Leptin Regulates Nutrient Reward via Galanin and Orexin Neurons

Amanda Laque

Louisiana State University and Agricultural and Mechanical College

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LEPTIN REGULATES NUTRIENT REWARD VIA GALANIN AND OREXIN NEURONS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

Amanda Laque
B.S., Louisiana State University, 2008
May 2014
This dissertation is, in part, dedicated to my parents Patricia and Albert Laque Jr., whose constant love and support has been the motivational force throughout my graduate studies. To their children, they have always been our biggest fans. Words cannot express how truly grateful I am for all of your sacrifices that has allowed me to pursue the life of my choosing.

Above all, I dedicate this dissertation to my beloved husband and best friend, Ryan Burke. He has been my rock throughout the most challenging of times and my strongest advocate when achieving my goals. He has always believed in my abilities beyond the belief I had in myself, and this journey would have been impossible without him in my corner. I am sincerely thankful for his unconditional love and support.
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<td>3rd Ventricle</td>
</tr>
<tr>
<td>4V</td>
<td>4th Ventricle</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-amphetamine regulating transcript</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic-AMP</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
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<td>DMV</td>
<td>Dorsal motor nucleus of the vagus nerve</td>
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<tr>
<td>exPFA</td>
<td>Extended perifornical area</td>
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<td>FG</td>
<td>Fluorogold</td>
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<tr>
<td>fx</td>
<td>Fornix</td>
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<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>GalR1</td>
<td>Galanin receptor 1</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>JAK2</td>
<td>Janus kinase 2</td>
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<tr>
<td>LC</td>
<td>Locus coeruleus</td>
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<tr>
<td>LepRb</td>
<td>Long-form of the leptin receptor-b</td>
</tr>
<tr>
<td>LHA</td>
<td>Lateral hypothalamus area</td>
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<tr>
<td>MCH</td>
<td>Melanin-concentrating hormone</td>
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<tr>
<td>mt</td>
<td>Mammillothalamic tracts</td>
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<td>NAc</td>
<td>Nucleus accumbens</td>
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<td>NE</td>
<td>Norepinephrine</td>
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<td>Neuropeptide Y</td>
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<td>Nts</td>
<td>Neurotensin</td>
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<td>NTS</td>
<td>Nucleus of solitary tract</td>
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<td>ORX</td>
<td>Orexin</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PFA</td>
<td>Perifornical Area</td>
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<td>PKA</td>
<td>Protein kinase A</td>
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<td>POMC</td>
<td>Proopiomelanocortin</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>RER</td>
<td>Respiratory exchange rate</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling-3</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription-3</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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## LIST OF PRIMARY ANTIBODIES

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<th>Immunogen</th>
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<th>Manufacturer</th>
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<td>9131</td>
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ABSTRACT

Obesity has become a widespread concern to human health largely over the past three decades. It is thought that obesity is associated with the over consumption of calorically dense diets. The rewarding value of food is mediated through the mesolimbic dopamine system, though is less understood how appetitive control circuits relay information to existing reward circuitry. The adiposity signaling hormone, leptin, is a critical mediator of food intake and fat storage. Leptin signaling, via the long form of the leptin receptor (LepRb), is predominantly carried out within the hypothalamus. Leptin action specific to the lateral hypothalamic area (LHA) modulates reward function via direct and indirect inhibition of reward circuitry. Here, I have identified a novel leptin receptor population within the LHA that co-expresses the inhibitory neuropeptide galanin (termed Gal-LepRb neurons). To investigate the physiological function of leptin through Gal-LepRb neurons, we selectively deleted LepRb in galanin neurons (referred to as Gal-LepRbKO mice). In a two-bottle-choice paradigm, I assessed nutrient selection for isocaloric lipid and sucrose solutions. Interestingly, Gal-LepRbKO mice demonstrated a significant preference for the sucrose solution and decreased lipid intake compared to controls. Moreover, Gal-LepRbKO mice displayed stronger motivation to work for a sucrose treat. My data further indicate that Gal-LepRb neurons are inhibitory acting neurons that are stimulated by leptin. Gal-LepRb neurons strongly innervate local orexin neurons and noradrenergic neurons in the locus coeruleus (LC). Intriguingly, orexin neurons also strongly innervate the LC, and activation of orexin neurons
correlates with motivational and food-seeking behaviors. My data also show that orexin neurons express the G\textsubscript{i}-coupled GPCR galanin 1-receptor (GalR1), validating the cellular ability of orexin neurons to respond to galanin. In summary, we have characterized a novel population of LHA LepRb neurons and propose that leptin-mediated inhibition of orexin neurons, possibly via inhibitory galanin-GalR1 signaling, regulates the reward value of nutrients.
CHAPTER 1. GENERAL INTRODUCTION

1.1 LEPTIN

1.1.1 Leptin and energy balance. Leptin is a 16kD peptide secreted from adipocytes and largely communicates information concerning energy stores to the brain (Zhang et al. 1994, Elmquist, Maratos-Flier, et al. 1998, Friedman 1998). Circulating leptin levels are correlated with the amount of fat mass present within an organism (Frederich et al. 1995, Maffei et al. 1995) and fasting-induced drops in leptin levels elicits a strong feeding response (Maffei et al. 1995). Leptin plays a critical role in energy balance regulation as mice lacking the capability to produce leptin, known as ob/ob mice, are morbidly obese, show marked increases in food intake (hyperphagia) and exhibit numerous metabolic-associated dysfunctions (Zhang et al. 1994, Montague et al. 1997, Clement et al. 1998). Although ob/ob mice exhibit an extreme obesity phenotype, the absence of leptin triggers a starvation signal thereby inducing a potent feeding response. Accordingly, administration of recombinant leptin to ob/ob mice and leptin deficient humans fully reverses obesity and corrects co-morbidity metabolic effects (Halaas et al. 1995, Ozata, Ozdemir, and Licinio 1999, Farooqi et al. 2002, Farooqi 2008). When first discovered, leptin was expected to be the long anticipated cure for obesity since treatment with recombinant leptin reduced body fat mass in both lean and ob/ob mice (Halaas et al. 1995). However, this was not the case as diet-induced obese animals and obese humans exhibit markedly increased circulating leptin levels (Maffei et al. 1995, Considine et al. 1996, Frederich et al. 1995) and leptin
treatments only marginally decreased body weight in obese humans and rodents (Halaas et al. 1997, Heymsfield et al. 1999). These findings suggest a form of desensitization to leptin in the obese state. Therefore, it is important we unravel the diversity of leptin action in order to develop effective treatments to counteract or prevent obesity-induced leptin resistance.

**1.1.2 Leptin receptor signaling and distribution.** The leptin receptor gene encodes five alternatively spliced forms of the leptin receptor (Lep-Ra, Lep-Rb, Lep-Rc, Lep-Rd, and Lep-Re) (Tartaglia et al. 1995, Lee et al. 1996). The LepR-b form is considered the long form of the leptin receptor due to its long cytoplasmic domain containing various motifs required for leptin-mediated signaling. Mutations in the long form of the leptin receptor, LepRb, result in an obese phenotype that strongly resembles the ob/ob mouse (Li et al. 1998). Thus, the anorexigenic (appetite decreasing) effects of leptin are predominantly carried out by LepRb. Leptin-induced activation of the LepRb relies on the activity of janus kinase-2 (JAK2) which phosphorylates tyrosine residues associated with the receptor (Bjorbaek et al. 2001). *In vivo*, leptin-induced activation of the LepRb stimulates JAK2-mediated phosphorylation of the signal transducer and activator of transcription-3 (STAT3) as shown in Figure 1.1 (Vaisse et al. 1996). Phosphorylated-STAT3 (pSTAT3) then dimerizes and translocates into the nucleus prompting transcription of leptin responsive genes (Vaisse et al. 1996, Bjorbaek et al. 2001, Banks et al. 2000). Activation of STAT3 is crucial for leptin’s effects on body weight regulation, as mutations in tyrosine 1138 of the LepRb (the site of STAT3 phosphorylation; Figure 1.1) leads to hyperphagia and obesity (Bates et al. 2003). Leptin signaling via
LepRb also leads to tyrosine phosphorylation of the tyrosine phosphatase SHP-2 which decreases the activity level of JAK2 to down-regulate LepRb activation (Carpenter et al. 1998). Additionally, the induction of downstream leptin effector SOCS-3 (suppressor of cytokine signaling-3) is also involved in negative feedback to the activated LepRb and is largely implicated in leptin resistance (Bjorbaek et al. 1998).

Figure 1.1 Leptin-induced activation of pSTAT3 upon binding to the long form of the leptin receptor (LepRb).

In rodents and humans, leptin receptors are expressed in various nuclei throughout the central nervous system, but most prominent leptin action occurs within the hypothalamus. The hypothalamus is the brain region largely recognized
for energy balance regulation. Within the hypothalamus, LepRbs are expressed in the arcuate nucleus (ARC), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), paraventricular hypothalamus (PVN) and the lateral hypothalamus area (LHA) (Mercer et al. 1996, Fei et al. 1997, Elmquist, Bjorbaek, et al. 1998). As mentioned, the effects of leptin are generally associated with weight loss and reduced food intake (anorexia). However, it is now widely acknowledged that the anatomical location of LepRb-expressing neurons dictates a very specific contribution to whole body energy balance (Berthoud 2007, Dhillon et al. 2006, van de Wall et al. 2008, Morton et al. 2003, Gao and Horvath 2007). For example, proopiomelanocortin (POMC)-expressing LepRb neurons in the ARC are critical for glucose homeostasis (Leinninger 2011, Balthasar et al. 2004, Berthoud 2007, Elmquist et al. 2005, Morton et al. 2003, van de Wall et al. 2008), while glutamatergic (excitatory) LepRb neurons in the DMH are involved in body temperature regulation (Zhang et al. 2011). Recently, LepRbs in the lateral hypothalamus area (LHA) that express the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), have been highlighted for their role in reward function (Leinninger 2011, Leinninger et al. 2009). Taken together, the ability of leptin to regulate energy balance involves a complex integration of numerous and functionally distinct LepRb populations, many of which, have not been thoroughly characterized or possibly even identified.

1.2 LEPTIN INFLUENCES REWARD CIRCUITS

1.2.1 LHA-LepRb neurons and the mesolimbic dopaminergic system.

The LHA has long been established for regulating reward-driven behaviors and
LHA neurons are highly responsive to food cues (Rolls, Burton, and Mora 1976, 1980). Early intracranial self-stimulation experiments revealed that rats will continuously perform a licking task in order to activate an electrode implanted into the LHA (Nakamura and Ono 1986). This suggests that general stimulation of LHA neurons induces a form of pleasure. This effect also holds true for natural rewards as a large population of LHA neurons are stimulated when animals perform the same licking task for a glucose solution (Nakamura and Ono 1986). The LHA elicits these reward responses via activation of the mesolimbic dopaminergic system. Neurons in the LHA send dense projections to many limbic structures (ventral tegmental area, striatum, and pallidum) which act as critical regulators of motivated behavior. Namely, the mesolimbic dopaminergic system is comprised of dopamine (DA) neurons within the ventral tegmental area (VTA) that send long axonal projections to the nucleus accumbens (NAc; Figure 1.2). Activation of the VTA-DA neurons and subsequent DA release within the NAc (VTA→NAc dopamine release termed mesoaccumbal DA) is believed to be the pathway responsible for encouraging reward-driven and motivated behaviors (Goto and Grace 2005, Berridge and Robinson 1998, Pecina et al. 2003, Ostlund et al. 2011). Previous experiments showed that leptin administration dampened motivated responses for LHA intracranial self-stimulation (Fulton, Woodside, and Shizgal 2000). Thus, circuits governing reward-motivated behaviors appear to converge with leptin signaling pathways.

From a survival standpoint, it makes sense that pathways dictating nutrient intake would converge into the mesolimbic DA system to reinforce food seeking and
appetitive behaviors when energy stores are low. Due to their high-calorie content, sugar and fat-rich foods have evolved as potent stimulators of mesoaccumbal DA release (Hajnal, Smith, and Norgren 2004, Rada, Avena, and Hoebel 2005, Liang, Hajnal, and Norgren 2006). Thus, the evoked intensity of DA release is believed to encode the motivational properties (incentive value) for food rewards (Reilly 1999, Sclafani and Ackroff 2003, Wojnicki, Babbs, and Corwin 2010, Yoneda et al. 2007).

Likewise, the anorexigenic effects of leptin are mediated, in part, through the inhibition of the mesolimbic DA system. Electrophysiological studies in brain slice preparations demonstrated that leptin inhibits firing of VTA DA neurons (Hommel et al. 2006). In behavioral studies, leptin was shown to attenuate sucrose seeking and sucrose consumption in rats (Figlewicz et al. 2001, Figlewicz et al. 2006). Together, these observations suggest that leptin’s inhibitory influence over DA neurons acts to decrease the incentive value of a food reward. On the contrary, electrically stimulated DA release in the NAc is markedly reduced in leptin deficient ob/ob mice (Fulton et al. 2006). Therefore, the function of leptin is contradictory in the literature; suggesting either inhibitory or stimulatory leptin action on VTA DA neurons. Clarifying the role of leptin in the regulation of the mesolimbic dopaminergic system is critical to understand the mechanism by which leptin modulates nutrient reward.

Recent data indicates that LHA LepRb neurons project to VTA neurons and modulate food intake (Leinninger et al. 2009). The established LHA-LepRb circuit, shown in Figure 1.2, illustrates that leptin signaling in the LHA would directly modulate mesoaccumbal DA function. Whether leptin would induce its effects through inhibition or stimulation of mesoaccumbal DA release is matter that
remains controversial. Therefore, determining the specific contribution of LHA-LepRb neurons in the regulation the mesolimbic dopamine system would provide a functional pathway for understanding how the nutritional state of the body regulates reward-based feeding.

Figure 1.2 Schematic illustrating the established circuitry of LHA-LepRb neurons into the mesolimbic dopaminergic system. LHA-LepRb regulates the mesolimbic dopamine system both directly via projections to the VTA and indirectly via innervation of local orexin neurons.

1.2.2 Leptin regulates orexin’s neuronal activity. In addition to sending direct projections to the VTA, LHA LepRb-expressing neurons also innervate local orexin neurons as shown in Figure 1.2 {((Louis et al. 2010) and our own data detailed in Chapter 4}. Orexin neurons are exclusively found within the LHA where they are co-mingled but not co-expressed with LHA LepRb neurons {((Louis et al. 2010, Leinninger et al. 2009) and our data shown in Chapter 2}. Importantly, orexin neurons regulate various aspects of reward function largely via direct activation of the VTA-DA→NAc pathway (illustrated in Figure 1.2 (Peyron et al. 1998, Fadel and
Deutch 2002, Baldo et al. 2003)). Typically, orexin neurons are activated in response to low serum glucose levels (Cai et al. 1999, Moriguchi et al. 1999) as also seen during fasting (Sakurai et al. 1999), and activation of orexin neurons is strongly correlated with food/reward-seeking behaviors (Harris, Wimmer, and Aston-Jones 2005). Central administration of the orexin peptide increases the incentive motivation to work for sucrose pellets, while central (VTA) injections of orexin receptor antagonists block this effect (Choi et al. 2010). Activation of orexin neurons is also triggered by environmental cues that predict the arrival of a palatable food reward; a mechanism thought to enhance food anticipatory and seeking behaviors (Harris, Wimmer, and Aston-Jones 2005). Thus, the activity of orexin neurons, and subsequent mesoaccumbal DA response, relies on both nutritional signals regulating energy balance as well as environmental cues predictive of food rewards.

As mentioned, leptin is primarily considered a signal of nutritional status, but it also modulates reward function via inhibition of the mesolimbic DA system. This effect occurs, partly, via leptin-mediated inhibition of orexin neurons. In electrophysiological recordings from brain slice preparations, leptin application reduced the firing frequency of orexin neurons (Yamanaka et al. 2003), and this was observed by a reduction of excitatory inputs onto orexin neurons (Yamanaka et al. 2003, Cai et al. 1999, Horvath and Gao 2005). Leptin treatment also attenuates food/reward-driven behaviors that are associated with the activation of orexin neurons, as sucrose-seeking behaviors are significantly decreased following a leptin injection (Figlewicz et al. 2006). Since LHA LepRb neurons innervate local
orexin neurons, it seems highly likely that leptin action specific to LHA LepRb neurons would regulate food/reward-motivated behaviors via inhibition of orexin neurons. However, the precise LepRb population mediating this effect has yet to be identified.

1.3 CHARACTERIZATION OF CENTRAL GALANIN ACTION

As previously eluded to, the discrete distribution patterns of LepRb-expressing neurons throughout the brain suggest that each LepRb population contributes to distinct aspect of leptin action. This idea is reinforced by the fact that LepRb neurons exhibit differential molecular and neurochemical properties based on their anatomical position within the brain. Specific to the LHA/perifornical area (PFA) we have identified a novel subpopulation of LepRb neurons that co-express the neuropeptide galanin, which we refer to as Gal-LepRb neurons. Leptin mediates anorexigenic action via LepRb; however, central galanin signaling is often recognized for evoking orexigenic (appetitive) effects. Thus, we aim to understand how leptin regulates central galanin signaling via Gal-LepRb neurons and determine how galanin’s effects in this novel LepRb population would translate into aspects of feeding and body weight regulation.

1.3.1 Galanin introduction. Galanin is a 29 amino acid peptide that is widely expressed throughout the brain and periphery and functions in various physiological processes. Centrally, galanin elicits its inhibitory effects both directly as galanin decreases the spontaneous firing of neurons in the locus coeruleus (LC) (Seutin et al. 1989) and indirectly as presynaptic galanin action inhibits glutamate
release in the ARC (Kinney, Emmerson, and Miller 1998). Galanin’s inhibitory effects are mediated through the activation of any of its three known G-protein coupled receptors: GalR1, GalR2 and GalR3 (Figure 1.3). Specifically, GalR1 is widely expressed in the brain and galanin signaling via GalR1 has been recognized to modulate reward via palatable food selection and opioid-mediated reward function (Zorrilla et al. 2007, Holmes et al. 2012). GalR1 signals via G_i-pathway to reduce adenyl cyclase activity and thus induce the subsequent reduction in the cAMP→ protein kinase A (PKA)→ cAMP response element binding protein (CREB) pathway. Since galanin-GalR1 signaling decreases CREB phosphorylation, pCREB can be used as a marker to assess galanin’s involvement in reward circuits (Hawes, Narasimhaiah, and Picciotto 2006).

1.3.2 The role of galanin in macronutrient selection. Galanin signaling within the hypothalamus has prominent effects on food intake and fat deposition. In contrast to the anorexigenic-acting hormone leptin, centrally administered galanin stimulates feeding (Tachibana et al. 2008). Furthermore, when rodents are allowed to choose between distinct macronutrients, galanin evokes the preferential intake of fats over carbohydrates and proteins (Tempel, Leibowitz, and Leibowitz 1988). Consistently, galanin deficient mice or central injections of galanin receptor antagonists are shown to reduce fat consumption (Adams et al. 2008, Odorizzi et al. 2002). A positive feed-forward mechanism is thought to further drive fat intake, as the consumption of a high fat diet increases hypothalamic galanin gene expression (Leibowitz et al. 2004).
As mentioned, palatable food intake is partly regulated via galanin activation of GalR1. GalR1-deficient mice decrease their caloric intake when put on a high fat diet, despite their initial increased consumption in the 3-day acclimation to high fat (Zorrilla et al. 2007). Interestingly, GalR1 deficient mice show normal feeding behaviors on a standard chow diet, suggesting a specific role for galanin signaling via GalR1 in regulating fat intake.

1.3.3 The role of galanin in opioid-mediated reward. Galanin is also widely recognized for its role in reward and addiction pathways, specifically via modulation of opioid neurotransmission. Like galanin, opioids are potent stimulators of food intake and localized NAc injections of µ-opioid receptor agonists preferentially increases lipid consumption (Zhang, Gosnell, and Kelley 1998).
Central administration of galanin receptor agonists decreases opiate reward and attenuates opioid withdrawal symptoms (Zachariou, Parikh, and Picciotto 1999, Holmes et al. 2012). Consistently, galanin deficient mice experience both increased reward and withdrawal from opioids (Hawes et al. 2008), which appears to be largely regulated via GalR1 (Holmes et al. 2012). Galanin binding also seems to be high within dopaminergic areas of the brain (Hawes and Picciotto 2004), implicating a possible role for galanin in motivated feeding behaviors.

Previous reports investigating the interaction between leptin and galanin have been largely inconsistent; claiming leptin may decrease (Sahu 1998b, a) or increase (Cheung et al. 2001) galanin gene expression, or has no effect on galanin gene expression at all (Williams et al. 1991). Such discrepancies are due to poor visualization of galanin- and LepRb-expressing neurons via antibody detection methods. Therefore, determining their accurate co-expression patterns would be extremely difficult. Thus far, the role galanin plays in nutrient reward has not been investigated using novel state of the art technologies such as transgenic or Cre/LoxP genetic mouse models. To appropriately investigate a potential interaction between central galanin and leptin signaling, we implement neuron-specific Cre/LoxP technologies to generate genetic mouse lines for: 1). accurate visualization of galanin and LepRb neurons via green fluorescent protein (GFP) reporter lines, 2). galanin and LepRb neuronal circuit tracing studies 3). developing a galanin-specific LepRb Knock Out (KO) mouse line. Here we aimed to establish the importance of leptin action specific to galanin neurons for modulating energy balance and potentially properties of nutrient reward.
CHAPTER 2. LEPTIN RECEPTOR NEURONS IN THE MOUSE HYPOTHALAMUS ARE COLOCALIZED WITH THE NEUROPEPTIDE GALANIN AND MEDIATE ANOREXIGENIC LEPTIN ACTION

2.1 INTRODUCTION

Leptin controls energy homeostasis centrally via neurons that express the long form leptin receptor (LepRb). Several LepRb populations have been identified based on their anatomical location, co-expression of neuropeptides or other marker genes (Balthasar et al. 2004, Coppari et al. 2005, Dhillon et al. 2006, Hayes et al. 2010, Leinninger et al. 2009, van de Wall et al. 2008), which helped to clarify their commensurate contribution to full leptin function. In fact, most LepRb populations studied showed a significant, but surprisingly mild, contribution to full leptin function. However, the majority of LepRb subpopulations have not been studied in detail, largely due to the lack of specific markers for significant subpopulations of LepRb neurons.

Galanin is an orexigenic neuropeptide and galanin neurons are found throughout the brain, but a particularly dense population exists in the dorsomedial hypothalamus. However, orexigenic galanin action is mediated by the paraventricular nucleus (PVN), because PVN galanin injections most prominently increase food intake (Kyrkouli et al. 1990). The literature involving a possible interaction of galanin and leptin is very inconsistent. Some studies suggest contrary galanin and leptin action, because genetic deletion of galanin amplifies leptin-induced weight loss (Hohmann et al. 2003, Hohmann et al. 2004). This may involve

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leptin-mediated regulation of galanin gene expression, even though the experimental data is scarce and experiments are difficult to compare due to different experimental approaches, detection methods and anatomical areas investigated. This may explain the inconsistent results showing that leptin may decrease (Sahu 1998b, a) or increase (Cheung et al. 2001) galanin gene expression, or does not effect galanin gene expression at all (Williams et al. 1991).

Likewise, it is unclear if leptin directly or indirectly acts on galanin neurons, again demonstrated in controversial results showing that LepRb is either rarely (Cheung et al. 2001) or extensively (Hakansson et al. 1999, Iqbal et al. 2001) co-localized with galanin in the hypothalamus. Both LepRb- and galanin-expressing neurons cannot be easily visualized immunohistochemically. Galanin is rapidly transported into neuronal processes, so that immunohistochemical (IHC) staining for galanin cannot visualize cell bodies, unless animals are treated with colchicine to prevent vesicular galanin transport into processes (Perez et al. 2001). LepRb is expressed in low quantity, so that available LepRb specific antibodies are not sensitive enough to detect LepRb cell bodies, but LepRb-responsive neurons can be easily and reliably detected by leptin-induced phosphorylation of STAT3 (Faouzi et al. 2007, Munzberg, Flier, and Bjorbaek 2004, Zhang et al. 2011). However, such signaling pathways are blocked by colchicine treatment, so that LepRb and galanin cannot be easily detected together. Thus, in this study we aimed to reinvestigate the co-expression of galanin and LepRb and to start to evaluate if and how galanin may mediate physiological leptin action. To do this we used mice with transgenic green fluorescent protein (GFP) expression from the galanin promoter to
systematically characterize the existence, anatomical location and chemical nature of such Gal-LepRb neurons.

2.2 MATERIALS AND METHODS

2.2.1 Mouse strains. To visualize galanin expressing neurons we used male BAC transgenic mice (FVB background) with green fluorescent protein (GFP) expression under the control of the galanin promoter (Gal\textsuperscript{TgGFP}, Stock Tg(Gal-EGFP109Gsat, identification number 0163420UCD), which were obtained from the Mutant Mouse Regional Resource Center (MMRRC, \url{http://www.mmrrc.org}), a NCRR-NIH funded strain repository, and were donated to the MMRRC by the NINDS funded GENSAT BAC transgenic project (\url{http://www.gensat.org}). Gal\textsuperscript{TgGFP} mice and LepRb\textsuperscript{GFP} mice used in this experiment were bred in-house by crossing FVB wildtype mice with hemizygous Gal\textsuperscript{TgGFP} or by crossing double homozygous LepRb\textsuperscript{GFP} mice, respectively. Male LepRb\textsuperscript{GFP} mice on a mixed background were used to visualize LepRb neurons and were derived from the colony of Dr. Martin Myers (University of Michigan, Ann Arbor, MI) and has been described in detail elsewhere (Leshan et al. 2006). Male C57/B6 mice were obtained from Jackson Laboratory (Bar Harbor, MA). All animals were group housed at a 12:12h light: dark cycle with \textit{ad libitum} access to food and water unless stated otherwise. All animal breeding and experimental procedures were approved by the Institutional Animal Care and Use Committee and Pennington Biomedical Research Center.

2.2.2 Peripheral leptin treatment. To identify LepRb neurons via leptin-induced pSTAT3 induction, we treated Gal\textsuperscript{TgGFP} mice (n=3) or LepRb\textsuperscript{GFP} mice (n=2)
intraperitoneal (i.p.) with leptin (5 mg/kg body weight) and perfused them 1h later. The leptin dose and incubation time were used based on earlier studies showing that leptin-induced pSTAT3 greatly co-localized with LepRb-GFP expressing neurons (Faouzi et al. 2007, Leinninger et al. 2009, Leshan et al. 2009, Zhang et al. 2011). Leptin was obtained from Dr. Parlow (National Hormone and Peptide Program, http://www.humc.edu/hormones). For studies investigating leptin-induced cFos (as a surrogate of neuronal activity) in LepRb neurons, we treated LepRb<sup>GFP</sup> mice with PBS or leptin (5 mg/kg, i.p.) and perfused for brain fixation after 3 hours (n=3-4 per group). Following treatments animals were perfused as described below in section 2.2.7.

**2.2.3 In situ hybridization.** To verify correct transgenic galanin-GFP expression we compared the distribution of GFP expression with the known distribution of galanin mRNA expression. Galanin in situ hybridization (ISH) data were used from the Mouse Allen Brain Atlas (Seattle, WA) and were available via http://www.brain-map.org. Reproduction of ISH images was with permission of the Allen Institute for Brain Science.

**2.2.4 Chronic cannulations for central injections.** For central injections we chronically implanted single cannulas (Plastics One C353, Roanoke, VA) into the lateral ventricle (-0.3mm caudal, 1mm lateral and 2.1mm ventral to Bregma) or into the exPFA (-1.8mm caudal, -0.9mm lateral and -4.25mm ventral to Bregma in Gal<sup>TgGFP</sup> mice and -1.1mm caudal, 0.9mm lateral and 4.75mm ventral to Bregma in C57B6 mice). Gal<sup>TgGFP</sup> (n=2, for colchicine injection into the lateral ventricle, n=8 for intra exPFA cannulation) or C57/B6 mice (n=12) were deeply anesthetized with 1-
3% isoflurane/oxygen and the head was mounted on a stereotaxic frame (M1900Stereo
taxic alignment system, Kopf instruments, Tujunga, CA). An incision was
made to expose the skull and an access hole was drilled to insert the guide
cannula, which was secured in place with Loctite 454 (Fisher, Pittsburgh, PA) and
dental cement before the wound was closed with wound clips. A dummy cannula
was inserted into the guide and analgesia treatment was performed systemically
every 12 hours for 2 days with buprenorphine (10 mg/kg) and once locally to the
incision site with bupivacaine/lidocaine (2.5-12.5 mg/kg). Animals were recovered
for 1 week after surgery before injections were performed.

2.2.5 Colchicine treatment. To allow efficient visualization of neurotensin
(Nts) in GalTgGFP mice (n=2), we injected a single dose of freshly diluted colchicine
(1 µl of 10 µg/µl colchicine in sterile saline; Sigma, St. Louis, MO) into the lateral
ventricle. 24 hours post-colchicine injections mice underwent transcardiac
perfusion with 10% neutral buffered formalin for tissue fixation and brains were
extracted for further processing.

2.2.6 Central leptin treatment and measurement of metabolic
parameters. GalTgGFP mice and wildtype C57/B6 mice with unilateral exPFA
cannulation were habituated to the metabolic chamber for 3 days. After that, mice
were monitored daily for body weight and food intake and continuously for oxygen
consumption, carbon dioxide production and locomotor activity (CLAMS, Columbus
Instruments; Columbus, OH). After 3 days of baseline data collection, mice were
divided into two groups, one group (n=3-6) received a single leptin injection {dose:
20 ng/100 nl sterile phosphate buffered saline (PBS)} and the other group (n=3-6)
received 100 nl PBS, injections were performed at a rate of 10 nl/60 s to give time for volume absorption. Leptin doses were used that we found earlier to be sufficient (20 ng) or insufficient (1 ng) to produce a measureable leptin response after central intracerebroventricular (i.c.v.) injections (Faouzi et al. 2007). Data collection was continued for 4 days post-injection. Mice were allowed to recover for 1 week in home cages after the last day of measurements. The experiment was then repeated with a lower leptin dose (1 ng/100 nl) and groups were crossed over so that each mouse received leptin only once during the studies (1 or 20 ng). At the end of the study, animals were perfused and analyzed for correct cannula placement, and two mice were removed from the studies due to cannula misplacement (see arrow in Figure 2.11D).

2.2.7 Perfusion and immunohistochemistry. For perfusions animals were deeply anesthetized with an overdose of pentobarbital (60 mg/kg) and transcardiac perfusion with ice cold physiological saline for 30 seconds and ice cold 10% neutral buffered formalin (Sigma, St. Louis, MO) for 5 minutes. Brains were removed, post fixed overnight in fixative, transferred to 30% sucrose for 48 hours before cryosectioning into 4 representative series per brain of 30 µm sections on a sliding microtome. For immunohistochemistry (IHC) procedures, free-floating tissue sections were used. Nuclear peptides (cFos or pSTAT3) were stained first and developed with the diaminobenzidine (DAB) method. Staining with additional primary antibodies were performed (neuropeptides and GFP) and detected with fluorescent-labeled secondary antibodies, Alexa568 (neuropeptides) or Alexa488
For nuclear DAB IHC detection of pSTAT3 and cFos, the tissue was pretreated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxide, followed by a 10 minute treatment in 0.3% glycine in phosphate buffered saline (PBS), and an additional 10 minute treatment in 0.03% sodium dodecyl sulfate in PBS. After that, sections were blocked for 3 hours in 3% normal donkey serum in PBS/0.25% Triton X-100/0.2% sodium azide. pSTAT3 or cFos antibodies were added (rabbit anti-pSTAT3, 1:1000 or rabbit anti-cFos, 1:3000 in blocking solution) and incubated overnight at 4°C. On the next day, sections were washed with PBS, incubated with a biotinylated secondary donkey anti-rabbit antibody for 1 hour (1:1000, in blocking solution without sodium azide). A biotinylated-horseradish peroxidase (HRP) enzyme is pre-incubated (1 hour) with free avidin to form the avidin-biotin complex (ABC solution: per 10 mL PBS= 20 µL avidin solution + 20 µL of biotin solution) and sections are then incubated in the ABC solution (Vector Laboratories, Burlingame, CA) for 1 hour. Finally, the signal was developed by 1x DAB solution (Pierce DAB Substrate Kit, ThermoScientific; Waltham, MA) giving a brown precipitate. Sections were then washed with PBS and staining with additional primary antibodies was performed (neuropeptides and GFP) and detected with fluorescent-labeled secondary antibodies, Alexa 568 (neuropeptides), or Alexa 488 (GFP). Sections were mounted onto gelatin-subbed slides and cover slipped with Pro-Long Anti-fade mounting medium (Invitrogen; Carlsbad, CA) for further microscopy analysis.
2.2.8 Antibody characterization. The anti-pSTAT3 antibody stains a single band of 80kDa molecular weight on Western blots after stimulation with ciliary-neurotrophic-factor (CNTF; manufacturer’s technical information). The specific detection of leptin-induced pSTAT3 was originally characterized by comparison of pSTAT3 signal in vehicle versus leptin-stimulated brain sections by Western blots and IHC (Faouzi et al. 2007, Munzberg, Flier, and Bjorbaek 2004, Munzberg et al. 2003); in both cases leptin robustly increased pSTAT3 signals. Furthermore, induction of pSTAT3 by leptin was found in an anatomical distribution similar to in situ hybridization signals for LepRb (Munzberg, Flier, and Bjorbaek 2004, Munzberg et al. 2003, Faouzi et al. 2007) and conditional deletion of LepRb in steroidogenic factor 1 (SF1) or proopiomelanocortin (POMC) neurons resulted in SF1 and POMC specific lack of leptin-induced pSTAT3, respectively (Dhillon et al. 2006, Plum et al. 2006). Specificity of anti-CART and anti-galanin has been tested by omitting the primary antibody and pre-adsorbing the antibody with the immunogen, which resulted in all cases in a complete lack of staining for all sections (Dun et al. 2000, Sherin et al. 1998). Specificity of anti-MCH (melanin concentrating hormone), -orexin, -neurotensin and –TH (tyrosine hydroxylase) was verified by analysis of their typical distribution pattern as reported by others (Aston-Jones, Zhu, and Card 2004, Swanson, Sanchez-Watts, and Watts 2005). Anti-GFP antibodies were verified by immunohistochemical staining of brains from LepRb<sup>GFP</sup> reporter mice compared with control mice that do not express GFP (unpublished data) and by co-localization of LepRb<sup>GFP</sup> with leptin-induced pSTAT3 (Figure 2.3 and (Zhang et al. 2011)).
The anti-cFos antibody recognizes the 55kDa cFos and 62kDa v-Fos proteins and does not cross-react with the 39kDa Jun protein (manufacturer's technical information). Specificity of anti-cFos has been tested by omitting the primary antibody and pre-adsorbing the antibody with the immunogen, which resulted in all cases in a complete lack of staining in all sections (Elmquist et al. 1997).

2.2.9 Leptin induced gene expression. For assessment of leptin regulated gene expression in the exPFA of ob/ob mice (8-10 weeks old) were treated twice daily with leptin (i.p. 5 mg/kg body weight) or PBS over 3 days (n=6 per group). On the 3rd day the last injection was given in the morning and tissue was collected 2 hours later. Animals were anesthetized with pentobarbital (60 mg/kg) and decapitated for rapid brain removal. Brains were sliced in a pre-cooled rodent brain matrix for mice (Electron Microscopy Sciences, Hatfield, PA) consisting of 1mm, coronal dividers, and the exPFA and ARC were harvested from a 1mm coronal brain slice (approximately -1 to -2 mm caudal from Bregma). To obtain consistent tissue pieces we used the mammillothalamic tracts (mt) as landmarks to cut horizontal to the 3rd ventricle (defining the dorsal border of the exPFA). Another cut was made through the mt parallel to the 3rd ventricle (defining the lateral border of the exPFA) and a 3rd cut was made laterally to remove any remaining cortex/amygdala structures. No ventral cut was performed, maintaining the natural ventral base of the exPFA. The ARC piece was collected from the ventral portion of the remaining midline piece. RNA was isolated with the ToTALLY RNA kit (Ambion, Carlsbad, CA) and 350 ng RNA was used as template for the generation of cDNA
(RETROscript kit, Ambion, Carlsbad, CA). For quantitative PCR 10.5 ng template cDNA was used to determine gene expression using Taqman assays for SOCS3 (Mm00545913_s1), galanin (Mm01236508_m1) and GAPDH (Mm99999915_g1), the latter served as housekeeping gene. Fold induction of gene expression with leptin was analyzed with the $\Delta\Delta^{Ct}$ method (also known as the comparative Ct method) as determined by the following equation: $\Delta\Delta^{Ct} = \Delta^{Ct}$, treatment - $\Delta^{Ct}$, control. Here, the $\Delta^{Ct}$ treatment is the Ct value for the sample normalized to the endogenous housekeeping gene (GAPDH) and $\Delta^{Ct}$-reference is the Ct value for the control also normalized to the endogenous housekeeping gene.

2.2.10 Estimates of cell counts. We counted the number of LepRb neurons that were co-localized with cFos (as a surrogate for stimulated neurons) after PBS or leptin treatment to evaluate whether leptin would stimulate or inhibit exPFA LepRb neurons. Immunohistochemical staining for cFos and GFP (LepRb$^{GFP}$) was visualized with a fluorescent microscope (Olympus BX51) and images were taken with a digital camera (Olympus DP30BW) using appropriate filters for fluorophores or bright-field illumination for DAB stain. Images for cFos and GFP were taken at identical sites, superimposed, and pseudocolored using Olympus Software and Adobe Photoshop (Adobe Systems, San Jose, CA). Brightness and contrast were modified for all images to enhance visibility of nuclear pSTAT3 and cFos staining for figure preparation and cell counts, however, care was taken that all images within an experiment were modified with identical settings. For each brain (n=3-4 per group) images from 4 anatomical levels were taken, representing the rostro-caudal extension of the exPFA (from Bregma –1.8 to –2.1 mm, as seen in Figure
Because the exPFA consists of 3 anatomically different locations (including the DMH, perifornical area and lateral hypothalamus) that may respond differently to stimuli, we subdivided the exPFA into 3 sub-areas for analysis: lateral hypothalamus (LH), perifornical area (PFA) and the DMH. A vertical line through the fornix separated the LH and PFA. The DMH and PFA were separated by a vertical line based on the distribution of DMH LepRb neurons. The total number of LepRb\textsuperscript{GFP} neurons and cFos/GFP neurons were estimated by cell counts from digital images. The total number of GFP neurons within anatomical sites was analyzed to ensure comparison of similar GFP populations among animals. The number of cFos/GFP neurons was expressed as percentage of the total number of GFP neurons in the quantified area. The described cell counts are not intended to provide precise numbers of stimulated GFP neurons. They instead served as estimates for a mainly stimulatory or inhibitory action of leptin in LepRb neurons.

To evaluate the number and percentage of galanin-GFP/pSTAT3 neurons in the exPFA compared to the total number of galanin or pSTAT3 neurons, as well as to identify co-expression of CART with galanin-GFP/pSTAT3 neurons we counted cells from Gal\textsuperscript{TgGFP} mice (n=3) that were treated with 1 hour leptin (leptin administered 1 hour before perfusion) and every 4\textsuperscript{th} section was stained for pSTAT3, GFP and CART. For each brain 4 sections containing the exPFA were used to determine the number of total pSTAT3, total galanin-GFP, galanin-GFP/pSTAT3 double labeled neurons and galanin-GFP/pSTAT3/CART triple labeled neurons within the exPFA. The total number as well as percentage of galanin-GFP/pSTAT3 neurons based on total pSTAT3 or total galanin-GFP
neurons was expressed. For CART co-localizations the mean number of pSTAT3/GFP/CART neurons was expressed as a percentage of total pSTAT3/GFP neurons in the exPFA.

We further evaluated the extent of co-localization for galanin and neurotensin neurons in colchicine treated Gal$^{TgGFP}$ mice (n=2). While extensive co-localization was easily visible, we performed cell count estimates from four exPFA sections of a single brain. The number of total Nts neurons and Nts/GFP neurons in the exPFA were determined and Nts/GFP neurons were expressed as the percentage of total exPFA Nts neurons.

For orexin and MCH co-localization we evaluated the co-localization of pSTAT3/galanin-GFP with orexin and MCH from Gal$^{TgGFP}$ mouse brains (n=3). Because we did not observe any co-localization of pSTAT3 or galanin-GFP with orexin or MCH we did not perform cell counts and instead report the anatomical evidence for lack of co-localization with images.

2.2.11 Statistical analysis. Data were analyzed by student’s t-test (two group comparison) or by one-way repeated measure ANOVA followed by a Holm-Sidak test for pairwise comparisons. The statistical tests used are specified accordingly in the figure legends and a significance level of $p<0.05$ was used in all tests.

2.3 RESULTS

2.3.1 Verification of Gal$^{TgGFP}$ mice. To verify correct anatomical expression of galanin-GFP in Gal$^{TgGFP}$ mice we compared GFP expression to in situ hybridization of galanin mRNA expression in adult mice (in situ data kindly provided
by the Allen Brain Institute, Figure 2.1, A-D). Indeed, galanin-mRNA expression patterns throughout the brain are very similar; this is illustrated in detail for the hypothalamus (Figure 2.1, A and B) and locus coeruleus (Figure 2.1, C and D).

It is well known that adrenergic neurons in the locus coeruleus are highly co-expressed with galanin (Holets et al. 1988, Melander et al. 1986, Melander, Staines, and Rokaeus 1986, Olpe and Steinmann 1991). Thus, we further verified correct expression of galanin-GFP by demonstrating their co-localization with tyrosine hydroxylase (TH), which we used as a marker for adrenergic neurons in the locus coeruleus (Figure 2.1, E). We found that every galanin-GFP neuron co-localized with TH.

There are no known markers for galanin-expressing neurons in the exPFA. Thus instead, we stained brain sections from GalTgGFP mice with anti-galanin to detect galanin peptide as well as with anti-GFP to detect galanin-GFP expression (Figure 2.2). The overall expression pattern of galanin and galanin-GFP fibers was identical, but cell bodies could only be detected with galanin-GFP (Figure 2.2, A-C). Furthermore, confocal high magnification images convincingly showed that galanin-positive fibers highly co-localized with galanin-GFP (Figure 2.2, D-F) in the PFA. These data indicate that galanin-GFP expression correctly corresponds with galanin neuropeptide expression; thus, verifying GalTgGFP mice as a useful tool to study galanin expressing neurons.
Figure 2.1 Similar expression patterns of galanin mRNA and galanin-GFP expression in the hypothalamus and locus coeruleus. A., C.: *In situ* hybridization for galanin mRNA expression shows the distribution of galanin neurons in the hypothalamus (A.) and locus coeruleus (C.) of an adult mouse brain. Images were kindly provided by the Allen Brain Atlas Institute. B., C.: Immunohistochemical detection of galanin-GFP in Gal\textsuperscript{TgGFP} mice shows strong anatomical similarity to galanin mRNA expression with many galanin neurons in the hypothalamus (DMH, PFA and LH) (A. and B.) and the locus coeruleus (C. and D.). E. Double immunohistochemistry in Gal\textsuperscript{TgGFP} mice for galanin-GFP (green) and tyrosine hydroxylase (TH, red), a marker for adrenergic neurons, confirms the adrenergic character of galanin-GFP neurons in the locus coeruleus (E.). Note that galanin-GFP also visualizes neuronal projections of galanin neurons, which are nicely visible in the pre-coeruleus that is void of galanin cell bodies.
Figure 2.2 Co-expression of galanin (peptide) and galanin-GFP in synaptic boutons of neuronal processes. Double immunohistochemistry in Gal^{TgGFP} mice for galanin-GFP (green, A. and D.) and galanin peptide (red, B. and E.) shows similar distribution of neuronal processes in the DMH, PFA and LH (merge, C and F.). High magnification of the PFA under a confocal microscope (magnification is 63x, D. to F.) shows a complete overlap of galanin peptide containing synaptic boutons with galanin-GFP. Note, cell bodies are only visualized with galanin-GFP, but as expected not with galanin peptide.
2.3.2 Distribution of galanin-expressing LepRb neurons. The immunohistochemical detection of LepRb is difficult due to unspecific antibodies or lack of sensitivity. Leptin activates LepRb via the janus kinase-2/pSTAT3 signaling pathway, resulting in a robust induction of nuclear pSTAT3 that can be detected by western blotting as well as immunohistochemistry (Faouzi et al. 2007, Munzberg, Flier, and Bjorbaek 2004, Munzberg et al. 2003). We used LepRb\textsuperscript{GFP} reporter mice to demonstrate that LepRb neurons show no pSTAT3 signal when injected with PBS (Figure 2.3, A), while an i.p. injection with 5 mg/kg leptin for 1h resulted in a robust induction of nuclear pSTAT3 that highly co-localized with LepRb\textsuperscript{GFP} neurons (Figure 2.3, B). We further confirmed earlier findings that leptin-induced pSTAT3 co-localizes with about 80\% of LepRb\textsuperscript{GFP} neurons {Figure 2.3, C; (Leinninger et al. 2009, Zhang et al. 2011)}}, demonstrating that leptin-induced pSTAT3 is highly representative for LepRb neurons.

Galanin-GFP neurons are densely packed in the DMH, however, surprisingly only a small subpopulation in the ventral portion of the DMH (vDMH, Figure 2.4, A) co-expressed leptin-induced pSTAT3, marking LepRb-expressing neurons (galanin-GFP/pSTAT3 neurons). The majority of galanin-GFP/pSTAT3 neurons were located in the perifornical area and lateral hypothalamus, where about 20-44\%, respectively, of all pSTAT3 neurons are co-localized with galanin-GFP (Figure 2.4, A; Figure 2.5, A; Figure 2.6, B), and 17-28\%, respectively, of all galanin-GFP neurons were co-localized with pSTAT3 (Figure 2.4, A; Figure 2.5, A; Figure 2.6, B). Another significant population of galanin-GFP/pSTAT3 neurons was found in
the nucleus of the solitary tract; here about 25% of all pSTAT3 neurons co-expressed galanin (Figure 2.4, B and Figure 2.5, B).

We also carefully examined galanin-GFP neurons in the arcuate nucleus for potential co-localization with leptin-induced pSTAT3. Even though we found an occasional co-localization of galanin and pSTAT3, this was negligible compared to the large number of LepRb neurons found in the ARC (Figure 2.4, C). In Figure 2.5 the rostro-caudal distribution of galanin-GFP/pSTAT3 neurons in the hypothalamus (Figure 2.5, A) and brainstem (Figure 2.5, B) are shown.

Figure 2.3 Leptin-induced pSTAT3 is a marker for LepRb neurons. LepRb<sub>GFP</sub> reporter mice were treated with PBS (A.) or leptin (1 mg/kg body weight, B.) for 1 hour and fixed brain sections were stained for pSTAT3 (red) and GFP (green). C. Cell counts of double-labeled pSTAT3/LepRb<sub>GFP</sub> neurons in leptin treated LepRb<sub>GFP</sub> mice (n=2) to demonstrate that the majority of leptin-induced pSTAT3 highly correlates with LepRb<sub>GFP</sub> expression.
Figure 2.4 Immunohistochemical detection of Gal-LepRb neurons. Brain sections from transgenic Gal\textsuperscript{TgGFP} reporter mice were stained with double immunohistochemistry for Galanin-GFP (green) and leptin-induced pSTAT3 (red).

A. Representative image of the exPFA depicting double labeled Gal-LepRb neurons in the PFA (inset top left), ventral DMH (vDMH, inset top middle) and lack of Gal-LepRb neurons in the dorsal DMH (dDMH, inset top right). B. Representative image of the NTS with inset showing several Gal-LepRb neurons. C. Representative image of the ARC depicting some of the few Gal-LepRb neurons. Scale bars: A. 200 µm; B. and C. 100 µm
Figure 2.5 Distribution of Gal-LepRb neurons. Rostro-caudal distribution of LepRb neurons (green, pSTAT3) and Gal-LepRb neurons (red, pSTAT3/galanin-GFP) in the exPFA (A.) and NTS (B.) relative to Bregma.
2.3.3 Neuropeptidergic characterization of galanin-GFP/pSTAT3 neurons. Within the lateral hypothalamus orexin and melanin-concentrating hormone (MCH)-expressing neurons are two well-characterized neuronal populations that are both known to regulate energy homeostasis. However, neither galanin- nor LepRb-expressing neurons were co-localized with orexin (Figure 2.7, A) or MCH (Figure 2.7, B). Thus, galanin-GFP/pSTAT3 neurons are a distinct population from orexin and MCH neurons.

Cocaine-and-amphetamine-regulated-transcript (CART)-expressing neurons are well known in the ARC where they are co-expressed with POMC-
expressing neurons and are well known as anorexigenic neurons. Another CART population is found in the lateral hypothalamus and we found that about 56±1.2% (n=3) of all galanin-GFP/pSTAT3 neurons in the hypothalamus co-express CART (Figure 2.7, C and Figure 2.8, A).

Leinninger et. al. (2009) showed recently that a large portion of LH LepRb neurons (about 60% of all LH LepRb neurons) co-express the anorexigenic neuropeptide neurotensin. Therefore we wanted to investigate if galanin-GFP/pSTAT3 neurons would also co-express neurotensin. Indeed, most identified neurotensin neurons were co-localized with galanin (about 95±3% of all neurotensin neurons, n=2; Figure 2.7, D and Figure 2.8, B). Neurotensin/galanin co-localization was restricted to the perifornical area and LH, suggesting that the neurotensin-LepRb neurons, identified by Leinninger et. al. (2009) may indeed represent galanin-GFP/pSTAT3 neurons (galanin/neurotensin-LepRb neurons). However, neurotensin neurons were not found in the DMH and the few neurotensin neurons in the NTS rarely co-localized with galanin (Figure 2.7, E), suggesting that a subpopulation of galanin-GFP/pSTAT3 neurons is distinct from neurotensin-LepRb neurons.

Also, many galanin neurons in the LH did not co-localize with neurotensin. Because we were not able to study co-expression of galanin, neurotensin and LepRb neurons together (colchicine treatment distorts cell signaling events like STAT3 phosphorylation), we cannot entirely rule out that galanin-GFP/neurotensin neurons may specifically not express LepRb.
Figure 2.7 Gal-LepRb neurons in the exPFA are distinct from orexin and MCH neurons, but co-express CART and neurotensin. A-C (overviews) and a-c (magnified): GalTgGFP mice were treated 1h with leptin for detection of leptin receptor neurons via leptin-induced pSTAT3 (red, nuclear stain). Galanin-GFP is shown in green (A and B) or blue (C.) and sections were further stained for orexin (blue, A.), melanin concentrating hormone (blue, MCH, B.) or cocaine-and-amphetamine-regulated transcript (green, CART, C.). D (overview) and d (magnified): Colchicine treated GalTgGFP brain sections were co-stained for galanin (green, GFP) and neurotensin (red). Scale bar A-D: 200 µm E: Brainstem section of the colchicine treated GalTgGFP brain co-stained for galanin (green, GFP) and neurotensin (red). Scale bar E.: 100 µm
Figure 2.8 Quantification of CART and neurotensin neurons. A. Cell counts of total Gal/pSTAT3 neurons in the exPFA and compared to Gal/pSTAT3 neurons that also co-express the neuropeptide CART (n=3). B. Cell counts of total neurotensin neurons and neurotensin neurons that also co-express galanin (n=1).

2.3.4 Leptin increases exPFA galanin gene expression. We further tested if leptin may modulate galanin gene expression specifically in the exPFA. We compared the exPFA of chow fed ob/ob mice treated with either PBS or leptin over 3 days and indeed found that leptin treatment caused a moderate, but significant increase in gene expression of galanin (p<0.005, Figure 2.9, A). To confirm correct leptin stimulation we compared SOCS3 expression in the exPFA and ARC of PBS and leptin treated mice. Indeed leptin induced SOCS3 expression in the ARC (p<0.01, Figure 2.9, C) and exPFA, even though in the exPFA data did not reach significance (p=0.07, Figure 2.9, B).
2.3.5 Leptin stimulates exPFA LepRb neurons. Leptin can cause either a stimulation or inhibition of neuronal activity (Belgardt and Bruning 2010, Cowley et al. 2001, Munzberg et al. 2007), even though LepRb neurons in the LH have been mainly associated with stimulatory leptin action (Leinninger et al. 2009). Thus, we wanted to test if we could recapitulate leptin stimulation of LepRb neurons in all subdivisions of the exPFA, including the DMH, PFA and LH (Figure 2.10, p<0.03). We injected LepRb\textsuperscript{GFP} reporter mice with PBS or leptin, perfused brains were harvested 3 hours later. After IHC staining the number of LepRb neurons that were co-expressed with cFos as a surrogate for neuronal activity were quantified. As expected, we found that leptin stimulates LepRb neurons in all subdivisions of the exPFA (DMH, PFA and LH). Leptin stimulated cFos was found in about 30-35% of all LepRb neurons (Figure 2.10), thus indicating that leptin may also stimulate neuronal activity in galanin-GFP/pSTAT3 neurons.
2.3.6 The exPFA is sufficient for anorexigenic leptin action. Given the mixed neuropeptide expression of anorexigenic and orexigenic neuropeptides in galanin-GFP/pSTAT3 neurons, combined with a likely neuronal stimulation of galanin-GFP/pSTAT3 neurons, we wanted to confirm that leptin action in the perifornical area (PFA), where the majority of galanin-GFP/pSTAT3 neurons are found, indeed results in anorexia. Thus, we injected low leptin doses (20 ng and 1 ng) unilaterally into the PFA of GalTgGFP and C57B6 wildtype mice.

We confirmed that PFA leptin is sufficient to decrease body weight and induce anorexia in GalTgGFP mice (Figure 2.11, A-C) and C57B6 mice (data not shown). Both doses, 20 ng (data not shown) and 1 ng leptin (Figure 2.11, A-C) were found effective to reduce body weight (Figure 2.11, A; \( p^{\text{ANOVA}} < 0.01, p^{\text{Holm-Sidak}*} < 0.05 \)), 24h food intake (Figure 2.11, B; \( t\text{-test} < 0.05 \)) as well as respiratory exchange rate (RER, Figure 2.10, C; \( p^{\text{ANOVA}} < 0.05, p^{\text{Holm-Sidak}*} < 0.05 \)), even though 1ng leptin was ineffective in C57B6 mice, likely due to differences in leptin sensitivity of the background strain (C57B6 versus GalTgGFP mice on an FVB background).

Cannula placements in C57B6 (blue dots) and GalTgGFP (red dots) mice are shown schematically in Figure 2.11, D and cases of misplaced cannulas (see arrow in Figure 2.11, D) were removed from analysis. Figure 2.11, E shows an example of a cannula track in a GalTgGFP mouse using galanin IHC as landmark to show correct targeting of the fornix. Thus, we show that leptin action in the PFA, including galanin-GFP/pSTAT3 neurons, is sufficient to mediate anorexigenic leptin action in wildtype mice.
Figure 2.10 Leptin stimulates neuronal activity in exPFA LepRb neurons. LepRb\textsuperscript{GFP} reporter mice were treated once with leptin (5mg/kg BW, n=3) for 3 hours to allow time for cFos induction as a surrogate for neuronal activation. The number of cFos labeled LepRb neurons was estimated by cell counts and reported as percent of total LepRb neurons (A.). The total number of LepRb (B.) neurons was also determined to ensure comparison of similar neuronal populations. $p_{t\text{-test}}<0.03$. 
2.4 DISCUSSION

The aim of this study was to reinvestigate the existence and localization of LepRb neurons that co-express the neuropeptide galanin, which would characterize a new subgroup of LepRb neurons and would enable future molecular genetic studies to identify the importance of such a LepRb subpopulation for physiologic leptin action.

Here, we show convincing evidence that galanin-GFP/pSTAT3 neurons are found in two distinct brain locations, one in the hypothalamus and another in the...
brainstem. Leptin-induced pSTAT3 is an excellent marker of LepRb expressing neurons within the exPFA (DMH, PFA and LH), as shown in Figure 2.3 and earlier publications (Leinninger et al. 2009, Zhang et al. 2011), thus in the remaining discussion we will refer to galanin-GFP/pSTAT3 neurons simply as Gal-LepRb neurons.

Galanin is well known to induce food intake, thus, it is possible that galanin would not only serve as a genetic marker for a subset of LepRb neurons, but galanin release from Gal-LepRb neurons could also play a role to mediate physiological leptin actions on food intake. Surprisingly, our data support that leptin induces exPFA galanin expression and stimulates neuronal activity in exPFA LepRb neurons, conflicting with an orexigenic action of galanin in this neuronal population. We found that Gal-LepRb neurons further co-localize with anorexigenic neuropeptides like neurotensin or cocaine-and-amphetamine-regulated-transcript (CART) and intra-exPFA leptin clearly mediated anorexia and weight loss.

2.4.1 Technical considerations. We demonstrate that LepRb neurons co-express the neuropeptide galanin in two distinct populations found within the hypothalamus and brainstem. Similar to an earlier double in situ hybridization study that focused on co-expression within the ARC and DMH, we found no or only sporadic co-localization in the ARC and only a low percentage of co-localization in the DMH (Cheung et al. 2001). But our data contradict previous reports that showed extensive co-localization (60% of all hypothalamic LepRb neurons) in the mouse or ewe (Hakansson et al. 1999, Iqbal et al. 2001). We hypothesized that these discrepancies are due to different methods used that may have lacked specificity.
{LepRb IHC, (Hakansson et al. 1999, Iqbal et al. 2001)}. This is also supported by the fact that extensive co-localization of LepRb with MCH and orexin was reported (Hakansson et al. 1999, Iqbal et al. 2001). In contrast, our data (using leptin-induced pSTAT3) did not support any co-localization of LepRb with MCH or orexin, which is consistent with earlier published data (Leinninger et al. 2009). Thus, our data show convincingly that leptin can directly act on galanin neurons in the exPFA and the nucleus of the solitary tract in the brainstem. Furthermore, we identified that LepRb and galanin neurons are distinct from orexin and MCH neurons.

**2.4.2 LepRb neurons in the brainstem and lateral hypothalamus.** LepRb neurons in the brainstem and lateral hypothalamus (LH) both contribute to the regulation of energy homeostasis (Leinninger et al. 2009, Hayes et al. 2010). However, the neuropeptides involved in these leptin actions have not been completely characterized. Within the LH, a subpopulation of LepRb neurons co-expresses neurotensin (Leinninger et al. 2011). Our data show that exPFA neurotensin neurons almost entirely co-expressed galanin, even though compared to the total number of galanin neurons, neurotensin was only found in a small subset of galanin neurons. Thus, we conclude that the recently described population of neurotensin-LepRb neurons likely co-expresses galanin, even though technical limitations prevented us from specifically studying if Gal-LepRb neurons co-express neurotensin. Importantly, a subset of Gal-LepRb neurons (at least in the DMH and NTS) clearly does not co-express neurotensin.

Instead, we found that half of all exPFA Gal-LepRb neurons co-expressed the neuropeptide CART, suggesting a heterogeneous character of Gal-LepRb
neurons. Surprisingly, we found combined expression of typical anorexigenic (neurotensin, CART) and orexigenic (galanin) neuropeptides, thus conflicting with the classic model of orexigenic or anorexigenic neurons (Schwartz et al. 2000).

Within the exPFA, only LH LepRb neurons have been studied in more detail (Leinninger et al. 2009, Leinninger et al. 2011, Louis et al. 2010), characterized as the population distal-lateral to the fornix (Harris, Wimmer, and Aston-Jones 2005, Leinninger et al. 2009), thus excluding neurons in the perifornical area (PFA, surrounding the fornix) and dorsomedial hypothalamus (DMH, proximal-lateral to the fornix). LH LepRb neurons are sufficient to regulate body weight and food intake in leptin-deficient ob/ob mice. Here we show that exPFA leptin administration is sufficient to decrease body weight and food intake even in wildtype mice. Neither PFA nor DMH LepRb neurons have been studied in detail for their contribution to regulate energy homeostasis. Certainly, it will be necessary to further investigate the physiological importance of these specific Gal-LepRb populations for known leptin actions, such as food intake, energy expenditure and the rewarding value of food.

2.4.3 Anorexic leptin action via inhibitory galanin action? To understand how leptin may regulate body weight and food intake in the exPFA, we tested if leptin would regulate the co-expressed neuropeptides. Galanin generally increases food intake (Kyrkouli et al. 1990), suggesting that anorexigenic leptin action should inhibit galanin expression or block galanin release. Surprisingly, we found that leptin upregulated galanin gene expression moderately, but significantly, in ob/ob mice. Furthermore, leptin stimulates neuronal activity in exPFA LepRb neurons,
suggesting that leptin induces galanin release. It is not plausible that anorexigenic leptin action requires increased expression and synaptic release of an orexigenic neuropeptide. Similarly, galanin neurons co-expressed the anorexigenic neuropeptides neurotensin and CART, again conflicting with an orexigenic galanin action.

Galanin affects many biological systems from induction of food intake, reproduction to pain and addiction (Gundlach 2002, Hokfelt et al. 1999, Merchenthaler 2010, Picciotto 2010). Another prominent feature of galanin is its inhibitory action (Dong, Tyszkiewicz, and Fong 2006, Poulain, Decrocq, and Mitchell 2003, Xu, Zheng, and Hokfelt 2005). Interestingly, LH LepRb neurons are generally GABAergic (Leinninger et al. 2009), thus further supporting the known inhibitory leptin action on orexin neurons (Louis et al. 2010). Indeed, LH galanin injections had no orexigenic effects in rats (Kyrkouli et al. 1990). Thus, it is plausible to speculate that galanin acts as an inhibitory (instead of orexigenic) neuropeptide in Gal-LepRb neurons and that inhibitory galanin action plays an important role to mediate anorexigenic leptin action via the exPFA. Consistent with this, orexigenic galanin action has been attributed to galanin action with the PVN (Kyrkouli et al. 1990, Menendez, Atrens, and Leibowitz 1992, Tempel, Leibowitz, and Leibowitz 1988), while LH LepRb neurons do not project to the PVN, but rather project locally in the LH and to the VTA (Leinninger et al. 2009).

2.4.4 Leptin regulation of exPFA galanin gene expression. A correlation of leptin and galanin gene expression has been studied by others with very inconsistent results, showing that leptin decreased galanin in the hypothalamus of
wildtype mice (Sahu 1998b, a), had no effect on galanin expression comparing leptin deficient ob/ob mice and wildtype mice (Williams et al. 1991) or increased galanin expression specifically in the periventricular region of leptin treated ob/ob mice (Cheung et al. 2001). The presented results would support leptin induced gene expression in the exPFA, even though this was not a robust effect. exPFA galanin neurons may represent a heterogeneous neuronal population, that are co-expressed with several neuropeptides with or without LepRb. Thus, leptin may regulate galanin expression differentially in subpopulations of galanin neurons. Furthermore, it needs to be cautioned that mRNA expression may not reflect the quantity of functional galanin at the synapses or the amount of galanin that is indeed released into the synaptic cleft. Therefore, changes in mRNA galanin expression may simply not correlate with changes in behavior.

Leptin robustly stimulates cFos, a marker of neuronal activity, in exPFA LepRb neurons, suggesting increased neuropeptide release such as galanin. However, we could not determine if leptin-induced LepRb neurons indeed included Gal-LepRb neurons, and we cannot rule out that leptin may exclusively stimulate non-galanin LepRb neurons in the exPFA. Indeed, in the LH as well as other hypothalamic sites leptin exerts depolarizing and hyperpolarizing effects on individual LepRb populations (Cowley et al. 2001, Leinninger et al. 2009). Future studies are necessary to test if galanin indeed contributes to inhibitory leptin actions {e.g. inhibition of orexin neurons (Louis et al. 2010)} and to demonstrate how this affects anorexigenic leptin action.
The perifornical area and lateral hypothalamus are generally involved in reward behavior (Berthoud and Munzberg 2011) and regulate sleep and arousal via orexin neurons. Similarly, leptin (Fulton et al. 2006, Gaus et al. 2002, Laposky et al. 2008, Laposky et al. 2006, Leinninger et al. 2009, Lu et al. 2002), galanin (Hawes et al. 2008, Picciotto 2010), neurotensin (Cape et al. 2000, Geisler et al. 2006, Hoebel 1985, Jones 2004) and CART (Jaworski et al. 2008, Keating et al. 2010, Philpot and Smith 2006) have all been associated with the control of reward as well as arousal behavior. Indeed, neurotensin-LepRb neurons modulate the reward system via orexin neurons. Even though it is still unknown by which mechanisms leptin inhibits orexin neurons (Louis et al. 2010). The fact that subpopulations of exPFA Gal-LepRb neurons also co-express neurotensin or CART suggests that Gal-LepRb neurons may be involved in the regulation of reward and arousal possibly via orexin neurons that could be inhibited by leptin induced galanin release.

In summary, we present clear evidence that LepRb is expressed on galanin neurons in two distinct locations, one in the hypothalamus and another in the brainstem. Surprisingly, several Gal-LepRb neurons further co-localized with the anorexigenic neuropeptides neurotensin or CART. However, intra-exPFA leptin action clearly demonstrated that leptin mediates anorexia and body weight loss in the exPFA. Furthermore, leptin induced galanin gene expression in the exPFA of ob/ob mice, and induced neuronal activity in exPFA LepRb neurons, thus conflicting with an orexigenic galanin action in this system. Galanin has also been well known as an inhibitory neuropeptide (Dong, Tyszkiewicz, and Fong 2006, Poulain,
Decrocq, and Mitchell 2003, Xu, Zheng, and Hokfelt 2005), thus we speculate that
galanin may act alternatively as an inhibitory neuropeptide, rather than an
orexigenic neuropeptide in Gal-LepRb neurons.
CHAPTER 3. LEPTIN REGULATES NUTRIENT REWARD VIA GALANIN NEURONS IN THE LATERAL HYPOTHALAMUS

3.1 INTRODUCTION

The rate of obesity is increasing worldwide (Mokdad et al. 2003), and this effect is likely due to the surplus and accessibility of calorically dense foods in modern societies. Calorie-rich foods, namely those high in fat and sugar content, evoke a potent reward response when consumed. With the high availability of such rewarding foods it seems our current feeding behaviors have shifted from consuming nutrients solely for homeostatic survival purposes, to now eating foods based on the pleasure they evoke. Such alterations in nutrient selection behaviors that govern palatable food intake would indicate a mechanism currently driving the obesity epidemic.

Leptin is highly implicated in obesity research as leptin signaling through LepRb exerts its anorexigenic effects via reductions in body fat mass and food intake. It is now appreciated that anorexic leptin actions are also mediated in part by leptin’s ability to modulate the rewarding value of food (Fulton et al. 2006). Reward behavior is regulated by the mesolimbic dopaminergic system, which consists of dopaminergic (DA) neurons in the ventral tegmental area (VTA) as well as their projection into the nucleus accumbens (NAc). In this circuit DA release modulates reward-motivated behaviors in response to a variety of stimuli (Hernandez and Hoebel 1988, Knutson et al. 2001, Ikemoto and Panksepp 1999, Nicola et al. 2005). The reward value of nutrients such as sucrose requires stimulation of midbrain DA neurons and is enhanced by fasting, whereas this effect...
is abolished by central leptin action (Domingos et al. 2011). Indeed, LepRbs are expressed on midbrain DA neurons and leptin directly inhibits these LepRb-DA neurons (Hommel et al. 2006). Conversely, leptin deficient ob/ob mice show reduced NAc DA content and peripheral or intra-LHA leptin rather increases NAc DA content (Leinninger et al. 2009, Fulton et al. 2006). However, the exact neuronal circuits involved in stimulating DA release are not entirely clear.

We previously reported on a large population of LepRb neurons in the LHA (referred to as exPFA in Chapter 2) that co-express the inhibitory acting neuropeptide galanin (Gal-LepRb neurons). We also showed that LHA galanin expression was stimulated by leptin (findings reported in Chapter 2). Central galanin injections selectively increase fat intake, and deficiency in galanin or galanin-receptor-1 (GalR1) results in decreased dietary fat intake (Zorrilla et al. 2007, Adams et al. 2008). Galanin also regulates reward circuits by inhibition of mesolimbic DA neurotransmission (Ericson and Ahlenius 1999, Counts et al. 2002), inhibition of noradrenergic LC neurons (Pieribone et al. 1995, Seutin et al. 1989, Sevcik, Finta, and Illes 1993) and counteracts withdrawal signs from abusive drugs (Zachariou et al. 2003). Thus, we hypothesized that Gal-LepRb neurons may play a critical role in the regulation of nutrient reward and that this could be important for body weight regulation. To test this, we studied mice with conditional deletion of LepRb from galanin neurons (Gal-LepRbKO mice). Here we observed that disruption of leptin signaling in galanin neurons results in differential preferences for sucrose and Intralipid (fat emulsion solution) and causes increased body weight gain in the form of a late onset obesity phenotype.
3.2 MATERIALS AND METHODS

3.2.1 Animals. All mice were bred and housed in a climate-controlled room (22°C) on a 12-hour light/dark cycle with lights on at 7:00 A.M. and lights off at 7:00 P.M. Food and water were available ad libitum unless otherwise specified. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

Generation of Gal^Cre^ mice: An IRES-cre sequence was inserted into the 3’ end sequence encoding the murine galanin gene between the STOP codon and polyadenylation site; and a frt-flanked neo cassette was inserted downstream of the polyadenylation sites. The construct was electroporated into mouse ES cells (albino C57B6 background), screening for construct insertion and injection into C57B6 blastocysts to generate chimeras was performed by the institutional transgenic core facility. Chimeras were bred to C57B6 mice and coat color screening was used to determine germline transmission. Correct genotypes were further confirmed by PCR, initially using long, transgene over-spanning DNA products to ensure correct insertion into the genome.

Generation of Gal-LepRb^KO^ mice: Gal^Cre^ mice were further crossed with LepRb^fl/fl^ mice on the mixed background C57BL/6J, FVB background {(McMinn et al. 2004) and kindly provided by Dr. Streamson Chua, Albert Einstein College of Medicine}, which generated Gal^{cre/+}; LepRb^{fl/+} and LepRb^{fl/+} mice. Final breeding pairs consisted of Gal^{cre/+}; LepRb^{fl/+} and LepRb^{fl/fl} mice to generate control, wildtype (WT) LepRb^{fl/fl} mice and Gal-LepRb^KO^ mice Gal^{cre/+}, LepRb^{fl/fl} mice or Gal^{cre/cre}, LepRb^{fl/fl} mice.
Some Gal<sup>Cre</sup> experimental animals were further bred on the (B6.129X1-Gt(ROSA)26Sor<sup>YFP</sup>/J background (stock#006148)(Gal<sup>YFP</sup> reporter mice) and compared to transgenic Gal<sup>TgGFP</sup> mice, which we used earlier to identify and characterize Gal-LepRb neurons in the LHA (as described in Chapter 2). In some mice the frt-flanked neo cassette was removed from the genome by crossing Gal<sup>YFP</sup> mice with 129S4/SvJaeSor-Gt(ROSA)26Sor<sup>FLP1</sup>/J mice (stock#003946)(Gal<sup>-neo</sup>YFP reporter mice) to prove that reporter expression pattern was not compromised by the presence of the neo cassette.

Genotyping of experimental animals was performed by PCR assessing hetero- or homozygous presence of Gal<sup>Cre</sup> (cre forward: CCT CTC CCA AGC GGC CGG AGA ACC, cre reverse: CCG GCT CCG TTC TTT GGT GGC CCC TTC GCG,); wt-cre primers: 5'-TCC TGA GAC CAT GTC CAC TG (fwd), 5'-CTG CCA CTC CTG TGA TCT GA (rev)); and Lepr<sup>m</sup> (mLepr106: GTC TGA TTT GAT AGA TGG TCT T, mLepr105: ACA GGC TTG AGA ACA TGA ACA, mLepr65A: AGA ATG AAA AAG TTG TTT TGG). We used the latter primers also to screen for potential germline LepRb excision and such animals were excluded from the study.

**3.2.2 Metabolic phenotyping.** Male Gal-LepRb<sup>KO</sup> and WT mice (n=11-15) were weaned into a multi-housing environment at three weeks of age and given standard rodent chow ad libitum (Laboratory Rodent Diet 5001, Purina). Mice were individually housed at 4 weeks of age, food intake and body weight was measured weekly until twelve weeks of age and body weight gain was determined from 4-12 weeks of age. At 12 weeks of age body composition was determined by nuclear magnetic resonance (NMR; minispec-mq series Bruker Billerica, MA) before mice
were acclimated to the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Ohio) for 3 days. Energy expenditure and locomotor activity was monitored continuously over 4 days. In another cohort of aged 7-9 month old, male Gal-LepRb\textsuperscript{KO} and WT littermates (n=7) body weight was again evaluated. Differences between groups were statistically analyzed with a repeated measure 2-way ANOVA or a Student’s t-test.

3.2.3 Microdissection and qPCR. To determine differences in galanin and galanin receptor expression in the LHA, Gal-LepRb\textsuperscript{KO} and WT mice (n=10-13) underwent CO\textsubscript{2} asphyxiation before decapitation. Brains were rapidly removed and placed in a pre-cooled rodent brain matrix for mice consisting of 1mm, coronal dividers (Electron Microscopy Sciences, Hatfield, PA). After initial brain extraction, the rest of the microdissection procedure was conducted over ice. Ice-cold razor blades were used to cut 1mm thick brain sections in a rostral to caudal manner. Visual landmarks such as the mammillothalamic tract (mt), fornix (fx), and the anterior cingulate area (aca) were used to strategically dissect the LHA and the NAc (refer to the schematic in Figure 3.1) from coronal brain slices. LHA tissue (LHA 1 and LHA 2) pieces were combined and collected in 1.5mL centrifuge tubes, flash frozen in dry ice, and stored in -80°C until further processed.

RNA extraction, RT-PCR and qPCR procedures were previously described in Chapter 2. cDNA (10.5ng/reaction) was analyzed in triplicate via quantitative PCR for GAPDH (housekeeping gene; Mm99999915\_g1), Galanin (Mm01236508), GalR1 (Mm00433515), GalR2 (Mm00726392), and GalR3 (Mm00443617; TaqMan Universal PCR Master Mix; Applied Biosystems-4304437, Carlsbad, CA) using an
Fold induction of gene expression was calculated using the $\Delta\Delta$CT method as described in Chapter 2.

Figure 3.1 Dissection of the LHA and NAc based on visual anatomical landmarks. Red lines indicate cuts made for consistent tissue dissection as determined by visual landmarks. A. Dissection of the NAc (0.98mm from Bregma), using the aca to make a horizontal cut, followed by two lateral cuts to remove remaining cortex structures. B-C. Dissection of the LHA rostral (B. -1.22mm from Bregma) to caudal sections (C. -2.06mm from Bregma). The first cuts were made horizontally through the mt and the fx to separate LHA (1) and LHA (2). Next, vertical cuts through the fx separated LHA tissue from other hypothalamic structures (DMH, VMH, and ARC). Final cuts were made laterally to remove any remaining cortex/amygdala structures.

3.2.4 Test for nutrient preference and reward. To test if WT and Gal-LepRb$^{KO}$ mice like fat similarly, we offered a fat emulsion (10% Intralipid) for 1 hour
per day over 10 days in a single bottle to both genotypes to compare their cumulative fat intake. To assess their preferences for different palatable foods we further compared their intake of isocaloric solutions of sucrose (25% sucrose) and Intralipid (10%) in a two-bottle-choice test. Mice were allowed to consume either solution (water was removed) for 1 hour over 10 days, and bottle positions were alternated every other day to prevent side preferences. Statistical differences were analyzed via 3-way repeated-measure ANOVA, followed by a post-hoc analysis to analyze consumption over time or by 3-way ANOVA to compare absolute intake after 10 days or during the first 4 days of experiment.

To analyze food-motivated/ reward-seeking behavior, mice were trained to run down a continually lengthening runway to reach a goal box containing a sweet treat (fruit loop), which is known as “incentive runway” (Pecina et al. 2003). Briefly, mice were maintained on a restricted feeding schedule (ad libitum food from 12:00-4:00 P.M., food removed from 4:00-12:00 P.M.) to enhance training success. Prompting runway training, mice were allowed overnight access to fruit loops in their respective home cages to avoid any neophobic responses to the reward. The incentive runway training procedure was conducted in a staggered schedule, allowing mice one-day recovery between training sessions to reduce stress. There were a total of 13 training sessions, with each session consisting of 5 runway-running trials (all sessions were conducted between 8:00 A.M. and 12:00 P.M., refer to schematic outlined in Figure 3.2). Sessions 1-3 were strictly acclimation trials, where the mice were granted access to the goal box with the fruit loop treat present. Runway training began on Session 4 and mice were placed at a starting
distance of 15 cm from the goal box. With each subsequent session the runway length was increased in 15 cm increments until reaching a maximal distance of 75 cm. In all sessions, mice were allowed 30 seconds to consume the fruit loop after completion of the runway task. Video footage of each session was recorded for slow motion analysis of running speed, number and duration of pauses, and reversals.

![Diagram of the incentive runway setup](image)

**Figure 3.2** Incentive runway measures reward-motivated behaviors. For runway running sessions mice were placed in enclosed start box (at the indicated distance from the goal box) for 30s. The start box barrier was then removed and mice were able to enter the runway and proceed to the goal box containing the fruit loop reward (animals were allowed 30s to consume the reward after each trial). Completion speed was assessed as the time from which the animal entered the runway until entering the goal box. Sessions were conducted in a staggered schedule (1 day training, 1 day recovery), and each session consisted of 5 runway running trials

**Sessions 1-3:** Goal Box acclimation with Fruit Loop (5min)
**Session 4:** Runway running
- Distance: 15 cm
**Session 5:** Runway running
- Distance: 30 cm
**Session 6:** Runway running
- Distance: 45 cm
**Session 7:** Runway running
- Distance: 60 cm
**Session 8-13:** Runway running
- Distance: 75 cm

(5 trials/session)

3.2.5 **Perfusions and immunohistochemistry.** Gal^{TGFP}, Gal^{YFP}, and Gal^{neo}YFP mice and a separate cohort of Gal-LepRb^{KO} and WT mice were all
administered a single leptin injection (5mg/kg, i.p.) and perfused exactly one hour later. Fixed brains were extracted post-fixed, and cryoprotected as described in detail in Chapter 2.

Galanin-GFP expression comparison: To visualize Gal-LepRb neurons, brain sections were immunohistochemically processed as described in Chapter 2 for leptin-induced pSTAT3 (anti-pSTAT3, 1:1000; DAB) and successively counterstained for GFP (anti-GFP, 1:1000; fluorescent). Sections were mounted onto gelatin-subbed slides and cover slipped with Pro-Long Anti-fade mounting medium (Invitrogen; Carlsbad, CA). Galanin-GFP expression in the LHA and NTS of transgenic GalTgGFP mice (which was described earlier in Chapter 2) was used to identify and compare the distribution of Galanin-Cre driven GFP expression in GalYFP mice and Gal(neo)YFP mice. Additionally, corresponding LHA and NTS sections from GalYFP and Gal(neo)YFP mice were used to identify accurate leptin-induced pSTAT3/ GFP co-expression (Gal-LepRb neurons), as those previously described in transgenic GalTgGFP mice.

Confirmation of proper LepRb deletion in Gal-LepRbKO mice: LepRb-expressing neurons were visualized via immunohistochemical procedures described in Chapter 2 for DAB labeling of leptin-induced pSTAT3 expression and further processed as described below.

3.2.6 Estimate of cell counts. Microscopy imaging procedures have been previously described in Chapter 2. For quantification of leptin-induced pSTAT3 in the LHA and NTS brains from WT and Gal-LepRbKO mice (n=4-8 per genotype) that were treated with leptin for 1h were analyzed. For each area 2 sections containing
leptin-induced pSTAT3 were imaged using the Olympus BX51 bright-field microscope and Olympus DP30BW digital camera (Olympus; Center Valley, PA) (LHA=2 sections; NTS=2 sections). pSTAT3 signals were amplified by appropriate changes in brightness and contrast, making sure that adjustments remained consistent between sections and animals. The number of pSTAT3 positive cell nuclei was quantified and compared between groups with a Student’s t-test.

3.3 RESULTS

3.3.1 Generation and validation of Gal-LepRbKO mice. In order to understand the physiological importance of leptin action in galanin neurons we aimed to delete LepRb selectively from galanin neurons. Thus, we generated mice that expressed cre-recombinase from the native galanin locus and crossed them with LepRbfl/fl mice {kindly provided by Dr. Streamson Chua: (McMinn et al. 2004)} to generate Gal-LepRbKO mice (GalCre/+; LepRbfl/fl or GalCre/Cre; LepRbfl/fl) and WT littermates (Gal+/+; LepRbfl/fl, mouse construct schematic Figure 3.3, A). For validation of correct cre-expression in GalCre mice GalYFP mice were generated by crossbreeding with cre-inducible YFP reporter mice (B6.129X1-Gt(ROSA)26Sor1m1(EYFP)Cos/J, stock#006148). We compared GFP expressing neurons in transgenic GalTgGFP mice (Figure 3.3, left panels), which we described earlier in Chapter 2 to identify and characterize Gal-LepRb neurons in the LHA to GalYFP mice (middle panels) and Gal(-neo)YFP mice (right panels), where the frt-flanked neo cassette was removed from the genome by crossing GalYFP mice with
mice that express flp-recombinase ubiquitously (129S4/SvJaeSor-Gt(ROSA)26Sor^{lm1(FLP1)Dym}/J, stock#003946).

The distribution of hypothalamic Gal^{GFP} neurons was very similar in all mouse models (Figure 3.3). We also confirmed that galanin neurons correctly expressed LepRb, by visualizing leptin-induced pSTAT3 with immunohistochemistry (IHC). As expected, pSTAT3 positive galanin neurons were found in the PFA, LH and to a lesser extent in the ventral portion of the DMH (Figure 3.3, B) and another population was found in the NTS (Figure 3.3, C) in Gal^{TgGFP}, Gal^{YFP} and Gal^{(neo)YFP} mice. Galanin neurons with detectable leptin-induced pSTAT3 are further referred to as Gal-LepRb neurons. All remaining experiments were only performed with Gal^{YFP} mice.

To confirm efficient deletion of LepRb in Gal-LepRb^{KO} mice we compared leptin-induced pSTAT3 in Gal-LepRb^{KO} and WT littermates. Leptin-induced pSTAT3 was significantly decreased in the LHA and NTS, as seen in IHC images (Figure 3.4, B) as well as by cell counts of pSTAT3 positive neurons (Figure 3.4, C-D; n=4-5, p<0.01).

**3.3.2 Increased body weight gain in Gal-LepRb^{KO} mice.** To assess the metabolic phenotype of Gal-LepRb^{KO} mice we followed their body weight and food intake over 12 weeks after weaning (4 weeks, n=10-12). Similar to leptin and leptin receptor deficient mice, Gal-LepRb^{KO} mice show a significantly lower body weight compared to their WT littermates at 4 weeks of age (Figure 3.4, E; p<0.05). At 12 weeks of age body weights were undistinguishable between both groups (Figure 3.4, E). However, the body weight gain over 8 weeks was significantly increased in
Gal-LepRb$^{\text{KO}}$ mice compared to WT mice (Figure 3.4, F; p<0.05). Consistent with a slow but accumulating increase in body weight gain we found, in a separate cohort of aged (7-9 month old) mice, that Gal-LepRb$^{\text{KO}}$ mice were indeed significantly heavier than WT littermates (Figure 3.4, G; n=7-8, p<0.05). However, food intake (Figure 3.4, H), energy expenditure (Figure 3.4, I) or locomotor activity (Figure 3.4, J) could not be clearly attributed to these differences in body weight gain.

Figure 3.3 Similar galanin-GFP expression between Gal$^{\text{TgGFP}}$, Gal$^{\text{YFP}}$ and Gal$^{\text{(neo)YFP}}$ mice. A-C. GFP expression in coronal brain sections of Gal$^{\text{TgGFP}}$ mice (left panels) Gal$^{\text{YFP}}$ mice (middle panels) and Gal$^{\text{(neo)YFP}}$ mice where the frt-flanked neo cassette was removed (right panels). A. Hypothalamic expression Gal$^{\text{GFP}}$. Confirmation of galanin neurons (green) correctly expressing LepRb via visualization of leptin-induced pSTAT3 (red) in the LHA- B. and in the NTS- C.
Figure 3.4 Gal-LepRb\textsuperscript{K0} mice experience late-onset obesity. A. Generation of Gal-LepRb\textsuperscript{K0} mice (Gal\textsuperscript{Cre/+}, LepRb\textsuperscript{fl/fl} or Gal\textsuperscript{Cre/Cre}, LepRb\textsuperscript{fl/fl}); mouse construct schematic. Confirmation of efficient deletion of LepRb in Gal-LepRb\textsuperscript{K0} mice as visualized B. by leptin-induced pSTAT3 in Gal-LepRb\textsuperscript{K0} (KO) and control (WT) littermates and quantified via total pSTAT3 cell counts in the LH. C. Metabolic phenotyping was assessed via weekly body weight- E. and food intake- H. measurements over 12 weeks after weaning (4 weeks, n=10-12); where Gal-LepRb\textsuperscript{K0} mice showed a smaller weaning weight compared to WT mice (E. p<0.05), but no differences of body weight were observed at 12 weeks of age. The lower weaning weight translated into a significant increase of 8-week body weight gain in Gal-LepRb\textsuperscript{K0} mice (F. p<0.05), and late-onset obesity was observed in 7-8 month old Gal-LepRb\textsuperscript{K0} mice (G. n=7-8, p<0.05). No differences were observed in cumulative food intake (H. 4-12 weeks of age), energy expenditure (I. 12 weeks of age) or locomotor activity (J. 12 weeks of age).
3.3.3 Decreased LHA galanin signaling is associated with decreased fat intake. The inhibitory acting neuropeptide galanin has been shown to selectively modulate the ingestion of fat (Tempel, Leibowitz, and Leibowitz 1988) and we have shown earlier that leptin increases galanin expression in the LHA. Thus, we tested if lack of leptin action in Gal-LepRb$^{KO}$ mice similarly regulates gene expression of galanin or its receptors and if this would modulate fat ingestion. We analyzed mRNA expression in the LHA of WT and Gal-LepRb$^{KO}$ mice and found a 57% decrease in galanin expression (Figure 3.5, A; $p_{\text{t-test}}^{*}<0.01$) and a 30% decrease in GalR1 expression (Figure 3.5, B; $p_{\text{t-test}}^{*}<0.05$) within the LHA, while gene expression of all other galanin receptors (GalR2 and GalR3) were unchanged (Figure 3.5, B).

Thus, we further tested if this affected their fat ingestion. Naïve Gal-LepRb$^{KO}$ mice and WT littermates had access to a 10% Intralipid (fat emulsion) solution for 1 hour per day over 10 days. It had been reported by others that various concentrations of Intralipid solution are highly palatable for mice and the amount of Intralipid ingested correlates with the “liking” of the offered solution (Sclafani 2007). As expected, both WT and Gal-LepRb$^{KO}$ mice showed increasing Intralipid consumption over 10 consecutive 1-hour access sessions. However, Gal-LepRb$^{KO}$ mice consumed significantly less Intralipid solution over the 10 days compared to WT mice (Figure 3.5, C; $n=4-5$; $p_{\text{ANOVA}}^{*}<0.001$; *$p_{\text{Holm-Sidak}}^{*}<0.05-0.001$), indicating that they “liked” the Intralipid solution less than the WT mice.
Figure 3.5 Altered nutrient reward in Gal-LepRb\textsuperscript{KO} mice. A. LHA galanin mRNA expression is decreased in Gal-LepRb\textsuperscript{KO} mice (n=10-13, \*p\textsuperscript{t-test}<0.01). B. LHA galanin receptor 1 (GalR1) mRNA expression is decreased in Gal-LepRb\textsuperscript{KO} mice (n=10-13, \*p\textsuperscript{t-test}<0.05). C. Cumulative Intralipid intake is reduced in Gal-LepRb\textsuperscript{KO} mice during a 1 hour daily access of a single bottle of 10% Intralipid over a 10 period (n=5; p\textsuperscript{ANOVA}<0.001; \*p\textsuperscript{Holm-Sidak}<0.05-0.001). D. Cumulative sucrose intake is increased, but Intralipid intake is decreased in Gal-LepRb\textsuperscript{KO} mice during a 1h daily two-bottle-choice access of isocaloric 10% Intralipid and 25% sucrose solutions (both 1 kcal/ml) over a 10 period (n=5-7; p\textsuperscript{ANOVA}<0.0001; \*p\textsuperscript{Holm-Sidak}<0.05-0.001). E. Comparison of total sucrose and Intralipid intake over 10 days shows a clear preference of sucrose over Intralipid in Gal-LepRb\textsuperscript{KO} mice, while WT mice did not show preference for either solution (n=5-7, p\textsuperscript{ANOVA}<0.001; \*p\textsuperscript{Holm-Sidak}<0.01). F. Daily intake of sucrose and Intralipid during the first 4 days shows that sucrose preference is prevalent on day 1 in naïve Gal-LepRb\textsuperscript{KO} mice (p\textsuperscript{ANOVA}<0.05, \*p\textsuperscript{Holm-Sidak}<0.02).
Next we wanted to test if this behavior was selective for fat or if other palatable nutrients like sucrose were also less “liked.” In another cohort of naïve mice (n=6-9) we gave 1-hour daily access to a choice of isocaloric (1 kcal/ml) solutions of 25% sucrose and 10% Intralipid over 10 consecutive days. Again, Gal-LepRb<sup>KO</sup> mice consumed less Intralipid compared to WT mice, but in striking contrast Gal-LepRb<sup>KO</sup> mice consumed more of the isocaloric sucrose solution compared to WT animals (Figure 3.5, D; n=6-9; p<sub>ANOVA</sub> < 0.0001; *p<sub>Holm-Sidak</sub> < 0.05-0.001). While WT mice ingested equal amounts of Intralipid and sucrose solutions and thus had no preference for either of the solutions, Gal-LepRb<sup>KO</sup> showed a robust and significant preference for sucrose over lipids (Figure 3.5, E; n=6-9, *p<sub>t-test</sub> < 0.05). Therefore, deficiency of LepRb in galanin neurons differentially modulates the preference for sucrose and lipids. Importantly, the robust sucrose preference in Gal-LepRb<sup>KO</sup> mice was present from the first day of exposure to the solutions (Figure 3.5, F; p<sub>ANOVA</sub> < 0.05; *p<sub>Holm-Sidak</sub> < 0.02) demonstrating that this nutrient selection is not due to a learned behavior.

**3.3.4 Gal-LepRb<sup>KO</sup> mice work more for sugar rewards.** We further wanted to evaluate if the increased sucrose preference observed in Gal-LepRb<sup>KO</sup> mice would also affect their motivation to work for a sucrose food reward; which has been also termed “wanting” (Berridge, Robinson, and Aldridge 2009). The incentive runway has been successfully used in mice to evaluate their motivation to work for food rewards (Pecina et al. 2003) and is particularly useful in mice due to the simplicity of the learning task. Mice were trained to run a straight path with increasing runway length (up to 75 cm) to obtain a visible food reward (fruit loop).
Various aspects of runway performance were analyzed to compare food-motivated behaviors between Gal-LepRb\textsuperscript{KO} and WT mice respectively.

Indeed, we found that Gal-LepRb\textsuperscript{KO} mice showed an increased motivation to work for a sugary treat, which was observed as an increased completion speed to obtain the food reward (Figure 3.6, A; n=4; $p^\text{ANOVA}<0.05$; $p^\text{Holm-Sidak}<0.05-0.02$). Further analysis revealed that Gal-LepRb\textsuperscript{KO} mice had more incidences of direct, undistracted runs, while WT mice spend significantly more time with distractions such as pauses, falters and reversals (Figure 3.6, B and C; n=4; $p^\text{ANOVA}<0.05$; $p^\text{Holm-Sidak}<0.03$, $\#<0.06$; $p^\text{t-test}<0.03$, respectively). In addition, Gal-LepRb\textsuperscript{KO} mice also ran faster to obtain the sugary treat compared to their WT littermates (Figure 3.6, D and E; n=4; $p^\text{ANOVA}<0.03$; $p^\text{Holm-Sidak}<0.03$, **<0.02, $\#<0.08$; $p^\text{t-test}<0.03$, respectively).

3.4 DISCUSSION

Our data demonstrate that LHA leptin action - via galanin neurons – indeed robustly regulates nutrient reward. Dysregulation of this system, by deletion of LepRb from galanin neurons, enhances body weight gain and causes late onset obesity; strongly suggesting that nutrient reward behavior drives body weight gain. The concept that galanin controls macronutrient selection has been first introduced by Leibowitz and colleagues, where they found that central galanin injections selectively increases fat intake, while carbohydrate intake remained unchanged (Tempel, Leibowitz, and Leibowitz 1988). More recently, galanin’s effect on fat intake was further supported in galanin deficient mice, which showed a decreased ingestion of a high fat diet (Adams et al. 2008).
Figure 3.6 Gal-LepRb\(^{\text{KO}}\) mice work more for sugar rewards. In the incentive runway WT and Gal-LepRb\(^{\text{KO}}\) mice (n=4 each genotype) were tested for their completion speed (cm/sec= runway length [cm] /time from leaving start box till entering goal box [sec]) (A.; \(p^\text{ANOVA}<0.05; \*p^\text{Holm-Sidak}<0.05-0.01\)), the number of direct runs (in contrast to distracted runs) (B., C.; \(p^\text{ANOVA}<0.05; p^\text{Holm-Sidak}<0.03, \#=0.06; p^\text{t-test}<0.03\), respectively) and running speed (mean completion speed from all direct runs)(D., E.; \(p^\text{ANOVA}<0.03; p^\text{Holm-Sidak}<0.03, **<0.02, \#=0.08; p^\text{t-test}<0.03\), respectively). Each session consisted of 5 trials and sessions that were held every other day.
Similarly, GalR1 deficient mice show a decreased ingestion of high fat diet, even though their short-term consumption was initially increased (Zorrilla et al. 2007). In line with this, our data demonstrate that reduced galanin and GalR1 mRNA expression in the LHA is associated with decreased fat (10% Intralipid) consumption, supporting the idea that galanin action in the LHA via GalR1 importantly controls fat ingestion.

On the contrary, we observed that Gal-LepRb^KO^ mice exhibit a robust preference for sucrose over Intralipid, and even consumed significantly more than their WT littermates. Leptin deficiency in humans and rodents dramatically increases the rewarding value of all foods; which mimics the strong effect of fasting to enhance the rewarding value of food (Myers, Cowley, and Munzberg 2008). Reward is strongly influenced by midbrain dopamine (DA) neurons and optogenetic stimulation of DA neurons paired with water intake is strongly preferred over water without DA neuronal stimulation. However, DA stimulation alone is not able to override preferences for sucrose solutions (Domingos et al. 2011). This nicely demonstrates the importance of the DA system for reward, while pointing out that food rewards such as sucrose require additional regulatory circuits. Sucrose consumption stimulates DA neurons and metabolic states can either enhance or abolish DA responses. Thus, fasting enhances sucrose preference over non-caloric sweeteners, while leptin abolishes this effect.

Our data demonstrate that the robust sucrose preference further translates into enhanced work for a sweet treat. Gal-LepRb^KO^ mice perform the incentive runway with increased running and completion speed, and show fewer distractions
during the task performance. Leptin action in the LHA regulates the mesolimbic DA system by direct LepRb projections to the VTA (Leinninger et al. 2009) as well as innervation of orexin neurons (Louis et al. 2010). Moreover, orexin neurons also innervate the VTA to regulate DA neurons (Korotkova et al. 2003). Indeed, peripheral leptin injections and intra-LHA leptin increases DA content in the NAc of \textit{ob/ob} mice (Fulton et al. 2006, Leinninger et al. 2009). Similarly, orexin action in the VTA is known to stimulate DA release. However, leptin inhibits orexin neurons, which should decrease DA release in the NAc. Thus, determining the distinct circuit by which Gal-LepRb neurons mediate nutrient reward (either via projections to the VTA, orexin neurons etc.) may resolve some of these discrepancies encountered when LepRb function is generalized to the entire LHA-LepRb population.
CHAPTER 4: DEFINING GAL-LEPRB NEURONAL CIRCUIT: POTENTIAL SITES OF ACTION

4.1 INTRODUCTION

The lateral hypothalamus area (LHA) is a brain region well recognized for its role in reward function. Particularly, activation of LHA orexin neurons enhances reward-motivated behaviors, which is largely accomplished via orexin-induced stimulation of mesoaccumbal DA release. Palatable foods, high in sugar and fat, are perceived to be highly rewarding and generally evoke an increased motivational state to obtain them. And orexin mediates these reward responses as central orexin injections increase sucrose intake, while genetic or pharmacological blockade of orexin signaling decreases the rewarding value and intake of sucrose (Cason et al. 2010). Moreover, the activity of orexin neurons is highly correlated with sucrose seeking behaviors (Harris, Wimmer, and Aston-Jones 2005). Thus, the pathway responsible for negatively regulating orexin’s activity seems to be a crucial component in aspects of reward-based feeding and the development of obesity.

The anorexigenic hormone leptin is a likely candidate for opposing orexin’s effects, as leptin treatment decreases the rewarding value of sucrose and attenuates sucrose seeking behaviors (Figlewicz et al. 2006, Figlewicz et al. 2001). Moreover, leptin has been shown to inhibit the activity of orexin neurons, but how this is accomplished has not been determined. We do know that leptin is not capable of directly inhibiting orexin neurons, as we confirmed that LHA LepRb neurons are not co-expressed with orexin neurons ((Louis et al. 2010, Leinninger et al. 2009) and our data shown in Chapter 2). LHA LepRb neurons were observed to innervate orexin
neurons (Leinninger et al. 2011, Louis et al. 2010), suggesting that leptin may indirectly inhibit orexin neurons to regulate nutrient reward. Importantly our data shows that leptin modulates the reward value of nutrients via LHA Gal-LepRb neurons. We observed that mice exhibiting targeted LepRb deletion exclusively in galanin neurons (which we refer to as Gal-LepRbKO mice) exhibit a robust preference for sucrose over lipids. Interestingly, Gal-LepRbKO mice demonstrated enhanced food-motivated behaviors for a sugary treat, indicative of increased orexin activity.

In order to determine the precise pathway by which leptin signaling in Gal-LepRb neurons modulates nutrient reward, we employed a cell-specific viral tracing method to study anterogradely-labeled neural projection sites specific to either galanin and LepRb neurons in the LHA. Here we show that Gal-LepRb neurons do, in fact, innervate local orexin neurons. In line our hypothesis that Gal-LepRb neurons mediate leptin’s inhibitory influence over orexin neurons, the absence of leptin signaling in galanin neurons (Gal-LepRbKO mice) translates into increased activation of orexin neurons. Interestingly, Gal-LepRb neurons do not project to the VTA as previously reported for LHA-LepRb neurons, however, they were found to innervate the noradrenergic locus coeruleus (LC); a nucleus that had not been associated with leptin action. Importantly, our data demonstrates that Gal-LepRb neurons are a distinct LHA LepRb population that modulates the reward value of nutrients by eliciting an inhibitory tone onto orexin neurons.
4.2 MATERIALS AND METHODS

4.2.1 Animals. All mice were bred and housed in a climate-controlled room (22°C) on a 12-hour light/dark cycle with lights on at 7:00 A.M. and lights off at 7:00 P.M. Food and water were available ad libitum unless otherwise specified. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center. Gal\textsuperscript{Cre} and LepRb\textsuperscript{Cre} mice (described in Chapter 2 and 3) were used for cre-driven/cell-specific expression of anterograde adeno-viral tracing experiments to investigate projection sites of Gal-LepRb neurons. Gal\textsuperscript{YFP} mice (described in Chapter 3) were used to identify the presence of retrogradely labeled Gal-LepRb neurons from the LC (to confirm adenoviral anterograde findings). Finally, a cohort of Gal-LepRb\textsuperscript{KO} mice and their WT littermates (described in Chapter 3) were used to assess potential differences in orexin gene expression and neuronal activity.

4.2.2 Acute tracing studies. Adenoviral constructs: To define anterograde projections from galanin- and LepRb-expressing neurons in the LHA, we employed the use of molecular tracers with the cre-inducible system (for galanin- and LepRb-specificity) and adenoviral stereotaxic injection (for anatomic specificity) using two adenoviral vectors, Ad-iZ/EGFPf and Ad-iN/WED. For the generation of Ad-iZ/EGFPf, the floxed β-geo cassette from pCALL was excised and inserted upstream of the MCS from pShuttleCMV (He et al. 1998) to generate pShuttle-iZ. The coding sequence of EGFP from pEGFP (Invitrogen) was amplified with the addition of sequences encoding a COOH-terminal farnesylation by standard PCR and subcloned into the MCS of pShuttle-iZ to generate pShuttle-iZ/EGFPf, which
was purified, linearized and utilized to generate the Ad-iZ/EGFPf adenovirus as previously described (Leshan et al. 2009, Leinninger et al. 2009). Ad-iZ/EGFPf results in cre-inducible expression of farnesylated EGFP to mark cell bodies and processes of cre-expressing neurons, while the viral injection spread can be monitored by β-galactosidase (β-gal) expression in non-cre expressing neurons. For the generation of the Ad-iN/WED construct, the β-gal-encoding region of the β-geo fusion was deleted in pShuttle/iZ. Thus, generating pShuttle/iN, thereby decreasing the size of the construct to promote proper viral packaging with larger inserts. The coding region of WGA along with an IRES element and the coding sequence for farnesylated DsRed was then subcloned into the MCS of pShuttle/iN vector to generate pShuttle-iN/WED. Vector DNA was purified, linearized and utilized to generate the cre-inducible WGA adenoviral vector (Ad-iN/WED). Cre-dependent WGA expression from Ad-iN/WED was verified in vitro by immunohistochemical staining for WGA in infected HEK293 cells transfected with a vector encoding cre recombinase as determine previously (Louis et al. 2010). Ad-iN/WED results in cre-inducible expression of DsRed and wheat germ agglutinin (WGA). WGA is further anterogradely and transsynaptically transported into second order neurons, while DsRed remains in first order cre-expressing neurons.

For surgical procedures of acute central injections mice were deeply anesthetized with 1-3% isofluorane/oxygen and the head was mounted on a stereotaxic frame (M1900 Stereotaxic alignment system, Kopf instruments, Tujunga, CA). The surgical area was prepared by shaving and sterilizing the scalp of the mouse with 70% ethanol and nolvassan. Ophthalmic ointment was next
applied to the animal’s eyes to provide lubrication throughout the surgical procedure. Starting slightly behind the eyes, a midline sagittal incision about 2-3 cm long was made to expose the skull. Hydrogen peroxide was rubbed directly onto the skull to remove connective tissue and provide a means to adequately visualize and define Bregma. Bregma was then used as a reference point to determine the injection location for the stereotaxic coordinates of the LHA (X -0.9mm, Y -1.3mm, Z -5.2mm from Bregma) in Gal\textsuperscript{Cre} and LepRb\textsuperscript{Cre} mice, or the LC (X -0.8, Y -5.4, Z -3.7mm from Bregma) in Gal\textsuperscript{YFP} mice. An access hole was drilled to insert the guide cannula. A stainless steel guide cannula with dummy (Plastics One, Roanoke, VA) was lowered to the appropriate depth, and the injector was loaded with the respective tracing agent using a 0.5ul Hamilton syringe (Hamilton, Reno, NV). The filled injector was then inserted into the guide cannula and tracers were infused into the respective brain areas at a rate of 10nl/20s.

For galanin and LepRb adenoviral anterograde tracing experiments, Gal\textsuperscript{Cre} and LepRb\textsuperscript{Cre} mice received a LHA-targeted injection of either the Ad-iZ/EGFPf or Ad-iN/WED adenovirus (250-350nl, 2-6x10^{12} PFU/ml). After the infusion was complete, the injector was removed, the hole filled with bone wax and skin was closed with wound clips. Mice were subcutaneously administered the analgesic, Carprofen (10 mg/kg: two injections within 24 hours of surgery), and administered an injection localized to the incision of Buvidiocaine/Lidocaine mixture (2.5 mg/kg). Mice were allowed to recover on a heating pad and then single-housed in a biohazard containment room. After 7-10 days incubation time, mice were perfused,
fixed brains were extracted, cryoprotected (as described in Chapter 2) and processed for immunohistochemical analysis as described.

For injection of the non-specific retrograde tracer (FG), the cannula was inserted into the LC (based on above coordinates) of GalYFP mice and 10nl of a 2%FG solution was injected over 20 seconds. The injector was then removed, the hole filled with bone wax and skin was closed with wound clips. Mice were subcutaneously administered the analgesic, Carprofen (10 mg/kg: two injections within 24 hours of surgery), and administered an injection, localized to the incision, of Buvidiocaine/Lidocaine mixture (2.5 mg/kg). Mice were allowed to recover on a heating pad and then single-housed in clean cages. 3-5 days post-injection, animals were administered a single i.p. leptin injection (as described below) and perfused exactly 1 hour later. Fixed brains were extracted, cryoprotected (as described in Chapter 2) and processed for immunohistochemical analysis.

4.2.3 Immunohistochemistry. Refer to page xi for detailed primary antibody information, and Chapter 2 for DAB and fluorescent immunohistochemical detection methods. For Ad-iZ/EGFPf axonal tracing experiments, detection of cre-inducible fluorescent expression of the farnesylated enhanced green fluorescent protein (EGFPf; detected via anti-GFP antibody 1:1000) was used for visualization of anterogradely labeled axon fibers from LHA galanin and LepRb neurons. The extent of viral spread in the LHA was immunohistochemically detected via non-cre specific β-gal expression (Ad-iZ/EGFPf; anti- β-gal, 1:1000).

For Ad-iN/WED neuronal tracing experiments, detection of cre-inducible fluorescent expression of DsRed (anti-DsRed, 1:1000) was used for visualization of
primary (cre-expressing) galanin and LepRb neurons, whereas fluorescent detection of WGA (detected via anti-GFP antibody, 1:1000) allowed the visualization of both primary and second order (synaptically coupled) neurons from LHA galanin and LepRb neurons. Further, we determined that LHA galanin neurons innervate the adrenergic neurons of the LC via neuronal co-expression of galanin-derived WGA and tyrosine hydroxylase (anti-TH, 1:1000) within the LC.

Leptin-induced STAT3 phosphorylation: Detection of leptin receptors was visualized via phosphorylation of the downstream leptin signaling transcription factor STAT3. To assess if Gal-LepRb neurons, specifically, innervate the LC, GalYFP mice were injected with the retrograde tracer FG targeted to the LC as described above. 3-5 days following, mice were injected with 5-mg/kg dose of leptin (i.p.). Precisely 1 hour after leptin treatment, animals were perfused for brain fixation. GalYFP mice were confirmed for accurate LC-targeted FG injections (n=2) via fluorescent FG (anti-FG, 1:1000) expression in the LC. In the LHA, immunohistochemical detection of triple labeled neurons positive for leptin-induced pSTAT3 (anti-pSTAT3, 1:1000; detected via DAB), FG (anti-FG, 1:1000; fluorescently labeled) and GFP (anti-GFP, 1:1000; fluorescently labeled) confirmed this projection.

To assess differences in baseline activation of orexin neurons in Gal-LepRbKO and WT mice, brain sections were co-stained for orexin-A (anti-orexin, 1:1000; fluorescently detected) and nuclear cFos (anti-cFos, 1:3000; DAB detection). Neurons co-expressing cFos and orexin are indicative of an activated orexin neuron. Sections were mounted onto gelatin-subbed slides and cover
slipped with Pro-Long Anti-fade mounting medium (Invitrogen; Carlsbad, CA) for further microscopy analysis described below.

4.2.4 Estimate of cell counts. Microscopy procedures have been previously described in Chapter 2. For quantification of baseline activity of orexin neurons, brain sections of WT and Gal-LepRb\textsuperscript{KO} mice (n=5) were analyzed for cFos/orexin co-expression. Four sections containing the entire population of LHA orexin neurons were identified for each animal and organized anatomically in a rostral to caudal manner. The total number of orexin neurons and the number of cFos expressing orexin neurons was manually counted as described in Chapter 2. Differences between groups were evaluated with a 2-way ANOVA and posthoc-test.

4.2.5 Microdissection and qPCR. Microdissection procedures have been described in Chapter 3. For Gal-LepRb\textsuperscript{KO} and WT mice, the mt and fornix were used for visual landmarks to strategically dissect the LHA (Figure 3.1, B-C). LHA tissue pieces were combined and collected in 1.5mL centrifuge tubes, flash frozen in dry ice, and stored in -80°C until further processed.

RNA extraction, RT-PCR and qPCR procedures are detailed in Chapter 2. cDNA (10.5ng/reaction) was analyzed in triplicate via quantitative PCR for GAPDH (housekeeping gene; Mm99999915\_g1), orexin (Mm04210469\_m1), OX1(Mm01185776\_m1), OX2 (Mm01179312\_m1); TaqMan Universal PCR Master Mix; Applied Biosystems-4304437, Carlsbad, CA) using an Applied Biosystems 7500 Real-Time PCR System. Fold induction of gene expression was calculated using the $\Delta\Delta$CT method as described in Chapter 2.
4.3 RESULTS

4.3.1 Gal-LepRb neurons innervate orexin neurons and the LC. To understand how Gal-LepRb neurons may regulate the observed effects on natural reward behavior, we aimed to identify the anatomical sites and neurons that were innervated by both galanin and LepRb neurons from the LHA.

We first injected the adenoviral construct Ad-iZ/EGFPf into the LHA of LepRb$^{\text{Cre}}$ or Gal$^{\text{Cre}}$ mice, which resulted in a cre-specific expression of a farnesylated enhanced green fluorescent protein (EGFPf) and a non-cre dependent β-galactosidase expression from all adenovirus infected cells (Figure 4.1, A-G). The farnesylation anchors EGFPf to the cell membrane, which enhances the visualization of thin axonal processes, the β-galactosidase expression, is used to track the spread of overall virally infected cells. In a second step, we injected another adenoviral construct, Ad-iN/WED, that resulted in the cre-dependent expression of wheat germ agglutinin (WGA) and the fluorescent protein DsRed. WGA can be transported anterogradely and transsynaptically into 2nd order neurons, while DsRed is restricted to 1st order neurons (here LepRb or galanin neurons; Figure 4.1, G-L).

Correctly targeted Ad-iZ/EGFPf injection into the LHA of LepRb$^{\text{Cre}}$ (Figure 4.1, A) or Gal$^{\text{Cre}}$ (Figure 4.1, D) mice was immunohistochemically visualized by staining for EGFPf (green) and β-galactosidase (red). EGFPf positive cell bodies represent LepRb or galanin neurons and many fibers are seen from LepRb and galanin neurons projecting locally within the LHA (Figure 4.1, A and D, respectively). Similarly, Ad-iN/WED injection into the LHA of LepRb$^{\text{Cre}}$ or Gal$^{\text{Cre}}$
mice, respectively, results in WGA labeling within the LHA, where 1st order neurons are identified by WGA and DsRed expression (= yellow neurons) and many 2nd order neurons that are only positive for WGA (= green neurons) (Figure 4.1, G and J). Thus, Gal-LepRb neurons intensely innervate local LHA neurons.

We further confirmed that LHA LepRb neurons strongly innervate the VTA (Figure 4.1, B and H) as had been reported earlier (Leinninger et al. 2011, Louis et al. 2010). In contrast, we rarely found projections or second order labeled neurons to the VTA from LHA galanin neurons (Figure 4.1, E and K), suggesting that Gal-LepRb neurons are unlikely to substantially innervate the VTA.

Instead we found that both – LHA LepRb and galanin neurons - strongly innervated the LC (Figure 4.1, C and F), a site that had not yet been associated with leptin function. With Ad-iN/WED we found many 2nd order neurons labeled from LHA galanin neurons (Figure 4.1, L), and we also found an occasional 2nd order labeling from LHA LepRb neurons (Figure 4.1, I), however, 2nd order tracing from LHA LepRb neurons was not observed in all cases of injected animals. Thus, we further injected the retrograde tracer fluorogold (FG) into the LC of GalYFP mice, and analyzed the brains for the existence of leptin-induced pSTAT3 neurons co-localized with GalGFP and retrogradely labeled with FG from the LC (=FG/pSTAT3/GalGFP positive; Figure 4.2). Galanin is well known to be co-expressed in the majority of LC neurons (Moore and Gustafson 1989) so that the GalGFP signal served as a great visual outline for the LC and to verify the accuracy of FG injections (Figure 4.2, A).
Figure 4.1 Gal-LepRb neurons project to the LC, but not the VTA. A. Injection of Ad-iZ/EGFPf into the LHA of LepRb<sup>Cre</sup> mice shows the spread of infected neurons in red (β-galactosidase expression). Cre-recombination removes β-galactosidase expression and induces EGFPf expression (green, representing LepRb neurons). LHA LepRb neurons project into the VTA (B.) and LC (C.). D. Injection of Ad-iZ/EGFPf into the LHA of Gal<sup>Cre</sup> mice, again with spread of infected neurons in red (β-galactosidase expression) and neurons with cre-expression in green (EGFPf). LHA Gal neurons do not project into the VTA (E.), but strongly innervate the LC (F.). G. Injection of Ad-iN/WED into the LHA of LepRb<sup>Cre</sup> mice (n=7) shows infected first order neurons by co-expression of cre-inducible DsRed (red) and wheat germ agglutinin (WGA, green)(yellow labeling). The anterograde and transsynaptic tracer WGA is further transported into second order neurons indicated by single WGA labeling (green) within the LHA (G.), VTA (H.) and LC (I., only observed in one animal). J. Injection of Ad-iN/WED into the LHA of Gal<sup>Cre</sup> mice (n=6) shows local first order neurons (yellow) and second order neurons (green) are found in the LHA (J.) and LC (L.), but not the VTA (K.).
Indeed, we found many triple labeled FG/pSTAT3/Gal$^{GFP}$ neurons surrounding the fornix (Figure 4.2, B) supporting the projection of Gal-LepRb neurons to the LC. Neurons in the LC are the major source of central norepinephrine and express tyrosine hydroxylase (TH). Thus, we further confirmed that galanin-derived WGA labeled LC neurons are indeed co-localized with TH (Figure 4.3, C).

LepRb neurons in the LHA innervate local orexin neurons (Louis et al. 2010), thus, we further tested if the WGA labeled 2$^{nd}$ order neurons from LHA LepRb or galanin neurons might in fact be orexin neurons. Indeed, we found that many WGA labeled from LHA LepRb and galanin neurons were co-stained with orexin (Figure 4.3, A and B, respectively). Importantly, we showed earlier that neither LepRb nor galanin neurons are co-localized with orexin (data shown in Chapter 2) demonstrating that Gal-LepRb neurons innervate orexin neurons.

Figure 4.2 Gal-LepRb neurons innervate the LC. Gal$^{YFP}$ mice were injected the retrograde tracer fluorogold (FG, red) into the LC (A), and within the LHA brains were analyzed for the existence of leptin-induced pSTAT3 (red) neurons co-localized with Gal$^{GFP}$ (blue) and retrogradely labeled with FG (green) from the LC (B).
4.3.2 Galanin mediated inhibition of orexin neurons. LHA galanin and GaR1 expression was decreased in Gal-LepRb\textsuperscript{KO} mice (Chapter 3) and galanin is known for its potent inhibitory action in many neuronal systems, including the hypothalamus (Dong, Tyszkiewicz, and Fong 2006). Thus, if galanin would be significantly involved to mediate inhibitory leptin action onto orexin neurons, we predicted that Gal-LepRb\textsuperscript{KO} mice would show increased activation of orexin neurons. To test this we analyzed the brains of Gal-LepRb\textsuperscript{KO} mice and WT littermates for their basal level of cFos (as a surrogate for neuronal activation) in orexin neurons.

Figure 4.3 Gal-LepRb neurons innervate local orexin neurons and noradrenergic LC neurons. A. In LepRb\textsuperscript{Cre} mice injected with Ad-iN/WED into the LHA many WGA neurons are co-expressed locally with orexin. B. In Gal\textsuperscript{Cre} mice injected with Ad-iN/WED into the LHA many WGA neurons are co-expressed locally with orexin and within the LC WGA neurons co-expressed TH, depicting noradrenergic neurons (C.).
Indeed, we found that specifically the rostral portion of orexin neurons from Gal-LepRb$^{KO}$ mice had a higher percentage of cFos positive orexin neurons compared to WT littermates (Figure 4.4, A-E; n=5, $p^{\text{ANOVA}}<0.003$, *$p^{\text{Holm-Sidak}}<0.05$). This was due to an increased number of cFos/orexin neurons (Figure 4.4, D), while the total number of orexin neurons was unchanged or decreased (Figure 4.4, E). This was further confirmed with qPCR, where LHA orexin gene expression remained unchanged between WT and Gal-LepRb$^{KO}$ mice (Figure 4.4, G). Interestingly, within the LHA the gene expression of orexin receptor 2 (OX2), but not orexin receptor 1 (OX1), was significantly decreased (Figure 4.4, G; *$p^{t-test}<0.02$), suggesting that increased activation of orexin neurons may preferentially act via OX1 in the LHA. Furthermore, we establish that orexin neurons are co-labeled with GalR1 (Figure 4.4, F), further supporting that circulating (baseline) leptin levels provide an inhibitory tone onto orexin neurons that is mediated by galanin$\rightarrow$GalR1 signaling (Figure 4.4, H).

4.4 DISCUSSION

Our data outline a novel neuronal circuit where Gal-LepRb neurons directly innervate orexin neurons as well as noradrenergic neurons in the LC, but were not found to substantially innervate the VTA directly. While leptin stimulates inhibitory acting galanin expression in the LHA the lack of leptin action in galanin neurons robustly decreased galanin expression, and resulted in enhanced activation of orexin neurons.
Figure 4.4 Enhanced orexin activation in Gal-LepRb\(^{KO}\) mice. IHC stain of nuclear cFos (red nuclear stain, as a surrogate for neuronal stimulation) and orexin-A (green stain) in WT (A) and Gal-LepRb\(^{KO}\) mice (B). C. Percentage of total orexin neurons co-labeled for orexin and cFos in WT and Gal-LepRb\(^{KO}\) mice; extending from the most rostral portion of the orexin population (Section 1) to the most caudal portion (Section 4). \(p^{\text{ANOVA}}<0.05; {^*p^{\text{t-test}}<0.05, n=5}\). Total number of cFos/orexin positive neurons (D) and total orexin cells (E) were not significantly different between Gal-LepRb\(^{KO}\) and WT mice. F. IHC stain of orexin-A (red) and GalR1 (green) showed orexin neurons expressed the GalR1. G. Gene expression analysis of microdissected LHA tissue showed no difference in orexin or orexin receptor 1 (OX1) mRNA, however orexin receptor 2 (OX2) was significantly decreased (\(p^{\text{t-test}}<0.05, n=10-13\)). H. Hypothesized circuit establishing leptin-mediated inhibition of orexin neurons via galanin\(\rightarrow\)GalR1 signaling.
In line with this observation, orexin is known to stimulate sucrose intake (Baird et al. 2009), while orexin receptor deficient mice have reduced sucrose intake (Matsuo et al. 2011). Pharmacological blockade of orexin receptors partially prevents fasting-induced increases of palatable food intake (Rodgers et al. 2001). Thus, the observed increase in sucrose consumption in Gal-LepRb\textsuperscript{KO} mice (Chapter 3) and increased activation of orexin neurons suggests that leptin action on LHA galanin neurons mediates fasting induced sucrose consumption.

Importantly, Gal-LepRb neurons strongly innervate noradrenergic neurons in the LC. Similarly, the LC is the major projection sites of orexin neurons (Peyron et al. 1998) and control both arousal and reward (Sakurai and Mieda 2011). The LC is the sole NE source for many central sites (e.g. prefrontal cortex, PFC), while other sites (e.g. hypothalamus) receive additional NE input from the brainstem (NTS). Leptin treatment decreases and leptin deficiency increases hypothalamic NE levels, while activation of the α-2-adrenoceptor substantially increases hyperphagia in leptin deficient obese mice (Kutlu et al. 2010, Currie and Wilson 1991, 1992, 1993). Altogether, innervation of such prominent nuclei that regulate reward-driven behaviors provides an intriguing circuit for future investigation of LHA-leptin action.

Importantly, our data shows that Gal-LepRb neurons utilize a novel LHA LepRb circuit where leptin acts to inhibit orexin neurons via leptin-evoked galanin→GalR1 signaling in orexin neurons.
CHAPTER 5. CONCLUSION

5.1 GAL-LEPRB NEURONS ARE A NOVEL LEPRB POPULATION

Through the investigation of the LHA-LepRb subpopulation, as characterized by LepRb neurons co-expressing the inhibitory neuropeptide galanin (Gal-LepRb neurons), our data establishes that Gal-LepRb neurons mediate anorexigenic leptin action via modulation of reward circuits that govern nutrient intake. Gal-LepRb neurons are stimulated by leptin and leptin-induced stimulation of galanin neurons elicits inhibitory effects onto synapsing neurons either via the inhibitory neurotransmitter GABA or galanin→GalR1 mediated signaling. We found that Gal-LepRb neurons are a distinct population from two well-established orexigenic-influencing LHA populations: the melanin concentrating hormone (MCH) and orexin neuronal populations. However, we did identify additional subpopulations within the Gal-LepRb population. We show that 30% of Gal-LepRb neurons were found to additionally express the anorexigenic peptide cocaine-amphetamine regulated transcript (CART), as well as, a considerable portion of galanin neurons that co-expressed neurotensin (Nts).

Recently, Leinninger et al., reported on findings that characterized a LHA LepRb subpopulation co-expressing Nts (Nts-LepRb neurons). Like Gal-LepRb neurons, Nts-LepRb neurons innervate local orexin neurons, however, Nts-LepRb neurons were shown to directly project to the VTA (Leinninger et al. 2011). Importantly, Gal-LepRb neurons are distinguished from Nts-LepRb neurons, as Gal-LepRb neurons were not observed to project to the VTA. Furthermore, we show that Gal-LepRb neurons innervate the noradrenergic neurons of the LC, a finding
that was also not reported for Nts-LepRb neurons. The LC also receives dense projections from orexin neurons (Peyron et al. 1998) and is largely recognized for influencing arousal, addiction and withdrawal pathways (Rasmussen et al. 1990, Nestler 1992, Kutlu et al. 2010).

Importantly, the LC was a nucleus that had yet to be identified as a potential target of leptin action, and provides an intriguing circuit for investigating the role of Gal-LepRb neurons in aspects of arousal, addiction and nutrient selection. For example, we know that the LC is the sole norepinephrine (NE) source for many central sites. Leptin deficient ob/ob mice exhibit increased hypothalamic NE levels while leptin treatment decreases hypothalamic NE levels. Moreover, ob/ob mice given a low dose of the α-2-adrenoceptor agonist clonidine were observed to selectively increase carbohydrate intake {while protein and fat intake remained unaltered (Currie and Wilson 1991)}. This would implicated a potential role for leptin in regulating NE-mediated nutrient selection, possibly via leptin→Gal-LepRb-mediated inhibition of the LC. Our data indicates that Gal-LepRb neurons represent a novel LHA LepRb population capable of mediating homeostatic mechanisms of energy balance and reward function as proposed in the illustrated Gal-LepRb circuit schematic shown in Figure 5.1.

5.2 GAL-LEPRB NEURONS MEDIATE APPETITIVE BEHAVIORS VIA REWARD CIRCUITS

To establish the physiological importance of Gal-LepRb neurons we developed a mouse model with a targeted deletion of LepRb specific to galanin neurons; termed Gal-LepRbKO mice. We show that Gal-LepRbKO mice exhibit
reward system as largely observed by their differential preferences for palatable sucrose and lipid solutions and their enhanced food-motivated behaviors on the incentive runway. There are two predominant systems governing behaviors associated with food rewards: the mesolimbic dopamine system and the opioid system. To gain further insight as to how these reward pathways were altered, we compared expression profiles of markers specific to each system in Gal-LepRb\textsuperscript{KO} and WT mice. Indeed, we found that Gal-LepRb\textsuperscript{KO} mice have reduced DA content within the NAc (Figure 5.2) as well as decreased µ-opioid receptor gene expression within the NAc and LHA (Figure 5.3).

Figure 5.1 Schematic of proposed Gal-LepRb circuit.
Figure 5.2 Gal-LepRb$^{KO}$ mice have decreased dopamine content in the NAc. HPLC detection of total dopamine content from microdissected NAc tissue (methods described in Chapter 3) in Gal-LepRb$^{KO}$ and WT mice (n=7-8; p<0.05).

Figure 5.3 µ-opioid receptor gene expression is decreased in the LH and NAc of Gal-LepRb$^{KO}$ mice. µ-opioid receptor mRNA fold change from microdissected tissue from the LH and NAc (n=10-13; p<0.05) in Gal-LepRb$^{KO}$ and WT mice. (TaqMan probes: GAPDH, housekeeping gene= Mm99999915_g1; µ-opioid receptor Mm01188089_m1). Fold induction of gene expression was calculated using the $^{\Delta \Delta}CT$ method (refer to Chapter 3 for complete microdissection techniques and Chapter 2 for qPCR methodology).
It is now widely acknowledged the properties that determine the reward value of nutrients are mediated by two distinguishable mechanisms: reward “liking” and reward “wanting” (Berridge, Robinson, and Aldridge 2009). In this theory “liking” refers to the pleasantness or hedonic value of food and is primarily associated with opioid neurotransmission. On the other hand, “wanting” refers to the incentive value or motivational properties of a given stimulus, which is primarily a function of the mesolimbic dopaminergic system. Central pathways that regulate “liking” and “wanting” behaviors are highly relevant for studying the underlying mechanisms linked to diet-induced obesity. For instance, in rodent models of diet-induced obesity higher concentrations of sucrose and lipid solutions are required to achieve similar “liking” responses as those observed in lean individuals (Shin et al. 2011). Therefore in obesity, a much higher threshold must be reached to attain the expected pleasure response from palatable foods. In contrast, obesity-induced changes in both palatable food “liking” and “wanting” responses are reversed by leptin (Shin et al. 2011, Davis, Choi, and Benoit 2010, Figlewicz 2003, Figlewicz et al. 2004, Figlewicz and Benoit 2009).

**5.2.1 Food-motivated “wanting” and the reward deficiency hypothesis.**

Activation of the mesolimbic dopaminergic system, and largely dopamine release in the NAc (mesoaccumbal DA pathway) plays a critical role in the reinforcing and motivational aspects attributed to reward “wanting” (Berridge and Robinson 1998, Ostlund et al. 2011, Pecina et al. 2003). Palatable foods high in sugar are potent stimulators of dopamine release (Hajnal, Smith, and Norgren 2004, Rada, Avena, and Hoebel 2005). We report that Gal-LepRb\(^{KO}\) mice exhibit a marked increase in
their sucrose intake over lipids and demonstrate greater “wanting” for a sugary treat on the incentive runway. However, we found that NAc DA content was significantly reduced in Gal-LepRb\textsuperscript{KO} mice compared to WT mice. This finding seemed somewhat contradictory to our behavioral findings as we predicted DA content, if anything, would be increased in Gal-LepRb\textsuperscript{KO} mice. In light of this, we found that decreases in mesoaccumbal DA signaling, do not always translate into reduced reward-motivated behaviors. In fact, deficiencies in dopamine signaling are highly correlated with addictive-like behaviors and are observed by the strong “wanting” for a reward reinforcer (Blum et al. 2000, Comings and Blum 2000). This phenomenon is interpreted by the reward deficiency hypothesis, which explains that reward deficient individuals (a genetically or environmentally-induced down regulation of DA signaling) typically experience a heightened sense of unpleasantness even in presence of a rewarding stimulus. In order to alleviate the perceived negative state, they generally demonstrate greater reward-seeking and reward-taking behaviors as an attempt to restore mesoaccumbal DA signaling. Therefore, it is possible that the reduced NAc dopamine content observed in Gal-LepRb\textsuperscript{KO} mice is perceived as a reward deficit and thus translates into their enhanced food-motivated behaviors. As mentioned, Gal-LepRb neurons project to and inhibit orexin neurons. In parallel, fasting-induced decreases in circulating leptin would likely diminish leptin’s inhibitory influence. With the removal of this inhibitory block, orexin neurons increase their activity and thereby stimulate mesoaccumbal DA release. In the case that orexin neurons are kept in a chronic state of activation, as evident in Gal-LepRb\textsuperscript{KO} mice, a deficient reward response is likely to manifest
due to an exhausted mesoaccumbal DA system. However, more precise methodology to quantify aspects of dopamine signaling (such as release, re-uptake, synthesis, etc.) in Gal-LepRb\textsuperscript{KO} mice is necessary to fully support this hypothesis.

5.2.2 Macronutrient selection and “liking”. The pleasantness or the hedonic value attributed to food “liking” is governed by opioid neurotransmission. Particularly signaling via the \(\mu\)-opioid receptor within the NAc has been shown to increase food intake with a predominant effect on increasing fat consumption (Zhang, Gosnell, and Kelley 1998). Hypothalamic galanin administration also preferentially increases fat intake (Tempel, Leibowitz, and Leibowitz 1988) and galanin is known to influence \(\mu\)-opioid receptors via GalR1 signaling in reward pathways (Holmes et al. 2012).

Here we report that Gal-LepRb\textsuperscript{KO} mice show decreased fat consumption as assessed in a two-bottle choice test (when paired with sucrose or given alone). In parallel with the increased fat consumption observed in animals after administration of either galanin or \(\mu\)-opioid receptor agonists, we show that LHA galanin, GalR1 and \(\mu\)-opioid receptor gene expression is decreased in Gal-LepRb\textsuperscript{KO} mice. Hence, the down regulation of \(\mu\)-opioid receptors and galanin signaling (specifically galanin signaling via GalR1) likely contributes to the decreased “liking” of fats observed in Gal-LepRb\textsuperscript{KO} mice. In contrast, Gal-LepRb\textsuperscript{KO} mice demonstrated a robust preference for sucrose in the two-bottle choice test. The differential preference exhibited by Gal-LepRb\textsuperscript{KO} mice specifies that lipids are less “liked” and highlights a potential mechanism by which galanin and \(\mu\)-opioid receptor signaling selectively defines the pleasantness perceived when fats are consumed. Thereby suggesting
the existence of diverging circuits, which would selectively govern the hedonic properties of sucrose or fats, respectively. Hence WT mice, with normal galanin and μ-opioid receptor function, will demonstrate equal “liking” of sucrose and fats (as observed in two-bottle choice test) because the competition between the diverging circuits governing fat and sucrose intake is matched accordingly. The existence of dissociable pathways influencing sucrose intake has also been suggested, where the mesoaccumbal DA system drives sucrose “wanting,” in a manner that is independent from the hedonic “liking” component of sucrose (Pecina et al. 2003). As observed with drugs of abuse that induce abnormally high DA release, drug addicts report higher ratings of drug “wanting,” but no longer experience pleasure or “like” the drug (Evans et al. 2006). Similarly, addictive-like behaviors (comparable to “wanting”) have been reported in models of sugar bingeing but not observed for fat-bingeing (Hoebel et al. 2009, Avena, Rada, and Hoebel 2009). This suggests that the behaviors mediating lipid consumption are dictated through a pathway independent from “wanting,” and potentially involve the galanin and opioid systems. In summary, neural circuits guiding food choice are clearly not the result of one prevalent pathway. Instead, mechanisms driving the intake of select macronutrients seems to be differentially regulated through distinct signaling pathways that function to encourage food selection based on the needs of the body.

5.2.3 Implications for orexin neurons in the Gal-LepRb pathway. Orexin has been implicated in the activation of central hedonic hotspots that increase “liking,” as well as enhancing “wanting” for food rewards. Numerous reports provide evidence that show the activation of orexin neurons (and when orexin is centrally
injected) evokes reward-driven, locomotor responses (food-seeking, “wanting”) associated with increased mesoaccumbal DA release (Choi et al. 2010, Thorpe et al. 2005, Sharf et al. 2010, Harris, Wimmer, and Aston-Jones 2005). However, through a distinguishable circuit, the pleasantness or “liking” of palatable foods is amplified via opioid signaling within distinct hedonic “hotspots”. Projections from orexin neurons to one such “hotspot,” the ventral pallidum, holds implications for the divergent actions of orexin to regulate different aspects of nutrient reward. A recent study showed that injections of the orexin peptide into the ventral pallidum induced a marked increase of positive “liking” reactions to sucrose solutions, but had no effect sucrose “wanting” (Ho and Berridge 2013). Thus, orexin signaling within this distinct hedonic nucleus influences opioid-mediated “liking” responses. Moreover, μ-opioid receptors are expressed on orexin neurons (Georgescu et al. 2003), and endogenous opioids are required for orexin-mediated feeding (Clegg et al. 2002). Indeed, the activation of NAc μ-opioid receptors amplifies “liking” reactions and increases food intake. μ-opioid receptor activation in the NAc also increases the activity of orexin neurons. However, when orexin receptors in the VTA are blocked, food intake is no longer increased by NAc μ-opioid receptor activation (Zheng, Patterson, and Berthoud 2007). Taken together, orexin neurons appear to serve as a hub for the integration of signals relaying nutritional status (i.e. fasting/ or low leptin) and information regarding the value of a food reward. This signal convergence is then translated into specific appetitive behaviors based orexin’s distinct anatomical projection targets to effect both reward “liking” (via μ-opioid receptors in hedonic nuclei) and reward “wanting” (via mesoaccumbal DA pathway).
In light of this, we know that leptin reduces palatable food “liking” and “wanting” behaviors, and these effects are most likely the outcome of leptin-mediated inhibition of orexin neurons. Consistent with previous findings (Leinninger et al. 2009, Louis et al. 2010), we confirmed that LepRbs are not expressed on orexin neurons, demonstrating that leptin inhibits orexin neurons indirectly. But the LepRb population that mediates this inhibitory leptin effect onto orexin neurons is unknown.

The here presented work shows convincing evidence that Gal-LepRb neurons in the LHA mediate an inhibitory effect onto orexin neurons. As we found that abolishing leptin signaling exclusively in galanin neurons increases the activation level of orexin neurons. Consistent with this, Gal-LepRbKO mice show enhanced motivation to obtain a sugary food reward on the incentive runway. Our data further establishes a functional circuit responsible for mediating these effects. We show that leptin stimulates GABAergic Gal-LepRb neurons, promoting synaptic release of the inhibitory neuropeptide galanin (and/or the inhibitory neurotransmitter, GABA). Gal-LepRb neurons innervate local orexin neurons. Additionally, we determined that orexin neurons express the Gi-coupled GPCR galanin 1-receptor (GalR1), validating the cellular ability of orexin neurons to respond to inhibitory galanin action. Altogether, these data demonstrate that leptin-mediated galanin release from LHA Gal-LepRb neurons mediates the inhibitory tone onto orexin neurons. Our findings reveal that Gal-LepRb neurons are a novel LHA LepRb population that regulates the rewarding value of nutrients via control of orexin neurons.
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VITA

Amanda Laque was born on March 26\textsuperscript{th} in Metairie, Louisiana to Patricia and Albert Laque Jr. Amanda grew up in Luling, Louisiana and attended Mimosa Park and Lakewood Elementary. She graduated with honors from Hahnville High School in 2004. Amanda began her undergraduate career in the fall of 2004 at Louisiana State University, where she majored in Biological Sciences. In the fall of 2007, Amanda joined the laboratory of Dr. Thomas Gettys at Pennington Biomedical Research Center as an undergraduate student worker where she worked part time through the remainder of her undergraduate studies. In the fall of 2008, Amanda graduated with her Bachelor of Science from Louisiana State University. She began a career in research as a Research Associate in the laboratory of Dr. Heike Münzberg at the Pennington Biomedical Research Center in the spring of 2009. In the spring of 2011, Amanda entered the graduate program at Louisiana State University in the Department of Biological Sciences. Amanda anticipates graduating with her doctor of philosophy degree in Biological Sciences on May 16, 2014 and plans to pursue a scientific career in academia.