Direct and Indirect Innervation and Modulation of the Mesolimbic Dopamine System by Leptin Responsive Neurons

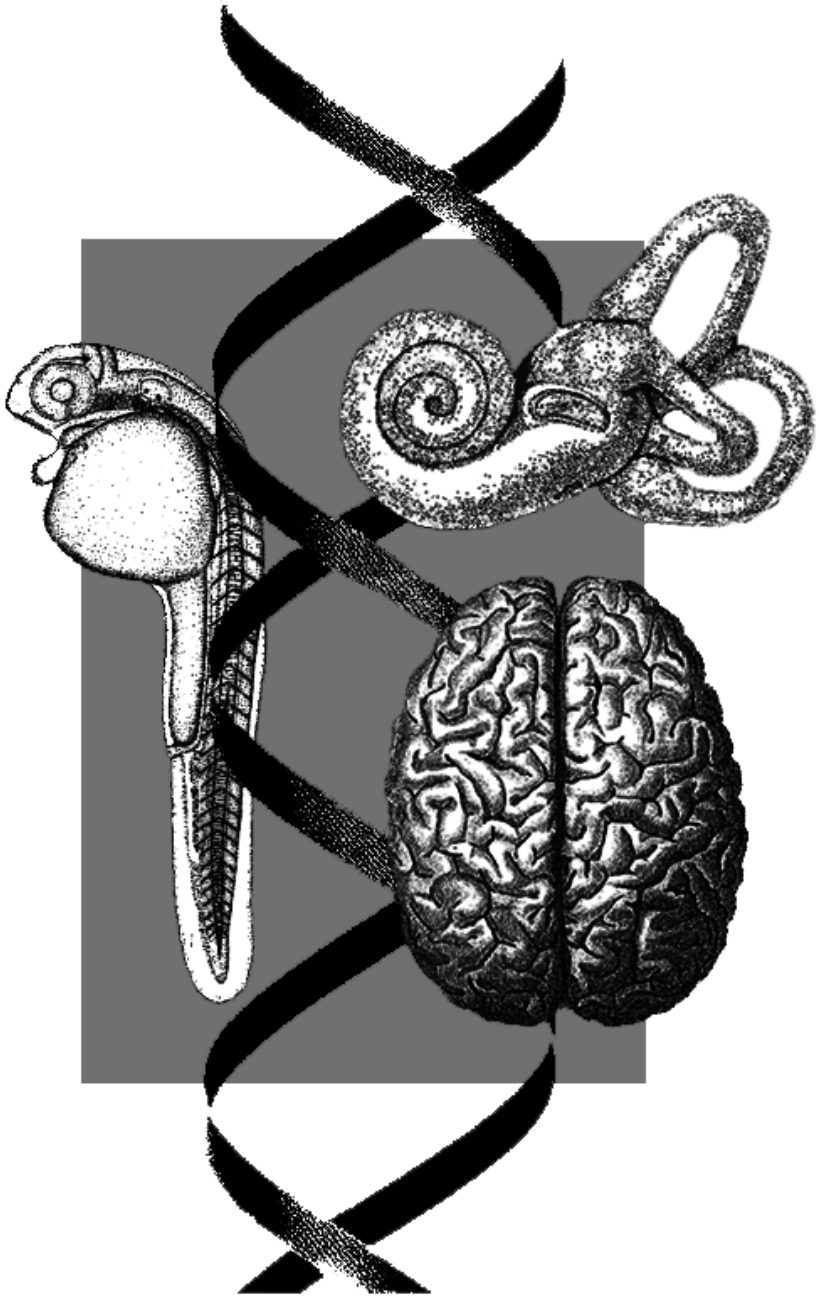
by

Darren M. Opland

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2011

Doctoral Committee:

Associate Professor Martin G. Myers Jr., Chair
Professor Jill B. Becker
Professor Kent C. Berridge
Professor Robert T. Kennedy
Professor Audrey F. Seasholtz



Dedicated to my Parents

Acknowledgements

I would like to acknowledge Dr. Martin G. Myers Jr. for his mentoring, support and for giving me the opportunity to work and learn in his laboratory. I would also like to thank the whole Myers lab for all their help and support over the years: Rebecca, Scott, Gwen, Eneida, Gina, Justin, Miro, Heike, Ryoko, Christa, Megan, Josh, Mike, Meg, ZZ, you all rock!

To Rebecca Leshan and Gina Leinninger for their productive collaboration and their invaluable training. I could not have done this without you.

I would also like to acknowledge the support of my whole family, especially my parents, without whom none of this would be possible and my brothers for all their support over the years. To the Badgers and all my friends for being the best and making sure my time in Ann Arbor was unforgettable.

One more shout out to Christa Patterson and Megan Greenwald-Yarnell for keeping me on the straight and narrow for the past few years. They made sure I was working, made sure I was eating, made sure I was healthy, were incredibly huge helps during the worst of it and have always been great friends.

To Gina (Louise) for being so good to me for the past few years.

Thanks to Avrim Eden for his advice and help.

Thanks: Rich Hume, Huda Akil, Gina Poe, Audrey Seasholtz, Peggy Gnegy, Bob Thompson and my entire thesis committee for all their council over the years

Table of Contents

List of Figures

List of Abbreviations

3v	third ventricle
ac	anterior commissure
acp	anterior commissure, posterior nerve
Aq	central aqueduct
BLA	basolateral amygdaloid nucleus
CART	cocaine- and amphetamine-regulated transcript
CeA	central amygdala
DA	dopamine
EW	Edinger-Westphal nucleus
f	fornix
GABA	gamma-aminobutyric acid
GAD1	glutamate decarboxylase 1
IF	interfascicular nucleus
IP	interpeduncular nucleus
IPAC	interstitial nucleus of the posterior limb of the anterior commisure
LHA	lateral hypothalamic area
LV	lateral ventricle
MCH	melanin concentrating hormone
ml	medial lemniscus
NAc	nucleus accumbens
Nts	neurotensin
opt	optic tract
OX	orexin
PAG	periaqueductal gray
POA	preoptic area
RLi	rostral linear nucleus
SNc	substantia nigra pars compacta
VTA	ventral tegmental area

ABSTRACT

Direct and Indirect Innervation and Modulation of the Mesolimbic Dopamine System by Leptin Responsive Neurons

by

Darren M. Opland

Chair: Martin G. Myers Jr.

Obesity is a burgeoning problem and a major risk factor for the development of Type-2 diabetes, cardiovascular disease, and cancer. This is likely caused by coupling of environments promoting an obesogenic lifestyle with biological systems that evolved to maintain body weight by responding to rewarding properties of food. It is crucial to understand systems that link energy balance and reward as we seek to define mechanisms that drive overeating and obesity.

A major breakthrough in our understanding of energy homeostasis came with the discovery of the adipose-derived satiety factor, leptin. Leptin's primary action in the brain is in the mediobasal hypothalamus where much of its anorectic effects are mediated. Recent research has shown that hypothalamic leptin signaling is inadequate to account for all of leptin's actions in the brain. There are several leptin receptor-expressing neural populations that are may mediate

reward sensation as they interact with elements of the mesolimbic dopamine system.

Our goals were to interrogate leptin-responsive neural populations that interact with the mesolimbic dopamine reward system (MLDA) in an attempt to better understand the relationship between energy status and reward. We used novel leptin receptor (LepRb) specific tract tracing system to identify the circumscribed projection pattern of ventral tegmental area (VTA) LepRb neurons and their downstream targets. Additionally we investigated LepRb neurons in the lateral hypothalamic area (LHA) which are known to project locally to orexin neurons and indirectly to VTA dopamine (DA) neurons. Using molecular mouse models with deficient LepRb expression in LHA neurons we assessed how leptin acts through the LHA to modulate reward sensitivity. We also studied the role of the neuropeptide neurotensin in mediating LHA LepRb neural. We found that VTA LepRb neurons project to and regulate GABAergic CART neurons in the central amygdala where they presumably regulate limbic function while LHA LepRb neurons modulate striatal behaviors. These findings confirm the supposition that individual LepRb neural populations regulate distinct aspects of central leptin signaling as a whole.

Advancing our knowledge of how systems that maintain energy balance interact with reward processing brain regions is an important step to our combating the development of obesity.

Chapter 1

Introduction*

Obesity is a burgeoning problem, not only in the United States but throughout the world. In America alone, obesity rates almost doubled from the late 1970's to the year 2000 and almost 1 in 4 adults in America today are considered obese [1]. Obesity is a major risk factor for the development of Type 2 diabetes, cardiovascular disease, and cancer. While genetics might play a role in the predisposition to an overweight body type, the rate at which waistlines are expanding worldwide far exceeds what can be causally attributed to alterations in genes alone.

The problem lies with environments that promote an obesogenic lifestyle: we are constantly surrounded by readily-available, palatable, energy-dense foods in conjunction with prevalent leisure activities that are more sedentary than in the past. Presumably systems that have evolved over time to regulate and maintain body weight can be co-opted by energy dense, highly rewarding food to promote ingestive behaviors. A more detailed understanding of what factors promote or inhibit appetite, especially in response to environmental factors, is key to development of successful strategies to combat this worldwide trend towards

* This work has previously been published: Opland DM, Leinninger GM, Myers MG Jr. (2010) Modulation of the mesolimbic dopamine system by leptin. Brain Res. 2010 Sep 2;1350:65-70. Epub 2010 Apr 22

obesity. Pharmacologic therapies to decrease appetite would be useful in treating obesity, diabetes, and related metabolic disorders, but our limited understanding of the neural and molecular mechanisms that regulate these processes has hindered the development of truly effective long-term therapies. Hence, it will be crucial to understand the systems that link energy balance to reward, and especially food reward, as we seek to define mechanisms that drive overeating and obesity.

This link between nutritional status and reward is, if anything, more relevant than ever today. Not only does food restriction enhance motivation for (and relapse to) drugs of abuse, underlining the relevance of this interaction for the understanding of addiction, but the reward value of food itself promotes overeating and likely contributes to the development and maintenance of obesity [2, 3]. Food has long been known as a powerful behavioral modulator (i.e. being used to train animals) since before recorded history; however mechanistic study of the pathways linking feeding and motivation is relatively recent. Behaviorist Edward Thorndike used food reward to motivate hungry animals learning to escape from early operant chamber [4], suggesting that incentive properties of food could aid in directing learning processes. In the 20th century Clark Hull proposed Drive Theory [5], suggesting that an organism's overall motivation to obtain a reward is controlled by 3 factors: previous experience with the reward, size or value of the reward, and a separate overall drive component. This potentially dissociable drive component could be modulated by factors like

deprivation, placing energy balance in context as a powerful behavioral modulator.

1.1 The regulation of energy intake and expenditure.

Animals regulate and maintain energy homeostasis over the long term: body adiposity is generally controlled within a given range for each individual, and alteration in energy stores provokes a countermanding response [6]. Not only does weight loss provoke increased feeding and decreased energy utilization, but forced overfeeding blunts volitional food intake and increases metabolic energy expenditure. Within energy balance, there are two overarching control variables- energy intake and energy expenditure. Overall energy expenditure includes basal metabolic rate, which is set by hormones such as thyroid hormone (among others), as well as the autonomic nervous system. Activity also contributes to energy utilization, as does the expenditure of energy on other functions, such as reproduction, lactation, etc. Decreased energy (fat) stores diminish energy utilization via each of these processes in order to maintain energy homeostasis.

The other side of the coin, energy intake, is mediated solely by the uptake of calories through eating and drinking. Many variables influence the amount of food consumed, however, including recent food intake, stomach distension, time of day, the perceived palatability of food, illness, etc [7]. Broadly speaking, however, two processes control feeding- satiation, which causes meal termination, and the incentive salience of food that promotes the initiation and

continuation of feeding. Both of these processes are influenced by energy balance- not only does weight loss delay satiation during meals, but it increases the incentive value of food measured in several ways, including the amount of work one is willing to perform in order to obtain food.

In 1954 Edward Stellar put forth the Dual Control Theory of Feeding suggesting that distinct brain regions, the ventromedial hypothalamus (containing the Arcuate (ARC) and ventromedial hypothalamic (VMN) nuclei) and lateral hypothalamic area (LHA) represented opposing “satiety” and “hunger” centers in the brain, respectively [8]. Lesions of the LHA cause animals to stop feeding and lose weight, while lesions of the satiety center promote hyperphagia and rapid weight gain [9]. It is now clear that other regions contribute to each of these processes: for instance, important signals from additional hypothalamic centers, as well as the hindbrain, contribute to satiety [10-12]. Furthermore, while the LHA is clearly important to feeding behavior, the early LHA lesion studies disrupted the medial forebrain bundle, through which course the axons from dopamine- (DA)-containing midbrain neurons that appertain to the mesolimbic DA system [3].

The mesolimbic DA system- encompassing DA neurons in the ventral tegmental area (VTA) plus their projections and neural targets in the striatum, amygdala, prefrontal cortex, and elsewhere- mediates the incentive salience of food and other rewards [2, 13, 14]. The reinforcing and addictive properties of both natural rewards (e.g., food, sex) and artificial rewards (drugs of abuse) are generally mediated by their effects on the mesolimbic DA system. Interestingly,

feeding status and body energy stores not only modulate the reward value of food, but of other reinforcers, as well: caloric restriction not only increases the incentive salience of food, but promotes drug-taking, relapse to drug-taking, and the amount of work an animal will do to obtain drugs [2, 15].

1.2 *Molecular components of energy balance*

The understanding of molecular processes that contribute to energy balance stemmed from the detailed study of spontaneously obese rodent models. In 1905 the first obese mouse model, the *Yellow* (A^y/a) or *agouti* mouse, was first described by Lucien Cuenot [16]. His pioneering work on genetics suggested that the *agouti* mutation was limited to a single gene and further study of this mouse model lead to the discovery, almost 100 years later, of the melanocortin pathway [17, 18]. In 1949 a spontaneous mutation in the mouse colony at Jackson Laboratories in Bar Harbor, Maine lead to the discovery of the autosomal recessive *obese* (ob/ob) mutation [19]. Less than two decades later a phenotypically similar mouse was identified with a mutation designated *diabetes* (db/db) due to the development of stark diabetes in addition to the phenotypic obesity associated with the *obese* mouse [20]. Early parabiosis studies confirmed that the missing factor in ob/ob mice was freely circulating whereas the mutated gene in db/db mice was likely a receptor for the ob/ob circulating factor [21, 22].

The molecular study of food intake leapt forward in 1994 with the positional cloning of the mutation resulting in obesity in the $Lep^{ob/ob}$ mouse; the

perturbed gene was dubbed ‘leptin’, from the Greek root *leptos* meaning ‘thin’ [23]. Leptin, a circulating hormone made and secreted by white adipose tissue, was found to have profound anorexigenic effects. A strong link was soon drawn between serum leptin levels and food intake as well as the fact that circulating leptin levels directly reflected relative adipose tissue stores [24-26]. Within a year it was demonstrated by several research teams that the mutation underlying obesity and diabetes in the db/db mouse was contained in the gene encoding the leptin receptor [27-30].

1.3 Leptin Receptor Structure and Signaling

The leptin receptor (LepR), encoded by the *diabetes* (db) gene, is a member of the type-I cytokine receptor family. The genetic product is alternately spliced into at least 6 different isoforms (LepRa – LepRf) that fall into three categories: short, long and secreted. The secreted isoform of LepR, LepRe, seems to play a role in modulating the amount of circulating leptin [31]. The membrane bound receptor isoforms (LepRa – d and LepRf) share the same extracellular and transmembrane domains containing one functional external leptin binding site [32]. The major difference between the membrane bound isoforms of LepR lie in their intracellular domain where the one long form of the receptor, LepRb, contains functional motifs that activate and interact with cellular signaling pathways whereas the other isoforms, with short intracellular domains, lack this ability.

The long form of LepR is responsible for mediating leptin's role in regulating food intake and energy expenditure [33]. Binding of leptin causes a conformational change in the receptor that brings intracellular LepRb-associated Janus kinase 2 (Jak2) molecules together, allowing them to phosphorylate and activate each other. Activated Jak2 leads to the phosphorylation of 3 important tyrosine residues: Y985, Y1077 and Y1138. Phosphorylation of the tyrosine residues activates several different signaling cascades, each of which is responsible for different aspects of leptin receptor signaling [34-36]. Recruitment of signal transducer and activator of transcription-3 (STAT-3) by Y1138 induces suppressor of cytokine signaling 3 (SOCS3) and mediates many of leptin's energy expenditure actions [36].

Since leptin production is essentially confined to adipocytes and is directly proportional to total fat mass, leptin levels largely reflect long-term energy stores. However, leptin levels do fluctuate in a more transient fashion, showing broad circadian rhythms with leptin levels highest when an animal is inactive (night for diurnal organisms) as well as increasing briefly after large meals. In order for fluctuating leptin to have relevance to central signaling pathways, mechanisms must exist to aide in the transport of leptin from peripheral blood stores to the CNS. Short isoforms of LepR are found to line microvessels that make up the blood-brain barrier in rodents and have been proposed to facilitate the transport of circulating leptin to the brain. Additionally the median eminence, located neighboring the mediobasal hypothalamus and other circumventricular organs

may permit entrance to the CNS for large peptides that are otherwise excluded by the blood-brain barrier.

1.4 Sites of Leptin Action

Central leptin action is responsible for mediating many processes including the maintenance of energy balance and behaviors affected by nutritional status [37, 38]. Sensitivity to circulating blood sugar levels and glucose homeostasis in general are mediated by populations of LepRb neurons in the arcuate nucleus (ARC) [39]. Leptin acts in an opposing fashion on two populations of neurons in the ARC that can be distinguished by their co-expression of different peptides. Leptin activates one set of neurons that express pro-opiomelanocortin (POMC) [40] and cocaine- and amphetamine-regulated transcript (CART) [41] whereas it inhibits a second population of ARC neurons that co-express agouti-related protein (AgRP) and neuropeptide-Y (NPY) [42]. Both POMC/CART and AgRP/NPY neurons innervate neurons in other hypothalamic targets such as the paraventricular nucleus (PVH) and the lateral hypothalamus (LHA) [43-46]. Additionally, POMC neurons produce and release α-melanocyte stimulating hormone (α–MSH) an agonist at melanocortin-4 receptors (MC4R's) [47] whereas AgRP is an inverse agonist at the same receptors [48]. This further supports the existence of a primary central axis for a potent feeding control system in the hypothalamus, with leptin activated POMC/CART neurons opposing feeding and leptin inhibited AgRP/NPY neurons stimulating feeding.

Leptin receptor signaling has been implicated in the function and release of growth hormones and has shown to be important in controlling the proper growth and development of prenatal organisms and their central nervous systems [49-51]. Additionally, LepRb signaling in the ventral premammillary nucleus (PMV) and other brain regions have been shown to be important in the control of reproduction [52]. These and other functions of leptin in the brain have been demonstrated to be the result of distinct neural subpopulations of leptin receptors, suggesting that individual clusters of LepRb in the brain each have important functional roles. Consequently, it might be possible to parse the individual role that each LepRb-containing brain region plays in behavioral processes by studying how leptin action at each of these sites affects particular behavioral responses.

1.5 Leptin and Reward

Leptin also contributes to the regulation of reward. Leptin attenuates responding for lateral hypothalamic self stimulation (LHSS) [53, 54]- electrical stimulation of the LHA that presumably causes release of dopamine to the nucleus accumbens (NAc, the striatal structure most closely associated with reward–like behaviors in operant paradigms). Leptin also attenuates conditioned place preference (CPP) to sucrose in food-restricted rats [55, 56] and decreases responding for sucrose on a progressive ratio operant paradigm [57]. Additionally, leptin blunts the reinforcing properties of several drugs of abuse in rodent models [58, 59]. Together these studies suggest that leptin suppresses

the incentive value of a variety of natural and artificial rewards; one proposed mechanism for this finding was the possibility that leptin suppresses mesolimbic DA release [14]. Indeed, loss of mesolimbic DA abrogates feeding in normally hyperphagic *ob/ob* (leptin-null) mice [60], and leptin decreases both basal and feeding-evoked extracellular dopamine levels in the NAc of rats [61]. Leptin has also been shown to increase the activity of TH and the dopamine transporter (DAT) as well as enhance amphetamine-stimulated DA efflux in the NAc of rats, suggesting potential mechanisms through which leptin could stimulate MLDA function [62].

In addition to these classical reward and feeding behaviors, leptin also modulates other behaviors associated with the mesolimbic DA system: Mice with low leptin levels display a variety of depressive and anxiety behaviors, and leptin treatment reverses these [63, 64]. Evidence from humans also suggests the importance of leptin in modulating the mesolimbic DA system and reward behaviors. Both leptin-deficient and calorically-restricted, weight-reduced human patients display increased hedonic drive for food, which is reversed by leptin; functional imaging studies have revealed that this leptin treatment alters the activity of a number of brains areas associated with reward and the mesolimbic DA system [65, 66]. Interestingly, obesity in humans, which is often defined as "leptin-resistance", is closely associated with increased occurrence of mood and anxiety disorders [67].

To understand how leptin might modulate mesolimbic DA function, a number of investigators have examined the possibility of LepRb-expressing

neurons in the mesolimbic DA system, revealing the expression of LepRb in a subset of DA neurons in the VTA, the source of mesolimbic DA cell bodies, as well as in the substantia nigra (SN) [68]. Hommel, et al., utilized a number of approaches to examine the potential roles for VTA leptin action in the regulation of feeding and mesolimbic DA function [69]. Direct leptin infusion to the VTA decreased food intake. Also, AAV-siRNA- mediated knockdown of LepRb expression in the VTA increased food intake and sensitivity to food reward, as measured with sucrose preference testing. Leptin was found to modestly hyperpolarize VTA DA neurons, as well. Thus, one model suggests that leptin might directly inhibit VTA LepRb-expressing DA neurons to mediate the effects of leptin on the mesolimbic DA system [14, 69].

1.6 Multiple distinct effects of leptin on the mesolimbic DA system.

Work from several decades ago, prior to the description of leptin and its receptor, had examined mesolimbic DA-mediated behaviors in the spontaneously obese Zucker *fa/fa* rat- the phenotype of which is now known to stem from a mutation in the gene encoding the leptin receptor [70]. In operant conditioning paradigms, these animals pressed levers for food at a much greater rate and consumed more food when the number of lever presses per food reward was low- consistent with the dramatic hyperphagia of these animals. Interestingly, however, the *fa/fa* rats stopped pressing the lever for food much earlier when response ratios were raised (i.e., when animals had to work hard to obtain the food reward [71]). This result reveals decreased incentive salience of food in

leptin receptor deficient animals, suggesting decreased striatal DA in the absence of leptin action. Furthermore, this result is unlikely to result solely from the obesity of these animals, since animals with genetic or lytic lesions of ventromedial hypothalamic pathways demonstrated increased rates of responding under a variety of reinforcement paradigms [71-74].

Indeed, Fulton, et al., directly examined the function of the mesolimbic DA system in Lep$^{ob/ob}$ animals, demonstrating that the VTA of these animals contain less tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthesis) in the VTA, and that DA content is decreased in the VTA and NAc of these animals [75]. Consistent with the attenuated operant responding for food by the *fa/fa* animals and the decreased mesolimbic DA content of the Lep$^{ob/ob}$ animals, the Lep$^{ob/ob}$ mice also demonstrate a severely blunted response to the DA-releasing drug, amphetamine [75]. Several days of systemic leptin treatment reversed the changes in TH expression, DA content, and amphetamine responsiveness of these animals. Consistent with these data, Roseberry, et al., while unable to detect effects of direct leptin action on the firing of VTA neurons, demonstrated decreased vesicular DA stores in VTA DA neurons of Lep$^{ob/ob}$ mice [76].

Thus, some data suggest that direct leptin action on LepRb-expressing VTA neurons controls food intake and reward, perhaps by decreasing the firing of VTA DA neurons. Leptin deficiency also decreases overall DA levels in the VTA and NAc, and these changes are restored by leptin, suggesting a role for leptin in the modulation of mesolimbic DA content, as well. Thus, leptin appears to

control at least two distinct variables in mesolimbic DA system function: 1) the firing of neurons and 2) the DA content of neurons.

DA neurons in the VTA project to numerous brain regions, including limbic structures such as the extended amygdala, cortical targets in the prefrontal cortex, and the hippocampus, in addition to their striatal targets such as the NAc [3, 13, 14]. Alternately-projecting midbrain DA neurons have recently been subdivided into distinct populations based on their VTA location and different electrophysiological properties [77-79]. These findings have lead to the suggestion that midbrain DA neural populations are more heterogeneous than previously thought, such that they can be parsed apart not only by anatomical and electrophysiological differences but also by their role in contributing to complex mesolimbic behavioral phenotypes.

The existence of anatomically and electrophysiologically diverse VTA DA neuron populations could underlie the multiplicity of dopamine function throughout the brain. Further characterization of distinct populations of VTA DA neurons, including assigning behavioral and physiological roles for these neurons is integral in fully understanding the varied roles that DA plays in the CNS. Our hypothesis is **that VTA LepRb neurons comprise a distinct subpopulation of VTA DA neurons with a circumscribed projection pattern and unique role in modulating physiology and behavior**. Our interrogation of this system using novel tract-tracing techniques and molecular tools to elucidate the distinct projections from VTA LepRb neurons and their function could identify a specific neural circuit as a potential therapeutic target for pharmacological treatment of

eating disorders. Data from these studies is discussed in detail in Chapter 2 and subsequent conclusions from these studies are further discussed in the conclusion chapter.

1.7 A leptin-regulated LHA circuit controls mesolimbic DA content

A number of lines of evidence link the LHA to the control of the mesolimbic DA system, including the reinforcing potential of electrical stimulation of the LHA and the apathetic nature of animals with LHA lesions [3, 80]. While some of these effects could be attributable to mesolimbic fibers of passage that course through the LHA, it is also clear that multiple groups of LHA neurons project to major mesolimbic centers to control DA action and reward [3, 81, 82]. Among other, less well-characterized neural populations, the LHA contains large populations of widely-projecting neurons that express melanin concentrating hormone (MCH) or orexin (OX) [81, 83]. Among their other projections, MCH neurons innervate the NAc to promote feeding. Some OX neurons innervate the VTA, and acute OX injection promotes feeding, as well as modulating DA neurons and the incentive value of drugs of abuse. While some opposite effects are observed in mice null for OX signaling [84, 85], OX clearly promotes important effects on the mesolimbic DA system.

Recent work from our laboratory revealed a set of LepRb-expressing LHA neurons that are distinct from MCH and OX cells [86]. Several lines of evidence suggest potentially important roles for these LHA LepRb neurons in feeding and the regulation of the mesolimbic DA system. In addition to projecting locally

within the LHA, many of these LHA LepRb neurons directly innervate the VTA. Furthermore, intra-LHA leptin treatment of $Lep^{ob/ob}$ animals decreases feeding, while promoting VTA TH expression and increasing NAc DA content. In contrast, intra-VTA leptin fails to modulate TH expression. Thus, leptin action via LHA LepRb neurons appears to represent a major controller of overall mesolimbic DA content.

Other populations of LepRb neurons may play roles in the mesolimbic DA system and reward processing- including LepRb neurons of the substantia nigra (SN), linear raphe (RLi) and Edinger-Westphal nuclei adjacent to the VTA, among others. Clearly, leptin also modulates the mesolimbic DA system by less direct means, involving additional synapses. Indeed, lateral hypothalamic melanin concentrating hormone (MCH) and orexin (OX) neurons project to the NAc and VTA, respectively, and modulate the mesolimbic DA system and feeding [3, 81, 82]. Neither of these leptin-inhibited populations of LHA neurons express LepRb, however [86], and leptin must act trans-synaptically to regulate MCH and OX neurons.

Additional research in our laboratory has begun to approach the interaction between LHA LepRb neurons and other LHA neural populations [87]. Through use of a Cre-inducible wheat germ agglutinin (WGA) based adenoviral trans-synaptic tract tracing system and a LepRb-specific WGA-EGFP reporter mouse ($LepRb^{WGA}$) it is possible to study neurons that make synaptic contact with individual LepRb populations. These molecular tools show that while some LHA LepRb neurons project directly to the dopaminergic neurons in the VTA,

they also densely innervate local OX containing neurons. Additionally, leptin action in the LHA is sufficient to regulate expression of several OX-related genes suggesting that the LepRb neuron – OX neuron connections are physiologically relevant. This suggests another potentially interesting manner in which leptin can modulate MLDA function. Since LHA LepRb neurons are depolarized by leptin and are GABAergic it is possible that leptin acts in the LHA to inhibit OX neurons thereby indirectly affecting downstream VTA DA neurons.

The potential for LHA LepRb neurons to indirectly affect the function of the MLDA suggests another means by which central leptin action can influence reward mediated behavioral processes. Further research into this area could be critical in understanding the complexity of leptin-MLDA interaction. Since VTA DA neurons may receive direct input from LHA LepRb neurons and indirect input from leptin-responsive OX neurons there might be bimodal regulation of the VTA by LHA leptin. Our hypothesis is that **LHA LepRb neurons can regulate the function of striatal-projecting VTA DA neurons and striatal-specific behaviors and that specific neurotransmitters in these neurons mediate specific aspects of mesolimbic function. We will assess the role of LHA LepRb neurons using a molecular mouse model lacking LepRb expression in a subset of LHA neurons as well as by assessing the role that LHA neuropeptides play in this circuit.** We predict that altered signaling through a leptin/LHA-VTA-NAc circuit will have a behavioral phenotype that corresponds to compromised striatal function, such as changes in locomotor activity or reward sensitivity. In this regard, our utilization of behavioral paradigms to further

interrogate MLDA function in molecular mouse models lacking LepRb signaling in select populations of neurons will give us further insight to the role that leptin signaling plays in reward processing and how this might affect ingestive behaviors.

Chapter 2

VTA Leptin Receptor Neurons Specifically Project to and Regulate CART Neurons of the Extended Central Amygdala[2]

2.1 *Introduction*

The adipose-derived hormone, leptin, conveys the adequacy of nutritional reserves to the CNS, where it acts to permit energy expenditure, decrease feeding, and modulate a number of other behaviors; low leptin levels promote opposite responses [11, 88-92]. Leptin acts via the long form of the leptin receptor (LepRb) on specific populations of CNS neurons to mediate most leptin action [93, 94]. LepRb-expressing neurons lie in numerous regions involved in the regulation of energy balance, including mediobasal hypothalamic (MBH) "satiety centers" (e.g. the arcuate nucleus (ARC)), as well as the lateral

[2] The work described here has been previously published: Leshan RL*, Opland DM*, Louis GW, Leinninger GM, Patterson CM, Rhodes CJ, Münzberg H, Myers MG Jr. (2010) Ventral tegmental area leptin receptor neurons specifically project to and regulate cocaine- and amphetamine-regulated transcript neurons of the extended central amygdala. J Neurosci. 2010 Apr 21;30(16):5713-23. In these studies co-author Dr. R.L. Leshan generated midbrain LepRb population figure and data as well as half of the Ad-iz/EGFPf injections to the VTA and FG to the NAc and CeA (Figures 1B-F, Figures 6-7, Figure 8, Figure 11). Dr. G.W. Louis generated the iZ/WAP transgenic mouse used in Figure 8 and Figure 14. Dr. G.M. Leinninger helped microdissect tissue used for Figure 12. Dr. CM Patterson generated the data on pSTAT3 in VTA. Dr. C.J. Rhodes packaged the viral constructs into adenovirus. Dr. H Münzberg assisted with perfusions and feedback on the manuscript. We thank Amylin Pharmaceuticals for the generous gift of leptin; we thank Dr. Yoshihiro Yoshihara, RIKEN Brain Science Institute, Japan for the gift of the WGA plasmid, Dr. Corrinne Lobe, Toronto, Canada for the iZAP plasmid and Yuchio Yanagawa, Gunma University, Japan for the generous gift of the GAD-GFP mice.

hypothalamic area (LHA), the midbrain, and the brainstem [11, 12, 52, 69, 95-98].

A number of aspects of leptin action in the MBH are beginning to be unraveled, including the role of leptin in regulating LepRb/pro-opiomelanocortin (POMC)-expressing neurons and their opposing LepRb/agouti-related protein/neuropeptide Y (AgRP/NPY)-expressing neurons in the ARC [89-92, 99]. These neurons regulate satiety and thus mediate an important component of the anorectic response to leptin, as well as modulating energy expenditure and aspects of glucose homeostasis. Many data suggest that the action of leptin on these LepRb-expressing MBH neurons only accounts for a fraction of leptin action, however [11, 98-102]. Indeed, MBH LepRb neurons represent a minority of LepRb-expressing neurons in the brain [11, 103]. Thus, populations of LepRb neurons in other brain areas must play crucial roles in leptin action.

In addition to regulating satiety, leptin regulates the incentive value of food and other rewards, as well as suppressing depression and anxiety-like behavior [53, 63, 64, 81, 95]. The mesolimbic dopamine (DA) system, which arises from DAergic neurons in the ventral tegmental area (VTA), mediates important aspects of incentive salience for food, as well as contributing to other aspects of emotion and behavior [3, 13, 81]. These VTA DA neurons project to the prefrontal cortex (PFC) and to limbic structures, such as the striatum (including the nucleus accumbens (NAc)) and the extended amygdala complex. While the historical tendency has been to consider the VTA DA neurons *en bloc*, a variety of recent observations suggest differing projection patterns, gene expression,

regulation and electrophysiologic properties for distinct subsets of these cells [77, 79, 104].

Leptin modulates DA-dependent measures of food and drug reward, and LepRb-expressing VTA neurons as well as VTA-regulating LHA LepRb neurons have been described [53, 69, 95, 96, 98]. Many questions remain regarding the sites and mechanisms whereby leptin might influence the mesolimbic DA system, however, and the direct projections from LepRb neurons into and within the mesolimbic DA system have not been examined systematically. Also unknown are the potential distinctions between LepRb-expressing and other (non-LepRb) VTA neurons. In this study, we examine neural mechanisms by which leptin may control the mesolimbic DA system by revealing the distribution of LepRb neurons and their projections in mesolimbic brain regions. We show that the CeA and its extension in the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) represent the main components of the mesolimbic DA system that are directly innervated by LepRb neurons, that leptin promotes CeA CREB phosphorylation, and that LepRb projections into the extended amygdala arise mainly from LepRb neurons in the VTA. In contrast, VTA LepRb neurons do not significantly innervate the NAc. Furthermore, LepRb neurons synapse with CART neurons in the CeA, and leptin suppresses CeA CART expression in leptin-deficient mice, suggesting that leptin action via VTA LepRb neurons modulates CeA function.

2.2 *Materials and Methods*

2.2.1 Materials.

Recombinant mouse leptin was the generous gift of Amylin Pharmaceuticals (La Jolla, CA). Fluorogold-equivalent, hydroxystilbamidine, was purchased from Biotium (Hayward, CA). Rabbit anti-pCREB was from Cell Signaling Technologies (Beverly, MA), rabbit anti-cFos was from Calbiochem (EMD Biosciences/Merck, Darmstadt, Germany), rabbit anti-Fluorogold was from Chemicon/Millipore (Billerica, MA), chicken anti-GFP was from Abcam (Cambridge, MA), rabbit anti-CART was from Phoenix Pharmaceuticals (Belmont, CA), goat anti-WGA was from Vector Laboratories (Burlingame, CA) and goat anti-β-gal was from Biogenesis (Poole, UK). Normal donkey serum and biotinylated donkey anti-rabbit were purchased from Jackson ImmunoResearch (West Grove, PA). Alexa 488-conjugated donkey anti-rabbit, Alexa 488-conjugated goat anti-chicken, and Alexa 568-conjugated goat anti-rabbit were purchased from Invitrogen (Carlsbad, CA). ABC Vectastain Elite kit was purchased from Vector Laboratories (Burlingame, CA). All other immunohistochemical supplies were purchased from Sigma.

2.2.2 Animals.

Lep$^{ob/ob}$ animals were purchased from Jackson labs. All other animals were housed and bred in our colony and according to guidelines approved by the

University of Michigan Committee on the Care and Use of Animals. Mice were given *ad libitum* access to food and water and were housed in groups of 2-4 until surgery, after which animals were individually housed. *Lepr*$^{cre/cre}$ (LepRb^{Cre}) and *Lepr*$^{cre/cre}$*;Gt(ROSA)26Sor*$^{tm2Sho/tm2Sho}$ (LepRb^{EGFP}) mice have been described and were generated by intercrossing homozygous animals within our facility [52, 105]. *Gt(ROSA)26Sor*^{tm1mgmj} (a.k.a., *Gt(ROSA)26Sor*^{EGFPf} or ROSA26-EGFPf) animals were produced and interbred with *Lepr*^{cre} mice to generate LepRb^{EGFPf} mice, as described (Leshan et al., 2009). GAD-GFP mice were bred in house and were a generous gift of Yuchio Yanagawa (Gunma University, Japan).

2.2.3 Generation of iZ/WAP mice.

The coding region for wheat germ agglutinin (WGA) was PCR-amplified from the pBluescript II SK-WGA plasmid (the generous gift of Dr. Yoshihiro Yoshihara, RIKEN Brain Science Institute, Japan) and inserted into the iZ/AP vector (the generous gift of Dr. Corrinne Lobe, Toronto, Canada (Allen et al., 2006)) downstream of the CMV promoter-driven floxed β-geo cassette. The resulting pCALL2-WGA/AP (iZ/WAP) plasmid was submitted to University of Michigan transgenic core for production of transgenic embryonic stem cell clones. Four hundred and eighty clones were screened for single copy number by qPCR for neo sequences and also screened for β-gal expression via immunocytochemical staining (β–gal staining kit, Roche. Five ES clones were expanded and rescreened, and three ES clones were injected into blastocysts and implanted into pseudopregnant females. The resulting chimeric male

progeny were bred to C57 females for the determination of germline transmission (by brown coat color) and PCR for the presence of Neo. Several F1 *iZ/WAP* mice from each ES clone were perfused and screened for CNS β-gal expression by IHC using antibodies against β-gal. One *iZ/WAP* line was determined to express the transgene ubiquitously in the CNS, and was chosen for further study. Subsequent *iZ/WAP* litters were genotyped by PCR utilizing oligos derived from WGA sequence (Forward: AATGAGAAAGATGATGAGCACC; Reverse: AGGTTGTTCGGGCATAGCTT). *iZ/WAP* mice were bred to mice containing *Lepr*^{cre} in order to generate LepRb^{WGA} mice expressing WGA in LepRb neurons.

2.2.4 Tract tracers and Stereotaxic surgery for microinjection.

The generation of Ad-iZ/EGFPf and the production of concentrated, purified adenoviral stocks were as described [106]; Leshan, 2009 #8859}. For all tract tracing experiments, LepRb^{Cre} or LepRb^{EGFP} mice were anesthetized using isofluorane and placed in a stereotaxic frame. After exposing the skull, a guide cannula with stylet was lowered into the target regions. Coordinates (from bregma) used for each brain region were as follows: VTA (AP –3.2mm, ML –0.5mm, DV –4.3mm), ICV (AP –0.6mm, ML –1.0mm, DV –2.2mm), IPAC (AP –0.5mm, ML –2.5mm, DV –4.6mm), CeA (AP –1.2mm, ML –2.8mm, DV –4.8mm), NAc (AP +1.0mm, ML –1.4mm, DV –4.8mm). The stylet was removed and replaced by an injector and either 10-20 nl of 2% fluorogold-equivalent (Sigma) to LepRb^{EGFP} mice or 200-250 nl of Ad-iZ/EGFPf to LepRb^{Cre} mice was injected to the tissue using a 500nl Hamilton syringe at a rate of 50 nl/ 30 sec. After 10

minutes for absorption of tracer, the injector and cannula were removed from the skull and the incision was sutured. Mice were then individually housed for either 3 days (FG-mediated retrograde tracing) or 5 days (Ad-iZ/EGFPf-mediated anterograde tracing) before perfusion and processing.

2.2.5 Perfusion and Immunohistochemistry.

Perfusion and immunohistochemistry were performed as previously described [107]. Briefly, mice were deeply anesthetized with a lethal dose of intraperitoneal pentobarbital (150 mg/kg) and transcardially perfused with sterile PBS and then either 4% paraformaldehyde or 10% formalin. Brains were removed, postfixed overnight and dehydrated in a 30% sucrose solution. Following cryoprotection, brains were sectioned into 30 μm coronal slices, collected in four consecutive series and stored at –20 °C until further use.

For IHC, sections were pretreated with ice-cold methanol, 0.3% glycine and 0.3% SDS before blocking. Sections were then incubated with primary antibodies [either chicken anti-GFP (1:1000), goat anti-βGal (1:1000), mouse anti-tyrosine hydroxylase (1:200), rabbit anti-pCREB (1:100), rabbit anti-CART (1:1000), or goat anti-WGA (1:1000)] overnight at 4°C. Detection of primary antibodies was done either by immunofluorescence (anti-chicken-FITC, anti-rabbit Alexa 488, anti-mouse Alexa 568, anti-goat Alexa 568; all 1:200 dilution, Invitrogen) or using the avidin-biotin/diaminobenzidine (DAB) method.

2.2.6 Mouse microdissection and analysis by qPCR.

Leptin-deficient Lep$^{ob/ob}$ mice and their WT controls were treated and processed as previously described [98]. Briefly, following a baseline day which included handling and vehicle (PBS) injections mice were treated with either leptin (5 mg/kg, i.p.) or PBS for 24 hr during which food intake and body weight were measured. Mice were then anesthetized and their brains microdissected on a rodent coronal brain matrix (1mm divisions) and frozen on dry ice. RNA was prepared from microdissected tissue using TRIzol (Invitrogen), converted to cDNA using the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). cDNA was analyzed in triplicate via quantitative RT-PCR for *Gapdh* (housekeeping gene) and *Cart* (both as supplied from Applied Biosystems) using an Applied Biosystems 7500 Real-Time PCR System. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.2.7 Image Collection, Data Analysis and Statistics.

For anterograde and retrograde tracing experiments, pictures of identical regions of brain nuclei were taken using filters for Alexa 488 or Alexa 568 as previously described [107]. Confocal microscope images were taken on an Olympus Fluoview FV500 Laser Scanning Confocal Microscope. Using Adobe Photoshop (Abode Systems, San Jose CA) images were overlaid in different RGB channels such that dual-labeled cells would become apparent. For quantification of pCREB, sections were processed in parallel for the detection of

pCREB by DAB. Images of matched sections were taken under identical microscope conditions and opened using ImageJ software (NIH, Bethesda MD). All images were converted into binary files using a standard threshold value for each set of matched images. The ratio of total area above threshold within a selection area (a circle 288 pixels in diameter) was compared between treatment groups for each brain region. Student's t-test was used to determine significance for pCREB-IR area, one-way ANOVA with Bonferroni correction for multiple interactions was used to determine significance in CART transcript changes.

2.3 *Results*

2.3.1 LepRb-expressing midbrain neurons.

Reliable detection of LepRb protein in the mouse brain using LepR-specific antibodies remains problematic. In order to reliably identify LepRb-expressing CNS neurons, we thus crossed *Lepr*^{cre} mice (in which cre recombinase is expressed specifically in LepRb-neurons) onto the *Gt(ROSA)26Sor*^{tm2Sho} (a.k.a., ROSA26-EGFP) background, in which cre-mediated deletion of a LoxP-flanked (floxed) transcription-blocking Neo cassette results in the expression of enhanced green fluorescent protein (EGFP) from the virtually ubiquitously-expressed *ROSA26* locus (Figure 1A). The expression of cre recombinase from within the LepRb-specific mRNA generated by the *Lepr*^{cre}

allele predicts the LepRb-specificity of the cre-induced EGFP expression in $LepRb^{EGFP}$ mice, and EGFP-expression in these animals coincides with functional LepRb [52, 98]. EGFP expression in neural soma in the brains of these $LepRb^{EGFP}$ mice thus reveals and facilitates the study of LepRb neurons.

The presence of EGFP-immunoreactive (IR) cells in the midbrain of $LepRb^{EGFP}$ mice is consistent with previous reports of LepRb-containing soma in the VTA by the criterion of leptin-induced (LepRb-dependent, cell-autonomous) STAT3 phosphorylation (pSTAT3) [69, 96]. EGFP-IR neurons in the caudal midbrain are located predominantly in midline nuclei, including the Edinger-Westphal (EW) and linear raphe (RLi), as well as in the medial aspects of the VTA (Figure 1B, C; courtesy of Rebecca Leshan). The mid- and rostral portions of the midbrain contain a large number of EGFP-IR neurons in the DAergic portions of the VTA and the substantia nigra pars compacta (SNc), as well as in the same medial nuclei as in the caudal midbrain (Figure 1D-G; courtesy of Rebecca Leshan). 74.9% ±3.9 (SEM; n=6) of EGFP-IR cells in the VTA colocalize with tyrosine hydroxylase (TH)-IR, consistent with the DAergic nature of the majority of VTA LepRb neurons (courtesy of Rebecca Leshan). Of the TH-containing VTA neurons, approximately 6% expressed EGFP, suggesting that LepRb neurons represent a relatively small and potentially specialized subset of VTA DA neurons.

2.3.2 LepRb projections to the limbic regions of the mesolimbic DA system primarily target the extended central amygdala.

Since standard cytoplasmic EGFP reveals neural soma but poorly labels long projections (such as axons), we also utilized ROSA26-EGFPf mice, in which cre recombinase-mediated excision of a transcription-blocking cassette induces the expression of a farnesylated EGFP (EGFPf) from the *ROSA26* locus (Figure 1) [52]. Farnesylation drives EGFPf to the membrane, effectively labeling even very long axonal projections [52, 98, 108]. We crossed ROSA26-EGFPf animals to *Lepr*^{cre} mice in order to generate LepRb^{EGFPf} mice to facilitate the study of projections from cre-expressing LepRb neurons [52].

To determine the potential points of direct interaction between LepRb neurons and brain regions integral to the mesolimbic DA system, we examined the midbrain and extended amygdala/striatum of LepRb^{EGFP} and LepRb^{EGFPf} mice for the presence of EGFP-IR soma and projections, respectively (Figure 2; in conjunction with Rebecca Leshan). With respect to soma, in contrast to the midbrain, no EGFP-IR cell bodies were detected in the extended amygdala or striatum of LepRb^{EGFP} mice, suggesting that these regions do not contain LepRb neurons (Figure 2; in conjunction with Rebecca Leshan). This result is consistent with previous studies that revealed no evidence of LepRb-specific mRNA in these regions [97, 109], and consistent with our finding that these regions are devoid of pSTAT3-IR following treatment with leptin (data not shown). Thus, the midbrain contains all of the LepRb-expressing soma within the mesolimbic DA system itself. Other leptin-mediated inputs to this system must stem from projections into the mesolimbic DA system from LepRb neural soma that lie elsewhere, or less directly, via projections from second-order neurons.

To determine the regions of the mesolimbic DA system that receive direct projections from LepRb neurons, including those neurons residing elsewhere in the brain, we examined EGFP-IR in the midbrain, amygdala, and striatum of $LepRb^{EGFPf}$ mice (Figure 2; in conjunction with Rebecca Leshan). Within the midbrain, we observed EGFP-IR within the VTA, SN, and midline nuclei (EW, RLi) that contain EGFP-IR/LepRb soma in the $LepRb^{EGFP}$ animals. Since the EGFP-IR in these midbrain regions of $LepRb^{EGFPf}$ animals could derive from local LepRb neurons and/or projections from LepRb neurons located elsewhere in the brain, we examined potential LepRb projections into the VTA by examining colocalization of EGFP and fluorogold (FG) following intra-VTA FG injection in $LepRb^{EGFP}$ animals (Figure 3). This analysis revealed that, along with LepRb neurons in the lateral hypothalamic area (LHA) that project to the VTA [98] a few LepRb neurons in the periaqueductal grey (PAG) and hypothalamic preoptic area (POA), but not elsewhere in the brain, project to the VTA.

The limbic target regions of the mesolimbic DA system in $LepRb^{EGFPf}$ animals contained substantial EGFP-IR projections from LepRb neurons in the extended central amygdala- specifically, the CeA and its rostral extension- the IPAC. In contrast, other important rostral regions such as the nucleus accumbens (NAc) contained little EGFP-IR, although substantial EGFP-IR projections (and a few soma) were apparent in the adjacent bed nucleus of the stria terminalis (BNST) (Figure 2; in conjunction with Rebecca Leshan). Thus, the CeA and IPAC represent the major amygdala/striatal projection fields of LepRb neurons. Since the amygdala (including the CeA and IPAC) and striatum

contain no LepRb neurons, the EGFP-IR in these regions of LepRb^{EGFPf} animals must represent projections from distant LepRb neurons.

To examine the potential regulation of the midbrain and striatum/amygdala by leptin, we administered leptin (5 mg/kg, IP, 2 hours) to *Lep$^{ob/ob}$* (*ob/ob*) animals (which are leptin-deficient and highly leptin-sensitive) and examined the phosphorylation of CREB by IHC (pCREB-IR) (Figure 4). This analysis revealed that leptin promoted the several-fold induction of pCREB-IR in the VTA and CeA, but neither in the adjacent basolateral amygdala (BLA) nor the NAc, consistent with the leptin-mediated regulation of the CeA projection field identified in this analysis. No alteration of pCREB was observed in the IPAC, however (data not shown). Thus, LepRb neurons densely innervate and modulate the activity of the CeA.

2.3.3 VTA LepRb neurons primarily innervate the CeA and IPAC.

To determine the extent to which LepRb projections into the CeA and IPAC might derive from the well-known AgRP- or POMC-expressing LepRb neurons of the ARC, we examined AgRP- and POMC-IR and their potential colocalization with EGFP-IR in the extended amygdala and paraventricular hypothalamic nucleus (PVH) of LepRb^{EGFPf} mice (Figure 5). While this analysis revealed the expected copious colocalization of EGFPf with AgRP and POMC in the PVH (a major projection target of ARC neurons), the few AgRP- and POMC-IR axons in the amygdala largely lay outside the CeA and IPAC regions that are

densely innervated by LepRb/EGFPf projections, suggesting that the LepRb projections to the extended amygdala derive from LepRb neurons other than those in the ARC.

Conventional anterograde tracing studies have demonstrated the projection of VTA neurons into multiple limbic brain regions, including the NAc, extended amygdala, and other areas. Such studies do not differentiate the projections of LepRb-expressing cells from those of other VTA neurons, however. To define projections specifically from LepRb-expressing soma in the VTA, we thus utilized Ad-iZ-EGFPf, which merges the use of EGFPf-mediated tracing with the cre-inducible system (for LepRb-specificity) and adenoviral stereotaxic injection (for anatomic specificity) (Figure 1A) [52, 98]. We administered Ad-iZ/EGFPf into the VTA of $LepRb^{cre}$ mice and perfused them 5 days later for immunofluorescent analysis. While administration of Ad-iZ/EGFPf produced copious EGFPf-expression in $LepRb^{cre}$ mice (Figures 6-7); data collected in conjunction with Rebecca Leshan), no EGFPf expression was detected in wild-type animals (data not shown), confirming the cre-specificity of EGFPf expression.

Some Ad-iZ/EGFPf injections labeled LepRb neurons that were largely confined to the VTA (Figure 6; data collected in conjunction with Rebecca Leshan), while others tended to target LepRb neurons in midline structures, such as the RLi (Figure 7; data collected in conjunction with Rebecca Leshan), or included lateral areas, such as the SNc (Figure 8). Injections confined within the VTA revealed the dense innervation of the CeA and IPAC by VTA LepRb

neurons, along with the paucity of projections from LepRb VTA neurons to the NAc. Close examination of the EGFP-IR projections from VTA LepRb neurons into the CeA and IPAC revealed a “beads-on-a-string” appearance consistent with synaptic terminals in these projection fields. Thus, these data reveal that LepRb-expressing VTA neurons primarily innervate the CeA and IPAC, not the NAc.

While the distribution of axonal labeling from midline-centered injections overlapped substantially with that of VTA labeling, tracing from the midline midbrain LepRb neurons produced more widespread EGFP-IR projections (Figure 7; data collected in conjunction with Rebecca Leshan). In addition to demonstrating projections to extended central amygdala nuclei (CeA and IPAC), these midline injections demonstrated some modest innervation of the NAc relative to that observed in VTA-focused injections. These data suggest that the LepRb projections into the CeA and IPAC that are visualized in $LepRb^{EGFPf}$ mice arise from LepRb neurons throughout the midbrain, while the relatively small number of LepRb neurons that innervate the NAc may derive from midline midbrain structures, such as the RLi. Examination of projections from mice in which the viral injection included SNc (as well as VTA) labeling revealed substantial additional projections to the dorsal striatum (Figure 8), suggesting that many SNc LepRb neurons project to the dorsal striatum, as for the majority of SNc neurons.

2.3.4 Retrograde tracing experiments confirm the distribution of amygdala- and striatal-projecting LepRb neurons.

To examine these circuits more closely and to verify the projection patterns of VTA and midline midbrain neurons, we utilized retrograde tracing with fluorogold (FG) from the CeA, IPAC, and NAc in $LepRb^{EGFP}$ mice to determine the location of LepRb neurons that project to each of these regions (Figures 9-12). Following the injection of FG into the CeA, the midbrain demonstrated accumulation of FG predominantly in the VTA, with few FG-IR neurons seen in the RLi and SN (Figure 9; data courtesy of Rebecca Leshan). Many VTA LepRb neurons, primarily those clustered in the dorsal portions of the VTA, accumulated FG from the CeA. CeA FG failed to accumulate in EGFP-containing LepRb neurons outside of the VTA (data not shown), suggesting that essentially all LepRb projections to the CeA arise from VTA LepRb neurons. Triple-label immunofluorescence for EGFP, FG, and TH in these sections confirmed the expression of TH in some CeA-projecting VTA LepRb neurons, consistent with the DAergic nature of some of these neurons (Figure 10).

Following injection of FG into the IPAC, concentrated FG immunoreactivity was seen in lateral midbrain nuclei of these animals, especially the SNc and lateral portions of the VTA (Figure 11). No FG tracing extended to LepRb-containing midline nuclei. Substantial co-localization of EGFP and FG was seen in both the VTA and the SNc (Figure 11). As for CeA-labeled LepRb VTA neurons, triple-label immunofluorescence for EGFP, FG, and TH in these

sections confirmed the expression of TH in some IPAC-projecting VTA LepRb neurons, consistent with their DAergic nature (Figure 10).

Following FG injection in to the NAc, strong and extensive FG-IR was observed in the midbrain, including in the VTA, SN, and some midline nuclei (Figure 12; data courtesy of Rebecca Leshan). Interestingly, while EGFP-expressing LepRb VTA neurons were surrounded by VTA neurons that accumulated FG from the NAc, LepRb neurons were not among these NAc-projecting VTA neurons. In contrast, some RLi LepRb neurons accumulated FG from the NAc. Triple-label immunofluorescence for EGFP, FG, and TH in these sections failed to detect TH in NAc-projecting midbrain LepRb neurons (Figure 10). Thus, DAergic VTA LepRb neurons project to the CeA and IPAC, but not to the NAc, while midline midbrain LepRb neurons, such as those in the RLi, send a small number of projections to the NAc in addition to heavily innervating the extended central amygdala complex.

2.3.5 LepRb neurons synaspe with and regulate CART neurons.

To gain insight into the CeA neural targets of VTA LepRb neurons, we developed and utilized the *iZ/WAP* transgenic mouse strain, which mediates cre-inducible expression of the trans-synaptic tracer wheat germ agglutinin (WGA) under control of the CMV promoter (Figure 13A). Although this strain is similar in principle to a previously-described cre-inducible WGA line [110], the previous strain demonstrated little WGA expression in LepRb-expressing brain regions,

presumably as a consequence of the transgene insertion site. We crossed these new *iZ/WAP* mice to the LepRb^{cre} background to produce LepRb^{WGA} mice with WGA expression in LepRb neurons throughout the brain, thereby promoting WGA accumulation in the synaptic targets of LepRb neurons (Figure 13A). Examination of WGA-IR in these LepRb^{WGA} animals revealed the presence of WGA in regions receiving projections from LepRb neurons, including a dense cluster of WGA-IR neurons in the CeA (Figure 13B). In contrast, areas receiving few LepRb projections (including the BLA and NAc) revealed little WGA-IR, and mice lacking either the *iZ/WAP* or *Lepr^{cre}* allele displayed no WGA-IR (data not shown). Standard immunofluorescence for the neuropeptide, cocaine and amphetamine regulated transcript (CART) revealed the presence of WGA-IR in most CeA CART-IR neurons, suggesting that LepRb neurons form synapses with CART-expressing neurons in the CeA (Figure 13C-E). To examine the potential functional relevance of this circuit, we compared the expression of *Cart* mRNA in the CeA of wild-type or leptin-deficient Lep$^{ob/ob}$ animals following treatment with leptin (5 mg/kg, IP) or vehicle for 24 hours (Figure 13F). This analysis revealed the greater than 3-fold induction of *Cart* mRNA in Lep$^{ob/ob}$ relative to wild-type animals, and the normalization of CeA *Cart* mRNA by leptin treatment in Lep$^{ob/ob}$ mice. Taken together with the finding that VTA (but not other) LepRb neurons project to the CeA, these data suggest that VTA LepRb neurons form active synapses with CART-expressing CeA neurons and regulate *Cart* mRNA expression in these neurons. Additionally the majority (95% +/-1%) of CeA CART neurons express Gad1, which produces GABA (Figure 14), so GABA

signaling by CeA CART neurons also likely participates in the action of VTA LepRb neurons.

2.4 *Discussion*

Our use of LepRb-specific genetic and adenoviral systems reveals a limited set of direct interactions between LepRb neurons and brain regions of the mesolimbic DA system: The midbrain contains LepRb neurons as well as receiving projections from LepRb neurons of the LHA [98] as well as the PAG and POA. Most LepRb projections into the amygdala and striatum target the CeA and IPAC components of the extended central amygdala. These LepRb projections to the extended amygdala derive from the midbrain, including the VTA (Figure 15). VTA LepRb neurons project solely to the extended central amygdala, targeting the CeA and IPAC. Within the CeA, LepRb projections synapse with CART neurons, and regulate their gene expression. The LepRb neurons that originate in the midline nuclei of the midbrain (e.g., RLi) send a few projections to the NAc in addition to densely innervating the extended central amygdala. The CeA and IPAC receive the vast majority of projections from both VTA and midline midbrain LepRb neurons, however. This specificity of projections from LepRb VTA neurons fits well with other recent findings that describe discrete patterns of projection, gene expression, and functional properties for subsets of VTA DA neurons [77, 79, 104].

As for all experimental tools, the Ad-iZ/EGFPf and LepRb^{EGFPf} systems possess inherent limitations. The expression of EGFPf in the brains of transgenic LepRb^{EGFPf} mice is modest compared to that mediated by the higher copy number and stronger promoter system of the Ad-iZ/EGFPf, rendering it difficult to detect the relatively weak innervation of the NAc by LepRb neurons that could be observed with midline midbrain injection of Ad-iZ/EGFPf. Overall, the LepRb^{EGFPf} mice clearly reveal the much greater density of LepRb projections into the extended amygdala than the NAc, however. That leptin promotes CeA CREB phosphorylation and modulates CeA *Cart* expression suggests the functional relevance of this circuit for CeA physiology.

For Ad-iZ/EGFPf studies, the necessity of utilizing mice (specifically, transgenic animals with cre recombinase expression in LepRb neurons) with their small brains and resultant close spacing among midbrain nuclei limits the extent to which it is possible to isolate VTA relative to RLi LepRb labeling. The advantages of these systems, however, include the strict specificity for LepRb neurons, and the prospective interrogation of projections from LepRb neurons or anatomically-defined subpopulations of LepRb neurons. In this case, the use of the Ad-iZ/EGFPf system revealed the heretofore unsuspected dominant innervation of the CeA and IPAC by midbrain LepRb neurons, which were confirmed by standard tracing methods.

While others have previously demonstrated the presence of LepRb-expressing VTA neurons [69, 95, 96], the potential manner(s) in which LepRb VTA neurons might differ from other VTA neurons was not clear. Here, we

demonstrate the virtually exclusive innervation of the CeA and IPAC by LepRb VTA neurons, which contrasts with the predominant innervation of the NAc by the larger general population of VTA neurons. Although Fulton, et al., previously suggested that midbrain LepRb neurons innervate the NAc, the location of the LepRb neurons in question was not clear from the data shown [96]; based upon our present results, we surmise that the NAc-projecting LepRb midbrain neurons identified lie within the RLi or other midline areas of the midbrain, rather than from VTA DA LepRb neurons.

The laboratory of DiLeone has directly examined the role for midbrain LepRb action in long-term energy balance: Direct bilateral application of leptin to the midbrain of normal rats decreased food intake over 24 hours, and AAV-RNAi-mediated knockdown of midbrain LepRb in rats increased food intake, activity, and sucrose preference without altering body weight [69]. These data suggest a role for midbrain LepRb neurons in the modulation of feeding and activity. Our present findings regarding the projection patterns of VTA and midbrain LepRb neurons suggest the possibility that these LepRb neurons may also mediate behaviors not previously examined, however, and that previous data regarding the function of these neurons should be considered in this light.

In addition to promoting incentive salience, midbrain neurons in general (and VTA DA neurons specifically) also function in the modulation of anxiety behaviors and in learning related to aversive stimuli, and aversive signals from the VTA may be conveyed by specific subsets of midbrain neurons [111]. The well-known role of the CeA in anxiety and the behavioral response to aversive

stimuli suggests a potential role for amygdala-projecting VTA neurons in such reactions. It is therefore possible that LepRb VTA neurons primarily modulate aversive reactions and anxiety. Indeed, leptin decreases depression and anxiety-like behaviors in leptin-deficient and normal animals [63, 64]. Additionally, evidence that CeA-projecting LepRb neurons (i.e., VTA LepRb neurons) synapse with CeA CART neurons and regulate their *Cart* mRNA expression suggests that leptin may modulate CART-associated behaviors in the amygdala. Increased CeA *Cart* expression correlates with anxiety, depression, and stress responses under a variety of conditions [112-115].

While the effect of leptin on the firing and gene expression in LepRb VTA neurons remains unclear, due to the inability to record specifically from LepRb neurons, Hommel, et al., suggested that leptin hyperpolarizes VTA DA neurons [69, 76]. Indeed, our finding that leptin decreases *Cart* mRNA expression in the CeA not only suggests the functional relevance of this circuit, but is consistent with the notion that leptin decreases DA efflux into the CeA, since DA promotes *Cart* expression in the CeA and elsewhere [116]. One reasonable hypothesis thus suggests that leptin action via VTA LepRb neurons regulates the extCeA and CeA-directed behaviors. A great deal more work will be required to fully examine this issue, however.

Previous data also demonstrate that leptin promotes the expression of tyrosine hydroxylase (TH; the enzyme that catalyzes the rate-limiting step in DA synthesis) as well as increasing vesicular DA stores in the VTA and NAc [76, 96]. Recent data suggest that leptin action via LHA LepRb neurons plays a major role

in these effects, however [98] consistent with our present finding that the LHA contributes substantial LepRb projections into the VTA. The modulation of DA production and content in the VTA by this leptin-controlled pathway may contribute to the modulation of incentive value, the response to drugs of abuse, and other dopamine-dependent behaviors.

Clearly, leptin also controls the mesolimbic DA system by less direct means, involving additional synapses. Indeed, lateral hypothalamic melanin concentrating hormone (MCH) and orexin (OX) neurons project to the NAc and VTA, respectively, and modulate the mesolimbic DA system and feeding [117]. Neither of these leptin-inhibited populations of LHA neurons express LepRb, however [98], and leptin must act trans-synaptically to regulate MCH and OX neurons.

Overall, our data reveal a specific and circumscribed set of projections from LepRb neurons into the extended central amygdala, and that these projections stem primarily from midbrain (especially VTA) LepRb neurons. Based upon these data and our finding that leptin controls the activity and *Cart* gene expression in CeA neurons, midbrain leptin action likely controls an amygdala-specific subset of the functions ascribed to the larger mesolimbic DA system.

Chapter 3

Lateral Hypothalamic LepRb Neurons and Striatal Behaviors

3.1 Introduction

A quickly expanding body of literature supports the idea that extra-hypothalamic leptin action is integral to the overall effect of leptin on the CNS [97, 118]. One facet of leptin action in the CNS is modulation of reward systems that affect control over food intake. There exist several subpopulations of LepRb neurons that are candidates for control of the MLDA [119]. Work from our laboratory presented in Chapter 2 identified a novel population of LepRb neurons that originate in the VTA and project specifically to extended amygdala nuclei where they regulate the expression of CART peptide in GABAergic CeA neurons. Based on their physiology and projection pattern, these neurons are likely to be responsible for modulating anxiety-, fear- or stress-mediated responses, all limbic aspects of MLDA action [120-122]. Published data discussed at the beginning of Chapter 2 points to the fact that leptin plays a role in conditioned place preference for food and drug rewards as well as in break point under progressive-ratio reinforcement schedules, suggesting modulation of striatal DA signaling by leptin [55-57, 95]. It remains unclear as to how striatal dopamine neurons are regulated by leptin signaling and precisely how leptin acts through these neurons to modulate striatal components of reward, however.

One potential target by which leptin might modulate reward systems, the lateral hypothalamic area (LHA), has been the target of both ingestive behaviors and reward research for over 50 years. Contrasting roles for hypothalamic nuclei in regulating food intake and energy expenditure have been the focus of research since lesion studies in the 1950s. Low levels of electrical stimulation to the LHA induce feeding and excitotoxic lesions of the LHA lead to rodents that do not feed [123]). These early studies suggested that the LHA plays an important role in food intake, although the particular experimental techniques available during that era left it unclear as to the exact mechanisms by which this was possible [9]. In addition to lesion studies, early reward paradigms also utilized lateral hypothalamic self stimulation (LHSS) in which animals were shown to self-respond in order to receive low levels of electrical stimulation to the LHA, suggesting reward-related functionality for this brain region [124-126]. While these early studies were limited by the neuroanatomical confound that LHSS excites numerous brain regions and neural tracts that pass through or neighbor the LHA, a role for the LHA in modulating reward was a reasonable hypothesis.

More recent molecular analysis of the LHA identified several important populations of neurons that mediate both ingestive behaviors as well as modulate mesolimbic reward. Anatomically distinct populations of orexigenic neurons that contain either melanin concentrating hormone (MCH) or orexin (OX, also known as hypocretins) have been shown to project to the striatum or the VTA, respectively [127-131]. Signaling through these systems has been shown to affect both feeding as well as several different measures of reward [132-135].

While MCH was originally isolated as a peptide inhibitor of α-MSH-induced pigment change in salmon scales [136], it was thereafter shown to increase feeding and block the anorectic effect of α-MSH when administered centrally. The bulk of MCH neurons project to the NAc, where they synapse on neurons that express both D1- and D2-dopamine receptors [137, 138]. Administration of MCH potentiates cocaine-induced locomotor activity in mice and antagonists of the MCH receptor MCHR1 attenuate responding for cocaine and block cue-induced reinstatement [138]. Mice lacking MCHR1 expression have increased DAT expression and increased evoked dopamine release suggesting dysregulation of the MLDA and the importance of MCH signaling in proper function [139-141].

However important orexin neurons may be in food intake and reward, another crucial role seems to be to mediate alertness, arousal and sleep cycling [142, 143]. Orexin itself induces feeding, and mice lacking orexin, apart from being mildly hypophagic, are narcoleptic and have decreased overall activity levels [144, 145]. OX neurons project to the VTA and potentiate the response of VTA DA neurons to drugs of abuse suggesting that they play an important role in modulating reward systems [146].

Importantly, leptin action affects both populations of LHA neurons: leptin inhibits *Mch* expression, inhibits firing of OX neurons and blocks fasting-induced activation of OX neurons [84, 147-149]. Counterintuitively, leptin also increases *Ox* expression, suggesting that leptin has a complex interaction with the orexinergic system and can affect signaling through this pathway at numerous

levels. Until recently, however, the neural mechanisms by which leptin modulated LHA OX neurons was unclear.

Recent work from our laboratory has elucidated this interaction through identification of an additional functionally-relevant population of LHA neurons [86]. Leptin receptor-expressing neurons within the LHA are an anatomically distinct set of neurons from MCH and OX neurons that project to dopaminergic neurons in the VTA. This population of LepRb neurons contains GABA. Leptin administration to the LHA causes a decrease in food intake and body weight. Direct infusions of leptin to the LHA also increase expression of TH in the VTA and increase DA content in the NAc. A significant subpopulation of LHA LepRb neurons are depolarized by leptin. These initial findings suggest that the LHA is a likely candidate for approaching the mechanisms by which leptin can modulate striatal MLDA function.

Further work from our laboratory elucidated the LepRb circuit that originates in the LHA [150]. Using a LepRb specific viral-based tract tracing system, it was shown that LHA LepRb neurons have local projections within the LHA as well as direct projections to the VTA. LHA LepRb neurons lie in synaptic contact with OX, but not MCH, neurons. Leptin in the LHA regulates expression of OX mRNA. Thus, leptin action in the LHA can modulate the MLDA both directly as well as indirectly, through the OX system.

The neuropeptide, neurotensin, is co-expressed in about 60% of the LHA LepRb neurons, suggesting a role for this subpopulation of leptin responsive

neurons in modulating the MLDA [151-154]. Neurotensin (Nts) has a well established role in the control of feeding, as well as modulating the MLDA. Nts exerts opposite effects on dopamine transmission in the brain: injection into the NAc causes neuroleptic-like effects, whereas injection into the VTA causes psychostimulant-like effects, suggesting that the effects of Nts are predominantly determined by whether it is acting presynapticly or postsynapticly. Nts itself is anorexigenic when administered ICV and hypothalamic Nts expression is decreased in the $Lep^{ob/ob}$ mouse, suggesting a potential role for the neuropeptide in regulating food intake and leptin action [155, 156].

Of utmost important to our research is the fact that Nts only colocalizes with LepRb neurons in the LHA (not elsewhere in the brain), providing a specific marker for the LHA LepRb neurons (our unpublished data). This peptide is expressed primarily in LHA LepRb neurons that project locally to LHA OX neurons (and through them indirectly to the VTA), with the bulk of the remaining non-Nts expressing LepRb neurons presumably projecting directly to the VTA (Figure 16). This proposed circuitry for LHA LepRb neurons and how they may modulate VTA DA neurons suggests that there is both a direct and indirect pathway to the VTA. Given that Nts is a specific marker of a LHA LepRb subpopulation we generated a molecular mouse model in which LepRb expression is ablated in Nts neurons ($LepRb^{Nts}$ KO mouse – see methods section for detailed description). $LepRb^{Nts}$ KO mice have increased body weight due to increased adiposity (Figure 17A,C-D, n = 25 for KO and Ctrl, $p < 0.01$, data courtesy of Gina Leinninger), have slightly increased food intake (Figure 17B, n =

25 for KO and Ctrl, $p < 0.05$ weeks 5 and 7, data courtesy of Gina Leinninger) and lower baseline locomotor activity compared to littermate control mice (Figure 17E, n = 25 for KO and Ctrl, $p < 0.01$, data courtesy of Gina Leinninger). This phenotype has similarities to another transgenic mouse, the OXR_2 knockout mouse, in that it has modest alterations in food intake, increased body weight and lower baseline activity levels [84]. This similarity supports our hypothesis that leptin action in the LHA importantly modulates OX neuron function. Additional evidence of the regulation of OX by LHA leptin neurons comes from quantitative analysis of mRNA expression in the LHA of $LepRb^{Nts}$ KO mice treated with leptin. Treatment with i.p leptin (5 mg/kg, 2 hours) can significantly increase OX mRNA in the LHA of WT mice (Figure 18, PBS = 1.69 ± 0.60; Leptin = 6.03 ± 1.85; n = 12,10 respectively; $p < 0.05$) but not in KO mice (Figure 18, PBS = 2.67 ± 0.84; Leptin = 2.20 ± 0.85; n = 14, 17 respectively; $p < 0.05$ compared to Control + leptin group). This implicates a potentially important role for LHA leptin signaling in the expression of LHA orexin. Regarding the MLDA, although the trend to increased DA and decreased DOPAC in the NAc of $LepRb^{Nts}$ KO mice is not significant, the DA:DOPAC ratio in the NAc is elevated (Figure 17F, Ctrl = 3.2 ± 0.23, n = 12; KO = 3.8 ± 0.18, n = 15, $p < 0.05$, data courtesy of Gina Leinninger). While not yet confirmed by other means, these data are consistent with the notion of decreased DA release and turnover in the NAc of these animals.

These LHA LepRb neurons are a likely candidate to mediate leptin's modulation of striatal function via the LHA-VTA-NAc circuit. **We hypothesize**

that leptin action via LHA LepRb neurons is integral for MLDA-mediated behaviors, particularly those dependant on striatal components of the MLDA. Additionally, Nts and orexin may mediate the effects of LHA leptin action on OX and VTA and NAc DA neurons as well a striatal behaviors. Elucidating these mechanisms would give us a better understanding of the relationship between energy status and reward systems allowing better targeting of therapeutic agents to control both ingestive behaviors as well as compulsive reward driven behaviors.

3.2 Materials and Methods

3.2.1 Materials.

Recombinant mouse leptin was the generous gift of Amylin Pharmaceuticals (La Jolla, CA). d-Amphetamine hemisulfate salt was purchased from Sigma Aldrich (St. Louis, MO).

3.2.2 Animals.

Lep$^{ob/ob}$ mice (on BL/6 background), C57BL/6 (WT) mice and NtsR1KO mice were purchased from Jackson Laboratories (Bar Harbor, ME). All other animals were housed and bred in our colony and according to guidelines approved by the University of Michigan Committee on the Care and Use of

Animals. Mice were given *ad libitum* access to food and water unless otherwise noted in experimental methods. All mice used in behavioral studies were single housed post-weaning (3 weeks old) for at least 2 weeks prior to testing to allow for habituation, otherwise mice were housed in groups of 2-5. *Lepr*$^{cre/cre}$ (LepRb^{Cre}) mice have been described previously and were generated by intercrossing homozygous animals within our facility *[157, 158].*

Mice were maintained on a 12:12 light-dark schedule (lights on at 06:00 h) and all behavioral testing was conducted between 08:00 h and 12:00 h. Thus behavioral responses were obtained during the natural nadir in serum leptin levels in mice [159]. Unless otherwise noted, different cohorts of mice were used for each behavioral test, except in the case of mice undergoing anxiety-testing paradigms (elevated-plus maze and light-dark box) which were performed on the same mice with 2 days in between testing. All behavioral testing was conducted in accordance to the guidelines of the University of Michigan Committee on the Care and Use of Animals.

3.2.3 Generation of the Nts-cre and LepRb^{Nts}KO mouse.

We modified the Nts IRES-Tau-GFP targeting sequence (a kind gift of Dr. Peter Mombaerts, [160]) to create mice that express cre recombinase in Nts-expressing cells (Nts^{cre} mice). Briefly, we replaced the Tau-GFP coding sequence with an IRES-cre cassette (previously described in Leshan et al 2006) downstream of the 3' non-coding region of the Nts mRNA. The resulting vector

(Nts^{cre} is similar to the Mombaerts vector except that Cre is expressed in Nts-expressing cells instead of Tau-GFP, and the Neo selection cassette is flanked by FRT sites for Flp-mediated removal instead of being floxed for removal by Cre (since successful targeting of the cre recombinase could theoretically delete the neo cassette and prevent the selection of the cells). The Nts^{cre} construct was then linearized and electroporated into mouse ES cells, which were seeded into 96-well plates, then expanded into larger 24-well plate cultures to increase DNA yield.

293 clones were obtained and screened by qPCR for loss of homozygousity, using primers and probe against Nts and NGF (Nts Forward: 5' TGA AAA GGC AGC TGT ATG AAA ATA A, Nts Reverse: 5' TCA AGA ATT AGC TTC TCA GTA GTA GTA GGA A, Nts Probe: 6FAM-CCA GAA GGC CCT ACA TTC TCA AGA GG-Tamra, NGF Forward: 5' TGC ATA GCG TAA TGT CCA TGT TG, NGF Reverse: 5' TCT CCT TCT GGG ACA TTG CTA TC, NGF Probe: 5' VIC-AGC GTT CTG CCT GTA CGC CGA TCA-TAMRA). Correct insertion prevents the targeted Nts allele from amplifying, so the detected gene copy falls from 2 to in any positive clones. Of the 293 original clones, 8 putative positives were re-screened by Southern Blot for final confirmation. One of the 8 clones was positive by both analyses, and was linearized and injected into mouse blastocysts to generate chimeras.

Chimeras were bred to C57/Bl6 animals to determine germline transmission. Germline transmission of ES cell-derived material was determined by coat color, while transmission of the targeted allele in progeny was

determined by qPCR for Nts (as described above) and conventional PCR for neo. Germline mice found to contain the Nts^{Cre} allele were crossed with *Gt(ROSA)26-Sor*^{tm2Sho} mice (Jackson Laboratory) to generate Nts^{GFP} reporter mice or with $Lepr^{flox/flox}$ mice (a generous gift from Dr. Streamson Chua) to generate $Lepr^{Nts}$KO mice (Nts^{Cre}- $Lepr^{flox/flox}$) and littermate controls ($Lepr^{flox/flox}$).

3.2.4 Energy expenditure, respiratory quotient and body composition analysis

Oxygen consumption (VO_2), carbon dioxide production (VCO_2), spontaneous motor activity and food intake were measured using the Comprehensive Laboratory Monitoring System (CLAMS, Columbus Instruments), an integrated open-circuit calorimeter equipped with an optical beam activity monitoring device. Mice were weighed each time before the measurements and individually placed into the sealed chambers (7.9" x 4" x 5") with free access to food and water. The study was carried out in an experimentation room set at 20-23 °C with 12-12 hours (6:00PM~6:00AM) dark-light cycles. Animals were allowed to stay for at least 48 hours in acclimation dummy chambers before transferred into the measuring chamber. The measurements were carried out continuously for 48~72 hours. During this time, animals were provided with food and water through the equipped feeding and drinking devices located inside the chamber. The amount of food of each animal was monitored through a precision balance attached below the chamber. The system was routinely calibrated each time before the experiment using a standard gas (20.5% O2 and 0.5% CO_2 in

N_2). VO_2 and VCO_2 in each chamber were sampled sequentially for 5 seconds in a 10 minutes interval and the motor activity was recorded every second in X and Z dimensions. The air flow rate through the chambers was adjusted at the level to keep the oxygen differential around 0.3% at resting conditions. Respiratory quotient (RQ), also known as respiratory exchange ratio (RER), was calculated as VCO_2 / VO_2. Total energy expenditure, carbohydrate oxidation, and fatty acid oxidation can be calculated respectively based on the values of VO_2, VCO_2, and the protein breakdown (which is usually estimated from urinary nitrogen excretion). Body fat, lean mass, and free fluid were measured using an NMR analyzer (Minispec LF90II, Bruker Optics). The measurement takes less than 2 minutes while conscious mice or rats were placed individually into the measuring tube with a minimum restrain. The machine is daily checked using a reference sample as recommended by the manufacture.

3.2.5 Behavioral Testing.

Anxiety testing paradigms were video recorded from above and later hand-scored by an independent observer blinded to the genotypes of mice being tested and verified for accuracy by a second observer (not blind).

3.2.6 Elevated Plus maze.

Thirty-seven mice (19 LepRb^{Nts} KO mice and 18 (Lepr$^{flox/flox}$) controls, lacking *Cre*) were tested in the elevated plus maze (EPM) as previously described [161]. The maze itself is elevated 51 cm from the floor and has four arms that are 54 cm long and 6 cm wide. The arms are situated in a cross shape with an 6 cm x 6 cm square platform in the center allowing access to all four arms. Two opposing arms are enclosed by 14 cm high clear acrylic walls (closed arms) with the other two arms left exposed (open arms). Following 1 hour habituation to the testing room, mice were gently placed in the center of the maze with the mouse facing an open arm and behavior was recorded for the subsequent 5 minute period. Dependent measures included: (1) time spent in the open arm and closed arm (center square considered an extension of the closed arms due to proximity of acrylic walls); (2) number of entries into both closed and open arms; (3) latency to enter the open arm from start of experiment and (4) percent open arm entries (open entries / total entries). An entry to an arm was considered when all four paws of a mouse entered a given arm. Testing occurred under dim lighting (100 lux). Any mice that fell from the testing apparatus were disqualified from scoring (n=6 mice, 3 from each genotype tested), except for measurement of latency to enter open arm.

3.2.7 Light-Dark box.

The light-dark (LD) box is a two-chamber apparatus that is 46 cm in length, 28 cm wide and with 31 cm tall walls, as previously described [161]. The light compartment, made of white acrylic, comprises two-thirds of the total LD box length and was maintained under dim lighting conditions at 100 lux during testing. The dark compartment, comprising one-third of the total LD box, was made of black acrylic and has a lid which is closed during testing. A small door (10 cm wide by 4 cm tall) is located in the middle of the wall separating the light and dark compartments. An entry to either chamber was considered when all four paws of a mouse entered that compartment.

A total of 37 mice were tested (19 $LepRb^{Nts}$ KO mice and 18 ($Lepr^{flox/flox}$) two days following EPM testing by placing them in the dark compartment and recording their subsequent behavior for 5 min. Dependent measures recorded were: (1) time spent in the light and dark compartments; (2) total number of transitions between the two compartments; (3) latency to enter the light compartment from the start of the experiment. No mice were excluded from scoring of this experiment.

3.2.8 Open field and amphetamine-induced locomotor activity.

Open field activity measures were made in a Digiscan Activity Monitor chamber that measures 41.5 cm long, 41.5 cm wide and 31 cm in height (Accuscan Instruments, Columbus OH). The chamber is made of white acrylic

and the behavioral testing room was maintained with low lighting (150 lux) during testing. Light-beam sensors line the x-, y- and z-axis of the chamber at 2.5 cm intervals allowing digitized recording of activity through quantification of successive beam breaks. Four different experiments were performed in the activity monitoring chambers, utilizing 2 slightly different behavioral paradigms:

3.2.9 Dose response for amphetamine-induced locomotor activity in Lep$^{ob/ob}$mice.

The first paradigm was used initially to ascertain the dose response curve for amphetamine induced activity in our mouse models. A total of n=16 each of C57BL/6 and Lep$^{ob/ob}$ mice were tested, with n=4 of each genotype at each amphetamine dose (0 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg). In the first testing paradigm activity was measured for a total of 150 minutes. Mice were placed in the center of the chamber at the start of monitoring and were given 30 minutes to habituate to the chamber (open field activity). Following habituation, all mice were given an i.p. injection of 0.9% saline followed by 30 minutes of activity monitoring to quantify any possible injection stress effects on locomotor activity. After the initial 60 minutes, all mice were given the test dose of amphetamine (0 mg/kg, 2 mg/kg, 4 mg/kg or 6 mg/kg) and activity was monitored for an additional 90 minutes. Quantified measurements were collected in 5 minute bins by the DigiPro Software Program (Accuscan Instruments, Columbus, OH). Total distance traveled by each mouse is calculated by the software rather than solely recording beam breaks as it is a more accurate measure of locomotor activity.

3.2.10 Amphetamine induced locomotor activity in LepRb^{Nts} KO mice.

A second testing paradigm was used for all subsequent locomotor activity experiments that allowed us to test both the effects of vehicle treatment as well as our test dose of amphetamine (4 mg/kg) in a within-subject design that saved on the total number of animals needed for testing. This test consisted of 210 minutes in the activity monitoring chamber: the first 60 minutes for habituation, the following 60 minutes for monitoring activity following vehicle (0.9% saline) treatment, followed by injection of the test dose of amphetamine after which activity is monitored for 90 minutes. This also allowed us to administer pharmacological inhibitors in conjunction with vehicle injections and study how they affect activity following amphetamine. In fasting studies, LepRb^{Nts} KO and littermate controls were deprived of food for 24 hours previous to testing while maintaining them on *ab lib* water.

3.2.11 Sucrose preference testing.

In order to measure sensitivity to reward we decided to put our mouse models through a two-bottle sucrose preference testing paradigm using a Volumetric Drinking Monitor (Columbus Instruments, Columbus, OH), as previously described [69]. The preference testing apparatus consists of modified home cage units that have two lixits located opposite and equidistant from the food hopper. All mice lived in the testing cages (which were the same as their

home cages except for the dual liquid delivery modification) during the 6 day long experiment. For the first 4 days, mice were trained to use the dual lixit system with water in both source bottles. Following training, one source bottle was switched from water to a 0.5% sucrose solution and testing was continued for 2 additional days. Each day, at 07:00, liquid consumption data was collected and lixit positions were switched so that we were able to determine if there was baseline preference for either lixit or for either side that liquid was delivered. If it was determined that there was lixit preference at baseline we paired the sucrose solution with the less preferred lixit (we were unable to correct for side bias in this paradigm, however only 1 animals tested showed a side bias during testing). The percentage of sucrose water consumed was expressed as a percentage of the total liquid consumption when reporting sucrose preference. Total liquid consumption during baseline testing days as well as during preference testing days was also calculated in order to quantify potential differences in liquid intake between mouse models.

3.2.12 *Data Analysis.*

Repeated measures ANOVA analysis was used to determine differences in amphetamine induced locomotor activity as well as to determine interaction between treatment and feeding state in these studies. Paired t-tests were used to quantify significant differences in sucrose preferences paradigms as well as for differences in gene expression and alterations in anxiety measures in light-dark

and open field tests. The Percent Relative Cumulative Frequency (PRCF) was used for analysis of VO2 and RQ as described in reference 5 using GraphPad Prism 4 software. Comparisions of PRCF curves were based on the 50th percentile values (EC50) and curve slopes.

3.3 Results

3.3.1 Leptin deficient Lep$^{ob/ob}$ mice have a blunted locomotor response to amphetamine

Amphetamine is used in research settings to release DA stores from DAergic terminals through blockade of reuptake as well as reversal of DA transport through the DAT. Amphetamine-induced elevation in extracellular DA in the striatum is associated with increased locomotor activity seen with psychostimulant drugs and is used experimentally as one way to measure MLDA function. In order to assess whether leptin interacted with MLDA function we quantified amphetamine-induced locomotor activity in Lep$^{ob/ob}$ mice. Previous researchers have reported Lep$^{ob/ob}$ mice have blunted locomotor response to amphetamine [96]; however we wanted to recapitulate these data and verify that this behavioral paradigm and AMPH dose would be appropriate for further studies. Figure 19A outlines the experimental protocol for the activity testing. Following a dose response curve experiment (data not shown) we found that 4 mg/kg amphetamine caused significant increase on total distance traveled in

both WT and Lep$^{ob/ob}$ mice compared to vehicle treated counterparts (Figure 19B; $p<0.001$ for 90 min. following amphetamine). However there was significantly reduced locomotor activation in Lep$^{ob/ob}$ mice relative to WT's ($p<0.002$ for 90 min. following amphetamine). These findings confirmed our initial hypothesis that there are important MLDA perturbations in mice with deficient leptin signaling and that these mice have a diminished locomotor response to amphetamine.

3.3.2 Behavioral Characterization of LepRb^{Nts} KO mice:

Since our hypothesis also addresses the functional role of LHA LepRb neuron signaling in overall MLDA function we utilized LepRb^{Nts} KO mice that lack LepRb expression in the subpopulation of LHA LepRb neurons that projects to and regulates LHA OX neurons and therefore are deficient specifically in signaling relevant to this neural circuit. To determine the effect of deficient leptin action via this circuit on MLDA function, we chose to use several behavioral paradigms whose output examines striatal or limbic function.

3.3.3 Blunted Amphetamine induced locomotor response in LepRb^{Nts} KO mice

In order to address whether alterations in leptin action via LHA Nts LepRb neurons in the LepRb^{Nts} KO would affect response to psychomotor stimulants, we assessed locomotor activity following 4 mg/kg amphetamine. This behavioral paradigm was very similar to what was used previously for the

$LepR^{ob/ob}$ mice, changing only the duration of the habituation period (now 60 minutes) and testing the vehicle condition in a within-subject manner: each animal received both vehicle and 4 mg/kg amphetamine in sequence following habituation (Figure 20A). During the habituation period (first 60 minutes in the open field activity chamber) *ad lib* fed $LepRb^{Nts}$ KO mice had lower locomotor activity than *ad lib* fed littermate controls (Figure 20B, $F(1,33) = 5.16$, $p = 0.03$ for 60 min. of habituation, all $n = 15$ except fed-KOs: $n = 20$). This data confirmed what was found during metabolic analysis of these mice as well as adding the fact that the differences in baseline activity are still existent in novel environments and in an open field in addition to home cage activity. Additionally, we found that *ad lib* fed $LepRb^{Nts}$ KO mice had reduced amphetamine-induced locomotor activity relative to fed littermate controls (Figure 20C, $F(1,33) = 4.64$, $p = 0.039$ for 90 min. post amphetamine).

Decreased locomotor activity and diminished response to the locomotor activating effects of amphetamine in $LepRb^{Nts}$ KO supports the notion that lack of LepRb signaling through LHA LepRb neurons impairs striatal function. In order to determine if LHA LepRb neurons could also modulate limbic function we next put the $LepRb^{Nts}$ KO mice through a battery of behavioral tests aimed to assess anxiety-like behaviors in rodents.

3.3.4 No anxiety-like behavioral phenotype in LepRb^{Nts} KO mice

Since our hypothesis suggests that LHA LepRb neurons primarily modulate striatal projecting VTA DA neurons we would not expect mice lacking leptin signaling in the LHA to have any obvious limbic phenotype, as evidenced by altered anxiety-like responses. The light-dark box test is based on a rodent's innate fear of light environments and animal exploration of the lit compartment of the test chamber is evidence of lower anxiety. Similarly, the elevated plus maze is derived from a rodent's innate aversion to open spaces and exploration of the open arm of the maze is indicative of less anxiety. We tested LepRb^{Nts} KO mice (n = 19) and their control littermates (n = 18) in both of these testing chambers spaced apart by two days in order to see if functional loss of LHA LepRb signaling caused altered anxiety-like behavior. The LepRb^{Nts} KO did not differ from control mice in their latency to enter the light compartment of the light-dark box (Figure 21A) or open arm of the plus-maze (Figure 21B), nor in the total time spent in the light (Figure 21C) or open arm (Figure 21D). Neither group of animals differed in their total transitions between light-dark compartments (Figure 21E), total entries into plus-maze arms (Figure 21F), or the percentage of open arm entries they made (Figure 21G).

3.3.5 Summary of behavioral phenotyping data from LepRb^{Nts} KO mice

When taken together, these data suggest that there are significant alterations to DA-dependant striatal behaviors in LepRb^{Nts} KO mice relative to

their littermate controls. Since these mice lack LepRb signaling within a subpopulation of LHA LepRb neurons, these findings support our initial hypothesis that LHA LepRb signaling is integral to striatal MLDA function. Additionally our data reveal no anxiety-like behavioral phenotype in $LepRb^{Nts}$ KO mice, suggesting that the LHA LepRb neurons do not strongly modulate the limbic-projecting VTA DA neurons which would be responsible for these types of behaviors.

3.3.6 Potential roles for Nts in the control of the LHA-MLDA circuit in response to energy balance

Our next goal was to determine a mechanism by which LHA LepRb signaling can affect MLDA functions. Considering the striatal phenotype that we observed in the $LepRb^{Nts}$ KO mice it seems likely that one or more of the neurotransmitters used by these neurons, GABA and Nts, play crucial roles. GABA is a significantly more ubiquitous neurotransmitter both throughout the brain as well as within the LHA than is Nts, making it more difficult to examine the role for GABA in this specific circuit. The distribution of Nts, on the other hand, is more limited and 60% of LHA Nts neurons contain LepRb. Given the multiple and somewhat dissimilar effects of leptin on OX neurons (leptin inhibits OX neurons firing while promoting OX expression), the activation of LHA Nts neurons by leptin, and the GABAergic nature of these neurons, we hypothesized that leptin-stimulated GABA release likely mediates the inhibition of OX neurons

firing, while Nts might promote increased OX expression (Figure 22). We therefore set out to test the hypothesis that **neurotensinergic signaling modulates striatal aspects of MLDA function in response to changes in energy balance, and that Nts might mediate these effects through the neuropeptide orexin.** We interrogated this system using a transgenic mouse knockout model that lacks one form of the Nts receptor, NtsR1. This receptor is most prevalent in the CNS and is thought to mediate much of neurotensin's action in the brain [162, 163]. Using these mice will allow us to verify the role that neurotensin signaling plays in modulation of OX expression in the LHA as well as how this affects downstream striatal MLDA function.

3.3.7 Role of NtsR1 in modulation of MLDA by leptin

The NtsR1KO mouse has been described by several different groups [163-166] with the general consensus being that there are no gross metabolic or growth differences between NtsR1KO and WT mice. While there are some differences amongst their findings, most agree that NtsR1 mediates the bulk of Nts action on body temperature, analgesia, anorexia and gastric motility. None of these research groups have done a detailed analysis of metabolism or reward sensitivity in these animals to examine the role of neurotensin signaling through NtsR1 on these measures. Since our hypothesis suggests that Nts action through NtsR1 is primarily responsible for mediating the effect of LHA LepRb

neurons on OX expression and subsequently on MLDA function, we thought it prudent to extend the characterization of this genetic knockout model.

3.3.8 Effect of NtsR1KO on metabolic measures

In order to generate a more detailed metabolic profile of NtsR1KO mice, we analyzed them for 4 days using the Comprehensive Lab Animal Monitoring System (CLAMS) under both fed and fasted conditions. Any metabolic changes seen under baseline (fed) conditions would give us information regarding the role of signaling through NtsR1 on metabolism and changes seen in fasted animals would give us insight into how NtsR1 signaling interacts with systems that respond to and control energy balance. Genetic disruption of NtsR1 signaling in the KO mice lead to an increase in VO_2 relative to WT mice when in a fasted state (Figure 23A, WT = 2986.3 ± 216.1, KO = 3681.9 ± 228.4, $p < 0.05$). The increase in oxygen consumption suggests that when energy balance is tipped towards a negative state the KO mice have an inability to lower their overall metabolic rate as a WT animal would. The NtsR1KO mice also trended towards increased overall activity measures relative to their WT counterparts when fed (Figure 23B, $p = 0.053$). This increase in activity in KOs was more pronounced during the light cycle in both fed and fasted animals to the point that it reached statistical significance. That NtsR1KO mice are more active when mice normally sleep suggests that they might also have disrupted circadian rhythms, an effect not dependant on energy balance (Figure 23B, fasted: WT = 900.7 ± 66.7, KO =

1259.2 ± 127.5, $p < 0.05$; fed: WT = 1068 ± 95.9, KO = 1398 ± 91.0; n = 8 for both). The NtsR1KO mice show no changes in body weight, fat mass or lean mass (Figure 23C-E). This overall metabolic profile for a mouse lacking NtsR1 signaling (increased arousal and metabolism including potential alterations in circadian function) could be a result of increased overall OX action due to decreased Nts action via NtsR1.

3.3.9 Increased sucrose preference in NtsR1KO mice

We used a two-bottle sucrose preference paradigm to measure overall sensitivity to natural rewards in NtsR1KO mice. This paradigm utilizes a mouse home cage with two lixits delivering liquid, one offers normal water and the other either water or a slightly supra-threshold dose of sucrose. This dose, a 0.5% sucrose solution, was found to be the lowest concentration of sucrose that WT mice developed preference for in a sucrose concentration curve study (data not shown). Preference was measured as a percentage of liquid consumed on the sucrose lixit relative to total liquid consumption and alterations in preference were expressed as changes in preference for the sucrose lixit relative to preference for the lixit during baseline testing (when water is offered on both lixits). Taking preference measures relative to baseline testing allowed us to normalize for individual preference for either lixit that occurred in some animals even though there were no persistent lixit or side biases to the preference testing apparatus

(data not shown). Low concentration sucrose solution produced a modest preference in WT mice (Figure 24B, 17.3 ± 4.8% over baseline). Interestingly, there was a significant increase in preference for sucrose in the NtsR1KO mice relative to WT mice (Figure 24B, 36.7 ± 1.8% above baseline; $p < 0.04$ relative to WT). Increased sensitivity to sucrose reward in NtsR1KO mice suggests an exaggerated reward response to positive stimuli and potential alteration in MLDA function.

3.3.10 Blunted response to amphetamine in fasted but not fed NtsR1KO mice

Amphetamine-induced locomotor response was assessed in NtsR1KO mice since psychomotor stimulant activity is indicative of striatal DA function. We found that *ad lib* fed NtsR1KO mice did not differ from WT mice in their response to amphetamine (Figure 25A). However, following fasting for 24 hours we saw a significantly blunted peak response to the locomotor activating effects of amphetamine in NtsR1KO mice during the first 45 min. post-amphetamine (Fig 25A, genotype-feeding interaction $F(1,39) = 3.3$, $p < 0.05$).

These data on altered sucrose preference and amphetamine sensitivity confirm that NtsR1 signaling is important in proper function of the MLDA. The contingency of some of these phenotypic differences in the KO mice on overall energy balance suggests that signaling through this receptor system might be important leptin mediated alteration of reward-sensitivity. These observations are potentially consistent with our hypothesis since the drop in leptin during

fasting in normal mice would cause a balanced activation of OX neurons with a decrease in OX expression, and the lack of Nts activation of NtsR1 in the NtsR1KO mice could upset this balance.

3.3.11 Orexin Receptor-1 antagonist pretreatment blunts amphetamine induced locomotor activity in NtsR1KO mice similarly to WT mice.

In order to determine whether or not NtsR1 signaling affects the MLDA via OX signaling, we assessed the effect of pretreatment with SB-334,867 (20 mg/kg, i.p.), a selective OXR_1 antagonist, on amphetamine-induced locomotor activity in fasted NtsR1KO mice and WT mice. Since the antagonist should inhibit OX neuropeptide signaling, augmented or blunted effects of SB-334,867 in NtsR1KO mice would suggest that the MLDA disturbances in NtsR1KO mice could results from altered OX action during negative energy balance. As expected with an OXR_1 antagonist, pretreatment with SB-334,867 causes a decrease in baseline levels of activity in both NtsR1KO and WT mice (Figure 25C, minutes 80 – 120, OX antagonist vs. vehicle, $F(12,1) = 3.93$, $p < 0.05$, n = 8 KO and n = 6 WT) indicative of depressed OX neuron activity causing decreased overall activity levels. Amphetamine-induced locomotor activity was similarly depressed in both NtsR1KO and WT mice following pretreatment with SB-334,867 (Figure 25C, OX antagonist vs. vehicle $F(37,1) = 11.70$, $p < 0.01$). That orexin receptor antagonists have similar effects on locomotor activity in NtsR1KO and WT mice, both at baseline as well as after administration of amphetamine,

suggests that alterations in OXR_1 signaling are unlikely to underlie the MLDA alterations in NtsR1KO mice. To further explore this possibility we investigated whether NtsR1 signaling could modulate OX mRNA expression in the LHA.

3.3.12 Altered gene expression in the LHA of NtsRKO mice

Tissue samples were microdissected from the LHA of NtsR1KO and WT mice that had been *ad lib* fed or fasted for 24 hours. These conditions were used in order to assess the effect of ablated NtsR1 signaling on OX expression at baseline conditions and in a state of negative energy balance. Since we have recently shown that both i.p. leptin and direct microinjections of leptin to the LHA have the ability to increase OX mRNA expression in the LHA of $Lep^{ob/ob}$ mice (Louis GL 2010), any alteration of this effect in NtsR1KO mice might suggest that NtsR1 signaling can regulate OX expression. Analysis of OX mRNA expression by qPCR revealed that there was a significant effect of fasting on OX expression in both NtsR1KO mice and WT mice (Figure 26A,B ; $F(1,28) = 4.33$, $p = 0.047$, WT-fed = 1.03 ± 0.10, WT-fast = 0.86 ± 0.09, KO-fed = 0.97 ± 0.03, KO-fast = 0.76 ± 0.01, n = 8 per group) in the LHA. These data in suggest that if Nts is regulating OX levels in the LHA it is not doing so via NtsR1-mediated mechanisms, and this OX-dependent mechanism cannot explain the MLDA alterations in NtsR1KO mice.

In order to see whether energy balance affected gene expression of known LHA LepRb neurotransmitters we measured *Nts* and *Gad1* mRNA

expression in LHA tissue microdissected from NtsR1KO and WT mice. We found that *Gad1* gene expression was unaffected at baseline in NtsR1KO mice, and that its expression was appropriately decreased by fasting, similar to WT mice (Figure 26B, $p < 0.01$, $n = 8$ per group). On the other hand, *Nts* expression was decreased in NtsR1KO relative to WT mice (Figure 26C, $p < 0.01$, $n = 89$ per group). These data suggest that NtsR1 signaling modulates Nts expression, but not the expression of either OX or GAD1, which are regulated by energy balance.

These findings have led us to revise our initial hypothesis regarding the mechanisms by which LHA LepRb neurons modulate MLDA function. Our initial hypothesis suggested that Nts would act via NtsR1 to increase OX expression, thereby modulating MLDA-dependent functions. The recently collected results seem to suggest that while Nts-NtsR1 signaling is important for proper function of the MLDA, it is unlikely that this effect is mediated through OX itself. In contrast, NtsR1 signaling appears to be important for the regulation of LHA Nts expression, suggesting a potential role for Nts-NtsR1 action in the control of LHA Nts neurons themselves.

3.4 Discussion

We have examined the role of LHA LepRb neurons for the modulation of the MLDA using a number of different molecular mouse models and a variety of techniques. Based on existing evidence and the initial findings of Gina Leinninger and Gwen Louis [87, 98] we had devised a proposed model for how LHA LepRb neurons interacted with other brain regions, partially by direct projections to VTA DA neurons and partially via local projections to LHA OX neurons. The LHA LepRb neurons use both GABA and Nts as signaling peptides and leptin action in the LHA was sufficient to modulate the expression of TH in the VTA and DA content in the NAc. These data lead us to hypothesize that LHA LepRb neurons could modulate MLDA-dependent behaviors that depend on striatal but not limbic dopamine release. Our data are consistent with this hypothesis. We further hypothesized that Nts-NtsR1 signaling might contribute to the regulation of MLDA function in states of altered energy balance, via the regulation of OX action. In this case, while we confirmed altered MLDA function in fasted NtsR1KO mice, differences in OXR_1 action did not underlie these differences. Furthermore NtsR1 was not involved in the control of OX epxressio, but did modulate Nts expression. We have therefore revised our model of LHA-Nts action to suggest that Nts controls gene expression in LHA Nts neurons to indirectly modulate OX neurons and/or MLDA function (Figure 27).

In order to further probe the how the local and projection neurons modulate downstream neurons we utilized several mouse lines, starting with the leptin deficient LepR$^{ob/ob}$ mouse. We confirmed that that these mice have a blunted response to the locomotor activating effects of amphetamine, suggestive of impaired MLDA function. These findings are in line with previous studies that show decreased vesicular DA stores in the NAc of leptin-deficient mice [76] and that these mice have reduced evoked NAc DA release [75]. While these effects have previously been attributed to VTA LepRb neurons [69, 75] our findings in Chapter 2 seem to indicate that VTA LepRb neurons are more integral to limbic function than to alterations in striatal DA dynamics. Since LHA LepRb neurons potentially modulate VTA DA neurons more generally and control DA content in the striatum, it seemed logical to consider their role in the striatal effects of leptin.

To directly study the role of LHA LepRb neurons on MLDA function we employed a mouse model that lacked LepRb expression selectively in LHA neurons, the LepRb^{Nts} KO mouse. This mouse model has been shown to be mildly hyperphagic, have increased body weight and lower baseline activity levels, suggesting that they have both metabolic and arousal differences at baseline. The LepRb^{Nts} KO mouse also has alterations in striatal function as evidenced by increased DA:DOPAC ratio in the NAc (our unpublished data). Since our current model suggests that LHA LepRb neurons modulate VTA DA neurons that project to striatal targets (as opposed to limbic targets) we would expect that the behavioral phenotype of these mice shows alterations in

behaviors related to striatal dopamine dynamics as opposed to a phenotype dominated by a limbic phenotype such as anxiety- or fear-like alterations.

When tested for amphetamine-induced locomotor response we found that $LepRb^{Nts}$ KO exhibited decreased responsiveness to the psychomotor stimulant. The open field activity monitor used in these studies was also a secondary measure of total locomotor activity in $LepRb^{Nts}$ KO mice that can be viewed in comparison with what is found in the confines of a metabolic chamber. One system more accurately models home cage activity levels since they are essentially the same housing structures (metabolic chambers) whereas the open field activity monitor is a much wider open space and is better suited for quantifying novelty environment induced activity. Data collected during the 60 minute long habituation period confirms the findings that $LepRb^{Nts}$ KO mice have lower overall activity levels, further strengthening the power of this finding and extending it to slightly different conditions.

The amphetamine-psychomotor phenotype of the $LepRb^{Nts}$ KO mice is thus similar to that of $Lep^{ob/ob}$ mice, although more modest in magnitude. It is not clear whether these differences results from the continued action of other MLDA-projecting LepRb neurons (e.g. the LHA-LepRb neurons that do not contain Nts), the diminished obesity of $LepRb^{Nts}$ KO mice relative to $Lep^{ob/ob}$ animals, or other effects.

Several perturbations in striatal DA neuron function, including decreased overall NAc DA content or increased DAT availability could potentially lower the

overall effect of amphetamine induced striatal dopamine release [167-170]. The elevated DA:DOPAC ratio (and trend towards increased NAc DA) in LepRb^{Nts} KO mice suggests that the decreased response to amphetamine in these animals is unlikely to result from decreased DA availability, but rather that altered DA responsiveness, metabolism, or transport may play a role. Further work will be required to determine the precise cause of the phenotype.

The use of elevated-plus maze and light-dark box as a means to assess anxiety behavior in mice lacking LepRb in the LHA showed that they have no obvious anxiety phenotype. This finding supports our initial hypothesis that LHA LepRb neurons modulate striatal-projecting VTA DA neurons, while limbic projecting LepRb-VTA DA neurons may play a dominant role in anxiety. In concordance with these findings an analysis of time spent in the center of the open field activity chamber during habituation period of the amphetamine-induced activity testing showed that LepRb^{Nts} KO mice were no different than control mice (data not shown). Overall our behavioral characterization of these mice supported the idea that LHA LepRb neurons are important in modulating striatal, not limbic, MLDA function. We also found evidence to support the contention that OX-signaling is important in the locomotor response to amphetamine.

We continued to investigate the role of LHA LepRb neurons in MLDA function by approaching to question of what neurotransmitter is most important for mediating their signaling. Since LHA LepRb neurons express both GABA and Nts both were candidates however the tools existed to answer questions about

neurotensin first. We proceeded by characterizing mice null for NtsR1 and how energy balance affected the phenotype of these mice. If Nts were important for OX action then a lack of Nts input from leptin-responsive neurons should modulate their activity and downstream behavioral output.

Initial studies of the NtsR1KO mouse demonstrated an interesting metabolic phenotype that suggested that this receptor plays an important role in modulating energy balance. While not differing from WT mice at baseline, NtaR1KO mice showed increased oxygen consumption relative to controls when in a fasted state. This suggests that these mice have a harder time lowering their metabolic rate when in a state of negative energy balance, an important defense mechanism for energy homeostasis. Additionally, we showed that NtsR1KO mice had increased overall homecage activity levels that were only significantly increased during the light phase. This effect persisted regardless of whether or not these mice were fed or fasted suggesting that they have impaired circadian control of activity levels. The increased activity levels are also suggestive of increased OX action during the light phase consistent with our initial hypothesis that Nts signaling through NtsR1 is important for modulating OX expression.

In order to assess behavioral output of the MLDA system in NtsR1KO mice we looked at their sensitivity to low concentration sucrose solutions using a two-bottle preference paradigm. Sensitivity to 0.5% sucrose was increased in NtsR1KO mice compared to control mice, suggesting that these mice have a lower threshold for reward sensing. Since leptin attenuates sucrose preference and OX increases intake of sucrose solutions it is a possibility that NtsR1KO

mice have increased sensitivity to sucrose because they lack the ability of leptin responsive LHA neurons from decreasing sucrose intake via NtsR1 in the LHA. The other possibility is that they have increased OX neuron activity in the LHA increasing their sucrose preference. A third possibility is supported by our proposed LHA LepRb neural circuitry: that LHA LepRb neurons using neurotensin as a signaling peptide normally attenuate preference for sucrose solutions by decreasing the activity of NtsR1 containing OX neurons.

Amphetamine induced locomotor activity was also assessed in this knockout mouse model as a means of looking at striatal dopamine function. We found no differences in amphetamine-induced activity on KO mice compared to WT mice under baseline conditions, although fasted KO mice showed a significant reduction in stimulant induced activity. This reliance on energy balance suggests that while NtsR1 is important in modulating MLDA action, its role is dependent on energy status.

The finding that OXR_1 antagonism has the same effect on baseline activity levels and amphetamine induced activity in fasted NtsR1KO mice and WT mice was the first strong suggestion that Nts modulation of OX function might not be the mechanism of action of Nts in leptin-responsive LHA neurons. The antagonist decreased overall activity levels as would be seen in the orexin-inhibited state of lower arousal and this effect extended to amphetamine stimulated activity levels. Our gene expression data that shows there is no alteration of regulation of OX expression in NtsR1KO mice further strengthens the assertion NtsR1 is not important for OX expression or function.

In contrast, LHA Nts expression is decreased in NtsR1KO mice. Our revised hypothesis for LHA LepRb neurons thus suggests that Nts functions to regulate LHA Nts neurons, and thus regulates OX neurons only indirectly, and does not control OX expression or availability. NtsR1 signaling is clearly important for MLDA function, although more work will be required to determine its role in LHA function and whether LHA-Nts action underlies the MLDA phenotype of NtsR1KO mice.

Chapter 4

Summary and Conclusions

A complete understanding of how leptin acts in the CNS to exert its effects has been an important goal of researchers since the peptide was cloned almost twenty years ago. Some of the most impactful leptin research to date has elucidated the mechanism of action of one site of leptin action, the mediobasal hypothalamus. The opposing action of leptin on two distinct populations of neurons in the ARC (inhibiting AgRP/NPY neurons and exciting POMC/CART neurons) is responsible for mediating some aspects of energy homeostasis. However, it is apparent that leptin signaling through ARC neurons is insufficient to explain the totality of leptin action in the CNS. For this reason an important current focus of leptin researchers is understanding how each leptin-responsive neural subpopulation functions to participate in the physiological and behavioral effects of leptin.

The use of LepRb reporter mice and the detailed characterization of pSTAT3 induction following leptin administration have identified numerous populations of leptin responsive neurons throughout the brain and in some peripheral tissues [97, 171-175]. Peripherally, LepRb's are found in a wide range of tissues from primary sensory cells such as taste buds and nasal epithelium to visceral tissue such as the intestinal epithelium. Leptin action on peripheral

tissues, even if not completely understood, is likely related to the primary function of each tissue, with specific and known functions often attributed to individual cell types throughout the body. Leptin signaling in the CNS is much more of a proverbial 'black box'; knowledge of how different brain regions or even how individual subtypes of neurons interact is limiting our capacity to explain the mechanism of leptin action.

4.1 Advanced molecular tools permit asking more detailed circuitry questions

In every scientific discipline there is a push to understand phenomena on a more detailed level. Nowhere is this more necessary than in neuroscience, where scientists still lack the tools and understanding to properly dissect and describe everything that is observed. This greatly limits the types of questions that can be asked and properly answered; innovation along the lines of advancing research tools and techniques is one avenue by which great impact can be made in many areas of scientific inquiry. Particular to the research undertaken here have been advances made in tracing neural projections in a subpopulation-specific manner and vast improvements in identifying whole neural circuits. The use of cre-mediated expression of molecular reporters that are packaged in viral vectors to allow for site-specific microinjection has vastly improved the power of neuroanatomical studies.

The initial driving question for Chapter 2 focused on VTA LepRb neurons and the sites to which they project relative to those of neighboring populations of

VTA neurons. Since there are several clustered LepRb populations in the midbrain, parsing which are responsible for which aspects of midbrain leptin action is a complicated task. Numerous injections of Ad-iz/EGFPf were necessary in order to produce enough VTA hits to be convinced that the LepRb neurons were in fact projecting to limbic targets, as opposed to initially presumed striatal brain regions that receive much DAergic innervation. Previous research suggested that these VTA LepRb neurons might be responsible for mediating changes in gene expression and DA release in the NAc as well as having a role in baseline locomotor activity levels [69, 96]. This particular array of functions would be anticipated if VTA LepRb neurons were projecting primarily to striatal targets, but not if the bulk of their projections went to targets in extended amygdaloid nuclei such as the CeA. Consistent with the cre-specific tract tracing data we noted that midline midbrain populations of LepRb neurons, specifically those with soma located in the Linear Raphe nucleus and the Edinger-Westphal complex, sent modest projections to rostral targets such as the BNST and NAc core. The potential for these and other LepRb neural populations to modulate striatal aspects of LepRb signaling while VTA LepRb neurons attend to limbic aspects of LepRb-mediated behavioral modulation could be a parsimonious explanation if behavioral or physiological data supported the hypothesis. The tract tracing and physiological evidence presented in Chapter 2 supports the notion that VTA LepRb neurons project to and regulate limbic targets, whereas midline midbrain LepRb populations (and potentially other subpopulations of

LepRb neurons) may participate in the control of striatal targets as well as limbic systems.

4.2 Separating limbic- from striatal-projecting elements of the MLDA

The MLDA modulates many behavioral processes, but is most commonly associated with goal-directed behaviors. In order to properly assess MLDA function in animal models, common experimental paradigms revolve around seeking and obtaining rewards (since they tend to elicit motivated behaviors) and activating motor systems that form loops through basal ganglia circuits [176-179]. Dopamine neurons originating in the VTA are the primary modulators of striatal output and are involved in signaling novelty or salience of a stimulus that is likely to become an object of attention [180, 181]. These behavioral outputs are different for VTA DA neurons that project to limbic targets such as the amygdala which typically involve modulation of anxiety and stress responses as well as complex behaviors such as withdrawal and relapse [182, 183].

Of particular relevance to leptin-related physiology are states of negative energy balance, when leptin levels fall and protective systems are activated in order to maintain metabolic norms. In extreme cases of low leptin levels, such as starvation, there is an imperative drive to restore positive energy balance by ingesting food which must overcome potential food-seeking related dangers such as increased risk of predation. In such a condition of negative energy balance, the rewarding properties of food are generally increased and the response to

food cues is altered. A complex interaction between these limbic and striatal aspects of motivated behaviors suggest that the MLDA evolved in part to maintain energy balance as a primary goal for all organisms.

Our emotional responses to food are also part of this complex regulation of food intake. Positive emotional valence attached to calorie-rich foods is so integrated into society that it has become part of the common lexicon. 'Comfort foods' are almost always desserts or other sweets with positive reinforcing properties like chocolates which contain caffeine. The post-ingestive effects of eating comfort foods are likely to strongly activate limbic circuitry as well as directly affecting reward processing in the brain. While many brain regions participate in these processes, their concerted modulation by foods suggests that a single energy balance-related peptide might be responsible for these changes.

If there is a single factor can control parallel circuits that mediate different aspects of reward processing such as salience and emotional valence then one of two things should be the case: it should either be possible to dissociate these systems or these systems should be interconnected enough that they are almost impossible to functionally separate. The fact that low levels of stress have been shown to induce feeding (ostensibly the 'comfort food' type of eating) and higher levels of stress being suppressive to feeding suggest that there can be dissociation of these systems based on the magnitude of external factors that affect and control food intake. Additionally, while the majority of people find eating to be an enjoyable and rewarding aspect of their lives, certain individuals with eating disorders such as anorexia nervosa or bulimia nervosa, have the

exact opposite emotional response to food and eating. In these individuals eating is strongly associated with feelings of guilt and negative self-worth and it could very well be the case that they have hyper- or hyposensitive limbic responses to ingestive signals.

4.3 Leptin and a hypothesized limbic circuit

Leptin action through a proposed limbic circuit would be to reduce anxiety, signaling that positive energy balance has been restored and that food seeking is less dire a need, reducing associated hunger-induced anxiety. In fact, leptin has been shown to have antidepressant-like effects when administered acutely [184]. Organisms that are chronically fasted or maintained at lower than normal body weight would possibly show mild emotional valence deficits relative to *ad lib* fed animals that would present as anxiety or depression until fed enough to stave off negative energy balance. Animals deficient in leptin signaling through this pathway ($LepRb^{DAT}$ KO mice if our proposed circuitry is correct) would be hard to distinguish from control mice unless the correct behavioral test was used. While not differing in pain, motor or learning paradigms these mice should show anxiety-like behaviors on traditional tests such as elevated plus maze, light-dark box and novel object tests. These mice might even have other affective processing changes such as suppressed response to conditioned fear stimuli. Additionally, leptin should be insufficient to treat anxiety-like behaviors in these mice like it should be able to in fasted mice.

The regulation of CART expression in the CeA by VTA LepRb neurons is a particularly intriguing finding when considering the hypothesis that this circuit is responsible for regulating emotional valence and anxiety responses. When administered to the CeA CART has been implicated as an anxiogenic compound in social interaction tests and as an antidepressant in forced swim tests [112, 113]. These findings, though opposite in nature, are strongly suggestive that CART and potentially a VTA LepRb-CeA CART neuron circuit mediate anxiety like responses. The opposing nature of CART responses in the CeA also hint at the notion that this system more complex than just a small circuit of DAergic projections and likely has a complex regulatory hierarchy that surrounds and responds to a variety of stimuli.

4.4 Heterogeneous nature of VTA DA neurons

Another appealing aspect of characterizing VTA LepRb neurons is that it comes at a time when researchers are pushing forth the proposition that large DA neuron populations such as those that exist in the VTA are not a homogenous as once thought [77, 79, 111]. Detailed tract tracing studies in combination with immunohistochemical and electrophysiological techniques are being used to parse and identify distinct subpopulations of VTA DA neurons. These subpopulations differ in their physiology, projection pattern and very likely mediate different facets of the complex behavioral phenotype that emanates from DAergic signaling in this region. If projections from the medial posterior portion

of the VTA project mainly to the prefrontal cortex, amygdala and were to mediate limbic processing and regulate attention and impulsive behaviors whereas laterally located DAergic projections head to the lateral shell of the accumbens and regulated processing salient stimuli associated with rewards it would help demystify the complexity of central DA action. Additionally, having unique markers for each subpopulation, be they a unique electrophysiological signature, the presence of LepRb's, or if they receive input from hypothalamic OX neurons, would make study of individual VTA DA subpopulations a more manageable task.

In order to fully understand how complex behaviors arise from the MLDA will take a concerted effort to parse whatever heterogeneous elements to this system exist. A thorough analysis of similarities and differences between signaling through VTA DA neurons and how these affect behavioral output of the MLDA would greatly advance our understanding of reward-mediated behaviors as well as how fear, anxiety and depression interact with motivation. Leptin-responsive elements of this analysis, be they LepRb-expressing VTA DA neurons or other populations of LepRb neurons that project to and regulate elements of the MLDA, may be a functionally minor contributor to this regulation. However important the contribution of leptin signaling is, improving our understanding of the leptin-MLDA relationship will give us a better chance of understanding the system as a whole.

4.5 Are there functionally separable elements of leptin signaling that interact with the MLDA

The two leptin-responsive circuits addressed here, VTA DA-LepRb neurons that project to limbic targets and LHA LepRb neurons that project to the VTA and to OX neurons and which regulate striatal DA dependent processes are the most likely sites of leptin-MLDA interaction. Regulation of MLDA function by these circuits might act to chronically adapt reward and learning processes to optimize the balance between food seeking and other behaviors. Maladaptive processes within these circuits could lead to dysregulation of food intake in the face of easy access to energy dense foods that are the hallmark of modern society. While these represent the major interfaces between leptin signaling and the MLDA, however it is unlikely that these are the only points at which they interact.

Expression of LepRb on sweet-responsive taste buds has the ability to inhibit transmission of sweet taste sensation from primary sensory neurons to their more central targets [185, 186]. A similar phenomenon is observed with LepRb expression in the nasal epithelium and how leptin can modulate sensitivity to food-related odorants [187-189]. Modulation of hedonic components of ingestive behaviors such as smell and taste would affect 'liking' of food, a dissociable aspect of motivated behaviors that forms a complex interaction with traditional MLDA goal-directed behaviors [134, 190-192]. These additional interface points between leptin and the MLDA certainly contribute to the overall regulation of ingestive behaviors however probably not to the extent of LHA and

VTA circuits that could prime DAergic circuits for substantial alterations in overall reward sensitivity and changes in motivated behaviors.

4.6 The value of leptin deficient mice as a model of leptin-induced MLDA dysfunction

Initial evidence for the association between leptin and reward came from the study of leptin deficient and leptin-receptor deficient rodent strains as well as lesion studies that targeted LepRb rich brain regions. Each of these model systems has obvious assets and limitations however the information gleaned from these early studies developed a strong knowledge base from which current researchers can draw. Gross alterations in reward sensitivity as well as the motivation to seek and obtain reward are clear from these studies, but it wasn't until the molecular identification of leptin that researchers truly started to target the molecular mechanisms that underlie this modulation of reward processing.

Leptin deficient $Lep^{ob/ob}$ mice have decreased TH expression in the VTA, decreased DA content in the NAc and impaired evoked-DA release in the NAc [76, 96]. Leptin replenishment in this mouse model has been shown to ameliorate these impairments, restoring to normality most MLDA deficits [76, 96]. In fact, leptin therapy is sometimes only efficacious in instances where low leptin levels already exist, regardless of whether caused by genetic mutation or by chronic food restriction. This suggests that leptin action is much more sensitive in a low-leptin state than when leptin levels are at normal or elevated levels,

potentially due to evolutionary pressures that defend against starvation yet have not ever had the need to develop defense against the upper limits of adiposity.

4.7 Role of LHA LepRb neurons in modulation of MLDA function

Recent data published by our lab suggest that LHA LepRb neurons act via two pathways to modulate VTA DA neurons: direct projections and indirect projections via LHA OX neurons. The major distinction between these two subpopulations of LHA LepRb neurons is the presence of the neuropeptide Nts, which colocalizes with locally projecting LHA LepRb neurons (indirect pathway) rather than direct projecting LepRb neurons. Since tracing data suggest that the majority of LHA LepRb neurons project locally we targeted the indirect LHA LepRb → LHA OX neuron → VTA DA neuron circuit for our initial research into the mechanism of action of LHA LepRb neurons. Since Nts only colocalizes with LepRb neurons in the LHA, we were able to selectively delete LepRb expression from the indirect projecting population by crossing an Nts^{cre} mouse with a $LepRb^{flox/flox}$ mouse. This gave us a functional model that allows us to study the role of leptin signaling through this one circuit in the absence of other leptin-deficient signaling.

The initial characterization of these mice showed that they had a metabolic phenotype similar to other models deficient in leptin signaling: hyperphagic, overweight and with lower overall activity levels [28, 193]. Not only did this confirm that LHA-LepRb Nts neurons are important in overall energy

homeostasis, but the phenotype was similar to that seen in orexin-deficient mice with the major difference being in regulation of sleep-cycling which is grossly affected in OX KO mice [194, 195]. This was also in agreement with our proposed circuit diagram for these neurons: projecting locally to OX neurons where they would exert their downstream influence through orexinergic influences. We also found impaired ability of leptin to increase OX mRNA expression in the LHA of $LepRb^{Nts}$ KO mice, suggesting that leptin signaing through these neurons is important for modulation of OX expression.

An additional MLDA component was also implicated in that $LepRb^{Nts}$ KO had increased DA:DOPAC ratio in the NAc, suggesting that they have impaired DA release and metabolism. Analysis of amphetamine-induced locomotor activity in $LepRb^{Nts}$ KO mice showed that they had a blunted response. Therefore, lack of leptin signaling through indirect projecting LHA LepRb neurons impairs responsiveness to amphetamine. The DA:DOPAC data suggest a change in DAT or vesicular DA rather than in total DA could be responsible.

Characterization of anxiety-like behaviors in these animals showed that they had no overt anxiety-like behavioral phenotype. This further strengthens our overall hypothesis model that VTA LepRb neurons are responsible for mediating anxiety-related aspects of leptin signaling whereas LHA LepRb neurons are implicated in modulation of striatal-projecting DA neurons and associated behaviors. In contrast, we would expect $LepRb^{DAT}$ KO mice (lacking LepRb in VTA and SN DA neurons) would have an anxiety-like phenotype whilst lacking

modulation of striatal behaviors such as responsiveness to the locomotor activating effects of amphetamine.

4.8 Role of Nts signaling in mediating LHA LepRb effects

Since LHA LepRb neurons that project locally and regulate OX neurons contain two different neuropeptides, GABA and Nts, we used a knockout mouse lacking NtsR1, the receptor most strongly associated with mediating central neurotensin effects, to assess many of the same measures as in the $LepRb^{Nts}$ KO mouse to see if lack of NtsR1 signaling had similar effect.

NtsR1KO mice had a metabolic profile (increased respiration during the dark cycle but only in fasted mice) that suggested that these mice had an inability to lower their metabolism when in a state of negative energy balance, as normal animals do. Along with impaired ability to compensate properly for times of decreased energy intake, these mice are also significantly more active during the light cycle suggesting that they have impaired sleep-wake regulation. The dysregulation of circadian rhythms in these animals could be as a result of impaired OX neuron activity however we are unable at this time to confidently make that assertion. The fact that these animals only show a metabolic phenotype when fasted suggests that some important NtsR1 mediated processes are only utilized when an organism's energy balance is perturbed.

The need to perturb NtsR1KO mice in order to see a phenotype also extended to their sensitivity to locomotor effects of amphetamine. When tested

under baseline conditions these animals showed no difference in amphetamine induced activity; however when fasted for 24 hours they showed impaired sensitivity. Since fasting typically increases an animal's sensitivity to drugs of abuse [196, 197] this impairment is opposite what might be expected. While it is not clear that the alterations seen in NtsR1KO mice are definitely the cause of LHA LepRb neuron signaling since these mice lack functional NtsR1 throughout the whole body, the dependence of some aspects of their phenotype in energy balance suggests that leptin might be a mitigating factor.

Pharmacological antagonism of OXR_1 was used to see if decreased OX activity in NtsR1KO mice could explain some of the behavioral phenotype in this animal. The net effect of orexin antagonism was the same in NtsR1KO mice and their control mouse counterparts, suggesting that if NtsR1 did affect MLDA function it likely did not do so via OX *per se*. Indeed, disruption of NtsR1 did not alter the expression of OX or its regulation by fasting, suggesting that Nts may not function primarily to regulate OX content or availability. In contrast, LHA Nts expression was dysregulated in the NtsR1KO mice, suggesting a role for Nts in the modulation of LHA Nts neurons themselves. How this affects the physiology of OX neurons and/or the MLDA remains unclear.

4.9 Future Directions

Important issues to examine in the future will be the response of OX neurons and the MLDA system to leptin (including the activity of OX neurons) in

the NtsR1KO mice. As well, it will be useful to examine other parameters of MLDA function (e.g. DAT activity) in $LepRb^{Nts}$ KO and NtsR1KO animals. It will also be helpful to develop methods to manipulate non-Nts LHA LepRb neurons, to determine their function in leptin action and MLDA regulation.

Follow-up studies in mice lacking VTA LepRb neurons ($LepRb^{DAT}$ KO mice) will elucidate the behavioral function of leptin action on these neurons and should include a full characterization of metabolic measures, anxiety like responses and reward sensitivity. We would initially predict, based on circuitry mapped in Chapter 2, that these mice would lack an overt reward phenotype and possible lack a metabolic phenotype but would likely have greatly altered stress and anxiety responses. Additional electrophysiological characterization of VTA LepRb neurons would also help to understand exactly how leptin modulated VTA DA neuron function and could help confirm their proposed circuitry based on electrophysiological profile.

Overall, our data are consistent with a multi-faceted regulation of MLDA function by leptin, with distinct neural populations controlling discrete aspects of MLDA function and behavior. Future work to more completely define mechanisms by which each population acts may identify crucial signals that contribute to reward-driven feeding, anxiety-driven feeding or that may represent therapeutic targets for the treatment of obesity or other eating disorders.

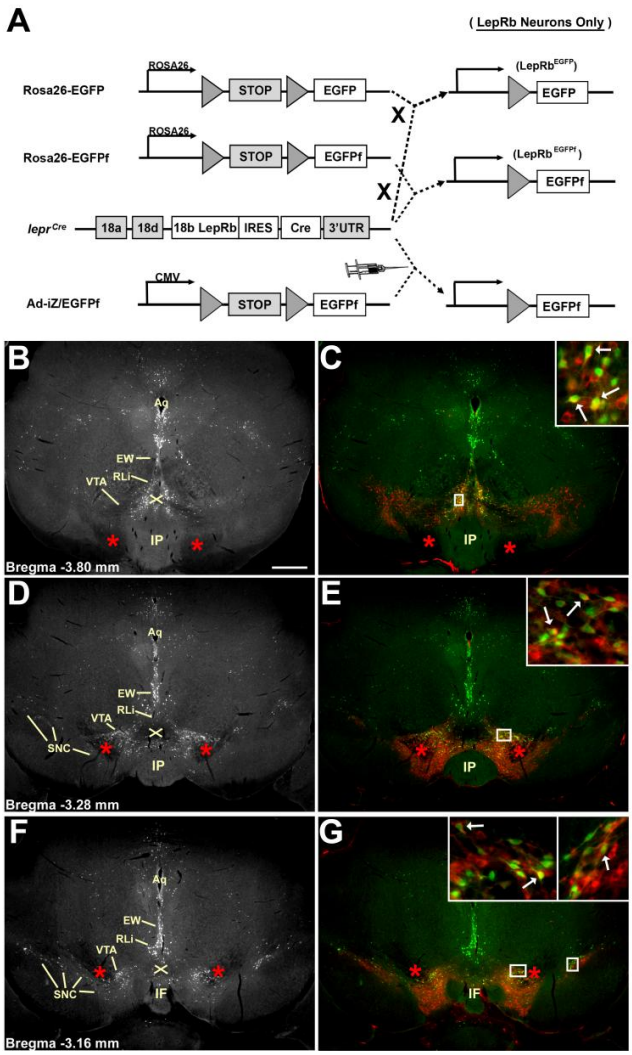


Figure 1. Mouse models and the visualization of midbrain LepRb neurons. (A) Schematic of methods for expression of EGFP or EGFPf in LepRb neurons. Combining *Lepr*^{cre} with *Rosa26-EGFP* or *Rosa26-EGFPf* alleles results in the stable expression of EGFP or EGFPf in LepRb neurons in LepRb^{EGFP} and LepRb^{EGFPf} mice, respectively (top). Additionally, injection into *Lepr*^{cre} mice of the adenoviral Ad-iZ/EGFPf promotes cre-mediated EGFPf expression in LepRb neurons surrounding in the injection site (bottom). (B,D,F) LepRb-expressing neurons revealed by EGPF-IR through the rostrocaudal extent of the midbrain of LepRb^{EGFP} animals. (C,E,G) Colocalization of EGFP-IR (green) and TH-IR (red) through the rostrocaudal extent of the midbrain of LepRb^{EGFP} mice. Insets show digital zooms of the boxed areas; arrowheads demonstrate examples of colocalized neurons. Red asterisks indicate the medial lemniscus; 'X' indicates the ventral tegmental decussation. The scale bar represents 200 μm.

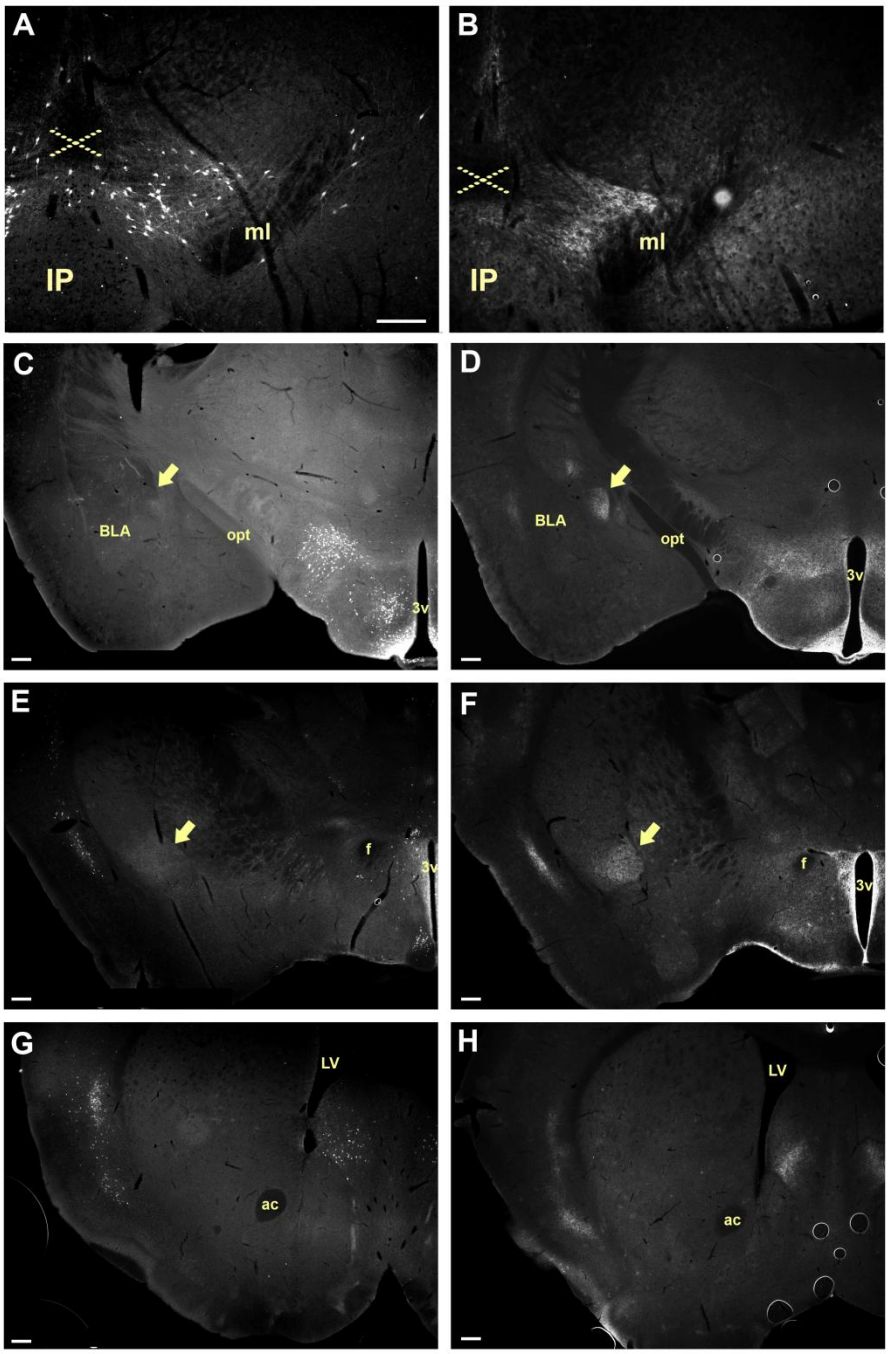


Figure 2. Detection of LepRb neurons and projections throughout the mesolimbic DA system in LepRb^{EGFP} and LepRb^{EGFPf} mice. Shown is EGFP-IR in the midbrain (A,B), hypothalamus and amygdala (C,D), rostral hypothalamus and IPAC, (E,F) and Striatum and BNST (G,H) of LepRb^{EGFP} (left panels) and LepRb^{EGFPf} (right panels) mice. Arrows in C, D indicate the CeA; arrows in E, F indicate the IPAC. Dashed 'X' indicates the ventral tegmental decussation. Scale bars are 200 μm.

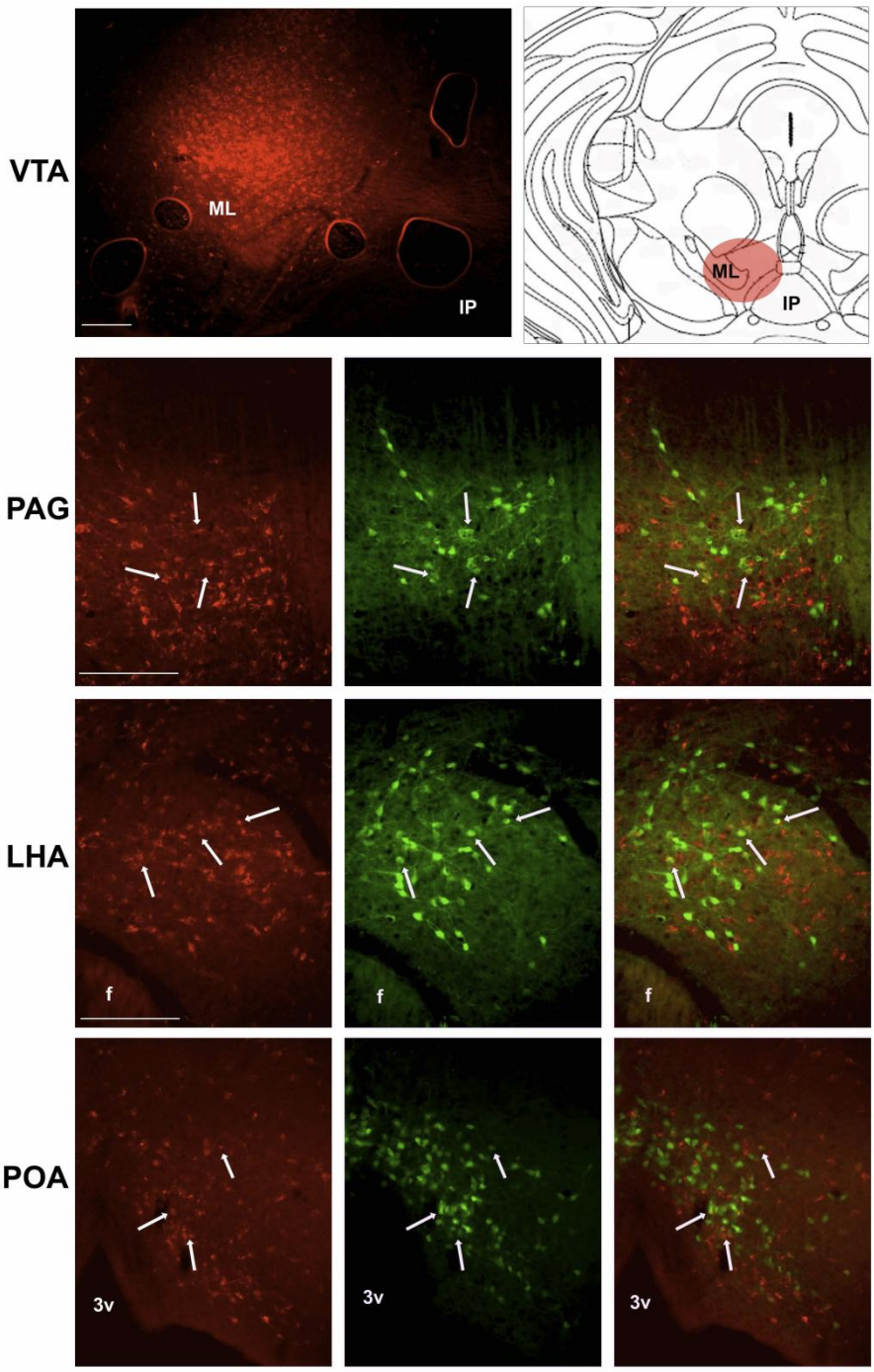


Figure 3. Retrograde tracing of VTA-projecting LepRb neurons in LepRb^{EGFP} mice. FG was stereotaxically injected into the VTA of LepRb^{EGFP} animals, and the brains were analyzed for FG-IR and EGFP-IR to determine sites of colocalization. Top panels- (Left)- FG-IR in the injection site and (Right) schematic of injection site in representative animal. PAG, LHA and POA panels show FG-IR (left), EGFP-IR (middle) and merged images (Right) from regions showing FG/EGFP colocalization. Arrows denote colocalized neurons. Red spot in schematic represents approximate FG injection plume. Scale bars are 100 μm.

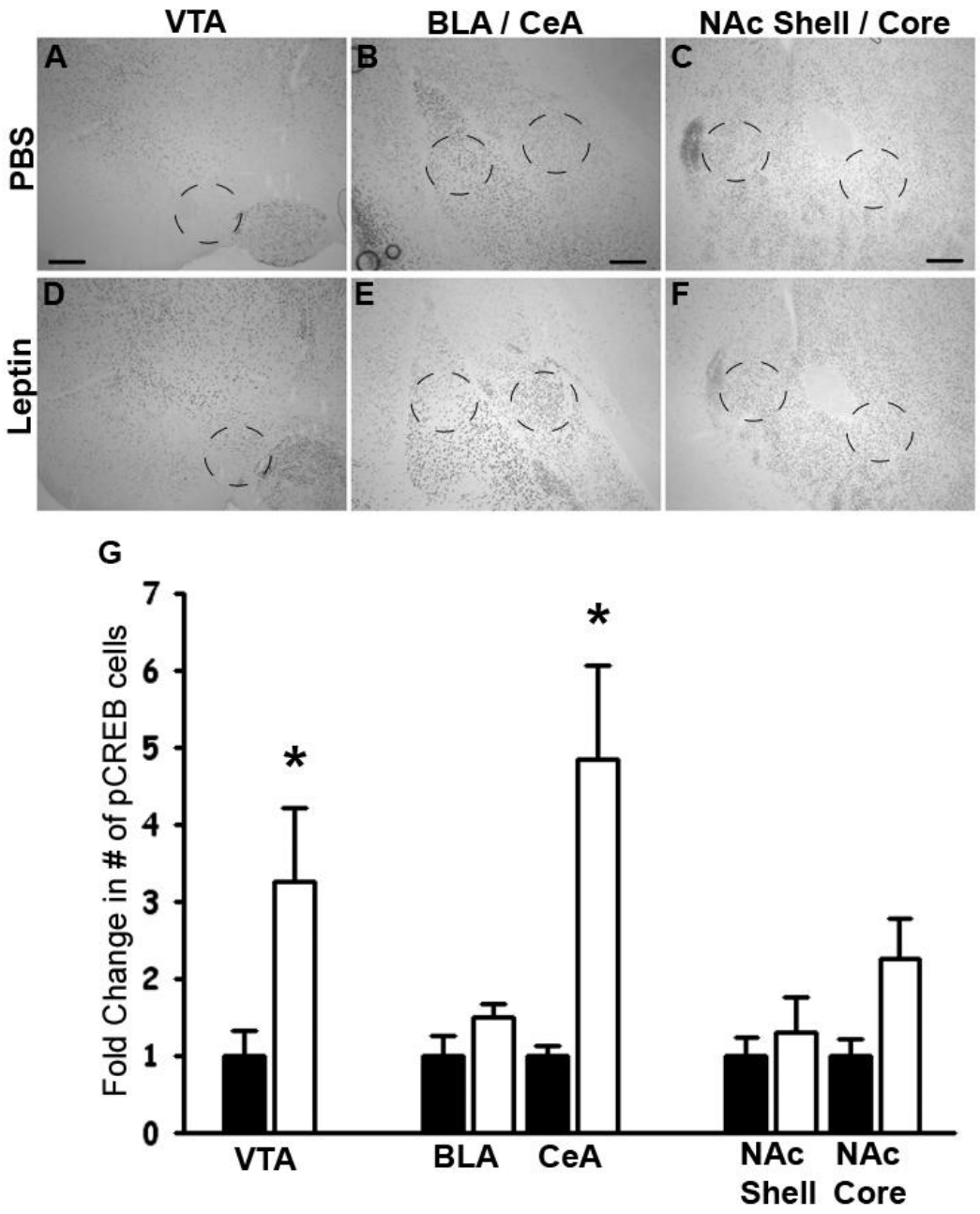


Figure 4. CREB phosphorylation in the midbrain, amygdala, and NAc of leptin-treated LepR$^{ob/ob}$ mice. Leptin-deficient LepR$^{ob/ob}$ mice were treated with leptin (5 mg/kg, i.p., 2 hrs) and perfused for the immunohistochemical detection of pCREB-IR. A-F show representative images of pCREB-IR in VTA (A,D), amygdala (B,E), and NAc (C,F) of vehicle (top panels) and leptin-treated (lower panels) animals. Circles denote regions analyzed for number of pCREB positive cells, which is plotted in (G). n = 6 for leptin-treated and 5 for PBS treated bars. * indicates $p < 0.05$. Scale bars are 100 .

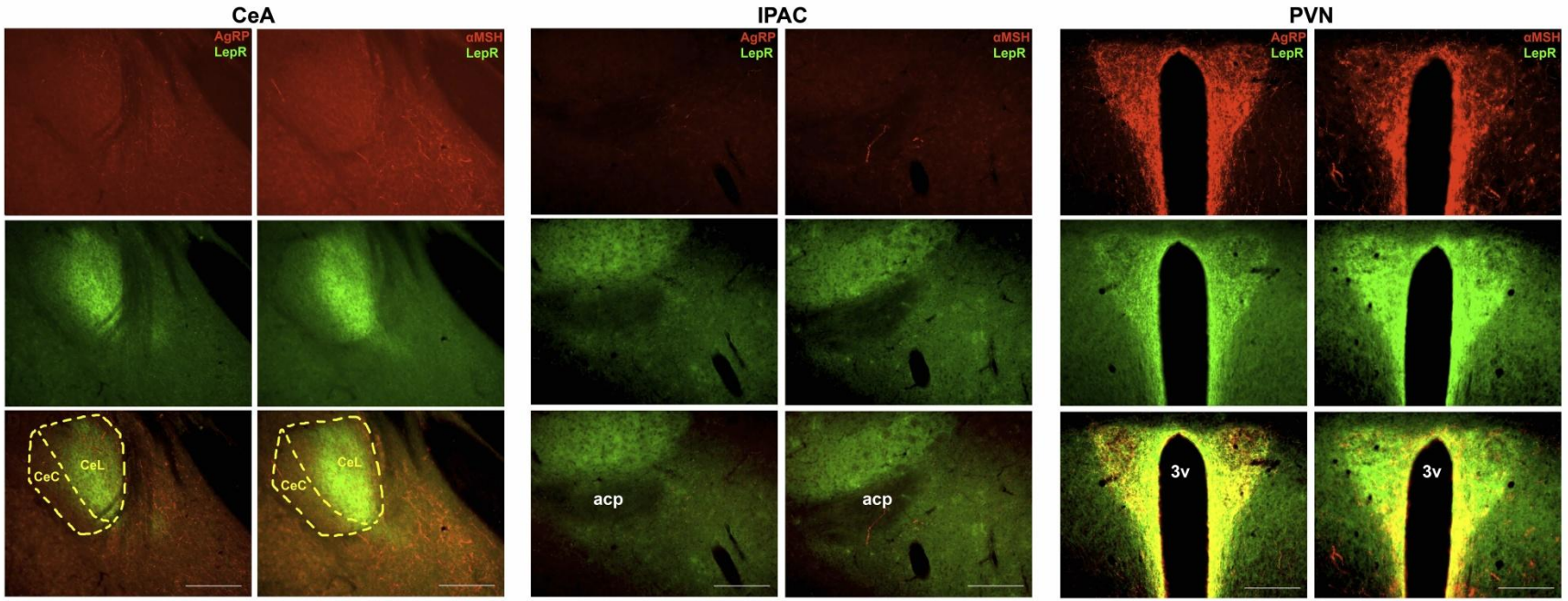


Figure 5. Overlap of EGFPf and AgRP- or POMC-IR in LepRbEGFPf animals. LepRbEGFPf animals were stained for EGFP (LepR; green) and AgRP or POMC (as indicated, red). Distribution of each is shown in the CeA (left panels), IPAC (middle panels), and PVN (right panels). Top panels represent red channel (AgRP or POMC), middle panels show EGFP-IR, and bottom panels show merged images. Scale bars are 200 μm.

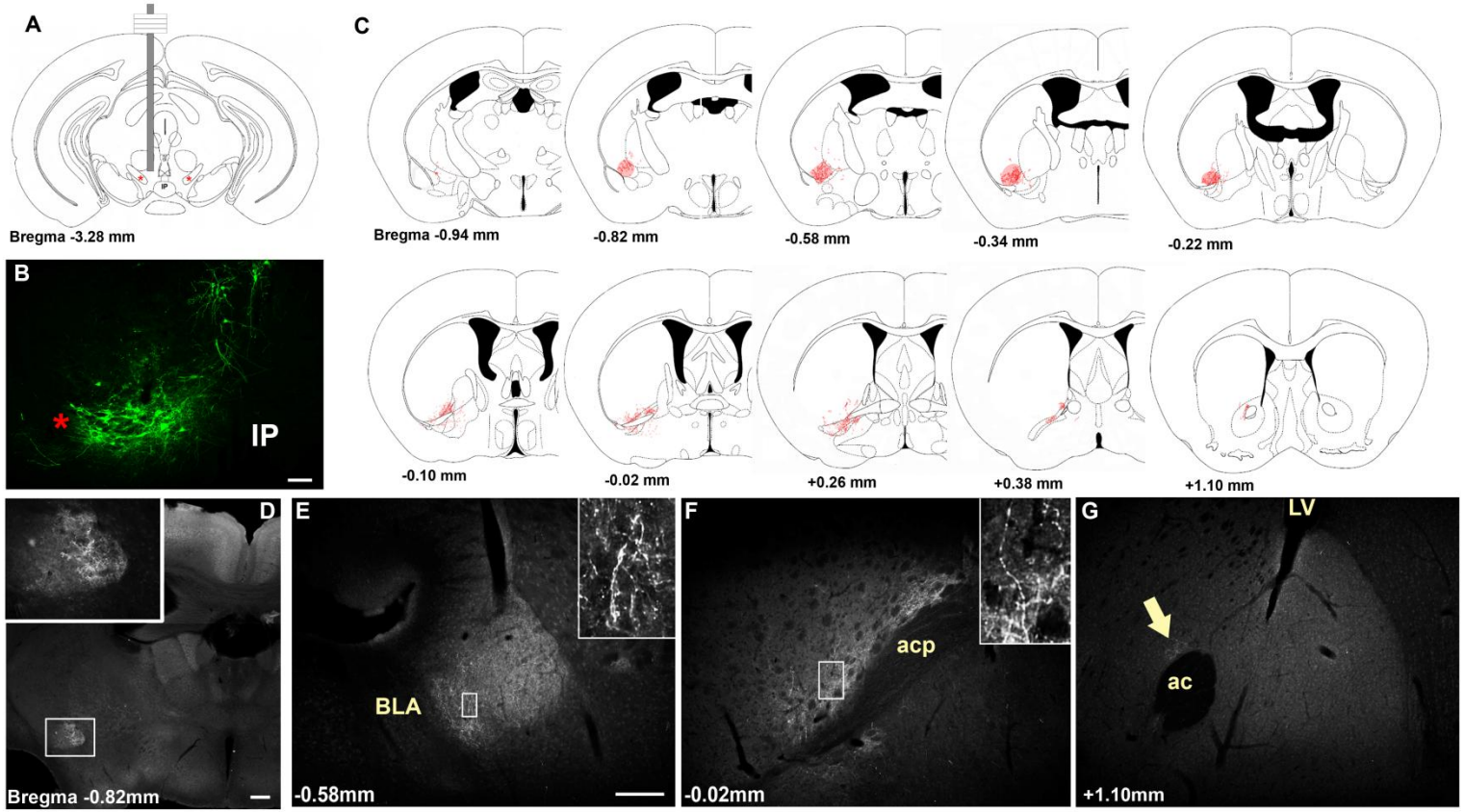


Figure 6. Representative Ad-iZ/EGFPf-mediated tracing of projections primarily from VTA LepRb neurons in *Lepr*^{cre} mice. Schematic (A) and EGFP-IR (B) of the VTA injection site in representative case. The appearance of rostral projections (red) in this animal is superimposed upon atlas sections from (Paxinos and franklin, 2001) in (C). (D-G) show EGFP-IR in various regions to which VTA LepRb neurons sent detectable projections. Insets represent digital zooms of boxed regions. Arrow in G indicates the small amount of NAc EGFP-IR observed in this and similar cases. Red stars indicate the medial lemniscus. Scale bars are 200 μm in B and D and 100 μm.in E-G.

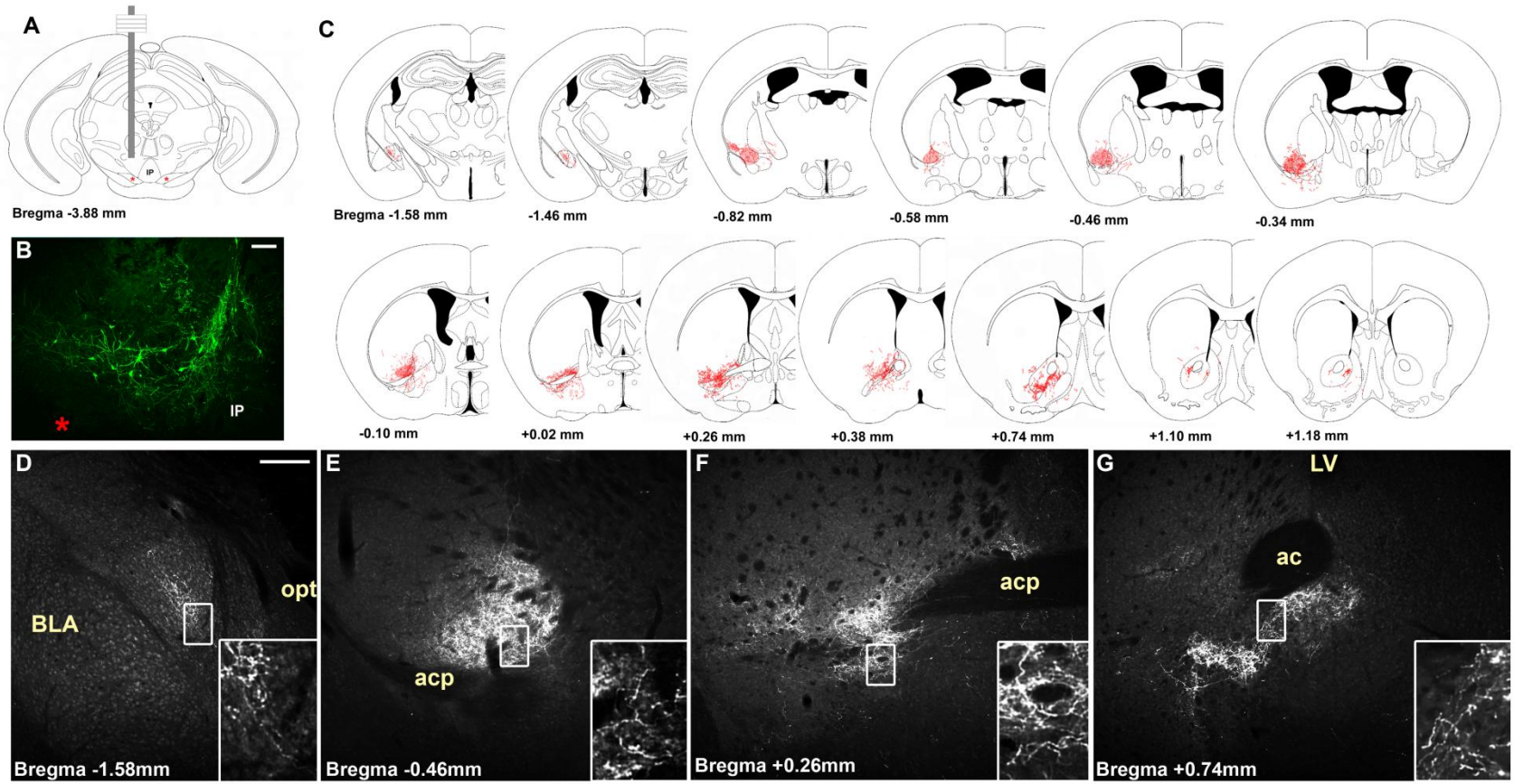


Figure 7. Representative Ad-iZ/EGFPf-mediated tracing of projections from VTA and midline midbrain LepRb neurons in *Lepr*^{cre} mice. Schematic (A) and EGFP-IR (B) of the midbrain injection site in representative case. The appearance of rostral projections (red) in this animal is superimposed upon atlas sections from(Paxinos and franklin, 2001) in (C). (D-G) show EGFP-IR in various regions to which midbrain LepRb neurons sent detectable projections. Insets represent digital zooms of boxed regions. Red stars indicate the medial lemniscus. Scale bars are 200 µm.

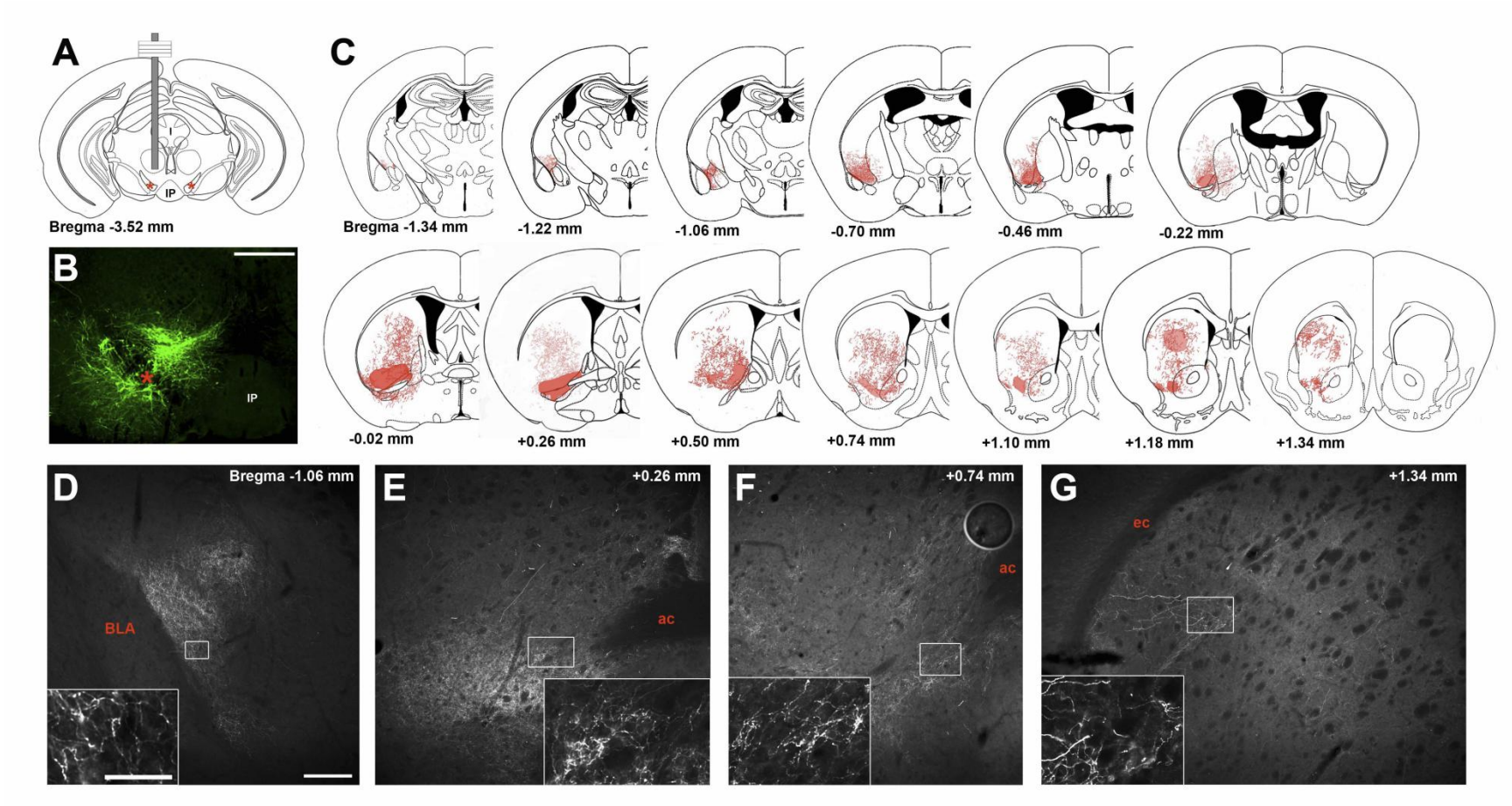


Figure 8. Representative Ad-iZ/EGFPf-mediated tracing of projections from VTA+SN LepRb neurons in *Lepr*$_{cre}$ mice. Schematic (A) and EGFP-IR (B) of the VTA+SN injection site in representative case. The appearance of rostral projections (red) in this animal is superimposed upon atlas sections from (Paxinos and franklin, 2001) in (C). (D-G) show EGFPIR in various regions to which VTA LepRb neurons sent detectable projections. Insets represent digital zooms of boxed regions. Red stars indicate the medial lemniscus. Scale bars are 200 μm in B and D and 100 μm in E-G.

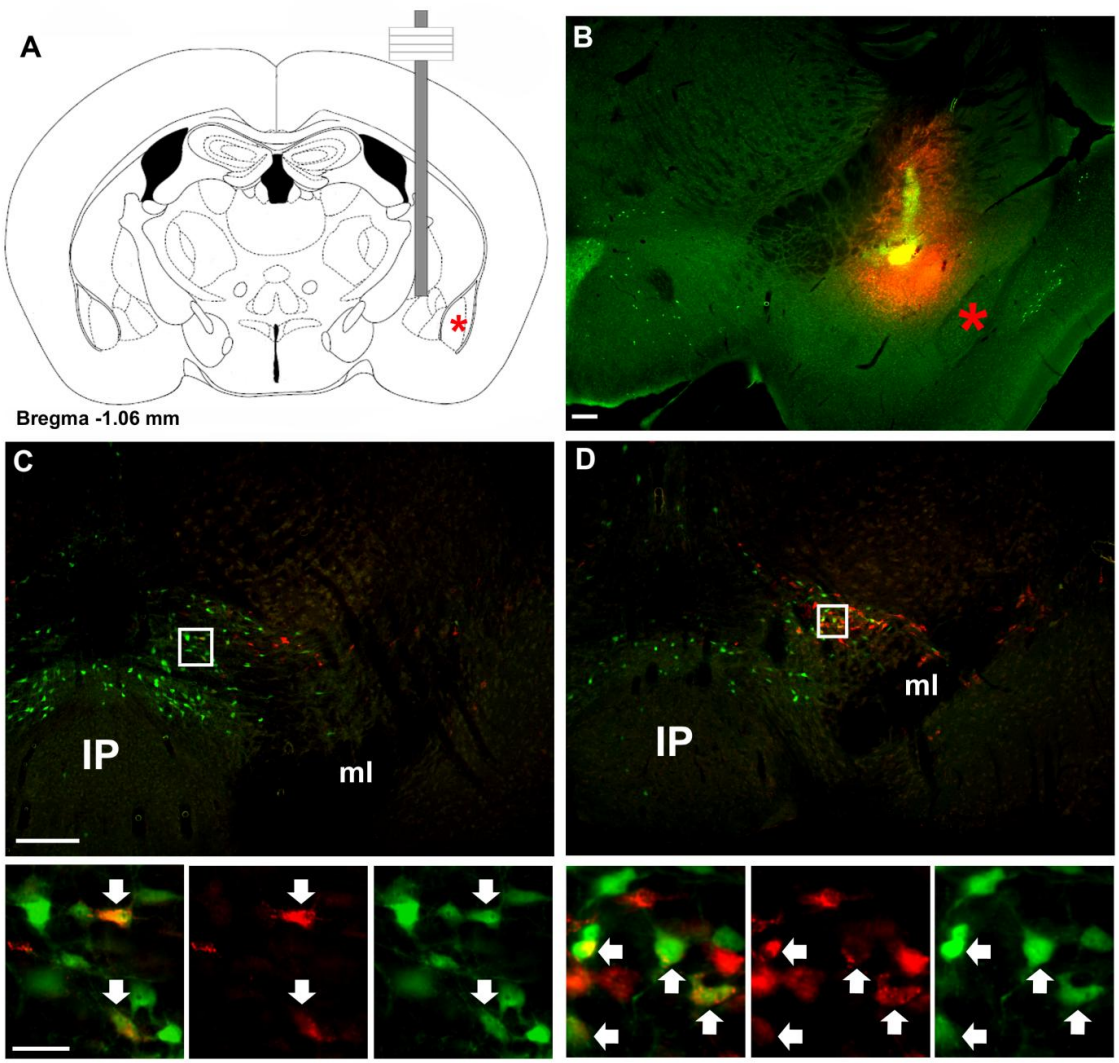


Figure 9. Retrograde tracing from CeA labels VTA LepRb neurons. The retrograde tracer fluorogold (FG) was stereotaxically injected into the CeA of LepRb^{EGFP} animals to determine the potential projection of VTA LeRb neurons to the CeA by colocalization of FG and EGFP-IR. (A) Schematic diagram and (B) fluorescent image (FG, red; EGFP, green) of CeA injection site in representative animal. (C,D) demonstrate distribution of FG- and EGFP-IR neurons at two different levels of the VTA. Images below are digital zooms of the boxed areas showing (left to right), merged images, FG-IR, and EGFP-IR. Arrows indicate colocalized neurons. Red star indicates the basolateral amygdala. Scale bars are 200 μm in B and C and 25 μm in inset panels.

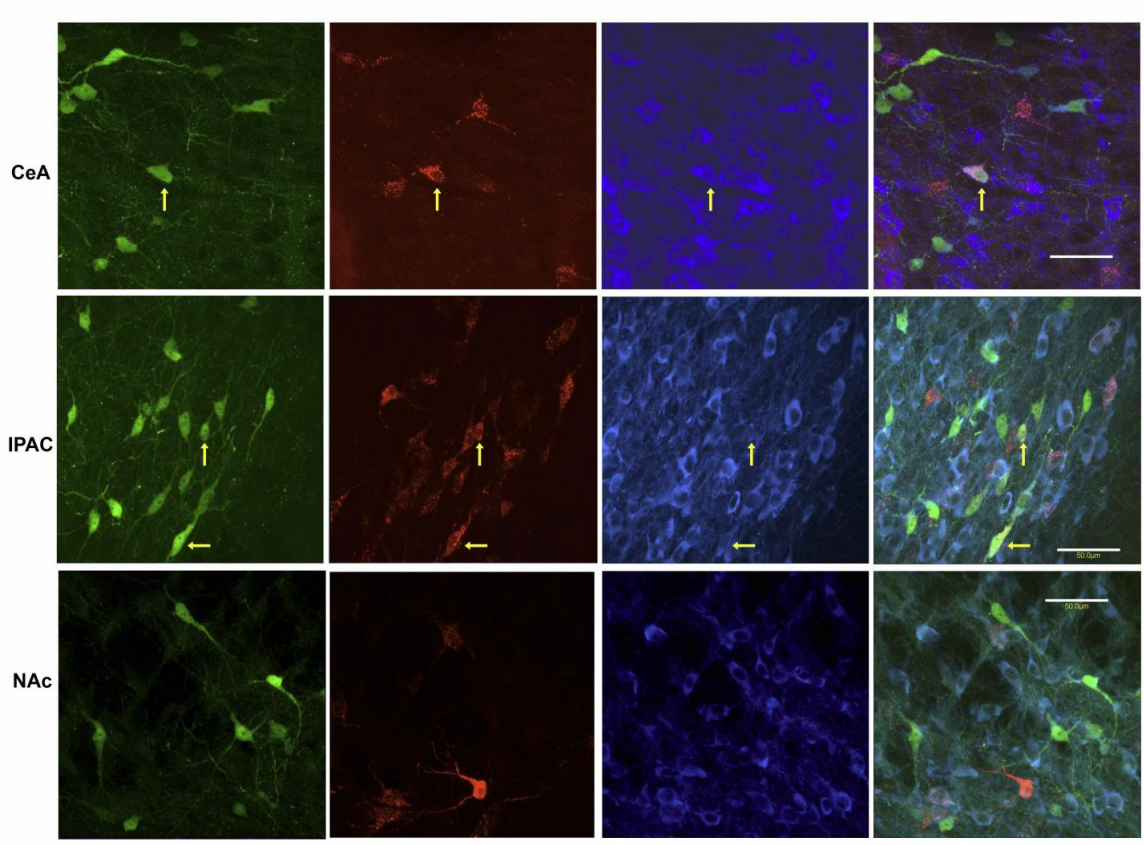


Figure 10. Accumulation of FG from the extended amygdala in TH-containing VTA LepRb neurons. Triple staining for EGFP (green, left panels), FG (red), and TH (blue) in the midbrain of LepRb$_{EGFP}$ animals that received FG into the CeA (top), IPAC (middle) or NAc (bottom) as in Figures 6-8 of the main text. Images were acquired by confocal microscopy. Rightmost panels show merged (3 channel) images. Yellow arrows indicate triple-stained neurons. Scale bars are 50 μm.

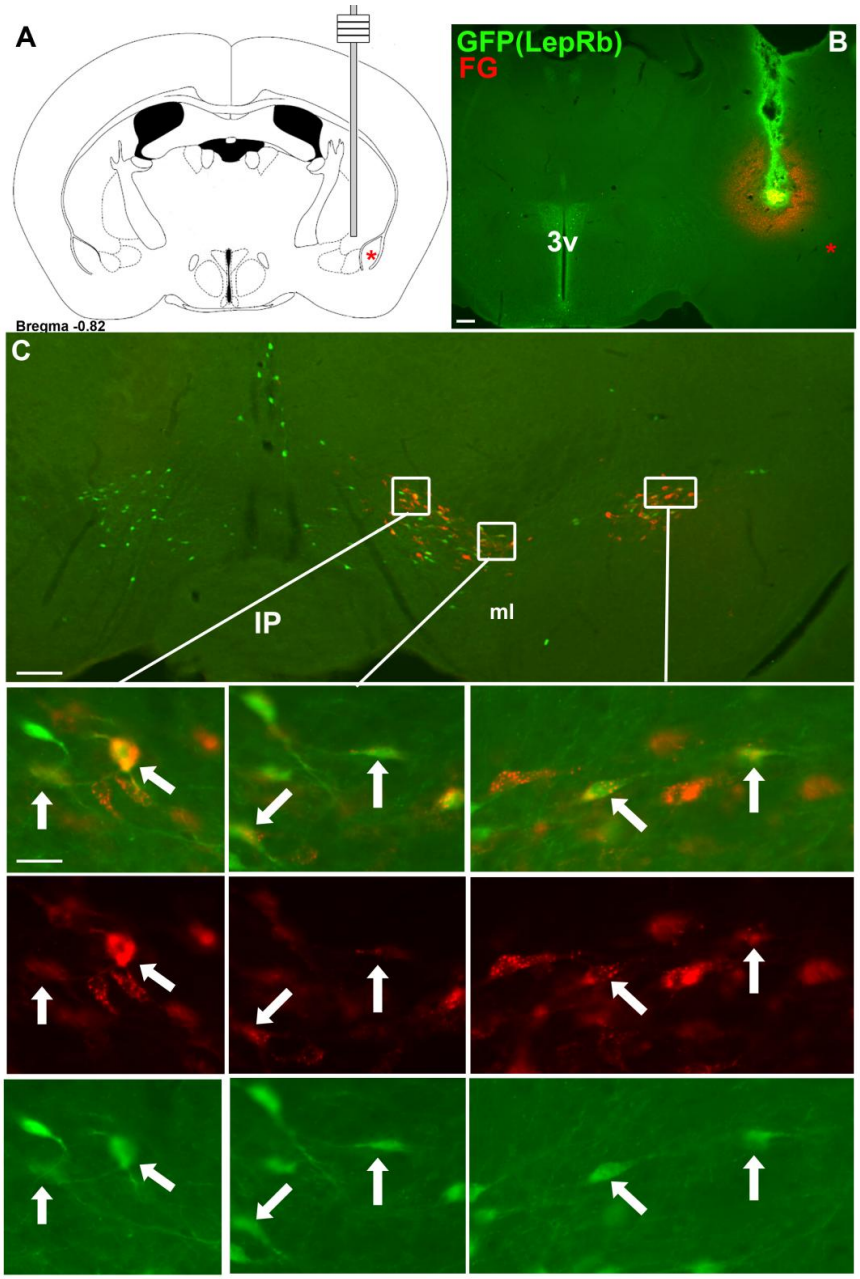


Figure 11. Retrograde tracing from IPAC labels VTA LepRb neurons. The retrograde tracer fluorogold (FG) was stereotaxically injected into the IPAC of $LepRb^{EGFP}$ animals to determine the potential projection of VTA LepRb neurons to the IPAC by colocalization of FG and EGFP-IR. (A) Schematic diagram and (B) fluorescent image (FG, red; EGFP, green) of IPAC injection site in representative animal. (C) demonstrates distribution of FG- and EGFP-IR neurons in the VTA. Images below are digital zooms of the boxed areas showing (top to bottom), merged images, FG-IR, and EGFP-IR. Arrows indicate colocalized neurons. Red star indicates the basolateral amygdala. Scale bars are 200 μm.in B and C and 20 μm in inset panels.

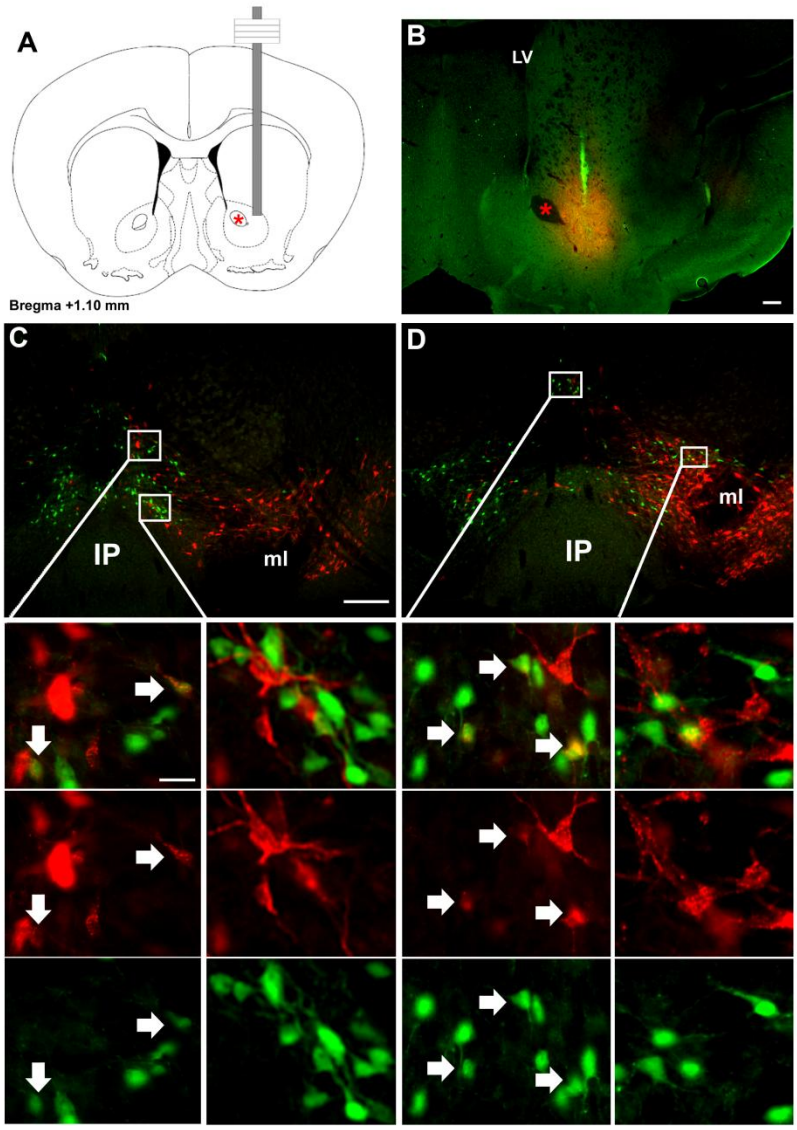


Figure 12. Retrograde tracing from NAc labels midline midbrain but not VTA LepRb neurons. The retrograde tracer fluorogold (FG) was stereotaxically injected into the NAc of LepRb^{EGFP} animals to determine the potential projection of VTA LeRb neurons to the NAc by colocalization of FG and EGFP-IR. (A) Schematic diagram and (B) fluorescent image (FG, red; EGFP, green) of NAc injection site in representative animal. (C,D) demonstrate distribution of FG- and EGFP-IR neurons in the VTA. Images below are digital zooms of the boxed areas showing (top to bottom), merged images, FG-IR, and EGFP-IR. Arrows indicate colocalized neurons. Red star indicates the anterior commissure. Scale bars are 200 μm in B and C and 25 μm in inset panels.

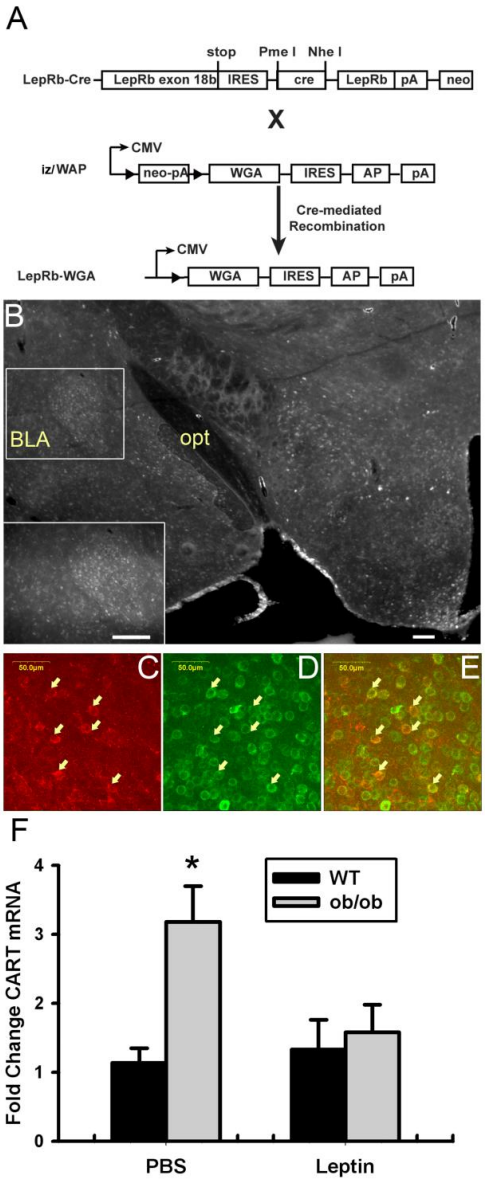


Figure 13. Identification of CART-expressing CeA neurons as targets of leptin action. (A) Schematic diagram showing the generation of LepRb-WGA mice. *Lepr*^{cre} mice were crossed with iZ/WAP transgenic mice to mediate the expression of the trans-synaptic tracer, WGA, in LepRb neurons. (B) WGA-IR in the hypothalamus and amygdala of a LepRb-WGA mouse. Inset: Higher-magnification image showing WGA-IR in the CeA. Scale bars are 200 µm. (C-E) WGA-IR (green, C), CART-IR (red, D), and merged (F) confocal images from the CeA of a LepRb-WGA mouse. Arrows indicate colocalized neurons. Scale bars as indicated. (E) Wild-type (WT) and leptin deficient LepR$^{ob/ob}$ mice were treated for with leptin (5 mg/kg, i.p.) or vehicle every 12 hours for 24 hours before dissection and mRNA extraction from the CeA. Expression of *Cart* mRNA was quantified by qPCR. n=9-10 per group, *p<0.05 compared to WT by ANOVA.

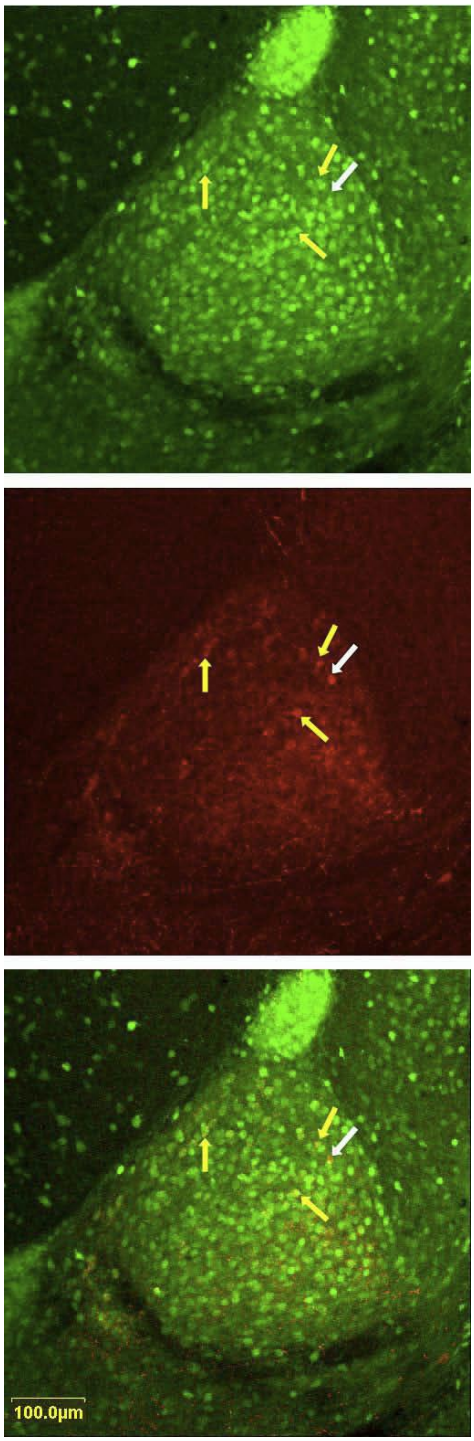


Figure 14. CART Neurons in the CeA are predominantly GABAergic. Sections from the CeA of *Gad1EGFP* mice were immunostained for GFP indicative of GABA neurons (green, top panel) as well as for CART (red, middle panel). Merged images (bottom panel) reveal colocalization of GFP-IR in CART neurons (yellow arrows). Quantification reveals that 95 ± 1% of CART neurons contain GFP-IR (n=5). White arrows indicate CART-only neurons. Scale bar is 100µM.

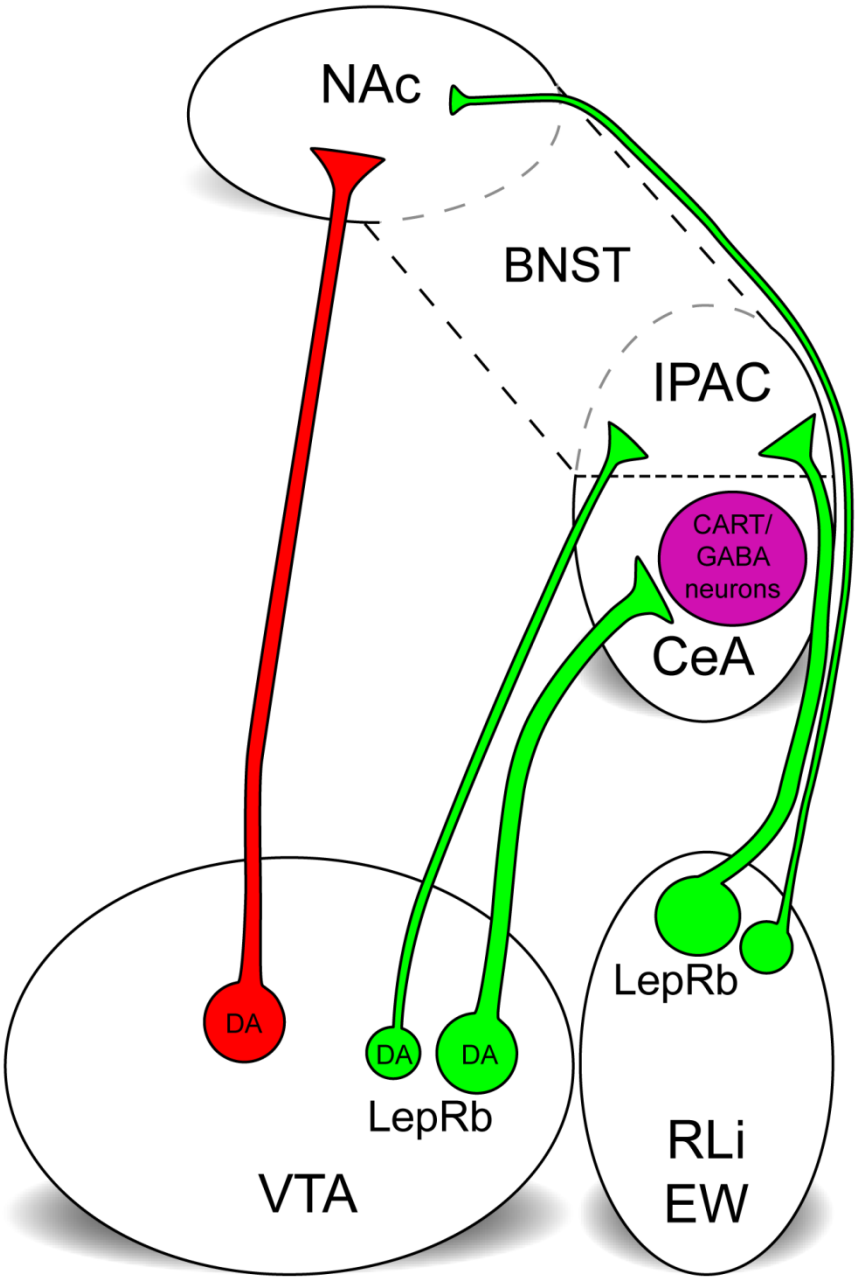


Figure 15. LepRb neurons originating in the midbrain have specific and circumscribed targets in striatal projection regions. Model describing projection patterns of LepRb neurons that originate in the VTA (green), which are primarily DAergic and project extensively to the CeA and IPAC; within the CeA these projections innervate and regulate CART neurons. LepRb neurons that originate in the midline RLi project primarily to the IPAC, but also send some projections to the NAc.

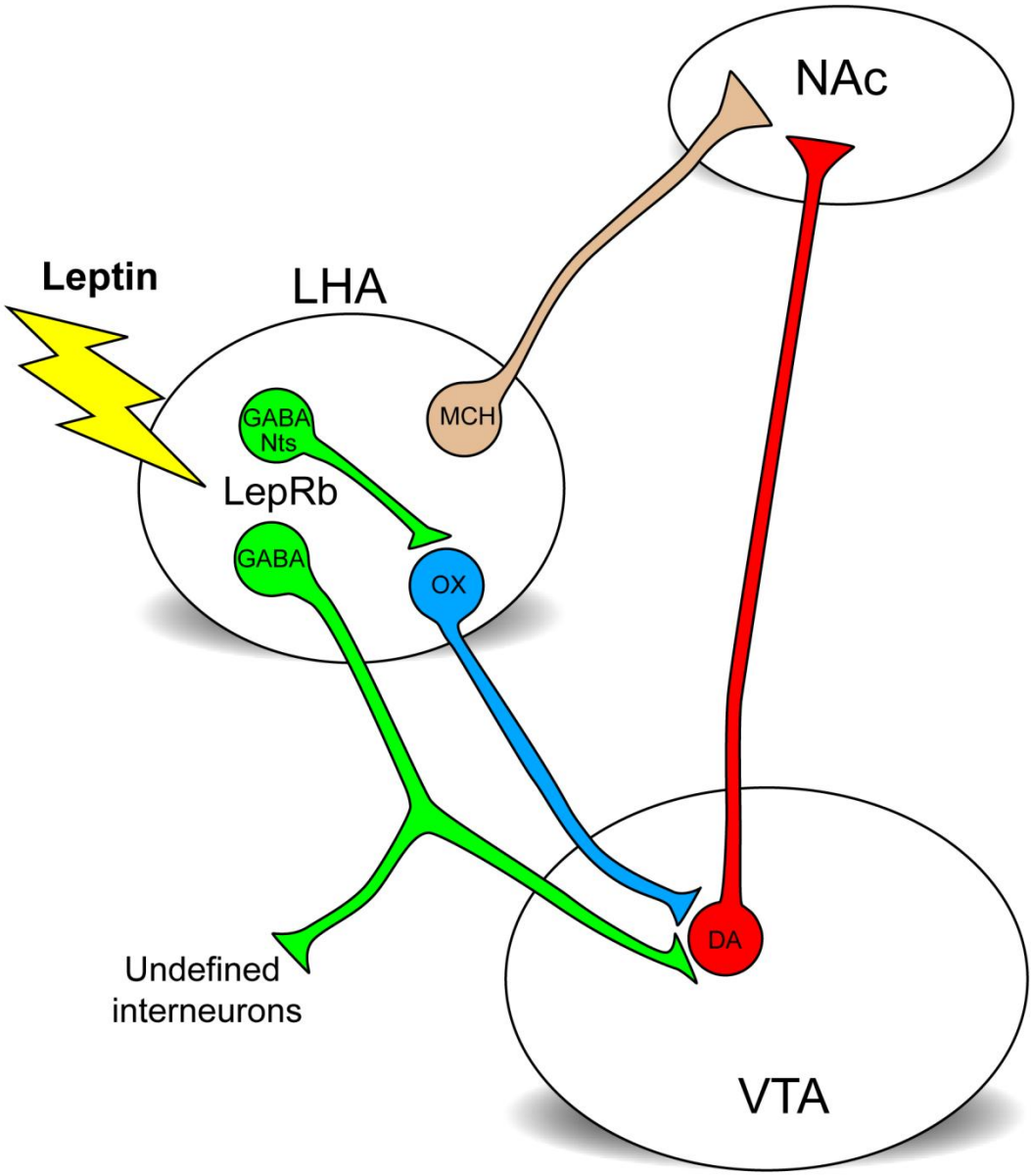


Figure 16. Proposed model for LHA LepRb neural circuitry. Based on previously published data from Leinninger GM et al (2009) and Louis GW et al (2010) we propose that there are two populations of leptin-responsive (LepRb, green) neurons in the LHA. One population projects directly to VTA dopamine (DA, red) neurons and the other project to LHA orexin (OX, blue) neurons that project to VTA DA neurons. We hypothesize that leptin regulates striatal components of the mesolimbic dopamine system through these bimodal projections to from LHA LepRb neurons to VTA DA neurons.

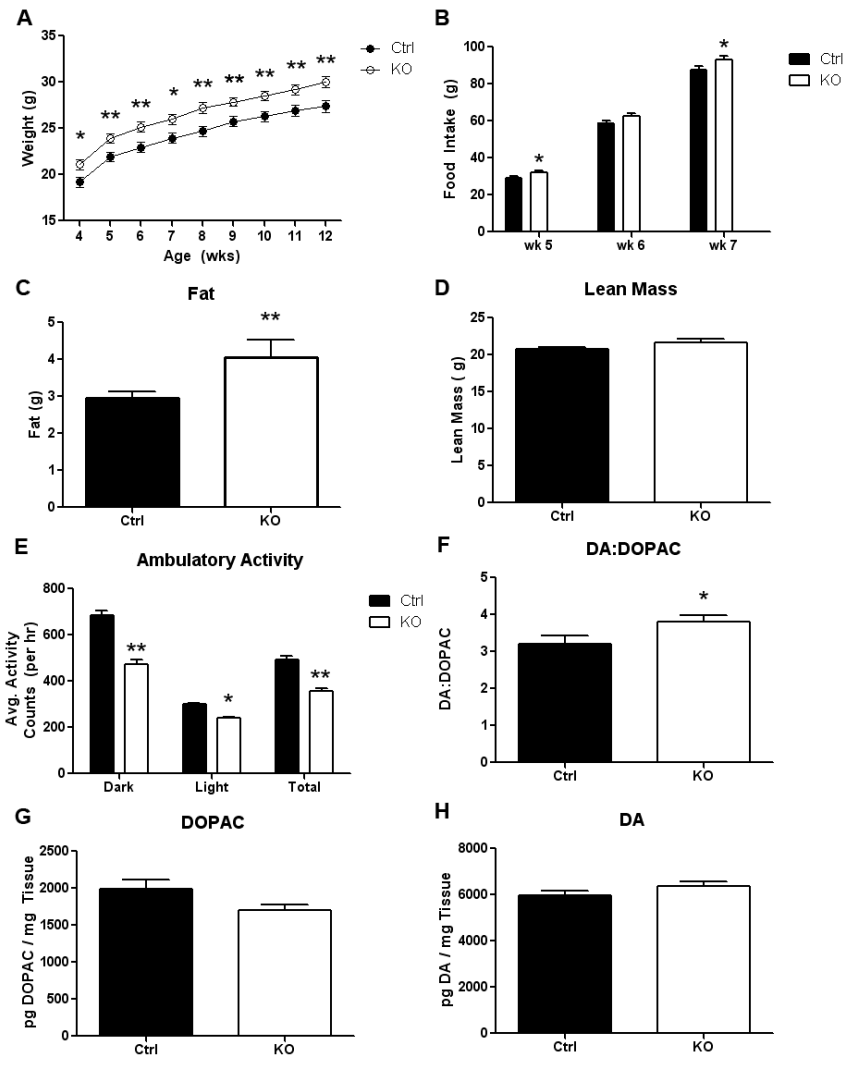


Figure 17. Physiological and body composition measurements from LepRb^{Nts} KO mice. Preliminary analylysis of LepRb^{Nts} KO mice shows that they have A) increased body weight (n = 25 for KO and Ctrl, $p < 0.01$) B) mild hyperphagia (n = 25 for KO and Ctrl, $p < 0.05$) C) increased fat mass (n = 25 for KO and Ctrl, Ctrl = 3.03 ± 0.28, KO = 4.41 ± 0.40) with D) no changes in lean mass relative to littermate controls. Additionally these mice have E) significantly decreased baseline locomotor activity (both in the light and dark cycle) suggesting a phenotype similar to what is seen in OX KO mice (n = 25 for KO and Ctrl, $p < 0.01$) and F) Increased DA:DOPAC ratio via HPLC suggests (Ctrl = 3.2 ± 0.23, n = 12; KO = 3.8 ± 0.18, n = 15, $p < 0.05$) that there are alterations in the MLDA in this molecular mouse model. G) DOPAC levels trend to lower in KO mice (Ctrl = 1987 ± 129, n = 12; KO = 1696 ± 75, n = 15, $p = 0.053$) while H) DA levels are not different. All graphs and data are mean ± SEM. Asterisks denote significant differences.

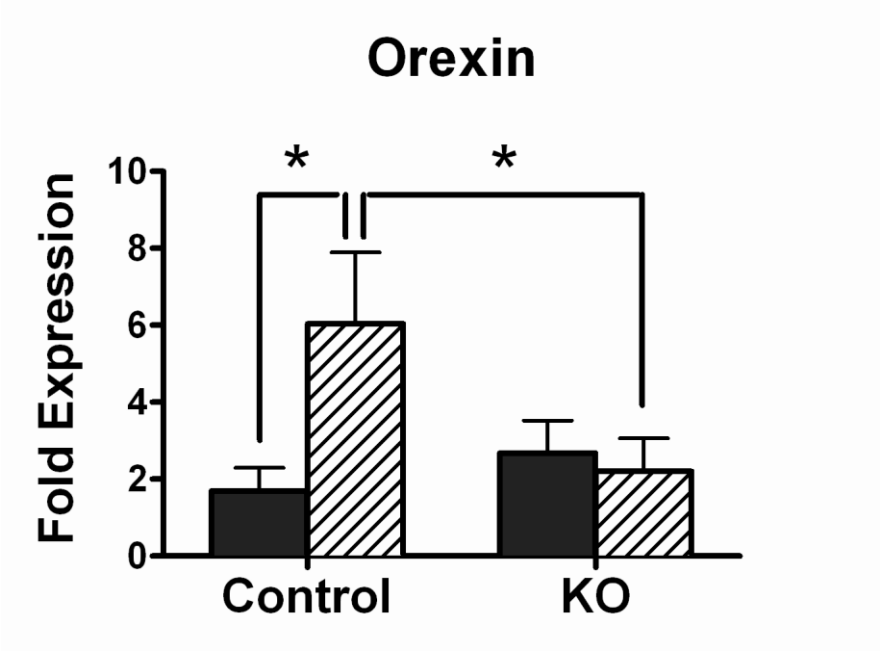


Figure 18. Lack of leptin induced increase in OX mRNA in LHA of $LepRb^{Nts}$ KO mice. Quantitative analysis of orexin mRNA expression in the LHA of $LepRb^{Nts}$ KO mice and control mice shows that i.p leptin (5 mg/kg) significantly increases OX mRNA expression in the LHA of control mice (PBS = 1.69 ± 0.60; Leptin = 6.03 ± 1.85; n = 12,10 respectively; $p < 0.05$) but does not do so in the LHA of KO mice (PBS = 2.67 ± 0.84; Leptin = 2.20 ± 0.85; n = 14, 17 respectively; $p < 0.05$ compared to Control + Leptin group). Graphs are mean ± SEM.

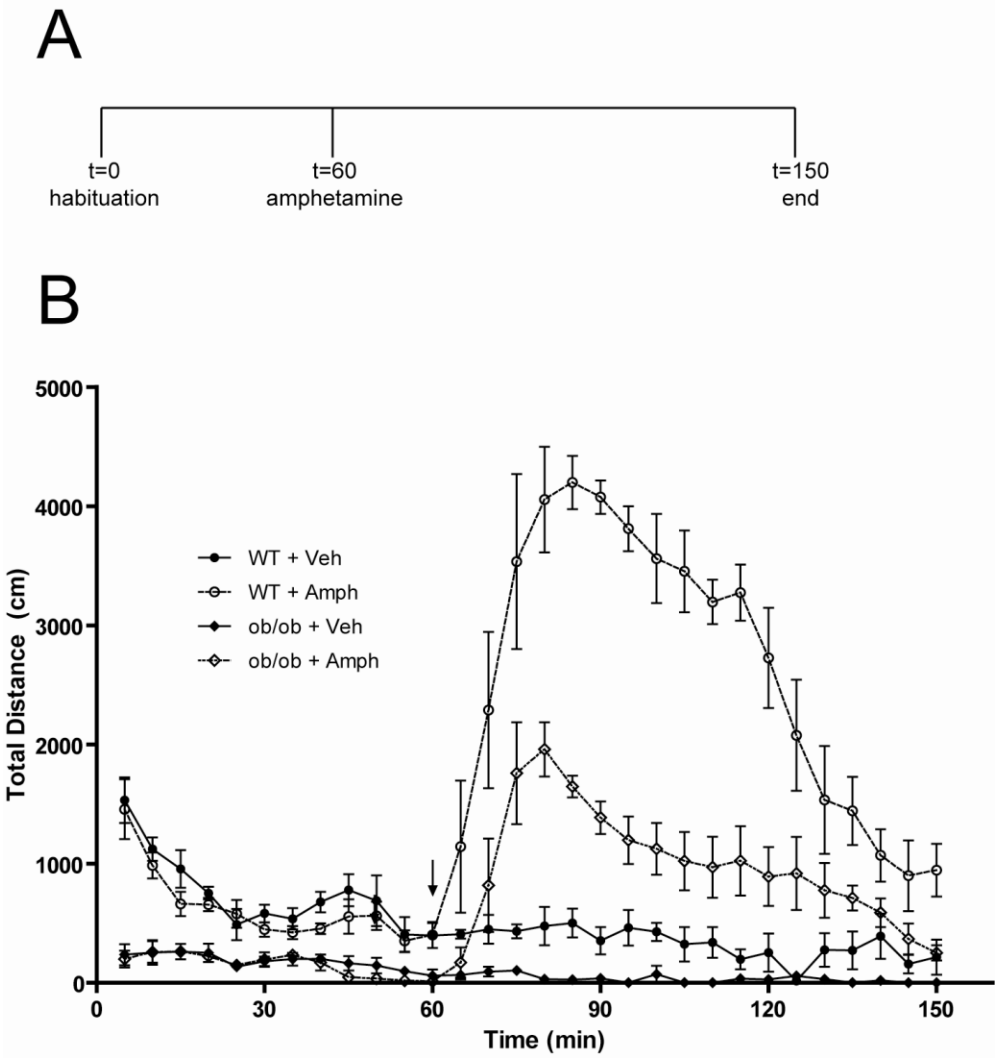


Figure 19. Blunted amphetamine-induced locomotor activity in *LepR*$^{ob/ob}$ mice. Experimental paradigm for initial amphetamine-induced locomotor activity studies (A) *LepR*$^{ob/ob}$ mice and WT controls were let to habituate for 30 minutes before receiving a sham injection (saline). Thirty minutes after the sham injection the test dose of amphetamine (4 mg/kg, i.p.) was administered and total distance traveled was recorded for an additional 90 minutes. (B) Blunted amphetamine-induced locomotor activity in LepR$^{ob/ob}$ mice relative to control mice (Wt-amph vs Ob-amph: $F(1,12) = 23.323$, $p<0.003$, $n = 4$ per group).

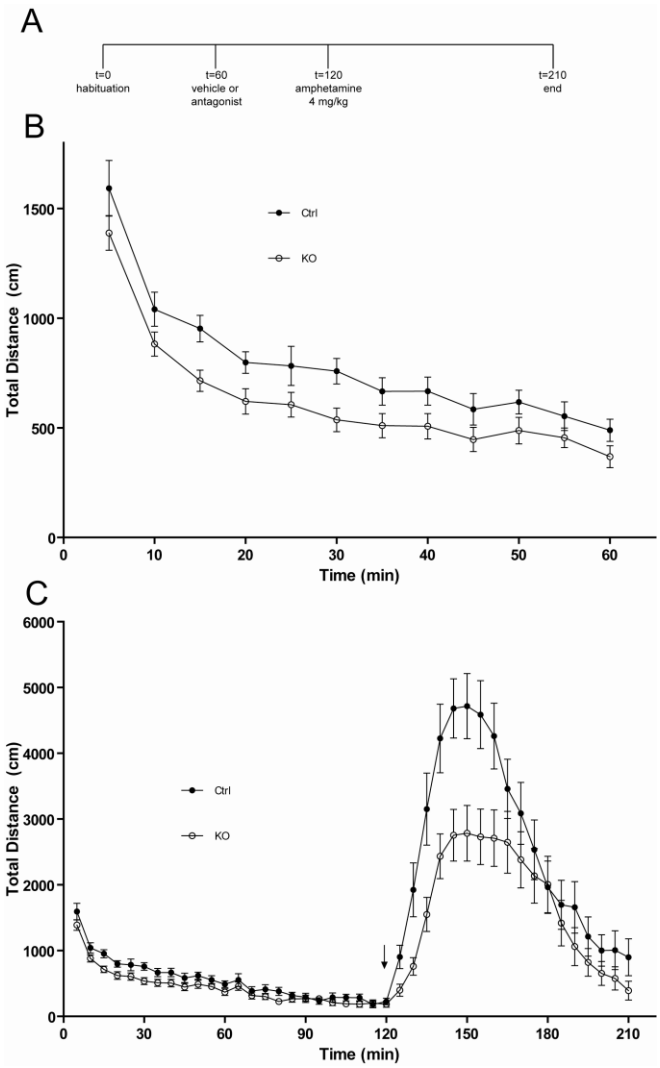


Figure 20. Decreased locomotor activity and blunted amphetamine response in LepRb^{Nts} KO mice. Open field activity testing was performed on LepRb^{Nts} KO (KO) mice and littermate controls (Ctrl) fed *ab lib* (fed) or fasted 24 hours (fast) in order to assess overall activity levels as well as amphetamine stimulated locomotion. (A) Alternate locomotor testing paradigm with longer habituation period and within-subject design for vehicle treatment before amphetamine-activity recording; also allows for treatment with pharmacological inhibitor During habituation to the activity chamber (B) LepRb^{Nts} KO mice had significantly lower activity than controls ($F(1,33) = 5.16$, $p = 0.03$ for 60 min. of habituation, all n = 15 except fed-KOs: n = 20). Following 4 mg/kg amphetamine (C) LepRb^{Nts} KO mice showed significantly decreased activity ($F(1,33) = 4.64$, $p = 0.039$ for 90 min. post amphetamine, same n as in (B)). All graphs are in 5 minute bins and show mean ± SEM. Arrow denotes amphetamine administration. (B) is an enlarged version of the first 60 minutes of (C).

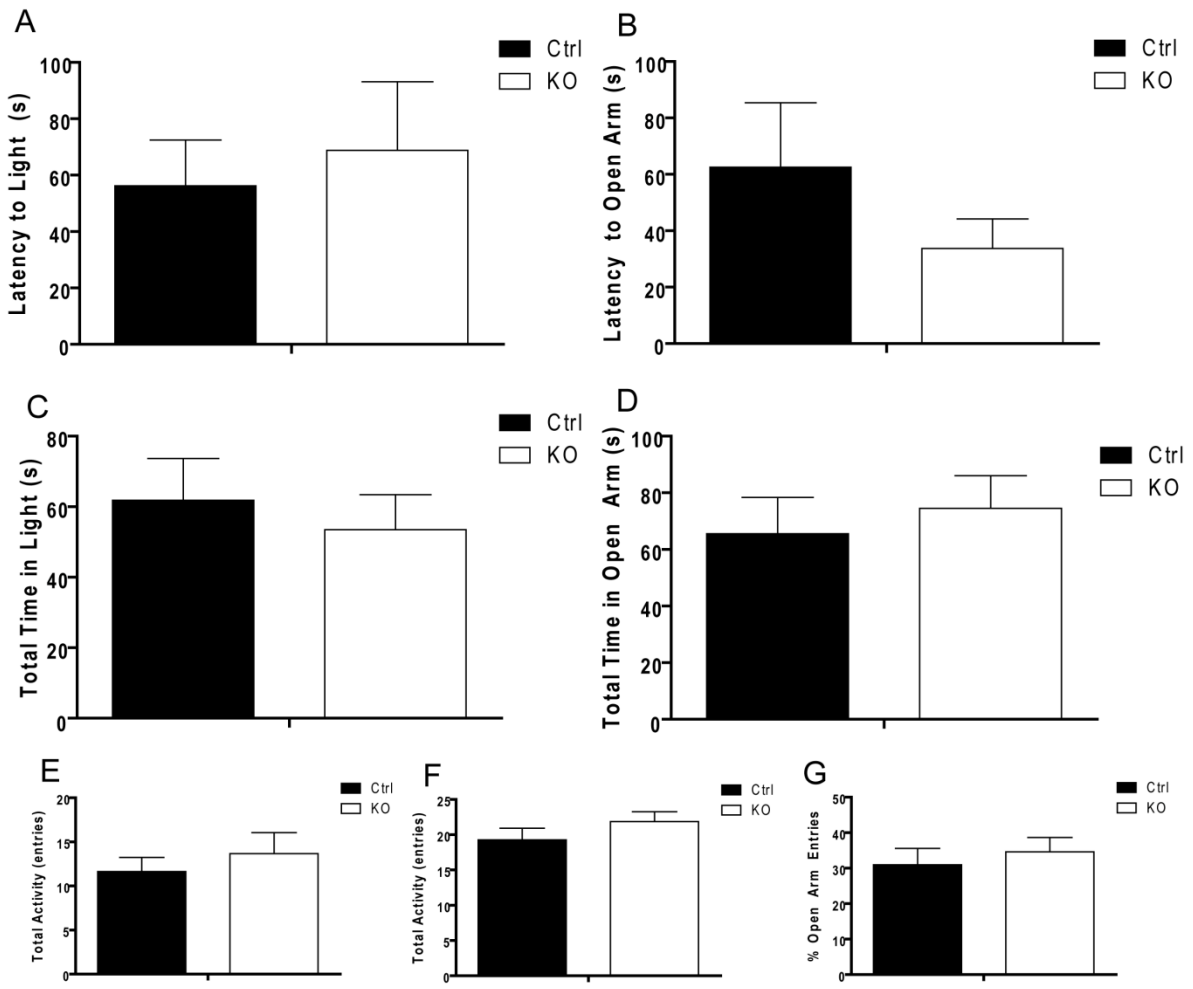


Figure 21. No anxiety-like behavioral phenotype in LepRb^{Nts} mice. Assessment of anxiety-like behaviors in LepRb^{KO} mice (n = 19) and controls (n = 18) using a two-chamber light-dark box (L-D) and elevated plus maze (EPM) show no overt differences in latency to enter the light in the L-D box (A) or open arm of the EPM (B). No differences were seen in total time spent in light compartment (C) or open arms (D). No changes in total activity in L-D box (total transitions between chambers, E) and EPM (total arm entries, F) were observed, nor was there a difference in the % open arm entries in the EPM (G).

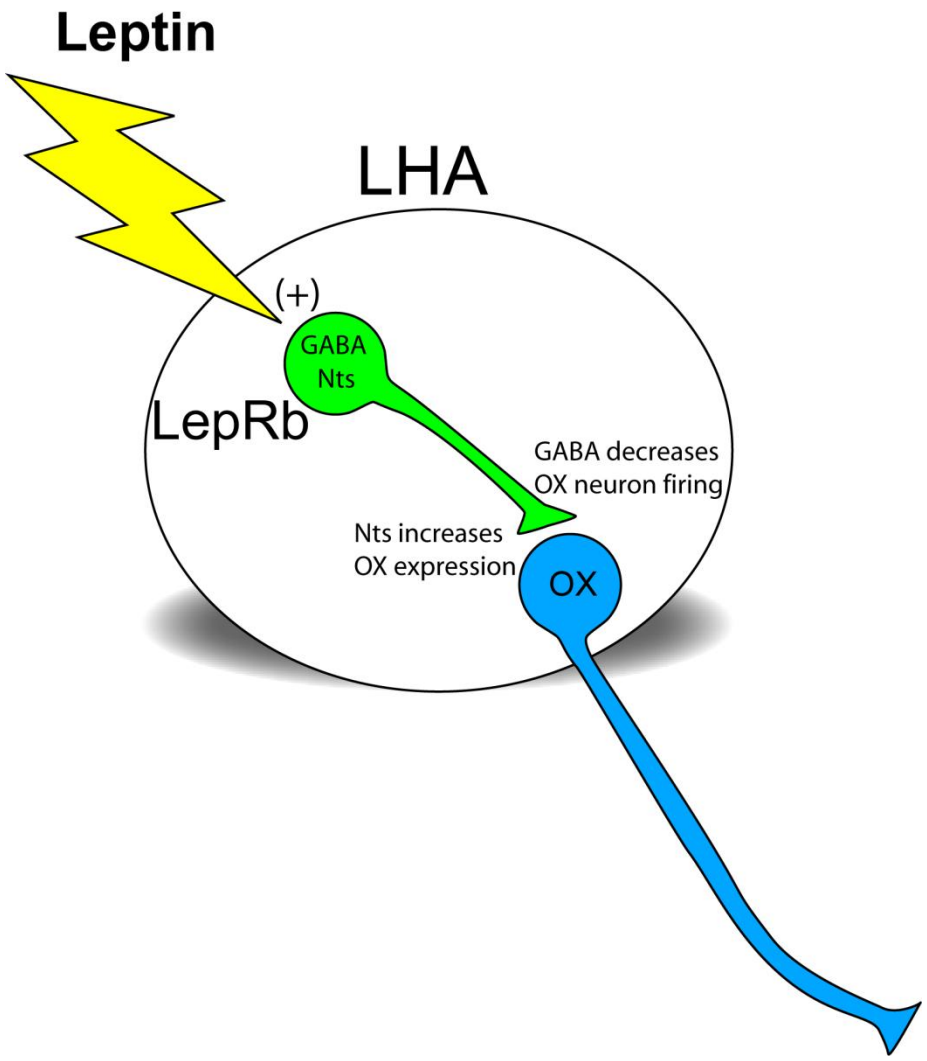


Figure 22. Updated hypothesis model of leptin action in the LHA. Our recent data suggest an explanation for the regulation of OX neurons by LHA LepRb action. We propose that leptin activation of LHA LepRb neurons causes GABA and Nts release onto downstream OX neurons: GABA release decreases the firing of OX neurons whereas Nts is responsible for mediating the expression of OX.

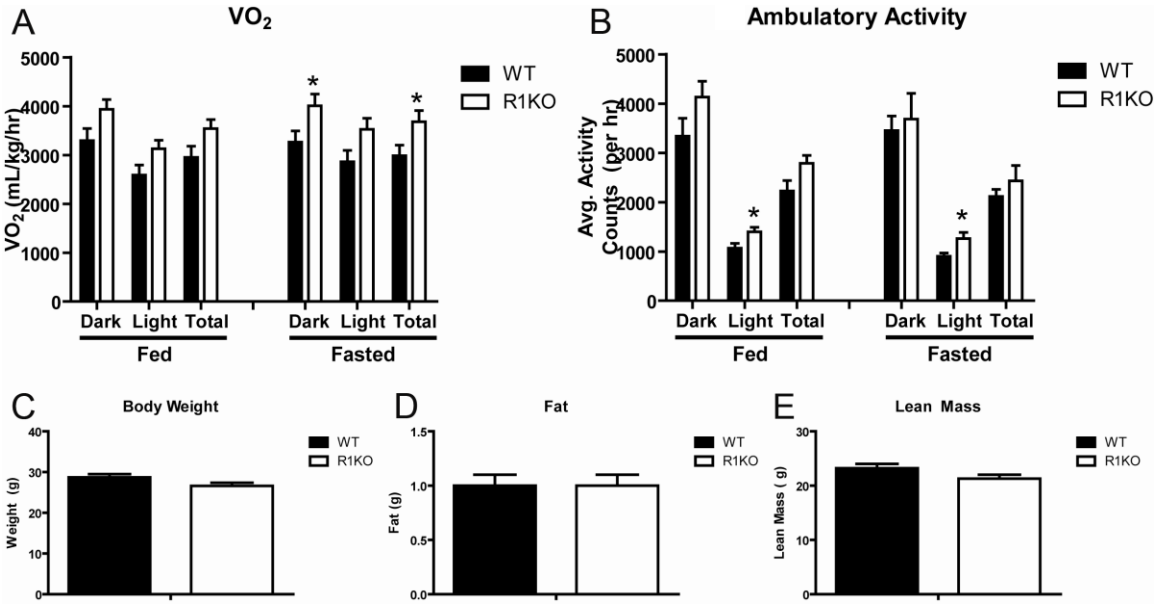


Figure 23. Metabolic analysis of NtsR1KO mice and WT shows altered respiration during active cycle and increased activity during light cycle.

Comprehensive metabolic testing of NtsR1KO mice (n = 8) and WT controls (n = 8) were performed using CLAMS chambers. (A) Maximum oxygen consumption (mL/kg/hr) measured in 20 minute bins for each 12 or 24 hour time period (light, dark or total) and was averaged for 3 consecutive days (fed condition) and then for during one full day of fasting (fasted condition) showing significantly increase aerobic capacity in fasted KO mice during the dark phase (WT = 2986.3 ± 216.1, KO = 3681.9 ± 228.4, $p < 0.05$). (B) Total x-axis ambulatory activity was also quantified and increased light cycle activity was seen in KO mice both in the fed and fasted conditions (fasted: WT = 900.7 ± 66.7, KO = 1259.2 ± 127.5, $p < 0.05$; fed: WT = 1068 ± 95.9, KO = 1398 ± 91.0; n = 8 for both). (C-E) There was no alteration in overall body weight, fat mass or lean mass in KO mice relative to WT mice. Graphs show mean ± SEM. Asterisks denote $p \leq 0.05$.

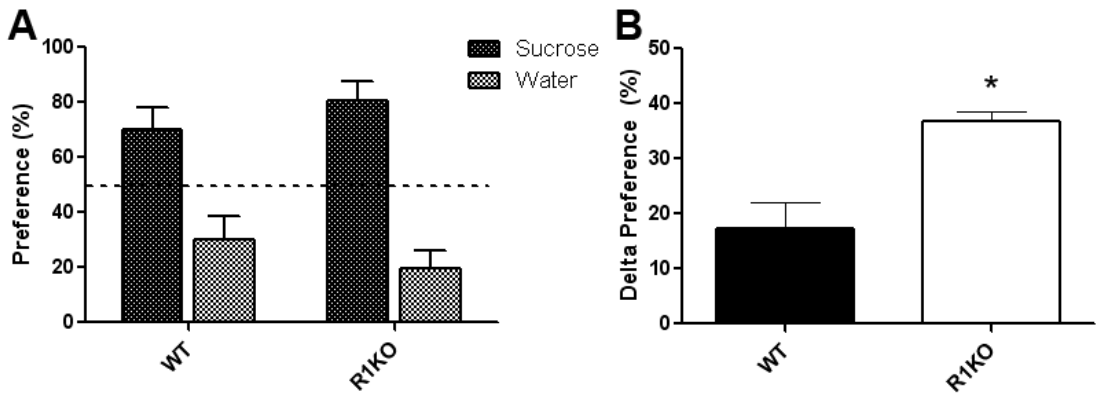


Figure 24. Increased preference for low concentration sucrose in NtsR1KO mice relative to WTs. A two-bottle sucrose preference testing paradigm was used to assess sensitivity to low levels of reward in NtsR1KO mice (n = 10) and WT controls (n = 10). (A) Overall preference for 0.5% sucrose increased in NtsR1KO mice with a significant increase in sucrose intake compared to baseline preference scores for the same lixit paired with sucrose (B) (WT = 17.3 ± 4.8%, KO = 36.7 ± 1.8%, $p < 0.05$). All graphs are mean ± SEM. Asterisks denote $p \leq 0.05$.

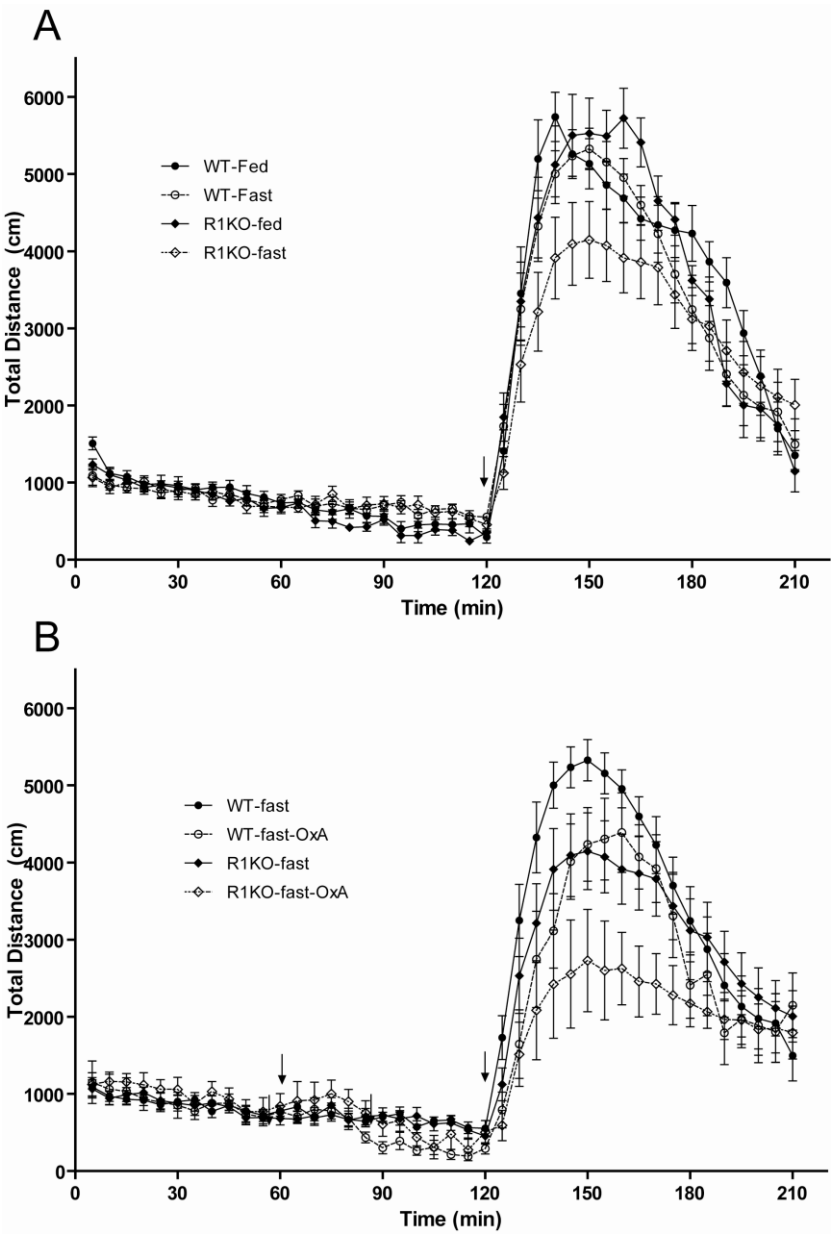


Figure 25. Altered sensitivity to amphetamine in NtsR1KO mice is not dependent on orexin activity. Amphetamine (4 mg/kg) induced locomotor activity was measured in NtsR1KO mice (n = 8 fed, n = 13 fasted) and WT controls (n = 8 fed, n = 14 fasted). (A) Fasted NtsR1KO mice showed blunted amphetamine response ($F(1,39) = 3.3$, $p < 0.05$ for 90 minutes following amphetamine) that was not seen in fed KO mice. (B) Pretreatment with SB-334,867, an OXR_1 antagonist (20 mg/kg, i.p., 1 hr) showed a significant decrease in activity 20 minutes after administration ($t = 80 – 120$, $F(12,1) = 3.93$, $p < 0.05$, n = 8 KO and n = 6 WT) and had a similar effect in reducing amphetamine induced locomotor activity in NtsR1KO and WT mice ((37,1) = 11.70, $p < 0.01$ for 90 minutes following amphetamine). Graphs are mean ± SEM and show 5 minute bins.

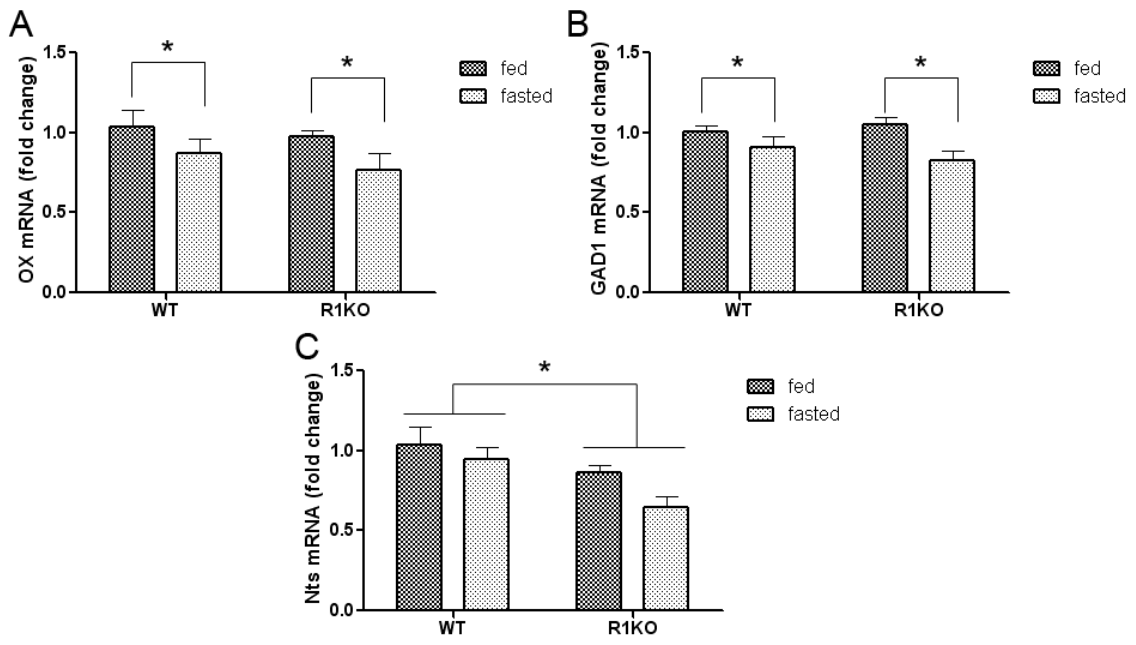


Figure 26. Decreased orexin, GAD1 and Nts mRNA in fasted NtsR1KO mice. Tissue was microdissected from the LHA of fed and 24-hour fasted NtsR1KO and WT mice and processed for mRNA extraction. Samples were assayed using qPCR for several genes. (A) Orexin mRNA levels were significantly decreased by fasting in both genotypes ($F(1,28) = 4.33$, $p = 0.047$, WT-fed = 1.03 ± 0.10, WT-fast = 0.86 ± 0.09, KO-fed = 0.97 ± 0.03, KO-fast = 0.76 ± 0.01, n = 8 per group). (B) GAD1 mRNA was also decreased by fasting ($F(1,28) = 3.49$, $p = 0.008$, n = 8). C) Levels of Nts mRNA were significantly decreased in NtsR1KO mice relative to WT controls ($F(1,28) = 0.465$, $p = 0.004$, n = 8 each). All graphs show mean ± SEM and asterisks denote $p \leq 0.05$.

.

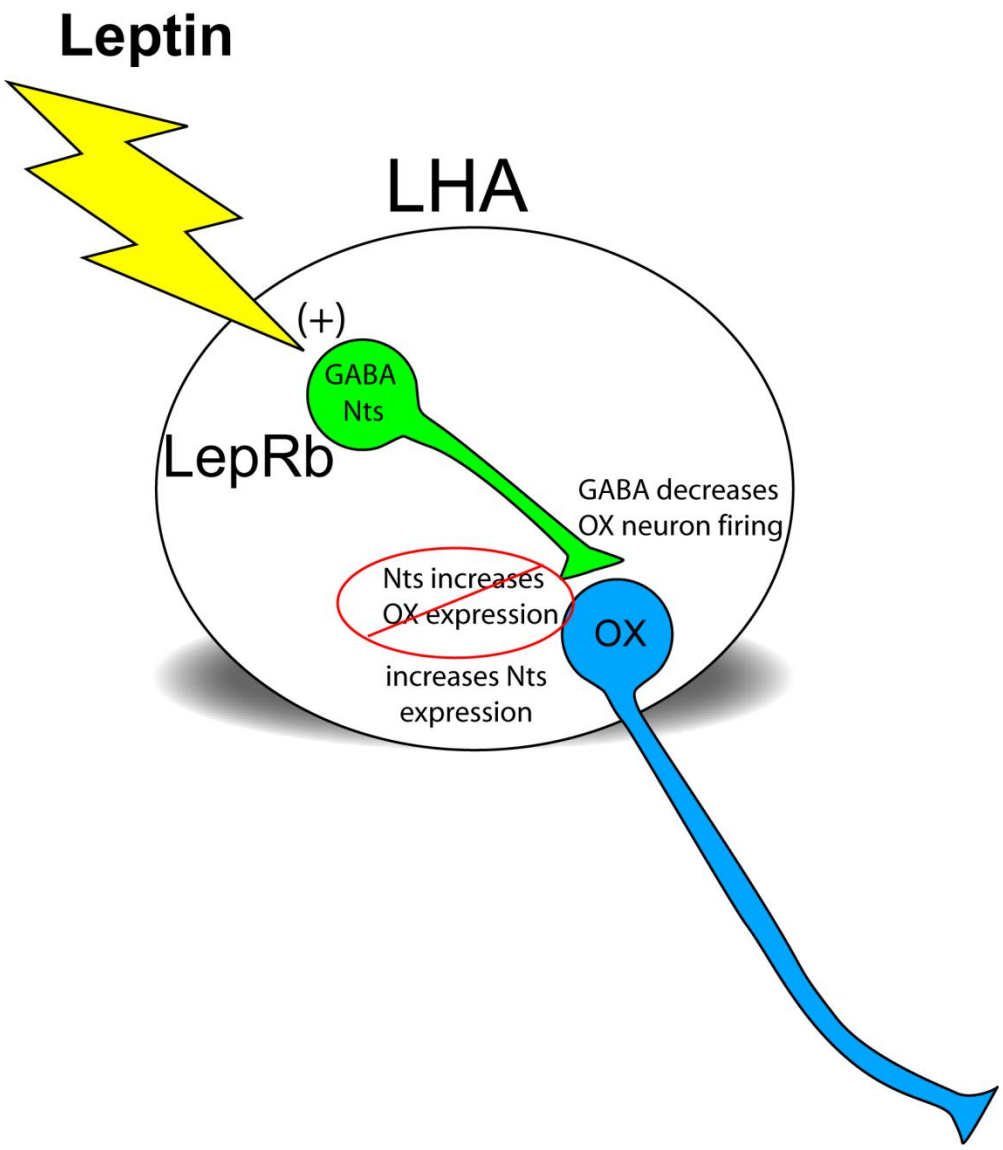


Figure 27. Updated model of leptin-responsive neural interactions with the MLDA. Updated model of LHA leptin action shows that leptin depolarizes GABA- and Nts-containing LepRb neurons. GABA release decreases the firing of downstream OX neurons. While we had hypothesized that Nts release would modulate OX expression, this now seems unlikely. Nts action via NtsR1 in the LHA likely mediates the expression of Nts itself.

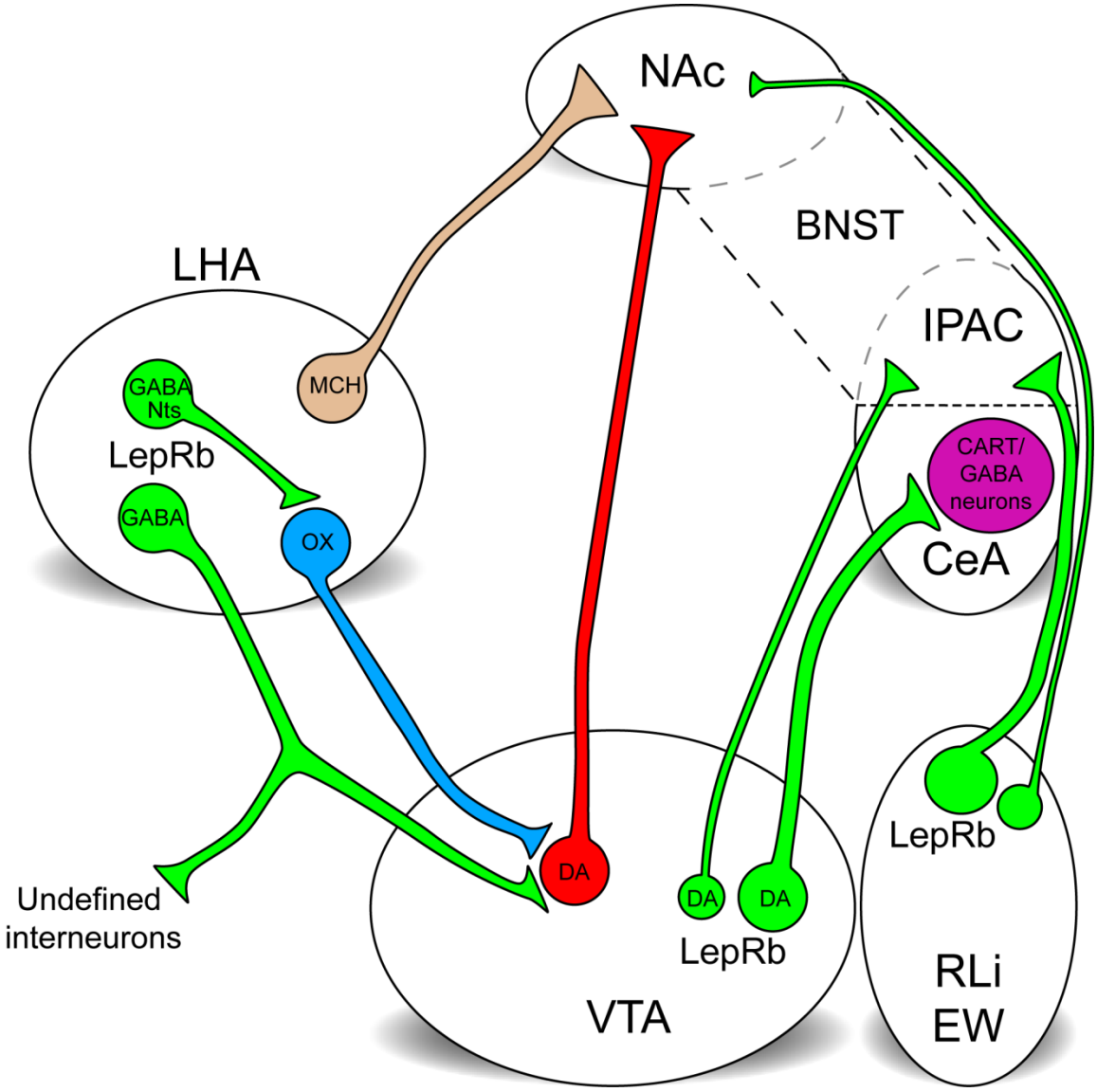


Figure 28. Overall model of leptin interaction with the MLDA. Complete model of sties of LepRb neuron interaction with elements of the MLDA. In the midbrain, LepRb neuron populations in the VTA project to limbic targets in the CeA and IPAC whereas midline projections from the RLi/EW project mainly to striatal targets in the IPAC and NAc. Separate populations of LepRb neurons in the LHA project locally to OX neurons and indirectly to VTA DA neurons where they can regulate gene expression and firing to modulate striatal DA function.

References

1. Flegal, K.M., et al., *Prevalence and trends in obesity among US adults, 1999-2000.* JAMA, 2002. **288**(14): p. 1723-7.
2. Kelley, A.E. and K.C. Berridge, *The neuroscience of natural rewards: relevance to addictive drugs.* J Neurosci., 2002. **22**(9): p. 3306-3311.
3. Kelley, A.E., B.A. Baldo, and W.E. Pratt, *A proposed hypothalamic-thalamic-striatal axis for the integration of energy balance, arousal, and food reward.* J Comp Neurol., 2005. **493**(1): p. 72-85.
4. Thorndike, E., *Some Experiments on Animal Intelligence.* Science, 1898. **7**(181): p. 818-824.
5. Hull, C.L., *Principles of behavior, an introduction to behavior theory*. 1943, New York,: D. Appleton-Century Company. x p., 1 l., 422 p., 1 l.
6. Ahima, R.S., et al., *Leptin regulation of neuroendocrine systems.* Front Neuroendocrinol., 2000. **21**(3): p. 263-307.
7. Berthoud, H.R., *Neural control of appetite: cross-talk between homeostatic and non-homeostatic systems.* Appetite, 2004. **43**(3): p. 315-317.
8. Stellar, E., *The physiology of motivation.* Psychol Rev, 1954. **61**(1): p. 5-22.
9. Teitelbaum, P. and E. Stellar, *Recovery from the failure to eat produced by hypothalamic lesions.* Science, 1954. **120**(3126): p. 894-5.
10. Berthoud, H.R., *Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance.* Obesity.(Silver.Spring), 2006. **14 Suppl 5**: p. 197S-200S.
11. Myers, M.G., Jr., et al., *The geometry of leptin action in the brain: more complicated than a simple ARC.* Cell Metab, 2009. **9**(2): p. 117-123.
12. Grill, H.J., *Distributed neural control of energy balance: contributions from hindbrain and hypothalamus.* Obesity.(Silver.Spring), 2006. **14 Suppl 5**: p. 216S-221S.
13. Nestler, E.J., *Is there a common molecular pathway for addiction?* Nat.Neurosci., 2005. **8**(11): p. 1445-1449.
14. Figlewicz, D.P. and S.C. Benoit, *Insulin, leptin, and food reward: update 2008.* Am J Physiol Regul Integr Comp Physiol, 2009. **296**(1): p. R9-R19.
15. Hao, J., et al., *Effects of central leptin infusion on the reward-potentiating effect of D-amphetamine.* Brain Res., 2006. **1087**(1): p. 123-133.

16. Cuénot, L., *Les races pures et leur combinaisons chez les souris.* Arch. Zool. Expér. Génér, 1905. **3**: p. 123-132.
17. Bultman, S.S., E.S. Michaud, and R.P. Woychik, *Molecular characterization of the mouse agouti locus.* Cell, 1992. **71**: p. 1195-1204.
18. Michaud, E.J., et al., *A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (Ay) mutation.* Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2562-6.
19. Ingalls, A.M., M.M. Dickie, and G.D. Snell, *Obese, a new mutation in the house mouse.* J Hered, 1950. **41**(12): p. 317-8.
20. Hummel, K.P., M.M. Dickie, and D.L. Coleman, *Diabetes, a new mutation in the mouse.* Science, 1966. **153**(740): p. 1127-8.
21. Coleman, D.L. and K.P. Hummel, *Effects of parabiosis of normal with genetically diabetic mice.* Am.J.Physiol., 1969. **217**: p. 1298-1304.
22. Coleman, D.L., *Effects of parabiosis of obese with diabetic and normal mice.* Diabetologia, 1973. **4**: p. 294-298.
23. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**: p. 425-432.
24. Saladin, R., et al., *Transient increase in obese gene expression after food intake or insulin administration.* Nature, 1995. **377**(6549): p. 527-529.
25. Maffei, M., et al., *Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects.* Nature Medicine, 1995. **1**(11): p. 1155-1161.
26. Frederich, R.C., et al., *Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action.* Nature Medicine, 1995. **1**(12): p. 1311-1314.
27. Tartaglia, L.A., et al., *Identification and expression cloning of a leptin receptor, OB-R.* Cell, 1995. **83**(7): p. 1263-1271.
28. Chua, J., S.C., et al., *Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor.* Science, 1996. **271**: p. 994-996.
29. Chen, H., et al., *Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice.* Cell, 1996. **84**(3): p. 491-5.
30. Lee, G.-H., et al., *Abnormal splicing of the leptin receptor in diabetic mice.* Nature, 1996. **379**: p. 632-635.
31. Huang, L., Z. Wang, and C. Li, *Modulation of circulating leptin levels by its soluble receptor.* J Biol Chem, 2001. **276**(9): p. 6343-9.
32. Fong, T.M., et al., *Localization of leptin binding domain in the leptin receptor.* Mol.Pharmacol., 1998. **53**(2): p. 234-240.
33. Kowalski, T.J., et al., *Transgenic complementation of leptin-receptor deficiency. I. Rescue of the obesity/diabetes phenotype of LEPR-null mice expressing a LEPR-B transgene.* Diabetes, 2001. **50**(2): p. 425-435.
34. Robertson, S.A., et al., *Regulation of Jak2 function by phosphorylation of Tyr317 and Tyr637 during cytokine signaling.* Mol Cell Biol, 2009. **29**(12): p. 3367-78.

35. Bjornholm, M., et al., *Mice lacking inhibitory leptin receptor signals are lean with normal endocrine function.* J Clin.Invest, 2007. **117**(5): p. 1354-1360.
36. Bates, S.H., et al., *LRb-STAT3 signaling is required for the neuroendocrine regulation of energy expenditure by leptin.* Diabetes, 2004. **53**(12): p. 3067-73.
37. Friedman, J.M. and J.L. Halaas, *Leptin and the regulation of body weight in mammals.* Nature, 1998. **395**(6704): p. 763-70.
38. Ahima, R.S., et al., *Leptin regulation of neuroendocrine systems.* Front Neuroendocrinol, 2000. **21**(3): p. 263-307.
39. Coppari, R., et al., *The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity.* Cell Metab, 2005. **1**(1): p. 63-72.
40. Schwartz, M.W., et al., *Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus.* Diabetes, 1997. **46**(12): p. 2119-2123.
41. Elias, C.F., et al., *Leptin activates hypothalamic CART neurons projecting to the spinal cord.* Neuron, 1998. **21**: p. 1375-1385.
42. Cone, R.D., et al., *The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis.* Int J Obes Relat Metab Disord, 2001. **25 Suppl 5**: p. S63-7.
43. Bouret, S.G., S.J. Draper, and R.B. Simerly, *Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice.* J Neurosci., 2004. **24**(11): p. 2797-2805.
44. Bouret, S.G., S.J. Draper, and R.B. Simerly, *Trophic action of leptin on hypothalamic neurons that regulate feeding.* Science, 2004. **304**(5667): p. 108-110.
45. Elias, C.F., et al., *Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area.* Neuron, 1999. **23**(4): p. 775-86.
46. Elmquist, J.K., *Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin.* Int.J Obes.Relat Metab Disord, 2001. **25 Suppl 5**: p. S78-S82.
47. Butler, A.A. and R.D. Cone, *The melanocortin receptors: lessons from knockout models.* Neuropeptides, 2002. **36**(2-3): p. 77-84.
48. Wilson, B.D., et al., *Physiological and anatomical circuitry between Agouti-related peptide and leptin signaling.* Endocrinol., 1999. **140**: p. 2387-2397.
49. Bates, S.H., et al., *STAT3 signaling is required for leptin regulation of energy balance but not reproduction.* Nature, 2003. **421**: p. 856-859.
50. Bouret, S.G., S.J. Draper, and R.B. Simerly, *Trophic action of leptin on hypothalamic neurons that regulate feeding.* Science, 2004. **304**(5667): p. 108-10.
51. Bouret, S.G. and R.B. Simerly, *Development of leptin-sensitive circuits.* J Neuroendocrinol., 2007. **19**(8): p. 575-582.

52. Leshan, R.L., et al., *Direct innervation of GnRH neurons by metabolic- and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus.* J Neurosci., 2009. **29**(10): p. 3138-3147.
53. Fulton, S., B. Woodside, and P. Shizgal, *Modulation of brain reward circuitry by leptin.* Science, 2000. **287**(5450): p. 125-128.
54. Fulton, S., et al., *Food restriction and leptin impact brain reward circuitry in lean and obese Zucker rats.* Behav.Brain Res., 2004. **155**(2): p. 319-329.
55. Figlewicz, D.P., et al., *Leptin reverses sucrose-conditioned place preference in food-restricted rats.* Physiol Behav., 2001. **73**(1-2): p. 229-234.
56. Figlewicz, D.P., et al., *Intraventricular insulin and leptin reverse place preference conditioned with high-fat diet in rats.* Behav.Neurosci., 2004. **118**(3): p. 479-487.
57. Figlewicz, D.P., et al., *Intraventricular insulin and leptin decrease sucrose self-administration in rats.* Physiol Behav., 2006. **89**(4): p. 611-616.
58. Shalev, U., J. Yap, and Y. Shaham, *Leptin attenuates acute food deprivation-induced relapse to heroin seeking.* J Neurosci, 2001. **21**(4): p. RC129.
59. Hao, J., d.V. Cabeza, and K.D. Carr, *Effects of chronic ICV leptin infusion on motor-activating effects of D-amphetamine in food-restricted and ad libitum fed rats.* Physiol Behav., 2004. **83**(3): p. 377-381.
60. Szczypka, M.S., M.A. Rainey, and R.D. Palmiter, *Dopamine is required for hyperphagia in Lep(ob/ob) mice.* Nat Genet, 2000. **25**(1): p. 102-4.
61. Krugel, U., et al., *Basal and feeding-evoked dopamine release in the rat nucleus accumbens is depressed by leptin.* Eur J Pharmacol, 2003. **482**(1-3): p. 185-7.
62. Perry, M.L., et al., *Leptin promotes dopamine transporter and tyrosine hydroxylase activity in the nucleus accumbens of Sprague-Dawley rats.* J Neurochem, 2010. **114**(3): p. 666-74.
63. Liu, J., et al., *Acute administration of leptin produces anxiolytic-like effects: a comparison with fluoxetine.* Psychopharmacology (Berl), 2009.
64. Lu, X.Y., et al., *Leptin: a potential novel antidepressant.* Proc.Natl.Acad.Sci.U.S.A, 2006. **103**(5): p. 1593-1598.
65. Farooqi, I.S., et al., *Leptin regulates striatal regions and human eating behavior.* Science, 2007. **317**(5843): p. 1355.
66. Rosenbaum, M., et al., *Leptin reverses weight loss-induced changes in regional neural activity responses to visual food stimuli.* J Clin Invest, 2008. **118**(7): p. 2583-91.
67. Simon, G.E., et al., *Association between obesity and psychiatric disorders in the US adult population.* Arch Gen Psychiatry, 2006. **63**(7): p. 824-30.
68. Figlewicz, D.P., *Adiposity signals and food reward: expanding the CNS roles of insulin and leptin.* Am.J Physiol Regul.Integr.Comp Physiol, 2003. **284**(4): p. R882-R892.
69. Hommel, J.D., et al., *Leptin receptor signaling in midbrain dopamine neurons regulates feeding.* Neuron, 2006. **51**(6): p. 801-810.

70. Chua, S.C., Jr., et al., *Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor.* Science, 1996. **271**(5251): p. 994-6.
71. Greenwood, M.R., et al., *Food motivated behavior in genetically obese and hypothalamic-hyperphagic rats and mice.* Physiol Behav, 1974. **13**(5): p. 687-92.
72. Hoebel, B.G. and P. Teitelbaum, *Hypothalamic control of feeding and self-stimulation.* Science, 1962. **135**: p. 375-7.
73. Beatty, W.W., *Influence of type of reinforcement on operant responding by rats with ventromedial lesions.* Physiol Behav, 1973. **10**(5): p. 841-6.
74. Vasselli, J.R., et al., *Development of food motivated behavior in free feeding and food restricted Zucker fatty (fa/fa) rats.* Physiol Behav, 1980. **25**(4): p. 565-73.
75. Fulton, S., et al., *Leptin regulation of the mesoaccumbens dopamine pathway.* Neuron, 2006. **51**(6): p. 811-22.
76. Roseberry, A.G., et al., *Decreased vesicular somatodendritic dopamine stores in leptin-deficient mice.* J Neurosci., 2007. **27**(26): p. 7021-7027.
77. Ikemoto, S., *Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex.* Brain Res Rev., 2007. **56**(1): p. 27-78.
78. Bjorklund, A. and S.B. Dunnett, *Dopamine neuron systems in the brain: an update.* Trends Neurosci, 2007. **30**(5): p. 194-202.
79. Lammel, S., et al., *Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system.* Neuron, 2008. **57**(5): p. 760-773.
80. Shizgal, P., S. Fulton, and B. Woodside, *Brain reward circuitry and the regulation of energy balance.* Int.J Obes.Relat Metab Disord., 2001. **25 Suppl 5**: p. S17-S21.
81. DiLeone, R.J., D. Georgescu, and E.J. Nestler, *Lateral hypothalamic neuropeptides in reward and drug addiction.* Life Sci., 2003. **73**(6): p. 759-768.
82. Harris, G.C., M. Wimmer, and G. Aston-Jones, *A role for lateral hypothalamic orexin neurons in reward seeking.* Nature, 2005. **437**(7058): p. 556-559.
83. Ludwig, D.S., et al., *Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus.* Am J Physiol, 1998. **274**(4 Pt 1): p. E627-33.
84. Funato, H., et al., *Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity.* Cell Metab, 2009. **9**(1): p. 64-76.
85. Hara, J., M. Yanagisawa, and T. Sakurai, *Difference in obesity phenotype between orexin-knockout mice and orexin neuron-deficient mice with same genetic background and environmental conditions.* Neurosci Lett, 2005. **380**(3): p. 239-42.
86. Leinninger, G.M., et al., *Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding.* Cell Metab, 2009. **10**(2): p. 89-98.

87. Louis, G.W., et al., *Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons.* J Neurosci, 2010. **30**(34): p. 11278-87.
88. Friedman, J.M., *The function of leptin in nutrition, weight, and physiology.* Nutr Rev, 2002. **60**(10 Pt 2): p. S1-14.
89. Morton, G.J., et al., *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-295.
90. Elmquist, J.K., et al., *Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis.* J Comp Neurol., 2005. **493**(1): p. 63-71.
91. Gao, Q. and T.L. Horvath, *Neurobiology of feeding and energy expenditure.* Annu.Rev.Neurosci., 2007. **30**: p. 367-398.
92. Berthoud, H.R., *Interactions between the "cognitive" and "metabolic" brain in the control of food intake.* Physiol Behav., 2007.
93. Cohen, P., et al., *Selective deletion of leptin receptor in neurons leads to obesity.* J Clin Invest, 2001. **108**(8): p. 1113-1121.
94. de Luca, C., et al., *Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes.* J. Clin. Invest., 2005: p. JCI24059.
95. Figlewicz, D.P., A.M. Naleid, and A.J. Sipols, *Modulation of food reward by adiposity signals.* Physiol Behav., 2006.
96. Fulton, S., et al., *Leptin regulation of the mesoaccumbens dopamine pathway.* Neuron, 2006. **51**(6): p. 811-822.
97. Scott, M.M., et al., *Leptin targets in the mouse brain.* J Comp Neurol., 2009. **514**(5): p. 518-532.
98. Leinninger, G.M., et al., *Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding.* Cell Metab, 2009.
99. Huo, L., et al., *Leptin-dependent control of glucose balance and locomotor activity by POMC neurons.* Cell Metab, 2009. **9**(6): p. 537-547.
100. DiMicco, J.A. and D.V. Zaretsky, *The dorsomedial hypothalamus: a new player in thermoregulation.* Am.J Physiol Regul.Integr.Comp Physiol, 2007. **292**(1): p. R47-R63.
101. Balthasar, N., et al., *Leptin Receptor Signaling in POMC Neurons Is Required for Normal Body Weight Homeostasis.* Neuron, 2004. **42**(6): p. 983-991.
102. Dhillon, H., et al., *Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis.* Neuron, 2006. **49**(2): p. 191-203.
103. Scott, M.M., et al., *Leptin targets in the mouse brain.* J Comp Neurol, 2009. **514**(5): p. 518-32.
104. Margolis, E.B., et al., *Midbrain dopamine neurons: projection target determines action potential duration and dopamine D(2) receptor inhibition.* J Neurosci., 2008. **28**(36): p. 8908-8913.

105. Leshan, R., et al., *Leptin receptor signalling and action in the central nervous system.* Obesity. (Silver Spring), 2006. **14**(Suppl 5): p. 208S-212S.
106. Morton, G.J., et al., *Arcuate nucleus-specific leptin receptor gene therapy attenuates the obesity phenotype of Koletsky (fa(k)/fa(k)) rats.* Endocrinol., 2003. **144**(5): p. 2016-2024.
107. Munzberg, H., et al., *Appropriate inhibition of orexigenic hypothalamic arcuate nucleus neurons independently of leptin receptor/STAT3 signaling.* J Neurosci., 2007. **27**(1): p. 69-74.
108. Zylka, M.J., F.L. Rice, and D.J. Anderson, *Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd.* Neuron, 2005. **45**(1): p. 17-25.
109. Elmquist, J.K., et al., *Distributions of leptin receptor mRNA isoforms in the rat brain.* J Comp Neurol, 1998. **395**(4): p. 535-547.
110. Braz, J.M., B. Rico, and A.I. Basbaum, *Transneuronal tracing of diverse CNS circuits by Cre-mediated induction of wheat germ agglutinin in transgenic mice.* Proc.Natl.Acad.Sci.U.S.A, 2002. **99**(23): p. 15148-15153.
111. Matsumoto, M. and O. Hikosaka, *Two types of dopamine neuron distinctly convey positive and negative motivational signals.* Nature, 2009. **459**(7248): p. 837-841.
112. Dandekar, M.P., et al., *Cocaine- and amphetamine-regulated transcript peptide plays a role in the manifestation of depression: social isolation and olfactory bulbectomy models reveal unifying principles.* Neuropsychopharmacology, 2009. **34**(5): p. 1288-1300.
113. Dandekar, M.P., et al., *Transient up-regulation of cocaine- and amphetamine-regulated transcript peptide (CART) immunoreactivity following ethanol withdrawal in rat hypothalamus.* Brain Res, 2008. **1240**: p. 119-131.
114. Dandekar, M.P., et al., *Importance of cocaine- and amphetamine-regulated transcript peptide in the central nucleus of amygdala in anxiogenic responses induced by ethanol withdrawal.* Neuropsychopharmacology, 2008. **33**(5): p. 1127-1136.
115. Hunter, R.G., et al., *Regulation of CART mRNA by stress and corticosteroids in the hippocampus and amygdala.* Brain Res, 2007. **1152**: p. 234-240.
116. Fagergren, P. and Y.L. Hurd, *Mesolimbic gender differences in peptide CART mRNA expression: effects of cocaine.* Neuroreport, 1999. **10**(16): p. 3449-52.
117. Bartke, A., et al., *Genes that prolong life: relationships of growth hormone and growth to aging and life span.* J Gerontol.A Biol Sci.Med Sci., 2001. **56**(8): p. B340-B349.
118. Myers, M.G., Jr., et al., *The geometry of leptin action in the brain: more complicated than a simple ARC.* Cell Metab, 2009. **9**(2): p. 117-23.
119. Leinninger, G.M., *Location, location, location: the CNS sites of leptin action dictate its regulation of homeostatic and hedonic pathways.* Int J Obes (Lond), 2009. **33 Suppl 2**: p. S14-7.

120. Carvalho, M.C., et al., *Central, but not basolateral, amygdala involvement in the anxiolytic-like effects of midazolam in rats in the elevated plus maze.* J Psychopharmacol, 2010.
121. de la Mora, M.P., et al., *Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: Structural and functional analysis.* Prog Neurobiol, 2010. **90**(2): p. 198-216.
122. Wand, G., *The anxious amygdala: CREB signaling and predisposition to anxiety and alcoholism.* J Clin Invest, 2005. **115**(10): p. 2697-9.
123. Coons, E.E. and H.A. White, *Tonic properties of orosensation and the modulation of intracranial self-stimulation: the CNS weighting of external and internal factors governing reward.* Ann N Y Acad Sci, 1977. **290**: p. 158-79.
124. Lorens, S.A., *Effect of lesions in the central nervous system on lateral hypothalamic self-stimulation in the rat.* J Comp Physiol Psychol, 1966. **62**(2): p. 256-62.
125. Mendelson, J., *Lateral hypothalamic stimulation in satiated rats: the rewarding effects of self-induced drinking.* Science, 1967. **157**(792): p. 1077-9.
126. Keesey, R.E. and E.P. Lindholm, *Differential rates of discrimination learning reinforced by medial versus lateral hypothalamic stimulation.* J Comp Physiol Psychol, 1969. **68**(4): p. 544-51.
127. Broberger, C., et al., *Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems.* J Comp Neurol., 1998. **402**(4): p. 460-474.
128. Balcita-Pedicino, J.J. and S.R. Sesack, *Orexin axons in the rat ventral tegmental area synapse infrequently onto dopamine and gamma-aminobutyric acid neurons.* J Comp Neurol, 2007. **503**(5): p. 668-84.
129. Moorman, D.E. and G. Aston-Jones, *Orexin/hypocretin modulates response of ventral tegmental dopamine neurons to prefrontal activation: diurnal influences.* J Neurosci, 2010. **30**(46): p. 15585-99.
130. Flier, J.S. and E. Maratos-Flier, *Obesity and the hypothalamus: novel peptides for new pathways.* Cell, 1998. **92**(4): p. 437-40.
131. Hahn, J.D., *Comparison of melanin-concentrating hormone and hypocretin/orexin peptide expression patterns in a current parceling scheme of the lateral hypothalamic zone.* Neurosci Lett, 2010. **468**(1): p. 12-7.
132. Thompson, J.L. and S.L. Borgland, *A role for hypocretin/orexin in motivation.* Behav Brain Res, 2010.
133. DiLeone, R.J., D. Georgescu, and E.J. Nestler, *Lateral hypothalamic neuropeptides in reward and drug addiction.* Life Sci, 2003. **73**(6): p. 759-68.
134. Berridge, K.C., et al., *The tempted brain eats: pleasure and desire circuits in obesity and eating disorders.* Brain Res, 2010. **1350**: p. 43-64.
135. Georgescu, D., et al., *The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate*

feeding behavior and forced-swim performance. J Neurosci., 2005. **25**(11): p. 2933-2940.
136. Kawauchi, H., et al., *Characterization of melanin-concentrating hormone in chum salmon pituitaries.* Nature, 1983. **305**(5932): p. 321-323.
137. Pissios, P., et al., *Dysregulation of the mesolimbic dopamine system and reward in MCH-/- mice.* Biol Psychiatry, 2008. **64**(3): p. 184-91.
138. Chung, S., et al., *The melanin-concentrating hormone system modulates cocaine reward.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6772-7.
139. Marsh, D.J., et al., *Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism.* Proc.Natl.Acad.Sci., 2002. **99**(5): p. 3240-3245.
140. Tyhon, A., et al., *Deletion of Melanin-Concentrating Hormone Receptor-1 gene accentuates D-amphetamine-induced psychomotor activation but neither the subsequent development of sensitization nor the expression of conditioned activity in mice.* Pharmacol Biochem Behav, 2008. **88**(4): p. 446-55.
141. Tyhon, A., et al., *Amphetamine- and cocaine-induced conditioned place preference and concomitant psychomotor sensitization in mice with genetically inactivated melanin-concentrating hormone MCH(1) receptor.* Eur J Pharmacol, 2008. **599**(1-3): p. 72-80.
142. Aston-Jones, G., et al., *A neural circuit for circadian regulation of arousal.* Nat Neurosci, 2001. **4**(7): p. 732-8.
143. Harris, G.C. and G. Aston-Jones, *Arousal and reward: a dichotomy in orexin function.* Trends Neurosci, 2006. **29**(10): p. 571-7.
144. Hara, J., et al., *Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity.* Neuron, 2001. **30**(2): p. 345-54.
145. Willie, J.T., et al., *Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes.* Neuron, 2003. **38**(5): p. 715-30.
146. Winrow, C.J., et al., *Orexin receptor antagonism prevents transcriptional and behavioral plasticity resulting from stimulant exposure.* Neuropharmacology, 2010. **58**(1): p. 185-94.
147. Qu, D., et al., *A role for melanin-concentrating hormone in the central regulation of feeding behaviour.* Nature, 1996. **380**(6571): p. 243-7.
148. Jo, Y.H., et al., *Integration of endocannabinoid and leptin signaling in an appetite-related neural circuit.* Neuron, 2005. **48**(6): p. 1055-66.
149. Mieda, M. and M. Yanagisawa, *Sleep, feeding, and neuropeptides: roles of orexins and orexin receptors.* Curr Opin Neurobiol, 2002. **12**(3): p. 339-45.
150. Louis, G.W. and M.G. Myers, Jr., *The role of leptin in the regulation of neuroendocrine function and CNS development.* Rev.Endocr.Metab Disord., 2007. **8**(2): p. 85-94.
151. De Witte, P., C. Heidbreder, and M. Gewiss, *Enhancement of mesolimbic rewarding brain stimulation by neurotensin injected into the accumbens, the subiculum, or the ventral tegmental area.* Ann N Y Acad Sci, 1992. **668**: p. 335-8.

152. Luttinger, D., et al., *The effect of neurotensin on food consumption in the rat.* Eur J Pharmacol, 1982. **81**(3): p. 499-503.
153. Stanley, B.G., B.G. Hoebel, and S.F. Leibowitz, *Neurotensin: effects of hypothalamic and intravenous injections on eating and drinking in rats.* Peptides, 1983. **4**(4): p. 493-500.
154. Glimcher, P.W., et al., *Neurotensin: a new 'reward peptide'.* Brain Res, 1984. **291**(1): p. 119-24.
155. Levine, A.S., et al., *Effect of centrally administered neurotensin on multiple feeding paradigms.* Pharmacol Biochem Behav, 1983. **18**(1): p. 19-23.
156. Rovere, C., et al., *Impaired processing of brain proneurotensin and promelanin-concentrating hormone in obese fat/fat mice.* Endocrinology, 1996. **137**(7): p. 2954-8.
157. Leshan, R.L., et al., *Direct innervation of GnRH neurons by metabolic- and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus.* J Neurosci, 2009. **29**(10): p. 3138-47.
158. Leshan, R.L., et al., *Leptin receptor signaling and action in the central nervous system.* Obesity (Silver Spring), 2006. **14 Suppl 5**: p. 208S-212S.
159. Sinha, M.K., et al., *Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects.* J Clin Invest, 1996. **97**(5): p. 1344-7.
160. Cohen, P., et al., *Selective deletion of leptin receptor in neurons leads to obesity.* J.Clin.Invest., 2001. **108**(8): p. 1113-1121.
161. Hebda-Bauer, E.K., et al., *Forebrain glucocorticoid receptor overexpression increases environmental reactivity and produces a stress-induced spatial discrimination deficit.* Neuroscience, 2010. **169**(2): p. 645-53.
162. Gully, D., et al., *Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor.* Proc Natl Acad Sci U S A, 1993. **90**(1): p. 65-9.
163. Pettibone, D.J., et al., *The effects of deleting the mouse neurotensin receptor NTR1 on central and peripheral responses to neurotensin.* J Pharmacol Exp Ther, 2002. **300**(1): p. 305-13.
164. Remaury, A., et al., *Targeted inactivation of the neurotensin type 1 receptor reveals its role in body temperature control and feeding behavior but not in analgesia.* Brain Res, 2002. **953**(1-2): p. 63-72.
165. Mechanic, J.A., et al., *Involvement of the neurotensin receptor 1 in the behavioral effects of two neurotensin agonists, NT-2 and NT69L: lack of hypothermic, antinociceptive and antipsychotic actions in receptor knockout mice.* Eur Neuropsychopharmacol, 2009. **19**(7): p. 466-75.
166. Kim, E.R. and T.M. Mizuno, *Role of neurotensin receptor 1 in the regulation of food intake by neuromedins and neuromedin-related peptides.* Neurosci Lett, 2010. **468**(1): p. 64-7.
167. Butcher, S.P., et al., *Amphetamine-induced dopamine release in the rat striatum: an in vivo microdialysis study.* J Neurochem, 1988. **50**(2): p. 346-55.

168. Sharp, T., T. Zetterstrom, and U. Ungerstedt, *An in vivo study of dopamine release and metabolism in rat brain regions using intracerebral dialysis.* J Neurochem, 1986. **47**(1): p. 113-22.
169. Zetterstrom, T., et al., *In vivo measurement of dopamine and its metabolites by intracerebral dialysis: changes after d-amphetamine.* J Neurochem, 1983. **41**(6): p. 1769-73.
170. Castaneda, E., J.B. Becker, and T.E. Robinson, *The long-term effects of repeated amphetamine treatment in vivo on amphetamine, KCl and electrical stimulation evoked striatal dopamine release in vitro.* Life Sci, 1988. **42**(24): p. 2447-56.
171. Leshan, R.L., et al., *Leptin receptor signaling and action in the central nervous system.* Obesity.(Silver.Spring), 2006. **14 Suppl 5**: p. 208S-212S.
172. Bjorbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery.* Recent Prog.Horm.Res., 2004. **59**: p. 305-331.
173. Morton, N.M., et al., *Leptin action in intestinal cells.* J Biol Chem, 1998. **273**(40): p. 26194-201.
174. Kawai, K., et al., *Leptin as a modulator of sweet taste sensitivities in mice.* Proc Natl Acad Sci U S A, 2000. **97**(20): p. 11044-9.
175. Sobhani, I., et al., *Leptin secretion and leptin receptor in the human stomach.* Gut, 2000. **47**(2): p. 178-83.
176. Spence, S.J., J.A. Silverman, and D. Corbett, *Cortical and ventral tegmental systems exert opposing influences on self-stimulation from the prefrontal cortex.* Behav Brain Res, 1985. **17**(2): p. 117-24.
177. Bardo, M.T., S.L. Bowling, and R.C. Pierce, *Changes in locomotion and dopamine neurotransmission following amphetamine, haloperidol, and exposure to novel environmental stimuli.* Psychopharmacology (Berl), 1990. **101**(3): p. 338-43.
178. Elverfors, A. and H. Nissbrandt, *Effects of d-amphetamine on dopaminergic neurotransmission; a comparison between the substantia nigra and the striatum.* Neuropharmacology, 1992. **31**(7): p. 661-70.
179. Angel, C., O.D. Murphree, and D.C. DeLuca, *The effects of chlordiazepoxide, amphetamine and cocaine on bar-press behavior in normal and genetically nervous dogs.* Dis Nerv Syst, 1974. **35**(5): p. 220-3.
180. Karreman, M., B.H. Westerink, and B. Moghaddam, *Excitatory amino acid receptors in the ventral tegmental area regulate dopamine release in the ventral striatum.* J Neurochem, 1996. **67**(2): p. 601-7.
181. Kiyatkin, E.A., *Dopamine in the nucleus accumbens: cellular actions, drug- and behavior-associated fluctuations, and a possible role in an organism's adaptive activity.* Behav Brain Res, 2002. **137**(1-2): p. 27-46.
182. McFarland, K., et al., *Limbic and motor circuitry underlying footshock-induced reinstatement of cocaine-seeking behavior.* J Neurosci, 2004. **24**(7): p. 1551-60.
183. Le Foll, B., et al., *Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice.* Eur J Neurosci, 2002. **15**(12): p. 2016-26.

184. Liu, J., et al., *Acute administration of leptin produces anxiolytic-like effects: a comparison with fluoxetine.* Psychopharmacology (Berl), 2010. **207**(4): p. 535-45.
185. Jyotaki, M., N. Shigemura, and Y. Ninomiya, *Modulation of sweet taste sensitivity by orexigenic and anorexigenic factors.* Endocr J, 2010. **57**(6): p. 467-75.
186. Shigemura, N., et al., *Leptin modulates behavioral responses to sweet substances by influencing peripheral taste structures.* Endocrinology, 2004. **145**(2): p. 839-47.
187. Taildeman, J., et al., *Identification of the nasal mucosa as a new target for leptin action.* Histopathology, 2010. **56**(6): p. 789-98.
188. Baly, C., et al., *Leptin and its receptors are present in the rat olfactory mucosa and modulated by the nutritional status.* Brain Res, 2007. **1129**(1): p. 130-41.
189. Getchell, T.V., et al., *Leptin regulates olfactory-mediated behavior in ob/ob mice.* Physiol Behav, 2006. **87**(5): p. 848-56.
190. Yeomans, M.R., J. Prescott, and N.J. Gould, *Acquired hedonic and sensory characteristics of odours: influence of sweet liker and propylthiouracil taster status.* Q J Exp Psychol (Colchester), 2009. **62**(8): p. 1648-64.
191. Hiroi, M., T. Tanimura, and F. Marion-Poll, *Hedonic taste in Drosophila revealed by olfactory receptors expressed in taste neurons.* PLoS One, 2008. **3**(7): p. e2610.
192. Berridge, K.C., *'Liking' and 'wanting' food rewards: brain substrates and roles in eating disorders.* Physiol Behav, 2009. **97**(5): p. 537-50.
193. Bates, S.H., et al., *LRb-STAT3 signaling is required for the neuroendocrine regulation of energy expenditure by leptin.* Diabetes, 2004. **53**(12): p. 3067-3073.
194. Mochizuki, T., et al., *Behavioral state instability in orexin knock-out mice.* J Neurosci, 2004. **24**(28): p. 6291-300.
195. Anaclet, C., et al., *Orexin/hypocretin and histamine: distinct roles in the control of wakefulness demonstrated using knock-out mouse models.* J Neurosci, 2009. **29**(46): p. 14423-38.
196. Carr, K.D., et al., *Evidence of increased dopamine receptor signaling in food-restricted rats.* Neuroscience, 2003. **119**(4): p. 1157-1167.
197. Cabeza, d.V. and K.D. Carr, *Food restriction enhances the central rewarding effect of abused drugs.* J Neurosci., 1998. **18**(18): p. 7502-7510.