH to 6.8 (7.4 if 1x PBS)
- Add water to make up to 2 l
**TAE Buffer** (Tris-acetate EDTA, 10x, pH 7.2)
- Add 48.4 g of Tris base, 11.4 mL of glacial acetic acid (17.4 M), and 3.7 g of EDTA to 800ml of distilled water
- Adjust pH to 7.2
- Make up to 1 l

**TBS** (Tris buffered saline, 10x, pH 7.6)
To make 2 l of 10x TBS:
- Add 121.2 g of Tris-HCl (Mw 157.6), 28.0 g of Tris base (Mw 121.1), 175.3 g of NaCl (Mw 58.44) to 1.7 l of distilled water
- Adjust pH to 7.6
- Add water to make up to 2 l

**TBS-TX**
Add 1 ml/l Triton-X100 to 1x TBS

**TBE Buffer** (Tris-Borate-EDTA, 10x, pH 8.3)
- Add 108 g Tris base, 55 g Boric acid, and 9.3 g EDTA to 500 ml distilled water and dissolve
- make to 1 l with water, pH is 8.3 and requires no adjustment

**TE buffer** (Tris-EDTA, pH 8.0)
- Add 1.211 g Tris base (10 mM) and 0.372 g EDTA (1 mM) to 500 ml distilled water and dissolve
- make to 1 l with water, adjust pH to 8.0 with HCl
Appendix
Appendix II

Map of custom 384 well micro fluid array cards
Neither Signal Transducer and Activator of Transcription 3 (STAT3) or STAT5 Signaling Pathways Are Required for Leptin’s Effects on Fertility in Mice

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The hormone leptin is critical for the regulation of energy balance and fertility. The long-form leptin receptor (Lepr) regulates multiple intracellular signaling cascades, including the classic Janus kinase-signal transducer and activator of transcription (STAT) pathways. Previous studies have shown that deletion of STAT3 or the closely related STAT5 from the brain results in an obese phenotype, but their roles in fertility regulation are not clear. This study tested whether STAT3 and STAT5 pathways of leptin signaling are required for fertility, and whether absence of one pathway might be compensated for by the other in a redundant manner. A Cre-loxP approach was used to generate 3 models of male and female transgenic mice with Lep-specific deletion of STAT3, STAT5, or both STAT3 and STAT5. Body weight, puberty onset, estrous cyclicity, and fertility were measured in all knockout KO mice and their control littermates. Knocking out STAT3 or both STAT3 and 5 from Lepr expressing cells, but not STAT5 alone, led to significant increase in body weight. All STAT3 and STAT5 single KO mice exhibited normal puberty onset and subsequent fertility compared to their control littermates. Surprisingly, all STAT3 and STAT5 double KO mice also exhibited normal puberty onset, estrous cyclicity, and fertility, although they had severely disrupted body weight regulation. These results suggest that, although STAT3 signaling is crucial for body weight regulation, neither STAT3 nor STAT5 is required for the regulation of fertility by leptin. It remains to be determined what other signaling molecules mediate this effect of leptin, and whether they interact in a redundant manner. (Endocrinology 154: 2434–2445, 2013)

Fertility in mammals is controlled by a neuroendocrine axis involving communication of hypothalamic GnRH neurons with the anterior lobe of the pituitary gland (1). GnRH stimulates the secretion of the gonadotropins LH and FSH, which in turn influence the gonads to produce the sex hormones estradiol, progesterone, and testosterone (2). The distinct tonic pulsatile and preovulatory surge modes of GnRH/LH are regulated by many effector neurons, forming the GnRH neuronal network. This network is modulated by various hormones. Leptin is one such hormone, which plays a crucial role in the metabolic regulation of fertility.

Leptin is a peptide hormone product of the ob gene, that is released by adipocytes in proportion to fat mass. Total absence of leptin (as occurs in ob/ob mice) or disruption in its receptor, leptin receptor (Lepr) (as in db/db mice), has been shown to cause severe obesity and infertility (3–8). Treating ob/ob mice with leptin normalizes their body weight (9) and also restores their fertility (10). Several Lepr-activated pathways have been shown to be involved in the regulation of energy balance and reproduction, including Janus kinase-signal transducer and activator of transcription (STAT) pathways (11), the phosphoinositol-3 kinase (PI3K) pathway (12), the ERK/MAPK cascades (13), the maximal target of rapamycin (mTOR) pathway (14), and the AMP-activated protein kinase (AMPK) (15, 16) pathway. Of these, STAT3 signaling has
received much attention, due to its broad expression in the body and pronounced activation in the hypothalamus following leptin administration in rodents (17). Two recent previous reports suggest that leptin also induces STAT5 activation in the hypothalamus (18, 19), although some other reports have not supported this (20–22).

Both STAT3 and STAT5 have been shown to play important roles in the regulation of energy balance, but their reproductive roles are less clear. The estrogen receptor α and β promoters contain response elements for the transcription factors STAT3 and STAT5 (23–25), and estrogen plays important roles in modulating both metabolism and reproduction. A complete neural knock-out (KO) of STAT5 results in severe obesity and infertility in mice (26). In apparent contrast to this finding, the contribution of STAT3 signaling to leptin actions was also examined by studying mice with disrupted leptin-induced STAT3 signaling (via a mutation in the STAT3-binding site on the Lepr). This mutation led to obesity but did not affect fertility (27). Neural KO of STAT5 also causes obesity (28) but the role of leptin-specific STAT3 signaling in the control of energy balance and fertility remains unclear. These findings indicate that leptin-induced STAT3 and STAT5 pathways may not each be absolutely required for normal fertility; rather it may be that they are sufficient for fertility and either pathway can be used in a redundant manner. Therefore, the current study aimed to examine the roles of STAT3 and STAT5 signaling pathways in leptin modulation of fertility in mice. Using transgenic KO mice with Lepr-specific deletion of STAT3, STAT5, or both STAT3 and STAT5, this study investigated the effects of these genotypes on body weight, puberty onset, estrous cyclicity, daily sperm production (DSP), and fertility over a 4-month breeding study. If these pathways act in a redundant manner, such that in the absence of one pathway leptin compensates by signaling through the other, it would be predicted that KO of either of these pathways alone would not affect fertility, but knocking out both pathways together would result in infertility.

Materials and Methods

Animals

Male and female transgenic mice were obtained from the University of Otago animal breeding facility. All mice were maintained on a C57BL/6 background. Mice were group-housed before breeding and had free access to standard rodent chow and water. Animals were kept under conditions of controlled lighting (12:12 light/dark cycle) and temperature (22 ± 1°C). All experimental protocols were approved by the University of Otago Animal Ethics Committee.

A Cre-loxP approach was used to generate a Lepr-specific KO of Stat3 and Stat5 genes. The generation and characterization of Lepr-Cre mice have been described previously (29–31). The Lepr-Cre transgenic mouse line expresses Cre recombinase and, when crossed with floxed mouse lines, leads to deletion of loxP-flanked DNA from all cells expressing Lepr (31). This mouse line was purchased from the Jackson Laboratories (BJ.129-Leprtm2(K)J/Rekcl) strain.

The generation of STAT3 flox mice has been described previously (32). This mouse line was obtained from Prof Tamas Hoovath, Yale University School of Medicine, New Haven, Connecticut. STAT5 flox mice were generated by first constructing separate STAT5b- and STAT5a-targeting vectors, which were subsequently used to generate the STAT5 flox mouse line (33). This mouse line was crossed with the Lepr-Cre-expressing mouse line to generate STAT5 single KO transgenic mice. This mouse line was kindly provided to us by Prof Dave Gravett, University of Otago, New Zealand, and Dr Lesley Henningshaus, Laboratory of Genetics and Physiology, Bethesda, Maryland. To visualize Lepr-Cre-expressing cells with the green fluorescent protein (GFP), Lepr-Cre mice were crossed with homozygous Cre-dependent GFP (ROSAS26::CAG::GFP) mice to produce Lepr-Cre-GFP mice. These mice were used to confirm that the Lepr-Cre cells were leptin-responsive, by colocalization of GFP with leptin-unbound STAT3 activation.

Generation and genotyping of KO animals

Lepr-Cre mice were crossed with animals homozygous for STAT3 flox (STAT3f/f) resulting in STAT3f/wt; Lepr-Cre mice; breeding these back to the STAT3f/f mice generated STAT3f/f; Lepr-Cre (hereafter referred to as STAT3 KO); females n = 7, males n = 7) and STAT3f/f; Lepr-wt (f/f) (hereafter referred to as STAT3f/wt); females n = 6, males n = 7) mice. STAT3 KO (females n = 7, males n = 7) and littermate controls (females n = 7, males n = 7) were bred using the same strategy. Finally, crossing STAT3f/f; STAT5f/f mice (generated by crossing the 2 floxed lines with each other for 2 generations) with Lepr-Cre mice generated STAT3f/wt; STAT5f/f; Lepr-Cre mice. Crossing these mice back to the double-flowered mice generated STAT3f/f; STAT5f/f; Lepr-Cre mice (hereafter referred to as double KO mice); females n = 7, males n = 7) and STAT3f/f; STAT5f/f; Lepr-wt (f/f) (hereafter referred to as STAT3f/wt; STAT5f/f; Lepr-Cre mice; females n = 7, males n = 7) mice. All mice were born at expected Mendelian ratios and were identified by PCR analysis of genomic tail-tip DNA using the following primers: Lepr-Cre: primer 1: GCCGCTGTCGCACTAAAATATG; primer 2: GACAACTGGACAAAAGCAG; primer 3: CAGAATCTGGAAAGATCT; primer 4: TCTAGGGATCTGCTGATCC; (wild-type, 324 bp, transgene, 102 bp, annealing temperature, 52°C); STAT3f/f: primer 1: ATTAGGACACTGGACAAAAGCAG; primer 2: ACXTGTACGTAGGTTGTGTC (wild-type, 490 bp, transgene, 520 bp, annealing temperature, 60°C); STAT5f/f: primer 1: GACAACTGGACAAAAGCAG; primer 2: GACAACTGGACAAAAGCAG; primer 3: AAGTTATCTGGACAAAAGCAG; (wild-type, 450 bp, transgene, 200 bp, annealing temperature, 38°C).

Puberty onset and estrous cyclicity assessment

All female mice were checked for vaginal opening from 25 days of age. Vaginal smears were collected from the day of vag
inal opening and assessed to determine cycle stage by vaginal cytology, until the first estrous was observed. To assess the progression of estrous cycle, daily vaginal smears were collected for 14 days from 7 weeks of age. All male mice were paired with mature wild-type C57BL/6j females at 5 weeks of age. Puberty onset in males was identified by backdating the gestation length (21 d) from the date of birth of the first litter. These pairings were then maintained for the breeding assessment.

Fertility

All fertility assessments were conducted by pairing the experimental mice with a C57BL/6j animal of the opposite sex for a period of 4 months. Litter size and frequency were scored for control and KO mice over this period and expressed as average number of pups per litter and number of litters per 120 days, respectively.

Perfusion and brain tissue collection

At the end of the breeding study all animals underwent an overnight fast to reduce endogenous circulating leptin levels before they were perfused for the collection of brains and assessment of leptin-induced STAT3 and STAT5 phosphorylation (pSTAT3 and pSTAT5) by immunohistochemistry. Mice were treated with 3 mg/kg sc bromocriptine methanesulfonate (Sigma-Aldrich, St Louis, Missouri) in 10% ethanol 4 hours before perfusion. This dopamine agonist was used to suppress endogenous prolactin and therefore prolactin-induced STAT5 activation (34). Mice were then treated with 1 mg/kg sc recombinant mouse leptin (National Hormone and Peptide Program, Torrance, California) in PBS 2.5% w/v. Two additional wild-type mice were treated with vehicle to provide an indication of basal pSTAT levels. All mice were deeply anesthetized with sodium pentobarbital (200 mg/kg ip) 2 hours following leptin/vehicle injections and were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were collected for immunohistochemical analysis, postfixed in 4% paraformaldehyde overnight, and then transferred into 30% sucrose for cryopreservation until they sank. Coronal sections of 30 μm thickness were cut through the hypothalamus at ±20°C. Three equivalent sections of series were collected, so that within each series consecutive sections were 90 μm apart.

Estimation of DSP

Following perfusion and brain collection, one testis from each control or double KO male mouse was removed, wet weight recorded, and stored at ~80°C for subsequent determination of DSP. DSP was determined similar to that described previously (35, 36). Testis fragments of about 25 mg were placed in 600 μL of a solution containing 0.05% NaCN, 0.01% sodium azide, and 0.05% Triton X-100 and homogenized for 10 minutes. Spermato- masts in stages 14 to 16 of spermatogenesis are resistant to homogenization and their nuclei were counted in 10 μL aliquots of homogenate using a hemacytometer. No morphological abnormalities in the sperm of the double KO were evident. These values were used to calculate the total number of spermato- masts per testis. Developing spermatids spend 4.84 days in steps 14 to 16 during spermatogenesis in mice (37). DSP was therefore calculated by dividing the values for the number of spermato- masts per testis by 4.84.

Figure 1. Cre-recombinase expressing cells are leptin-responsive in Lepr/Cre-GFP mice. A: mean (±SEM) percentage of Cre-recombinase expressing cells identified by green GFP labeling that showed evidence of leptin responsiveness (identified by labeling of red pSTAT3 immunoreactivity) in response to leptin (1 mg/kg ic) in male (black bar) and female (white bar) mice in hypothalamic regions. B-D: representative examples of GFP expression with leptin-induced pSTAT3 labeling in the region of the organum vasculosum of the lamina terminalis (D)/T(T) (B), arcuate nucleus (ARC) (C), and ventral premammillary nucleus (PMV) (D).-Based regions are magnified in the upper right of images (B-D). 3V: third ventricle; scale bar, 100 μm.

pSTAT3 immunohistochemistry

One series of brain sections was used for pSTAT3 immunohistochemistry to confirm the deletion of STAT3 signaling from Lepr-expressing cells of the KO animals. Free-floating sections were washed in 0.1 M Tris-buffered saline 3 times (this was repeated between all steps), incubated in 1 mM EDTA (pH 8.0) at 90°C for 15 minutes, in 0.3% hydrogen peroxide for 10 minutes, and then in monoclonal rabbit anti-pSTAT3 primary antibody (4G7; Cell Signaling Technology, Danvers, Massachusetts; 1:3000 dilution), in blocking solution (Tris-buffered saline containing 0.1% Triton X-100, 0.25% BSA, and 2% normal goat serum) for 24 to 48 hours. After this, sections were incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:1000; Vector Laboratories, Burlingame, California) for 1 hour, in Vector Elite ABC solution (Vector Laboratories) for 1 hour, and finally in a nickel-enhanced diaminobenzidine solution (Sigma-Aldrich) to visualize pSTAT3 immunoreactivity (blue-black nuclear staining) before mounting on glass slides. For pSTAT3 immunofluorescence, the primary antibody was followed by Alexa 568 goat anti-rabbit IgG (1:2000; Molecular Probes, Carlsbad, California); the sections were coveyslipped with Vectorshield and viewed and photographed using fluorescence microscopy.

pSTAT5 immunohistochemistry

One series of brain sections was used for pSTAT5 immunohistochemistry to confirm the deletion of STAT5 signaling from
LepR-expressing cells of the KO animals. Sections were stained similar to the chromogenic pSTAT3 procedure above, except that the EDTA solution was substituted for 0.01 M Tris-HCl (pH 10, 90°C for 5 min) and the primary antibody used was monoclonal rabbit anti-pSTAT3 primary antibody (type694; Cell Signaling Technology; 1:10,000 dilution).

pERK1/2 immunohistochemistry

One series of brain sections was used for pERK1/2 immunochemistry to determine whether deletion of STAT3 and STAT5 resulted in compensatory change in the tone of the MAPK signaling pathway, which is known to be used by leptin in the hypothalamus (11, 13). Sections were stained similar to the chromogenic procedures above, except that no high-temperature antigen retrieval step was required and the primary antibody used was polyclonal rabbit anti-pERK1/2 (hr202/yr204; Cell Signaling Technology; 1:3000 dilution).

Omission of primary antibodies resulted in a complete absence of staining; furthermore, leptin was unable to induce pSTAT3 in the STAT3 KO or pSTAT5 in the STAT5 KO animals, further validating the specificity of the antibodies.

Image analysis

Sections were viewed and photographed using light or fluorescence microscopy (Olympus BX45 microscope; Olympus, Tokyo, Japan) at ×20 magnification. The number of pSTAT3-, pSTAT5-, or pERK1/2-positive cells within the region around the organum vasculosum of the lamina terminalis (0.6 to 0.4 mm rostral to bregma), the arcuate, ventromedial, and dorsomedial nuclei (ARC, VMN, and DMN, respectively, all 1.5 to 2.0 mm caudal to bregma) and the ventral premammillary nucleus (PMV, 2.5 to 2.6 mm caudal to bregma) were counted with reference to the Franklin and Paxinos mouse brain atlas (38) using ImageJ analysis software (2 to 3 sections averaged to provide a single data point of counts per region per section for each animal). For fluorescent pSTAT3 and GFP labels, cells were deemed to be colocalized if a red pSTAT3 immunoreactive nucleus was observed within a green LepR-Cre-GFP cytoplasm.

Statistical analysis

Differences in body weights of control and mutant groups over time were analyzed by 2-way repeated measures ANOVA. All other statistical comparisons, which involved comparing a single endpoint between KO and control groups, were analyzed using Student t tests. Differences were considered significant if P < .05.

Results

LepR-Cre cells are responsive to leptin

We used LepR-Cre-GFP mice to confirm that the LepR-Cre cells were indeed leptin-responsive, by colocalization of GFP with leptin-induced STAT3 activation. In the ARC, the VMN, and the PMV, between 70% and 80% of GFP-expressing cells responded to leptin treatment by showing pSTAT3 in their nuclei. Colabeling of GFP and pSTAT3 was slightly lower in the DMN (63%), but markedly lower in the region around the organum vasculosum of the lamina terminalis (29%). There was no
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Figure 4. MAPK (pERK1/2) signaling is not altered in leptin-specific STAT3 or STAT5 KO mice. A, mean ± SEM number of pERK1/2-immunoreactive cells per section in medio basal hypothalamic nuclei in STAT3 single KO (A, middle bar), STAT5 single KO (B, middle bar), and STAT3 +5 double KO (A and B, right bar) mice (black bars) and their control littermates (white bars) following treatment with leptin (1 mg/kg sc). The gray bars show basal pERK1/2 levels in wild-type mice treated with vehicle instead of leptin. Data are pooled from male and female mice. pERK remained at basal levels (similar to those in vehicle-treated animals) in all animals. B, C, representative examples of pERK immunoreactivity in the ARC and VMN (B) and posterior ARC and PMV (C). Boxed regions are magnified in the upper right of images B and C. ARC, arcuate nucleus; PMV, premalitary nucleus; VMN, ventromedial hypothalamic nucleus; scale bar, 100 μm.

There was no difference in GFP-pSTAT3 colabeling between males and females (Figure 1).

Leptin-induced pSTAT3, pSTAT5, and pERK1/2 in the medio basal hypothalamus

Deletion of STAT3 and STAT5 signaling from LepR-expressing neurons was confirmed by immunohistochemical analysis of leptin-induced pSTAT3 and pSTAT5. Vehicle treatment in wild-type mice resulted in very low levels of pSTAT3 and pSTAT5 staining in all regions (Figure 2, A and F). As expected, treatment of control mice with leptin induced a marked increase in pSTAT3 and pSTAT5 staining in hypothalamic structures including the ARC, the VMN, and the PMV. Representative examples of staining in the ARC are shown in Figure 2, B and G. Immunohistochemical examination of pSTAT5 expression in the hypothalami of leptin-treated STAT5 KO mice demonstrated markedly decreased pSTAT5 immunoreactivity (Figure 2H), but did not affect pSTAT3 immunoreactivity (Figure 2G). Similarly, leptin-treated STAT3 KO mice had markedly decreased pSTAT3 counts (Figure 2D), but normal pSTAT5 levels (Figure 2I). In contrast, leptin treatment did not induce phosphorylation of STAT3 or STAT5 in double KO animals (Figure 2, E and J). A small amount of background staining was observed in all KO mice, similar to that of vehicle-treated mice. The lack of leptin-induced STAT3 and STAT5 phosphorylation in the KO animals confirmed the deletion of these specific pathways. The number of labeled pSTAT3 and pSTAT5 cells per section were counted in the ARC, VMN, and PMV regions and was significantly reduced in the KO animals compared with their control littermates (P < 0.001; Figure 3, A and B). To assess whether STAT3 or STAT5 KO resulted in compensatory changes in another common signaling pathway known to be upregulated in the hypothalamus, additional sections were also stained for...
Figure 5. Body weight of Leprf-specific STAT3 single KO male (A) and female (B), STAT3 single KO male (C) and female (D), and STAT3 + 5 double KO male (E) and female (F) mice. Values are mean ± SEM. Repeated measure analysis revealed that the effect of genotype on body weight was significant in male and female STAT3 single KO and double KO mice. STAT3 f (control) versus Leprf-Cre, STAT3 KO, STAT3 f (control), STAT3 f (Leprf-Cre), STAT3 KO; STAT3 KO and STAT3 f (control) versus STAT3 and STAT3 f (Leprf-Cre), double KO. * P < .05 vs controls at the same age.

pERK1/2. There was no evidence of up- or down-regulation of this signaling pathway in the ARC, VMN, and PMV regions in STAT3 and STAT5 single or double KO mice compared to controls; pERK1/2 cell counts did not differ from basal levels in any group (Figure 4). The data from male and female mice were pooled as no significant differences in pSTAT or pERK1/2 cell counts were observed between sexes.

**Effects of leptin-specific STAT3 and/or STAT5 deletion on body weight**

Body weight of all mice was measured every 2 weeks from weaning, except during the female mice breeding assessment. Consistent with previous studies (26, 27), male and female STAT3 KO mice in the current study were found to have significantly increased body weight compared to their control littermates (Figure 5, A and B). The effect of genotype on body weight was significant from 9 weeks of age in male mice. This confirms that the leptin-STAT3 signal is crucial for the regulation of body weight by leptin.

A complete neural KO of STAT3 has been shown to cause significant obesity in mice (28). However, the Lepr-specific STAT3 KO mice in the current study were found to have normal body weight regulation. No significant difference in body weight was observed between KO and control groups of STAT3 single KO mice (Figure 5, C and D), demonstrating that STAT3 is not required for leptin's effects on body weight regulation.

As expected, STAT3 + STAT5 double KO mice also displayed severely disrupted body weight regulation, similar to that seen in STAT3 single KO mice. All male and female double KO mice were found to be significantly heavier than their control littermates (Figure 5, E and F). Although severely overweight, the STAT3 single KO mice and the double KO mice were less obese than neural STAT3 KO mice (26), indicating the potential importance of STAT3 signaling in nonleptin neurons.

**Puberty onset**

All female STAT3 and STAT5 single KO mice exhibited normal puberty onset, with no significant differences in vaginal opening and first estrous compared to their control littermates (Figure 6, A and B). All STAT3 and STAT5 single KO male mice also exhibited no significant differences in puberty onset compared to their control littermates, although the STAT3 KO male mice had a nonsignificant trend toward delayed puberty onset (day 56) than controls (day 49) (P = .56) (Figure 6B).

Similar to the single KO mice, all double KO male and female mice in this study exhibited normal puberty onset compared to their control littermates (Figure 6C). These data indicate that leptin signaling through STAT3 and STAT5 pathways is not required for normal puberty onset in mice.

**Estrous cyclicity**

The estrous cycle of all female mice was evaluated by monitoring changes in vaginal cytology for 2 weeks. STAT3 and STAT5 single KO mice (Figure 7, A and B) and double KO mice (Figure 7C) cycled regularly, with no significant difference in cycle length compared to controls. Representative examples of the estrous cyclicity of STAT3
Similarly, all male and female STAT5 KO animals also exhibited normal fertility, with no significant differences in the number of litters produced and the average litter size (Figure 8B). These data indicate that STAT5 function is not required for leptin-mediated regulation of fertility.

In contrast to the hypothesis of this study, double KO mice were found to have normal fertility. No significant differences were observed in the average litter frequency or size between the KO and control groups (Figure 8C). All mice were able to produce at least 4 litters over the breeding assessment period. Thus, in contrast to d/db mice and neural STAT3 KO mice, the reproductive capacity of all double KO mice was essentially normal. Therefore, although STAT3 is known to play a key role in body weight regulation by leptin, the hypothalamic control of reproduction by leptin is likely to be regulated by signals independent of STAT3 and STAT5 in their absence.

**Testis weight and sperm production**

Despite an increase in body weight, the single testis weight of double KO mice did not differ significantly from control mice (95 ± 5 vs. 106 ± 5 mg, respectively; P > .1). Consistent with this, testis histology appeared qualitatively normal in all mice assessed. To determine if the sperm production of the double KO mice was altered, their DSP was calculated. All double KO mice assessed showed normal DSP compared with their control littersmates. At 6 months of age, control and double KO mice had 2.3 × 10^7 and 1.9 × 10^7 spermatozoids per testis, respectively, resulting in calculated DSP rates of 4.8 ± 0.6 × 10^6 and 4.1 ± 0.1 × 10^6 sperm per day (P > .01).

**Discussion**

This study addressed the roles of STAT3 and STAT5 signaling pathways in leptin-mediated regulation of fertility, and the possibility that they may interact in a redundant manner such that the presence of the other would be sufficient for this function. A Cre-loxP approach was used to generate either STAT3 and STAT5 single KO mice or STAT3 + STAT5 double KO mice, with STAT3 and/or STAT5 genes deleted specifically from all cells expressing LepRs. The results obtained do not support the hypothesis of redundant roles for these 2 pathways: remarkably, mice devoid of both signaling pathways displayed uncompromised fertility despite disrupted body weight regulation. This suggests that signaling pathways may be involved in this regulation.

A large body of evidence suggests that STAT3 plays a key role in mediating leptin’s effects on body weight regulation. Neural deletion of STAT3 (28) as well as discap-
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![Graphs and diagrams showing frequency of occurrence over 14 d for various conditions involving LepRb-STAT3 binding domain and STAT3 signaling in mice.](image-url)

Figure 7. Summary (A–C) and representative examples (D–I) of estrous cyclicity in STAT3 single KO (A, D, F, I) and STAT5 single KO (B, F, G) and STAT3 + STAT5 double KO (C, E, H, I) mice. Values in A–C are mean ± SEM frequency of occurrence over a 14-day period. Stage of the estrous cycle was determined by the predominant presence of either leukocytes, nucleated epithelial cells, or corded epithelial cells. There were no statistically significant difference between the control and KO groups. D, diestrus; E, estrus; M, metestrus; P, proestrus; STAT3 KO, control; STAT3 LepR-Cre, STAT3 KO; STAT5 KO, control; STAT5 LepR-Cre, STAT5 KO; STAT3 and STAT5 KO, control; STAT3 and STAT5 LepR-Cre, double KO.

Recent findings also suggest an important role for STAT5 in the regulation of feeding and energy expenditure. It has been shown that neural STAT5 KO mice exhibit significant obesity (60% increase in body weight compared to wild-type animals) with increased food intake as well as altered regulation of energy expenditure (28). The current study shows that leptin-specific deletion of STAT5 signaling has no effect on body weight regulation in mice. Therefore, although STAT5 signaling in the central nervous system (CNS) is crucial for the regulation of energy balance, the leptin-STAT5 signal is not required for body weight regulation. This suggests that STAT5 may mediate the effects of other cytokines or hormones in addition to leptin. Indeed, several lines of data suggest that STAT5 signaling is activated by other hormones such as prolactin (48, 49) and GH (50), both of which can act to increase body weight. As expected, all double KO male and female mice displayed significantly increased body weight. Because this phenotype was not observed in STAT3 KO mice, STAT3 appears to be the critical and predominant pathway for leptin-mediated body weight regulation.
The key objective of this study was to examine thoroughly the effects of STAT3 and STAT5 single or double KO on puberty onset and fertility, by pairing them with a wild-type animal of the opposite sex for a period of 4 months. Deletion of STAT3 or STAT5 signaling from LepR cells did not alter puberty onset, female estrous cyclicity, or adult fertility. Taken together, these data indicate that neither leptin-specific STAT3 nor STAT5 signaling is required for normal reproductive function, and that the hypothalamic regulation of reproduction by leptin can be regulated by STAT3- and STAT5-independent signals.

STAT3 signaling has previously been shown to be crucial for the regulation of fertility, as neutral (nestin-Cre) STAT3 mutant mice develop characteristic phenotypes of db/db mice (both obesity and infertility) (26). Although this might imply that STAT3 is responsible for mediating most leptin function in the CNS, the current study shows that LepR-specific deletion of STAT3 signaling does not affect fertility in mice. The obese yet fertile phenotype of our LepRb-specific STAT3 KO mice was much more similar to that of the LepRb-STAT3 binding domain mutant mice (27). This difference in phenotype between neural STAT3 KO mice and LepR-specific STAT3 KO mice could be explained by a few possibilities. For example, nestin expression begins as early as embryonic day 10.5 in neuronal progenitor cells, neural crest cells, and the undifferentiated neural epithelium (31, 52), thereby knocking out STAT3 function very early during embryogenesis. In contrast, it is not evident that LepR are expressed in the hypothalamus during such early development in mice (53–56). Therefore, STAT3 deletion would have occurred at a later developmental stage in the mouse model from this study. Furthermore, neural STAT3 mutants have STAT3 knocked out throughout the CNS, possibly affecting the early development of all brain cells. STAT3 KO mice from the current study are less likely to have these potential widespread developmental effects, as STAT3 was specifically knocked out from LepR-expressing cells. It is also worth noting that although both these studies were carried out on a pure C57BL/6 genetic background, it is likely that subtle genetic differences between the 2 mouse lines result in differential expression of modifier genes affecting the fertility of neural STAT3 KO mice. Perhaps the most likely explanation, however, is that STAT3 function in non-LepR neurons is important for fertility. This view is supported by previous findings that hormones such as GH (47), estradiol (43), and prolactin (34, 42) are also involved in regulating energy balance and fertility by signaling through STAT pathways. Thus, although STAT3 signaling by leptin may not be essential for the regulation of reproduction, STAT3 itself is required.

Contrary to our hypothesis of redundant actions of STAT3 and STAT5 pathways, double KO mice also had normal estrous cyclicity, testicular function, and adult fertility. This negative result is unlikely to be due to an incomplete deletion of STAT3 and STAT5 in the KO mice, because immunohistochemical analyses confirmed the inability of leptin to induce pSTAT3 and pSTAT5 expression in the KO mice. As prolactin has been shown previously to signal through STAT5, all mice were treated with bromocriptine (a potent inhibitor of prolactin) to suppress prolactin-induced STAT expression. The small amount of pSTAT3 and pSTAT5 expression observed in the KO mice
could have resulted from stimulation through other hormones such as GH or insulin in non-LepR cells. Furthermore, the fact that the STAT3 single KO mice and double KO mice develop obesity provides further confirmation that these mice indeed have STAT3 deletion from LepR neurones.

The normal fertility observed in STAT3 and STAT5 double KO mice compared to the infertility in dKO mice and neutral STAT3 KO mice could be explained by 3 possibilities. First, STAT3 and STAT5 may work through stimulation from hormones or cytokines in addition to leptin, and therefore, STAT signaling in non-LepR neurons may play a crucial role in the control of fertility. This hypothesis, however, does not explain the infertility of leptin (ob/ob) or LepR (db/db)-deficient mice. Second, leptin itself may not be the metabolic primary signal responsible for puberty onset and reproductive activity, but may act through unknown signaling pathways to modify the levels or activity of another factor, such as the hormone ghrelin (57), which acts on the reproductive axis. Finally, leptin signaling may occur through STAT3- and STAT5-independent intracelular signaling pathways in their absence to regulate reproduction. Although there is no evidence for hypothalamic leptin signaling via other STAT pathways, such as STAT1 (20, 21, 58), leptin exerts its widespread effects by interacting with multiple signaling factors and possibly through cross-talk between different signal transduction pathways. In this regard, we measured activation of ERK1/2 in hypothalamic regions to assess whether STAT3 or STAT5 KO resulted in compensatory changes in this MAPK signaling pathway, known to be used by leptin in the hypothalamus (11, 13). There was no evidence of up- or down-regulation of this signaling pathway in STAT3 and STAT5 single or double KO mice compared to controls. Other leptin-responsive signaling pathways include the insulin receptor substrate 2-mediated hypothalamic PI3K (15, 16), the AMPK pathway (59), and the mTOR pathways. Leptin, acting via the PI3K pathway, can acutely alter membrane potentials in hypothalamic proopiomelanocortin neurons (60) and in neurons located within the PMV (61), a region of the hypothalamus that is important for fertility in rodents (62). It is possible, therefore, that leptin could have maintained fertility in our STAT3 and STAT5 KO mice via a PI3K-mediated action on proopiomelanocortin and/or PMV neurons. In recent years, the mTOR pathway has also been suggested to have a potential role in linking energy balance and reproduction. Blockade of mTOR signaling with rapamycin has been shown to delay puberty, prevent leptin's stimulatory effects on puberty onset in food-restricted female rats, and also suppress kisspeptin expression (63, 64).

Cyclic AMP responsive element-binding protein-regulated transcription coactivator 1 (Crtc1) has been shown to be required for leptin's effects on energy balance as well as fertility. Leptin-deficient ob/ob mice have phosphorylated Crtc1 in the hypothalamus, whereas leptin administration increases the amount of dephosphorylated Crtc1. Crtc1 stimulates the expression of Carpt and Kif1b1 genes, which encode neuropeptides that modulate energy balance and fertility, and Crtc1 KO mice are obese and infertile (65). Another study using the same mouse line showed that Crtc1 is required for the hypothalamic expression of genes that mediate leptin's action on energy balance, but not on fertility (66). It is therefore likely that multiple pathways, possibly including but not limited to STAT3 and STAT5, act cooperatively or redundantly to mediate the central effects of leptin on fertility. Such pathways may have become up-regulated in our KO mice to compensate for the loss of STAT signaling; unfortunately, our assessment of such changes was limited to ERK1/2 signaling due to lack of further brain tissue series.

In conclusion, the results from this study clearly show that, whereas STAT3 is critical for bodyweight regulation, neither STAT3 or STAT5 is required for the regulation of fertility by leptin. This suggests that the hypothalamic control of fertility by leptin may involve the presence of multiple redundant pathways, possibly including STAT3 and STAT5. Determination of the signaling mechanisms underlying leptin's control of fertility is a fundamental step toward a better understanding of mechanisms of metabolic infertility.

Acknowledgments

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Leptin Signaling in GABA Neurons, But Not Glutamate Neurons, Is Required for Reproductive Function

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The adipocyte derived hormone leptin acts in the brain to modulate the central driver of fertility: the gonadotropin releasing hormone (GnRH) neuronal system. This effect is indirect, as GnRH neurons do not express leptin receptors (LEPRs). Here we test whether GABAergic or glutamatergic neurons provide the intermediate pathway between the site of leptin action and the GnRH neurons. Leptin receptors were deleted from GABA and glutamate neurons using Cre Lox transgenics, and the downstream effects on puberty onset and reproduction were examined. Both mouse lines displayed the expected increase in body weight and region-specific loss of leptin signaling in the hypothalamus. The GABA neuron-specific LEPR knock-out females and males showed significantly delayed puberty onset. Adult fertility observations revealed that these knock-out animals have decreased fecundity. In contrast, glutamate neuron-specific LEPR knock-out mice displayed normal fertility. Assessment of the estrous hypothalamic pituitary-gonadal axis regulation in females showed that leptin action on GABA neurons is not necessary for estradiol-mediated suppression of tonic inhibitory hormone secretion (an indirect measure of GnRH neuron activity) but is required for regulation of a full proovulatory-like lutetinating hormone surge. In conclusion, leptin signaling in GABAergic (but not glutamatergic neurons) plays a critical role in the timing of puberty onset and is involved in fertility regulation throughout adulthood in both sexes. These results form an important step in explaining the role of central leptin signaling in the reproductive system. Limiting the leptin-to-GnRH mediators to GABAergic cells will enable further research to focus on a few specific types of neurons.

Introduction

Circulating leptin is an important metabolic signal regulating food intake and energy expenditure. Leptin also forms a permissive modulator of fertility (Ahima et al., 1997; Nagatani et al., 1998). Reproduction is centrally regulated by the drivers of the hypothalamic-pituitary-gonadal (HPG) axis: the gonadotropin releasing hormone (GnRH) neurons. A significant body of research has been devoted to trying to understand how leptin’s effects are related to this reproductive axis. Leptin acts directly on the brain as an autocrine removal of leptin receptors (LEPRs) from forebrain neurons results in the same infertile and obese phenotype as does global mutation in the leptin gene itself (Sipe et al., 1995; Swerdloff et al., 1975; Quinnell et al., 2009). However, leptin affects GnRH neurons indirectly as they do not express LEPRs (Quinnell et al., 2009). To elucidate the identity of the neuronal network that must exist between responsive GABA and GnRH neurons, we investigated leptin signaling in the massive inhibitory and excitatory neuronal cell populations GABA and glutamate neurons. The GnRH neurons express both GABA and GABA receptor isoforms and binding of GABA to the GABA receptor causes an inhibition of pulsatile GnRH release (Toth et al., 2003; Zhang et al., 2008). Although GnRH is generally an inhibitory neurotransmitter, the effect of GABA receptor activation on GnRH neurons can be excitatory or inhibitory depending on the chloride concentration within the neuron (Kraievich, 1994; Herbstman and Niswender, 2011). Hypothalamic locations where GABAergic LEPR neurons are concentrated include the arcuate nucleus (Arc), dorsomedial nuclei (DMN), and lateral hypothalamus (OVX; Calo et al., 2001; Vong et al., 2011). Functionally, acute fasting (i.e., reducing leptin levels) alters GABAergic transmission to GnRH neurons and decreases GnRH neuronal activity (Sullivan et al., 2003; Sullivan and Moore, 2004), suggesting that leptin sensing GABAergic afferents integrate metabolic cues to modulate GnRH release.

Glutamate is the main excitatory neurotransmitter of the CNS. The cell bodies of GnRH neurons are innervated by glutamatergic terminals that stimulate GnRH release (Bourguignon et al., 1995; Kiss et al., 2003). Hypothalamic glutamatergic LEPR neurons can be found in the Arc, the ventral medial nucleus (VMM), and the ventral premammillary nucleus (PVM) (Kocsis, 2003; Vong et al., 2011). Lesion experiments indicate that PMV LEPR neurons are involved in leptin’s effects on fertility (Donato et al., 2006; Leshan et al., 2009). Hence, glutamatergic LEPR neurons form a likely leptin-to-GnRH candidate.

To specifically delete LEPRs from either vesicular GABA transporter (VGAT) or vesicular glutamate transporter 2 (VGluT2)
expressing neurons, targeting GABA and glutamatergic neurons, respectively. Cre-Lox transgenes was used. The effects of these deletions on fertility were studied in female and male animals. Specifically, puberty onset, adult fertility, and estradiol feedback on the HPG axis were assessed to determine which of these two major neuronal populations is primarily responsible for leptin's effects on reproductive function.

Materials and Methods

Animals. Female and male transgenic mice with Ylgat2 (Ylgat2-area-Cre) or Ygat (Ypar-cre-Cre) specific LEPR deletions were bred from transgenic lines as described by Yang et al. (2011) (on a mixed FVB and C57BL/6J background). These animals are hereafter referred to as GABA- and glutamate-specific LEPR knock-out. Homozygous LEPR floxed (Lepr^flox/flox) intermated were used as controls. Animals were group housed and pair fed with an animal of the opposite sex for fertility assessment. Mice were kept on a 12 h light to dark cycle (lights on at 0800 h), at constant room temperature (21 ± 1°C), with ad libitum access to water and standard rodent chow unless otherwise noted. The University of Ottawa Animal Ethics Committee approved all experimental animal protocols.

Tissue collection and preparation. For tissue collection animals were fasted overnight and injected subcutaneously with recombinant leptin (5 mcg/kg, National Hormones and Peptide Program) or vehicle (0.01% FBS, pH 7.8). Two hours after injection, animals were sedated and blood collected from the inferior vena cava before transcardial perfusion with 1% paraformaldehyde (0.1% FBS, pH 7.4). Brain tissue was prepared for immunohistochemistry, as previously described by Quevedo et al. (2011).

Immunohistochemistry and image analysis. Immunohistochemical labeling of leptin-induced phosphorylation of the leptin signaling molecule signal transducer and activator of transcription 3 (pSTAT3) was performed to determine loss of hypothalamic leptin responsiveness in the knock-out mice. To do so, we performed an antigen retrieval step (15 min, 1 mol sodium citrate buffer, pH 6.0, at 95°C), and used polyclonal rabbit anti-pSTAT3 as the primary antibody (1:1000; T-6419; Cell Signaling Technology). This was followed by a biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Laboratories), and labeling was visualized with 3,3'-diaminobenzidine (DAB; 30 min). A selection of antibodies was used to confirm the identity of the hypothalamic nuclei [lateral preoptic area (MPOA), lateral preoptic area (MPOA), medial preoptic area (MPOA), central arcuate (arcuate - 0.96 to -1.54 mm, lateral arcuate (arcuate - 1.46 to -1.94 mm), caudal arcuate (arcuate -2.96 to -2.54 mm), VMN, lateral hypothalamus, DMV, VMH] and the nucleus of the solitary tract (NTS). Positive leptin-responsive cells were counted when a clear circular and darkly stained nucleus was present. Counts were performed in photomicrographs of at least three tissue sections per area from each animal. The three Arcuate subregions were later merged to form a single bar on the graph (see Fig. 1A).

Fluorescent double label immunohistochemistry of GABA and vGAT was performed in control and GABA-specific LEPR knock-out male animals. Brain sections containing GABA neurons were incubated in polyclonal primary anti-GABA (1:2000; MAB2272, Millipore) and rabbit anti-vGAT (1:750; S6252, Millipore) primary antibodies. This was fol-
dowed by a direct labeling with fluorescent secondary antibodies, using goat anti-guinea pig Alexa 488 (1:500; Invitrogen) plus visualizing GABA and goat anti-rabbit Alexa 568 (1:500; Invitrogen) to label vGAT. Sections were mounted on microscope slides with VECTASHIELD (Vector Laboratories) for confocal microscopy (Zeiss LSM 710). Using an argon laser excited at 488 nm (GABA), a helium neon laser exciting at 543 nm (vGAT) and a 540 nm dichroic mirror objective lens, a 2-stack (0.5 um optical slices) was made of each individual GABA and vGAT stained cells and proximal processes. Omission of the primary vGAT antibody resulted in an absence of stained terminals. Twenty GABA neurons per animal were photographed and used for counting (10 within the medial septum population and 10 in the FCAO). Zeiss LSM image browser software was used to count the number of appositions where red pixels touched the green, for at least two consecutive optical slices) on the GABA immunostained images and in three 10 um segments progressing distally along the projections. This tool was deemed to measure the circumference of the soma. Where data were obtained from two projections per GABA neuron, these were averaged.

Leptin ELISA. Plasma leptin concentration of control and knock-out animals was measured by ELISA (Mouse Leptin ELISA Kit, Crystal Chem). The average coefficient of variation was 6.76% and the sensitivity of the assay was 0.8 ng/ml. All samples were run in duplicate on a single plate.

Positron emission tomography (PET) imaging. Female animals, pubertal onset was measured by vaginal opening along with the age at first estrus (based on vaginal cytology). From 26 to 45 of age, experimental (GABA- and glutamate-specific LEPR knock-out) and control mice were checked daily. Once vaginal opening was established, vaginal cytology was studied to assess signs of first estrus. Male mice were mated with adult wild-type C57BL/6J females from 36 to 40 of age. Next, subtracting 2 d (gestation period) from the date when their first litter was born, Males were kept in their breeding pairs to further assess adult fertility. To determine adult female estrous cyclicity, vaginal smears were taken for 10 (glutamate-specific knock-outs and controls) or 24 (GABA-specific knock-outs and controls) consecutive days starting at least 14 d after first estrus. Estrous cycle duration was calculated as the average time between two proestrous phases. Afterwards, female knock-out and control animals of both experimental groups were paired with adult wild-type C57BL/6J males to study fecundity.

Estimation of daily sperm production. After transcardial perfusion and brain collection, one testis from each control or knock-out male was removed, weighed, recorded, and stored at −80°C. Daily sperm production was subsequently determined as described previously with slight modifications (Robb et al., 1976; Singson et al., 2013). Briefly, testis fragments (25 mg) were homogenized in a tissue lyser (TissueLyser II, QIAGEN) for 10 min. Spermatozoa in stages 14–16 of spermatogenesis (LE and Clefmen) are resistant to homogenization, and their nuclei were counted in 10 μl aliquots of homogenate using a hemocytometer. Total count nuclei were divided by 4.44 because developing spermatozoa spend 4.44 d in stages 14–16 during spermatogenesis in mice.

Assessment of HPSC axis regulation by crenulized. Negative feedback assessment of estradiol was based on the experiments described by McCue et al. (2012). Briefly, GABA-specific LEPR knock-out and control mice were anesthetized with isoflurane and tail blood samples taken at baseline (day 0, luteinizing hormone (LH) were measured using a chronic slow-release 17β-estradiol subcutaneous implant (50 μg/kg, 11.26 mm long depending on body weight). After 12 d, another blood sample was taken (OVX). Animals were subsequently implanted with a chronic slow-release 17β-estradiol subcutaneous implant (50 μg/kg, 11.26 mm long depending on body weight). After 12 d, another blood sample was taken (OVX+implant). Subsequently, preovulatory-like lu-
testosterone (LH) surge induction was performed as described by Quevedo et al. (2009). On day 22, all mice received a bolus injection of estradiol benzoate (50 μg/kg, i.p.) 9 h before lights out. At lights out on day 23, final blood samples were taken. All blood samples were processed within 10 min of collection. Plasma was separated by centrifuga-
tion, immediately frozen, and stored at −20°C. The concentration of LH in the plasma was measured by radioimmunoassay, as described by Quevedo et al. (2009). The sensitivity of the assay (99% confidence in-	erval at 0 ng/ml) was 0.14 mg/ml, and the intra-assay coefficient of vari-
ation was 5.3%. All samples were analyzed to replicate within the same assay.

Statistical analysis. All results are presented as mean ± SEM. Student's t-tests were used to identify significant statistical differences (p < 0.05) between control and knock-out mice in the pSTAT3 cells counts, plasma leptin concentrations, daily sperm production, and other measures of fertility. The body weight and negative feedback data were compared using a two-way ANOVA with repeated measures and a Bonferroni multiple-comparison post hoc test. Pooling of LE and control groups was used in immunohistochemical, plasma leptin concentration and body weight comparisons because the two experiments were run concur-
rently and controls were identified produced.
Figure 1. Characterization of the knock-out model by labeling leptin-induced phosphorylated STAT3. A–F, Representative photomicrographs of staining in different hypothalamic areas, counted. A–C, IPNc and POA. B–D, Acc, VMN, LH, and DMN. E–F, Acc and PMV. The first column shows leptin-induced (1 mg/kg) pSTAT3 labeling in control animals. Second and third columns show pSTAT3 labeling in GABA- and glutamate-specific LEPR knock-out animals, respectively. J, Quantification of immunohistochemical staining (number of positive labeled cells per section) in control animals (black bars, n = 11), GABA-specific (gray bars, n = 10), and glutamate-specific (white bars, n = 8) LEPR knock-out animals. There was no difference between the pSTAT3 counts of the two control groups (t-test, n = 5) that are derived from the two different areas, therefore, they have been pooled in this graph. In all regions, experimental groups are compared with the control animals to show significant differences. All P values are calculated using a Student’s t-test. Scale bars, 50 μm. *p < 0.05.
Appendix

Results
Loss of leptin signaling in GABA and glutamate-specific LEPR knock-out neurons

To validate our transgenic knock-out models, we identified the loss of leptin-responsive cells using pSTAT3 immunohistochemistry. The Cre-Lox animal models used in this study disrupt leptin signaling by deletion of exon 17 of the Lepr gene. This exon codes for the part of the receptor responsible for JAK STAT signaling (Schärf et al. 2004); therefore, loss of phosphorylated STAT3 provides an accurate representation of where LEPR was deleted. The transgenic disruption of LEPR from either GABA or glutamate neurons resulted in a marked reduction of leptin-induced pSTAT3 immunoreactivity in the hypothalamic nuclei where these neurotransmitters are known to be strongly expressed (Vong et al. 2011). Removing LEPRs from GABA neurons caused a 42% reduction of leptin-induced pSTAT3 in the Arc (particularly in the medial and caudal Arc sections; the effect was not seen in the most rostral sections; Fig. 1E,F). Similar reductions were seen in the latero hypothalamic (49%) and the DMN (31%; Fig. 1E,F). Deletion of LEPRs from glutamate neurons caused a different pattern of pSTAT3 staining. Strikingly, pSTAT3 immunoreactivity was 80–97% reduced in the PMV and VMN, respectively (Fig. 1E,F). Significant decreases were also evident in the ventral pPOMC (47%; Fig. 1C) and MPA (47%; Fig. 1F) as well as the latero hypothalamic (30%; Fig. 1E,F) when glutamatespecific LEPR knock-out mice were compared with controls. Extra-hypothalamic LEPR deletion was addressed by counting pSTAT3-

Figure 2. Effects of leptin knock-out on body weight and plasma leptin concentrations. A, B: female and male GABA- and glutamate-specific LEPR knock-out and control animals body weight. Female GABA-specific knock-out animals (gray square) (n = 16) are significantly heavier than controls (black circle) (n = 10) at 4 weeks of age; glutamate-specific LEPR knock-out animals (white square) (n = 10) are significantly heavier than controls (black circle) at 4 weeks of age. Male GABA-specific LEPR knock-out animals (gray square) (n = 10) had a lower body weight from 4 weeks onward and glutamate-specific LEPR knock-out males (white square) (n = 6) by 2 weeks onward compared with controls (black circle) (n = 15). We were unable to gather body weight data for the female groups throughout the breeding cycle because females were at different stages of pregnancy. C: Plasma leptin concentrations of control animals (black bars; female n = 6, male n = 6); control significantly lower than GABA-specific knock-out animals (gray bars; female n = 4, male n = 4); in glutamate-specific (grey bars; male n = 6, female n = 6) there was significantly higher leptin than the control (dotted bars; female n = 4, male n = 4). *p < 0.05, **p < 0.005, ***p < 0.000.
Appendix

Figure 3. Puberty onset in GABA- and glutamate-specific LEPR knockout animals. A: GABA-specific LEPR knockout animals (gray bars; n = 7–10) exhibited delayed vaginal opening, first estrus, and male puberty onset when compared with controls (black bars; n = 7–10). In controls, vaginal opening, first estrus, and male puberty onset occurred at the same time in GABA-specific knock-out animals (black bars; n = 8–10) compared with control animals (gray bars; n = 8–10). C: Controls (VO) showed a significantly earlier estrus compared to GABA-specific LEPR knockout animals (gray) in both female and control animals. D: GABA-specific LEPR knockout animals (gray) had a significant effect on body weight in females (two-way repeated-measures ANOVA: F(12,232) = 27.16, p < 0.001) and in males (two-way repeated-measures ANOVA; F(12,204) = 6.50, p < 0.001) compared with controls. As previously described, LEPR knock-out from GABA neurons caused an obese phenotype (Vong et al., 2011). Post hoc testing revealed that female GABA-specific LEPR knock-out animals were significantly heavier than littermate controls from the age of 4 weeks onwards (Fig. 2A), whereas for males a significantly greater body weight was reached at 6 weeks of age (Fig. 2B). Glutamate-specific LEPR knock-out caused a milder metabolic phenotype. Females were significantly heavier than their littermate controls by 9 weeks of age (Fig. 2A). The males showed a significant weight difference from 12 weeks of age compared with control animals (Fig. 2B). These results are similar to the body weights described by Vong et al. (2011), with the GABA-specific knock-out being notably heavier than the glutamate-specific knock-out animals. Blood collected at the end of the breeding studies showed that plasma leptin concentrations were significantly elevated in both female and male GABA-specific LEPR knock-out animals (female; t(14) = 16.46, p < 0.0001 and male; t(13) = 9.85, p = 0.0023; Fig. 2C). In glutamate-specific knock-out animals, a significant increase in leptin concentration was only evident in male animals (t(13) = 4.56, p = 0.02; Fig. 2C).

Positive nuclei in the NTS. No significant differences were found between the GABA-specific knock-outs and controls (Fig. 1J); this was expected because several studies have shown that the NTS LEPR neurons are not GABAergic (Vong et al., 2011; Garfield, et al., 2012). The number of animals in the glutamate-specific LEPR knock-out group was low; nevertheless, a trend toward a reduced pSTAT3 response (albeit nonsignificant; n = 2; t(1) = 1.20, p = 0.24) was observed similar to what has been reported (Vong et al., 2011).

In vehicle-treated animals, the overnight fast was unable to reduce pSTAT3 immunoreactivity to the same extent in the GABA-specific LEPR knock-outs compared with the controls. This phenomenon is likely the result of the high circulating leptin found in these very obese animals (Fig. 2C).

The above-mentioned findings agree with previously described distributions of Vgat and Vglut2 mRNA in the hypothalamus (Vong et al., 2011), confirming that the LEPR deletions were indeed targeted to neurons expressing Vgat and Vglut2. There appeared to be very little overlap between the regions affected by GABA and glutamate LEPR knock-out. The lateral hypothalamus was an exception; both GABA- and glutamate-specific LEPR deletion reduced the numbers of neurons able to respond to leptin here.

Disrupted body weight regulation in both GABA- and glutamate-specific LEPR knock-out animals. Body weights of all knock-out and control groups were measured fortnightly. The genotype of the knock-outs had a significant effect on body weight in females (two-way repeated-measures ANOVA; F(12,232) = 27.16, p < 0.001) and in males (two-way repeated-measures ANOVA; F(12,204) = 6.50, p < 0.001) compared with controls. As previously described, LEPR knock-out from GABA neurons caused an obese phenotype (Vong et al., 2011). Post hoc testing revealed that female GABA-specific LEPR knock-out animals were significantly heavier than littermate controls from the age of 4 weeks onwards (Fig. 2A), whereas for males a significantly greater body weight was reached at 6 weeks of age (Fig. 2B). Glutamate-specific LEPR knock-out caused a milder metabolic phenotype. Females were significantly heavier than their littermate controls by 9 weeks of age (Fig. 2A). The males showed a significant weight difference from 12 weeks of age compared with control animals (Fig. 2B). These results are similar to the body weights described by Vong et al. (2011), with the GABA-specific knock-out being notably heavier than the glutamate-specific knock-out animals. Blood collected at the end of the breeding studies showed that plasma leptin concentrations were significantly elevated in both female and male GABA-specific LEPR knock-out animals (female; t(14) = 16.46, p < 0.0001 and male; t(13) = 9.85, p = 0.0023; Fig. 2C). In glutamate-specific knock-out animals, a significant increase in leptin concentration was only evident in male animals (t(13) = 4.56, p = 0.02; Fig. 2C).
Delayed puberty onset in GABA-specific LEPR knockout animals

To assess puberty onset, date of vaginal opening and first estrus in females were measured, whereas for males the age of first fertile mating was determined. Female mice with LEPR deleted from GABAergic neurons showed a significant, 7 day delay in vaginal opening ($t_{(12)} = 2.30, p = 0.027$), coupled with a 20 day delay in first estrus ($t_{(12)} = 10.31, p < 0.0001$; Fig. 3A,C). When male GABA-specific LEPR knockout animals and controls were mated with intact wild-type C57BL/6 female of reproductive age, they showed a significant delay in puberty onset. When the age at puberty was backdated, it revealed that the knock-out animals went through puberty $\sim$15 days later than the controls ($t_{(12)} = 3.40, p = 0.005$; Fig. 3A,D).

Puberty onset of glutamate-specific LEPR knockout females and males did not differ from controls (Fig. 3B). Both female knock-out and control animals showed vaginal opening at $\sim$2.7 d. In these animals, first estrus was evident $\sim$3 d later. Male glutamate-specific LEPR knockout animals did not show any significant difference in the timing of puberty onset either (Fig. 3B).

Reduced adult fertility in GABA-specific LEPR knockout animals

To assess adult fertility, we investigated female estrous cycles and measured fecundity in both sexes. Vaginal cytology was determined for 28 consecutive days beginning at day 60 in female GABA-specific LEPR knockout animals significantly less time was spent in proestrus ($t_{(12)} = 7.32, p < 0.0001$; Fig. 4A) and more time in estrus compared with controls ($t_{(12)} = 2.28, p = 0.026$). The time spent in met- or diestrus was not different between groups ($t_{(12)} = 0.63, p = 0.54$; Fig. 4A). Estrus cycle duration averaged $13.6 \pm 1.8$ d for knock-outs compared with $4.9 \pm 0.2$ d for controls, showing that GABA-specific LEPR knock-out resulted in significantly prolonged cycles ($t_{(12)} = 5.02, p < 0.0001$). For glutamate-specific LEPRknock-out females, there was no difference in the frequency of cycle stage compared with controls. On average, one day was spent in each of the proestrus, estrus phases and 2 d in met- and diestrus per cycle (Fig. 4C).

Fecundity of adult mice was assessed by litter size and frequency after pairing with wild-type mates. In the GABA-specific...
LEPR knock-out females, three animals had to be killed because of dystocia complications; and because of these welfare considerations, the experiment had to be terminated prematurely (after 38 d of mating). Only 30% (3/10) of GABA-specific LEPR knock-out females produced two litters within this time frame, a further four of the knock-out females delivered successfully but only had one litter. In contrast, all control females produced two litters after 38 d of mating. The interlitter interval for the multiparous three knock-out was not significantly different compared with controls (knock-out: 1.1 ± 0.28, p = 0.28; Fig. 4B). However, there was a significant delay in the time from parturition to first delivery for knock-out females (t = 4.2, p = 0.0005, Fig. 4B). The average number of pups in the litters that were born did not differ between groups (knock-out: 6 ± 0.6 pups per litter, t = 1.7, p = 0.09). Male knock-out and control animals were kept in their breeding pairs for 100 d after their first litter was born. The time between litters over this period was significantly greater in the knock-out group compared with the control animals (t = 2.7, p = 0.017, Fig. 4B). The average number of litters per litter was not different between groups (knock-out: 7.8 ± 0.7 and control: 7.8 ± 0.5 pups per litter; t = 0.01, p = 0.99). To assess whether this reduced fertility in the male animals was the result of lower sperm counts, daily sperm production was measured in GABA-specific LEPR knock-out males and controls. This revealed no significant differences between the two groups (control: 1.2 ± 10^8 ± 0.4 × 10^8; knock-out: 1.4 ± 10^8 ± 0.5 × 10^8 spermatozoa per testis per day, t = 0.32, p = 0.76). GABA-specific LEPR knock-outs and their controls were also assessed for litter frequency and time to first litter. After pairing with an infertile wild-type mate, both female and male knock-out and controls were proven to be equally fertile (Fig. 4D).

These results, together with the delay in parturition onset, indicate a critical role for GABAergic leptin-sensing neurons in the onset and maintenance of male and female fertility. Interestingly, the results show that LEPR signaling in glutamate (vglut2) neurons is not required for normal reproductive function.

GABA-specific LEPR knock-out animals have an impaired response to estradiol in a positive feedback paradigm

The experiments described above have demonstrated a significant delay in parturition onset and a reduction in fertility when LEPR signaling is disrupted in GABA neurons. To explore further the mechanisms of this decrease in fertility, we assessed the hypothalamic response to estradiol. Measuring the circulating concentration of LH in different estrous states provides a direct index of GnRH neuronal activity because GnRH is a potent stimulator of LH release. Estrogens inhibit GnRH LH secretion at most times during the female cycle (negative feedback); however, on the day before ovulation, a rising estradiol concentration triggers a massive preovulatory GnRH/LH surge (positive feedback), eventually causing ovulation (Herbison, 1998).

Intact control and GABA-specific LEPR knock-out mice had similar LH levels that increased significantly after OVX (controls t = 6.2; knock-out: t = 5.7, p < 0.0001) for both (Fig. 5A). The ability of estradiol to suppress LH levels was undiminished in GABA-specific LEPR knock-out compared with control animals (controls t = 5.0; knock-out: t = 5.27, p < 0.0001 for both; Fig. 5A). The ability of estradiol to suppress LH levels was undiminished in GABA-specific LEPR knock-out compared with control animals (controls t = 5.0; knock-out: t = 5.27, p < 0.0001 for both; Fig. 5A). The ability of estradiol to suppress LH levels was undiminished in GABA-specific LEPR knock-out compared with control animals (controls t = 5.0; knock-out: t = 5.27, p < 0.0001 for both; Fig. 5A).

At the time of lights out (Wintermantel et al., 2006), when the animals were subjected to this treatment, the knock-out animals had a 46% lower plasma LH concentration at the time of the preovulatory-like surge compared with controls (t = 2.49, p = 0.025; Fig. 5B). These data suggest that an impaired positive feedback mechanism may underlie the subtle phenotype seen in the GABA-specific LEPR knock-out mice.

The reduction in fertility of our GABA-specific LEPR knock-out animals could be a result of a difference in GABAergic wiring onto GnRH neurons. To investigate this, we counted GABAergic neurons and surrounding VGAT-positive terminals. We found that the corpus callosum (knock-out: 31.5 ± 0.78 μm²; controls: 32.3 ± 1.85 μm², t = 3.85, p = 0.042) and number (knock-out: 6.9 ± 1.32 GnRH neurons per section, t = 0.35, p = 0.74) of GABA neurons did not differ between knock-out and control animals. When the number of GABAergic projections onto the GnRH soma and projections were counted, there were no differences between the groups (Fig. 6), indicating that there are no detectable deficits in GABAergic inputs in the GABA-specific LEPR knock-out mice.
Discussion

Neuronal pathways that communicate leptin and other metabolic signals to the HPG axis are poorly understood. Here, LEPRs were selectively deleted from the principal inhibitory (GABA) and excitatory (glutamate) neuronal populations to identify whether these neurons mediate interactions between leptin and the reproductive axis. Using these validated mouse models, we conclude that leptin signaling in GABAergic neurons is critical for HPG axis functioning in both sexes, and surprisingly glutamatergic LEPR neurons are not.

Leptin signaling in the PMV is thought to play a major role in regulating fertility rather than metabolism. Lesion studies of this nucleus cause a disruption of estrous cyclicity in rats (Donato et al., 2009), and selective PMV LEPR reexpression in LEPR-null mice induces female puberty onset and some pregnancies (Donato et al., 2011). Additionally, PMV LEPR neurons (of which >80% are glutamatergic) are known to project to GnRH cell bodies, AVPV kispeptin neurons, and GnRH terminals in the median eminence (Rondini et al., 2004; Leushan et al., 2009; Donato et al., 2011; Louis et al., 2011). Although the present study showed a reduction of ~80% in PMV leptin signaling in the glutamate-specific LEPR knock-outs, it was unexpected to see that these neurons are not required for fertility. However, they may well participate in this role under normal conditions and even be sufficient to permit fertility in the absence of other brain LEPRs (Donato et al., 2011). Alternatively, the remaining ~20% of LEPR neurons in the PMV are not glutamatergic and may fulfill the leptin-to-GnRH role. In a recent paper by Leushan et al. (2012), removal of LEPR from neuronal nitric oxide synthase (nNOS) neurons, which are strongly represented in the PMV, caused a delay in puberty onset. Thus, PMV nitric oxide neurons might mediate leptin’s effects to the HPG axis. Interestingly, these nNOS LEPR knock-out mice were considerably more obese than our glutamate-specific LEPR knock-outs. This is probably because the nNOS LEPR knock-out targets Arc neurons in addition to those in the PMV (Leushan et al., 2012).

In contrast to the fully fertile glutamate-specific LEPR knock-out mice, male and female GABA-specific LEPR knock-outs exhibited markedly reduced fertility and the females had impaired estrous cycles. To try to identify the cause of subfertility in the GABA-specific LEPR knock-out mice, we assessed estradiol feedback in females and daily sperm production in males. The results of our estrogenic feedback experiment indicate that knock-out mice exhibit normal negative feedback regulation of LH but are impaired in their ability to mount a full preovulatory-like LH surge. This conclusion is consistent with the reduced occurrence of proestrus in these mice. Similar findings with regards to the LH surge have been reported in LEPR-deficient (in all forebrain neurons) and high fat diet-fed mice (Quennell et al., 2005; Sharma et al., 2013). As with the fertility data in intact animals, the concurrence of severe obesity with this effect means that caution must be exercised in attributing the disrupted preovulatory-like LH surge specifically to deficient leptin signaling in GABA neurons. Male 4530/60 mice completely lacking leptin produce relatively few mature spermatozoa, but this deficit appears to be the result of apoptotic effects of leptin deficiency at the gonadal level (Tahara et al., 2006; Danesi et al., 2015). Daily sperm production in our animals, which were not leptin deficient, did not differ between GABA-specific LEPR knock-out mice and controls, suggesting that our male mice had no such apoptotic effects. Perhaps their subfertility was instead the result of reduced sperm efficacy or to mating behavior deficits.

GABA is well known to be an important modulator of GnRH neurons, and the present results are consistent with the idea that leptin may communicate with GnRH through GABA. It is known that fasting alters GABA, receptor-mediated transmission to GnRH neurons, and leptin is able to modulate this change (Sullivan et al., 2005; Sullivan and Moenter, 2004). Similarly, GABA-specific LEPR knock-out mice in the study of Yong et al. (2011) were characterized by an increased inhibitory GABAergic tone upon Arc pro-opiomelanocortin neurons. Therefore, GABAergic input upon GnRH neurons may also be compromised in our GABA-specific LEPR knock-out mice, causing the subfertile phenotype. There are a few other candidates for providing the leptin-to-GnRH intermediate signal, in the form of neuropeptides that are potentially co-released from GABAergic LEPR neurons. First, the neuropeptide kispeptin is the most potent known stimulator of GnRH release and regulates the timing of puberty onset (Han et
Appendix

References


Additional, leptin is able to regulate kisspeptin expression (Quercoli et al., 2013). Disruption of the function of these cells would provide a convenient explanation for the inability of GABA-specific LEPR knock-out mice to mount a full LH surge in this study. However, conditional LEPR deletion in kisspeptin neurons or even reexpression in LEPR null mice has revealed that a direct action is neither required nor sufficient for fertility (Donato et al., 2011; Conzo et al., 2013). Therefore, kisspeptin function is reduced in these mice, it probably does not occur as a direct response to LEPR deficiency. Second, agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons in the Arc are affected by the knock-out of LEPR because these are GABAergic (Herbst et al., 1997). Both the AgRP and NPY neuropeptides have been linked to metabolic control of reproduction by direct actions onto GnRH neurons (Klevane et al., 2010; Boa and Herbston, 2012) and indirectly by inhibiting the stimulatory or mesolimbic stimulating hormone produced from the POMC gene (Israel et al., 2012). In both leptin-deficient ob/ob and LEPR-deficient db/db animals, ablation of AgRP neurons restores normal puberty onset and fertility (Israel et al., 2012; Wu et al., 2012). These experiments suggest that leptin-responsive AgRP/NPY/GABA neurons are likely to act as interneurones between metabolism and GnRH neurons, and thus suppress the HPG axis in absence of leptin or leptin signaling. We suggest that these LEPR/AgRP/NPY/GABA neurons are likely to be at least partly responsible for the subfertile phenotype observed in our GABA-specific LEPR knock-out mice. Last, there are galanin-like peptide (GALP) neurons in the Arc and caudal and amphetamineregulated transcript (CART) neurons in the DMH (that express LEPR and project to the area of the GnRH neurons (Leberleben et al., 2000; Takatsu, 2001; Ron- dini et al., 2004). Whether GALP and CART neurons corelease GABA has not been identified; therefore, it is unclear whether they have been targeted in our knock-out model.

Regardless of whether leptin’s permissive actions on the HPG axis occur via GABA inhibition or other predominantly inhibitory neurotransmitters such as NPY, deletion of LEPRs from GABA neurons might be predicted to cause upregulation of GABA/NPY function leading to an increased inhibitory tone on GnRH neurons. Alternatively, if leptin’s signals are relayed by stimulatory neurotransmitters (i.e., GALT and CART), and knowing that GABA has the ability to stimulate GnRH neuronal activity (Herbst and Moenter, 2011), then a reduction in the tone of these pathways upon GnRH neurons would be predicted in our knock-out model. It would be interesting to determine the nature of such changes in our GABA-specific LEPR knock-out mice.

The knock-out animals in which we observed a delay in puberty onset were also characterized by obesity and had high circulating leptin concentrations. Transgenic removal of exon 17 from the Lepr gene happens at early embryonic stages because expres expression is detectable from mid-gestation (Oh et al., 2005). Therefore, leptin receptor deletion in GABA neurons would have encompassed the second half of gestational development in all GABA-specific LEPR knock-out animals. The reproductive phenotype we observed in these animals could be an effect of defective wiring or the resulting obesity. However, early obesity or high circulating leptin is known to cause an advance in puberty onset rather than a delay (Ahima et al., 1997; Li et al., 2012). Additionally, HIV-1 axis functioning can be successfully stimulated by exogenous administration of gonadotrophins and progesterone, or of leptin in db/db animals (Smithberg and Run- ner, 1957; Chhabra et al., 1996). These studies indicate that neurotrophic deficits resulting from deficient leptin signaling throughout development are relatively minor, at least as far as fertility is concerned. Endocrinologist 1:45–61. DoGin, J. et al. GABA inputs were apparent in the GABA-specific LEPR knock-out mice because no detectable differences were noted in the number of GABA-positive axons in the GnRH neurons. Collective mouse results suggest that the obesity or any differences in neural wiring in our GABA-specific LEPR knock-outs are unlikely to be primarily responsible for their reduced fertility. Here, we have limited the first-order leptin-to-GnRH mediators to GABAAergic neurons. These experiments show that there is a pivotal role for LEPRs in GABA neurons, but surprisingly not in glutamatergic neurons, in metabolic regulation of male and female fertility. This serves to focus attention on GABA or a few known peptide codterminators, specifically NPY, AgRP, GALP, and CART as central neurotransmitters for the control of reproduction. Future studies should look at Orexin, GABA, and leptin-responsive, and send inputs to GnRH neurons to control fertility.