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Restriction of Food Intake by Dorsomedial Hypothalamus

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RESTRICTION OF FOOD INTAKE BY DORSOMEDIAL HYPOTHALAMUS

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Leptin deficient ob/ob mice eat voraciously and their food intake is markedly reduced by leptin treatment. Leptin acts in part by regulating the activity of AGRP neurons and POMC neurons in the arcuate nucleus and neurons in other brain regions. In these dissertation, I will describe how we identify novel neuronal populations that are regulated by leptin directly or indirectly. In order to identify novel sites of leptin action, we used phosphotrap, to molecularly profile leptin responsive neurons in the hypothalamus and brain stem. In addition to identifying several known leptin responsive populations, we found that neurons in Dorsomedial Hypothalamus (DMH) expressing GSBS are activated in ob/ob mice and suppressed by leptin treatment. Because ob mice are hyperphagic, we hypothesized that GSBS neurons would activate food intake. However excitation of GSBS neurons decreased food intake and body weight in ob/ob mice while chemogenetic inhibition of GSBS neurons increased food intake and body weight. The DMH regulates Food Anticipatory Activity (FAA) and in a scheduled feeding protocol that elicits increased consumption, mice also ate more when GSBS neurons were inhibited and less when they were activated without altering food anticipatory activity, body temperature and oxygen consumption. GSBS neurons do not express the leptin receptor suggesting that GSBS neurons in the DMH play a key role to restrict excessive food intake when consumption is increased and that leptin suppresses their activity indirectly by reducing food intake. These findings reveal that neural pathways activated by acute increases of food intake can restrain food intake independent of metabolic state. This finding has potential implications for an understanding of binge eating and other nutritional disorders.

Dedicated to my family for their unconditional love and unwavering support…

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Chapter 1: Introduction

Obesity and Overweight as Public Health Problems:

By reason of the scientifically-proven adverse health effects, the increasing prevalence of obesity is one of the most significant and complex health problems. Although obesity is a major concern for several countries, there is no country successfully tackling this issue (Global et al., 2014; Stevens et al., 2012). The number of people with a BMI over 25 is two times more than people with a healthy weight (Ogden et al., 2014). Although there are not many data points from countries outside the US and Europe, the prevalence of obesity increases in underdeveloped and developing countries because of the changes in the socio-economic environment (Global et al., 2014; Steven et al., 2012).

As obesity develops, the risk of developing some other diseases also increases. Association studies that are focusing on the link between obesity and other diseases are crucial to understand the devastating effects of obesity on health. Several association studies report that there is an association between obesity and other diseases, including diabetes, cardiovascular disease, colon, pancreatic, renal and gallbladder cancer in humans (Abdullah et al., 2010, Vainio et al., 2002; Larsson et al., 2007). Furthermore, evidence from different studies has suggested that there is a strong link between obesity and brain health. Some brain regions, including the hippocampus and frontal lobes, are smaller in children with obesity than their healthy counterparts. Overweight is also a predisposing factor of Alzheimer's disease (Anstey et al., 2011). However, exercise can blunt the effects of obesity on mental health in obese people by improving cognitive functioning (Lindholm et al., 2013).

Risk Factors in Obesity:

Energy expenditure and energy intake are two fundamental parameters in determining the change in body weight. Obesity starts to develop when energy intake is higher than energy expenditure. Therefore, explaining and understanding the control mechanisms of energy intake and energy expenditure is critical to solve the obesity epidemic. Factors controlling energy expenditure and energy intake can be grouped under two main headings, environmental and genetic factors (Hill et al., 2012).

The changes in the environmental factor in the last century worsen the obesity rate in the world. One of the most critical environmental factors that impact body weight is diet and its nutritional composition. Based on the researches trying to reveal

the role of diet in the development of obesity, the calorie restriction diet regimen is proposed as an effective way to maintain body weight and lose weight by decreasing energy intake (Hill et al., 2012). Also, the role of macronutrient composition of restriction diet in body weight regulation has been investigated by many studies. The results indicate that calories from different macronutrients exert different impacts on obesity and its comorbidities (Mozaffarian et al., 2011). In addition to daily amount of food consumption, an increase in daily activities is also associated with decreased body weight (Washington et al., 2008, Donnelly et al., 2009). In addition to daily activity, income is another environmental factor that impacts body weight. The prevalence of obesity is lower among wealthier individuals than individuals who live under poverty conditions (Levine et al., 2011). In addition to several other environmental factors that are increasing the risk of developing obesity, genetic factors are also playing a significant role in the development of obesity.

Genetics of Obesity:

In addition to the role of environmental factors in the development of obesity, genes are also involved in the regulation of body weight. Starting from the last century, researchers attempted to reveal genetic factors causing obesity (Pennington et al., 1953). Although it was not clear how genes regulate body weight in the early 20th century, Von Noorden is one of the first scientists who suggested some forms of obesity might have a genetic cause. To understand the impact of genes on obesity and to investigate whether obesity inheritable or not, several studies focusing on how similar the body weights of adopted twins to their biological parents compared to their adopted parents' body weight performed. (Stunkard et al., 1986). These studies revealed that the body weights of the adopted children are more similar to their biological parents' body weight compared to their adopted parents` body weight (Stunkard et al., 1986). These findings showed that obesity is not just a willpower issue that leads to overeating and under-exercising; instead, there is also a genetic contribution to the onset and development of obesity.

Genotyping tools and next-generation sequencing technologies have significantly revolutionized the field of genetics and became crucial to reveal the genetic cause of obesity. With the help of new techniques in genetics, it became plausible to identify single nucleotide changes in the genome that provides a better understanding of the genetic cause of obesity. While a mutation in a single gene can result in obesity (Monogenic obesity), it is more common than the cumulative effect of nucleotide changes in

different genes can increase the risk of developing obesity (polygenic obesity). Furthermore, in some diseases, there can be a manifestation of other phenotypes in the patients in addition to obesity. These diseases are classified as syndromic obesity.

Monogenic obesity:

In general, monogenic obesity can be a result of a mutation in a single copy of a gene or mutations in both copies of the gene. Mutation in several genes causes obesity. Leptin is one of the most well-known regulators of body weight, and several mutations in the leptin gene implicated in the development of obesity (Zhang et al., 1994, Zhang et al., 1997). Leptin is mainly expressed in adipose tissue by the LEP gene and secreted into the bloodstream that it can acts on the brain via its receptor, which is LepRb. While the length of the immature form of leptin is 167 amino acid, 21 amino acid (aa) peptide is cleaved to form 146 aa mature leptin (Zhang et al., 1994). Although mutations in leptin that can cause obesity are not prevalent, leptin is an effective treatment for obese patients with leptin mutation. Several studies have been performed to investigate the therapeutic use of leptin to treat obesity and metreleptin is approved by the FDA that can be used to treat congenital leptin deficiency and lipodystrophies (Mahmoodpoor et al., 2015). Furthermore, damaging mutations in the LEPR gene lead to obesity (Farooqi et al., 2007). The manifested phenotypes of obese patients with mutations in both copies of Lepr gene are similar to the leptin-deficient obese patients (Farooqi et al., 2007). Although leptin and leptin receptor regulates body weight and appetite via the same pathway, blood leptin level is much higher in Lepr deficient obese patients, resulting in leptin resistance in these patients (Farooqi et al., 2007). Therefore, metreleptin cannot be used to treat the complications of Lepr deficiency.

Proopiomelanocortin (POMC) is another gene that is involved in food-related behaviors. It has been shown that mutations in the POMC gene also result in obesity. Since cleavage products of POMC including Adrenocorticotropic hormone (ACTH), α-Melanocyte-stimulating hormone (α-MSH), and β-endorphins are essential to regulate critical signaling pathways in neurons, POMC deficiency can cause the manifestation of other phenotypes, as well (Lee et al., 2006; Challis et al., 2002).

Mutations in Melanocortin receptor 4 (MC4R) also lead to obesity. MC4R is a G protein-coupled receptor expressed in the hypothalamus (Mountjoy et al., 1997). In contrast to leptin and leptin receptor, dominant mutations in MC4R can lead to obesity and show an autosomal dominant mode of inheritance. Although several studies

are going on to develop a drug that targets MC4R, there is not any promising result so far (Fani et al., 2014; Chen et al., 2015).

Single-minded homolog of drosophila (SIM1) is a transcription factor that is highly expressed in the paraventricular hypothalamus. The paraventricular hypothalamus is a critical region for food-related behaviors (Michaud et al., 1998). Mutations in one or two copies of the SIM1 gene increase food intake (Holder et al., 2000). Furthermore, overexpression of Sim1 in mice prevents overeating observed in agouti yellow mice (Kublaoui et al., 2006).

Neurotrophins that are expressed in the brain and function as growth factors to regulate the central nervous system development and maintenance are also involved in food-related behaviors. Brain-derived neurotrophic factor (BDNF) is one of the critical neurotrophins expressed in the hypothalamus. People with a mutation in the BDNF gene may show early-onset obesity (Han et al., 2008). Furthermore, a mutation in the receptor of BDNF, Tropomyosin receptor kinase B (TRKB), has been associated with obesity (Yeo et al., 2004).

Syndromic Obesity:

Syndromic obesity is used as a term to define obesity with other phenotypes. The underlying genetic cause of syndromic obesity can be mutations in a single gene or chromosomal translocations that impact multiple genes. There are more than 100 diseases that obesity can be observed as a clinical manifestation. Some abnormal phenotypes, including intellectual disabilities and dysmorphic faces, have been observed in individuals with syndromic obesity (Kaur et al., 2017).

Bardet-Biedel Syndrome (BBS) is a genetic disorder that is inherited in the autosomal recessive form. In addition to obesity, some other phenotypes have been observed in patients with BBS, including renal dysfunction, retinal dystrophy, hypogonadism, and difficulties in learning (Forsythe et al., 2013). The phenotypes associated with BBS generally manifest themselves in the first ten years after birth (Forsythe et al., 2013). The disruption in the genes that are crucial for the function of immotile cilia can cause BBS. There are more than 15 genes that impact cilia function and lead to BBS (Forsythe et al., 2015)

Prader Willi Syndrome (PWS) is another genetic disorder that obesity can be seen as one of the manifested phenotypes. Some behavioral abnormalities and cognitive impairment can be accompanied by obesity (Elena et al., 2012). The genetic cause of

PWS is the inactivation of a specific region located on 15q11-13. (Sahoo et al., 2008). Although there are several genes in this region, including MKRN3(making 3), MAGEL2 (MAGE-like 2), NDN (necdin), NPAP1 (nuclear pore associated protein 1), SNURF-SNRPN (SNRPN upstream reading frame – small nuclear ribosomal protein 1) which are expressed in the hypothalamus and might have a role in the development of PWS, exact gene causing PWS is not known (Butler et al., 2011).

Obesity can be seen as a consequence of other chromosomal defects, including deletions of 1p36, 17p11.2, 6q16, 11p13. However, patients with deletions in these regions do not always show obese phenotype suggesting incomplete penetrance (D'Angelo et al., 2012)

Neuronal Regulation of Obesity:

Although several brain regions are crucial for regulating body weight, the most studied region in the brain is the hypothalamus. Several different nuclei in the hypothalamus are implicated in body weight regulation. Within the hypothalamus, two neural populations in the arcuate nucleus expressing Agouti Related Neuropeptide (AgRP) and POMC play an essential role in regulating body weight (Myers and Olson et al.,, 2012). Although the blood-brain barrier (BBB) limits molecules' diffusion from the bloodstream to the brain, there is no blood-brain barrier in median eminence where AgRP and POMC neurons are located. Since there is no blood-brain barrier in this part of the brain that controls selectivity, AgRP and POMC neurons can sense the changes in the bloodstream's nutritional status. Thus, these neurons are one of the first neuronal populations in the brain that respond to nutritional signals and transmit this information to other neuronal populations in other regions in the hypothalamus and the brain (Rodríguez et al., 2010).

When POMC neurons sense the nutritional signals and they are activated, the expression of POMC mRNA increases and this leads to decreasing food intake (Gropp et al., 2005; Balthasar et al., 2005). Immature POMC protein is cleaved to regulate other neuronal populations. α-MSH is one of the cleavage products of POMC protein that can bind and activate melanocortin 3 and 4 receptors (MC3/4R) (Könner et al., 2009). Although several brain sites express MC3/4R, paraventricular hypothalamic nucleus (PVN) is one of the main sites in the hypothalamus that MC4R is highly expressed (Gantz et al., 1993a,b; Liu et al., 2003). The release of α-MSH from the axonal projection of POMC neurons to PVH regulates the activity of MC4R neurons in the PVH by activating them. Activation of MC4R neurons in PVN results in inhibiting food intake and increasing energy expenditure (Tao et al., 2010; Krashes et al., 2016). In addition

to PVN, POMC neurons send their axons to the dorsomedial hypothalamus (DMH), the lateral hypothalamus (LH), and the ventromedial hypothalamus (VMH) (Kleinridders et al., 2009; Waterson and Horvath, 2015).

In contrast to POMC neurons, AGRP neurons lead to increased food intake when it is activated (Betley et al., 2013). Furthermore, fasting results in increased activity of AGRP neurons. Two peptides are secreted by AGRP neurons, AGRP and Neuropeptide Y (NPY) (Stanley and Leibowitz, 1984; Clark et al., 1984). NPY binds to Neuropeptide Y Receptor Y1 (NPYY1) (Yokosuka et al., 1999) and Neuropeptide Y Receptor Y5 (NPYY5) and activates them to increase food intake (Parker et al., 2000; Cabrele et al., 2000; McCrea et al., 2000). AgRP binds and inhibits MC3R and MC4R that blunts the activatory effect of α-MSH on MC3R and MC4R. In addition to NPY and AgRP, inhibitory γ-aminobutyric acid (GABA) is secreted from AGRP neurons to inhibit POMC neurons (Cowley et al., 2001). Furthermore, AgRP neurons send axonal projections to the PVN and bed nucleus of the stria terminals (BNST) to regulate body weight and food intake. While activation of axonal projections of AGRP neurons into PVN leads to increased food intake (Atasoy et al., 2012), the projection of Agrp neurons into the BNST regulates the activation of brown adipose tissue (BAT) (Steculorum et al., 2016).

The brainstem is another critical node for the regulation of body weight. Several neural populations in the brainstem are crucial to regulate food-related behavior, such as tyrosine hydroxylase (TH), proglucagon, cocaine- and amphetamine-regulated transcript (CART), and GABA. Furthermore, some neural populations express receptors of specific hormones that regulate satiety, such as leptin, ghrelin, glucagon-like peptide 1 (GLP1), and cholecystokinin (CCK) (Gil et al., 2011). In addition to sending and receiving projections from several brain regions that are important for regulating body weight, the gastrointestinal (GI) tract sends vagal inputs to the brainstem to transmit information about the nutritional status. Furthermore, vagal signaling plays a vital role in regulating food intake, meal size, and meal duration (Laskiewicz et al., 2001; Schwartz et al., 1999). The nucleus of the solitary tract (NTS) in the brainstem is the central region that receives vagal inputs from GI that send axonal projections into other areas of the brainstem and hypothalamus. A subpopulation of neurons in NTS expressing MC4R is regulated by released a-MSH from local POMC neurons and axonal projections from POMC neurons in the hypothalamus (Kishi et al. 2003, Palkovits & Eskay 1987). Injection of agonist and antagonist of MC4R in NTS leads to decreased food intake and increased food intake, respectively (Williams et al. 2000, Skibicka & Grill 2009b). Orexin and MCH neurons in LH also send projections to NTS. When orexin is injected

into the hindbrain, there is an increase in food intake. Neurons in PVH also send projections into NTS, and disruption of axon projections from PVH leads to increased body weight and food intake (Sawchenko & Swanson 1982, Luiten et al. 1985, Kirchgessner & Sclafani 1988). Furthermore, similar to median eminence in the arcuate nucleus, area postrema (AP) in the brainstem does not have BBB. Thus, neurons in AP can sense nutritional signals and hormones.

Role of glial cells:

Glial cells are the non-neuronal cell type in the brain that are providing support for neurons to maintain homeostasis. There are three different types of glial cells, microglial cells, oligodendrocytes, and astrocytes. After developing transgenic mice such as (hGFAP)-Cre and (Mbp)-Cre that can be used to understand the role of glial cells in the brain, glial cells' impacts on different diseases are investigated (Jakel et al., 2017). These studies showed that glial cells are essential to neuronal activity. Furthermore, the role of glial cells in obesity is investigated since it has been shown that there is an increase in the activity of microglial cells in the brain after three days of the high-fat diet treatment. The mRNA profile of microglial cells of mice treated with a high-fat diet for three months showed an increase in the expression of pro-inflammatory genes (Baufeld et al., 2016). Although the ablation of microglia does not display any effect on body weight or food intake during the chow diet (Sheridan et al., 2013), gavage feeding of the mice with the saturated fat solution leads to a decrease in chow intake (Valdearcos et al., 2014).

The role of astrocytes:

Astrocytes are a subclass of glial cells in the brain. They have crucial roles, including the maintenance of synapsis and the blood-brain barrier (Sofroniew et al., 2010). Since they locate very close to blood vessels, they can control the entry of hormones and other metabolites into the brain. Astrocytes also have a role in regulating glucose intake in the brain. Therefore the deletion of the insulin receptor in astrocytes decreases the glucose intake across the BBB (García-Cáceres et al., 2016). Furthermore, since astrocytes can store glycogen, they can provide glucose for the neurons when there is a decrease in glucose levels in the brain (Leloup et al., 1994). This helps neurons maintain their activity during hypoglycemia and high synaptic activity (Suzuki et al., 2011). In addition to glycogen, astrocytes can store ketone bodies. The release of ketone bodies during a high-fat diet activates the neuron populations decreasing food intake (Le Foll et al., 2016).

In the hypothalamus, astrocytes are one of the vital elements of energy metabolism. Leptin activates astrocytes via its receptor and regulates the connection between astrocytes and nearby neurons (Fuente-Martín et al., 2012). Furthermore, while high doses of leptin lead to anorexia, deletion of leptin receptor from astrocytes diminishes the leptin effect (Jayaram et al., 2013). Moreover, activated astrocytes in the arcuate nucleus release adenosine decreasing food intake by suppressing nearby AGRP neurons (Yang et al., 2015).

Obesity leads to an increase in the activation and proliferation of astrocytes in the hypothalamus (Buckman et al., 2013). Although there is an association between astrocyte activation and obesity, it is unclear whether activation of astrocytes leads to DIO and inflammation observed in the hypothalamus. Although activating astrocytes during high-fat diet treatment results in reducing BBB integrity, increasing the reward value of food, and worsen DIO (Yi et al., 2012), suppressing the signals that lead to inflammation in astrocytes does not improve DIO and worsen DIO at the early phase of high-fat diet treatment. (Buckman et al., 2015). These findings suggest that it is likely that while astrocyte activation has a protective role against DIO at the beginning of HFD treatment, long term HFD treatment diminishes the function of astrocytes and worsen DIO (Sofroniew et al., 2010).

The role of tanycytes in obesity:

Tanycytes are located between the peripheral circulation and hypothalamic parenchyma. They can facilitate and control the intake of hormones and nutrients into the brain. Since some of them are located in the third ventricular, they can lead to changes in the activity of hypothalamic neurons that are regulating food-related behavior via transporting hormones and nutrients into the brain (Langlet et al., 2019).

Leptin receptor expression in tanycytes also facilitates leptin transportation into the hypothalamus (Balland et al., 2014). Furthermore, tanycytes mediate the traffic of hormones and nutrients during fasting that can facilitate the adaptation to starvation (Langlet et al., 2013). Tanycytes are also proposed as the source of stem cells in the hypothalamus, and an increase in tanycytes` neurogenesis has been shown in the median eminence during high fat diet (HFD) treatment. When the proliferation of tanycytes near to median eminence is diminished via targeted radiation, weight gain slows down during HFD treatment (Lee et al., 2012). However, to understand whether tanycytes can cause DIO, further research needs to be performed.

Leptin signaling:

Human leptin consists of 146 aa, and its molecular weight is 16-kDa. Leptin is mainly expressed by adipose tissue and secreted into blood [Zhang et al., 1997). A low level of leptin expression has been detected in other tissues, including the stomach, bone marrow, ovary, and lymphoid tissue (Mantzoros et al., 2011). However, the role of leptin expression in these tissues is not well-known. The level of leptin in the blood is proportional to body fat mass (Maffei et al., 1995) and leptin functions by binding its receptor. There are six isoforms of the leptin receptor (LEPRa, b, c, d, e, and f) (Morris et al., 2009). Leptin binds to the N terminal extracellular domain of Lepr, and all six isoforms have this domain (Morris et al., 2009). Although they are identical at the N terminal part, Leprb is the only isoform that controls leptin signaling since it has a full-length c terminal domain (Morris et al., 2009). Leprb has three main domains, extracellular, intracellular, and a single membrane-spanning domain (Taga et al., 1997). Furthermore, it is mainly expressed in the brain, and leptin acts on the brain via binding Leprb (Schwartz et al., 1996).

Although Leprb does not have any enzymatic activity, Janus kinase 2 (Jak2), a cytoplasmic tyrosine kinase, binds to leptin receptors and exerts enzymatic activity (Taga et al., 1997). The binding of leptin to leptin receptor activates Jak2, leading to phosphorylation of tyrosine residues of downstream signaling molecules (Banks et al., 2000). In addition to the downstream pathway elements, Tyr985, Tyr1077, and Tyr1138 of Leprb are phosphorylated by Jak2 (Hekerman et al., 2005). These phosphorylated tyrosines act as a docking site for downstream signaling molecules. Although these three residues' phosphorylation is a key for leptin regulation, replacing these tyrosine residues with phenylalanine residues results in less severe glucose intolerance than db/db mice. This indicates that leptin can utilize other pathways to exerts its effect (Jiang et al., 2008).

Phosphorylation of Tyr1138 of Leprb is critical for leptin signaling to recruit SH2 domain of signal transducer and activator of transcript 3 (STAT3). Jak2 phosphorylates Stat3 that leads to the dimerization of STAT3 (White et al., 1997). Dimerized STAT3 translocates into the nucleus and binds its target genes' promoter regions as a transcription factor (Xu et al., 2007). It has been shown that the proper functioning of this pathway is very crucial to regulate body weight. A mutation that changes tyrosine residues 1138 into serine leads to increased food intake and obesity (Jiang et al., 2008). Furthermore, selective deletion of Stat3 from Leprb expressing neurons leads to obesity in mice (Gao et al., 2004).

One of the negative regulators of leptin signaling is the suppressor of cytokine signaling 3 (SOCS3). SOCS3 has a promoter binding site for Stat3, and activation of Stat3 by leptin signaling leads to an increase in SOCS3 expression. SOCS3 can also bind to a phosphorylated form of Tyr985 and compete with SH2 containing protein tyrosine phosphatase-2 (SHP2) to regulate the effect of leptin signaling by inhibiting the activity of the LEPRb/JAK2 pathway (Bjorbak et al., 2000).

In addition to STAT3, the activity of signal transducer and activator of transcript 5 (STAT5)is also regulated by leptin signaling. Phosphorylation of Tyr1138 and Tyr1077 of Leprb works together to recruit STAT5 and facilitate the activation and phosphorylation of STAT5 by JAK2 (Mütze et al., 2007). Similar to Stat3, Stat5 knockout mice result in obese phenotype suggesting Jak2/Stat5 pathways is crucial for leptin regulation of body weight. The importance of activation of STAT5 is further supported by the finding that shows increasing the activity of Stat5 results in a decrease in food intake in mice (Lee et al., 2008).

Furthermore, leptin is one of the regulators of the extracellular signal-regulated kinase (ERK) pathway (Rahmouni et al., 2009), and the effect of leptin on food intake is blunted after the inactivation of the ERK pathway (Rahmouni et al., 2009). The effect of leptin on ERK pathways is limited to the arcuate nucleus in the hypothalamus. Furthermore, reducing the ERK pathway's activity in the hypothalamus reduces the anorectic effect of leptin (Rahmouni et al., 2009).

Leptin exerts some of its effects via insulin receptor substrate (IRS)/ phosphoinositide 3-kinase (PI3K) signaling pathway (Niswender et al., 2001). IRS/PI3K pathway can be activated by leptin in the hypothalamus (Xu et al., 2005). Furthermore, brain-specific deletion of IRS2 and pharmacological inhibition of PI3K lead to obese phenotype and reduces the effect of leptin on body weight indicating the importance of this pathway in the regulation of body weight (Niswender et al., 2001)]. Forkhead Box O1 (FOXO1) is phosphorylated in multiple sites by AKT, a downstream pathway element of PI3K signaling, which is regulated by leptin (Kim et al., 2006). This phosphorylation inactivates FOXO1 and prevents its translocation into the nucleus (Taniguchi et al., 2006). When constitutively active FOXO1 is overexpressed in the arcuate nucleus, leptin's effect is blunted, resulting in increased food intake and body weight. Consistent with this, downregulating FOXO1 expression in the arcuate nucleus decreases body weight and food intake. (Kim et al., 2006). Furthermore, selective deletion of FoxO1 from POMC neurons leads to reduced food intake and body weight in mice (Plum et al., 2009).

IRS/PI3K pathway also regulates the activity of the mammalian target of rapamycin (mTOR)/ribosomal S6 kinase (S6K) pathway (Morris et al., 2009). Several studies demonstrate the significance of mTOR/S6K pathways in the regulation of body weight. Activation of mTORC1 by L-leucine leads to a reduction in food intake, while inhibition of mTORC1 by rapamycin results in increased food intake (Cota et al., 2006). When S6K is constitutively activated in the hypothalamus, mice become resistant to DIO. Since it has been shown that leptin activates mTORC1 that activates S6K in the hypothalamus, it is evident that this signaling cascade is vital for leptin regulation (Blouet et al., 2008). Although the effect of leptin signaling on several pathways has been investigated, how leptin resistance develops that leads to obesity is one of the critical questions in the obesity field.

Development of Leptin resistance:

One of the primary reasons for the development of obesity is leptin resistance (Morris et al., 2010). Although there is a high level of leptin in the bloodstream due to a high-fat diet or severe obesity, the amount of leptin in the brain does not proportionally increase (Banks et al., 1999). Defects in leptin transportation from the circulation into CSF via BBB are one of the mechanisms that may lead to leptin resistance (Banks et al., 1996). However, the importance of the impairment in leptin transportation is required to be further studied to understand its contribution to leptin resistance. Three main reasons show that leptin transportation may not be a key regulator for leptin resistance development. First of all, there are neural populations in arcuate nucleus AGRP and POMC can sense leptin in the bloodstream since there is no BBB in median eminence where AGRP and POMC neurons are located (Caro et al., 1996). Furthermore, ICV injection of leptin during HFD treatment does not prevent obesity (El-Haschimi et al., 2000). Lastly, leptin resistance may develop before impairment in leptin transportation (Banks et al., 2003).

Impairment in LEPRb signaling:

Signaling pathways that are regulated by leptin receptor are crucial for the development of leptin resistance. Impairments in these signaling pathways can result in leptin resistance. Localization of Leprb on the cell surface is required to start leptin signaling. In addition to the presence of Leprb on the cell surface, it is also detected in the Golgi apparatus and endosomes (Diano et al., 1998). However, the function of Leprb inside the cell is not well known. Several proteins are regulating Leprb trafficking. BBS proteins are one of those regulators. Therefore,

disruption in BBS proteins results in impairment of leptin trafficking that leads to impairment in leptin signaling and development of obesity (Rahmouni et al., 2008).

High diet treatment of mice results in leptin resistance. Several knockout mice studies identified genes that are vital elements of the leptin signaling pathway involved in the development of leptin resistance during high fat diet treatment. SOCS3 heterozygote knockout mice are more leptin sensitive and resistant to high-fat diet-induced obesity (Howard et al., 2004). The upregulation of SOCS3 in POMC neurons results in increasing body weight and leptin resistance (Reed et al., 2010). Furthermore, PTP1B, protein-tyrosine phosphatase 1B, knockout mice are resistant to diet-induced obesity, and specific deletion of PTP1b from neurons leads to increased energy consumption and a decrease in food intake (Tsou et al., 2012).

SH2B Adaptor Protein 1 (SH2B1) is also a key regulator of leptin signaling. Leptin signaling results in the binding of SH2B1 to Insulin Receptor Substrate (IRS) protein. This binding facilitates the phosphorylation of IRS proteins by Jak2 and prevents the dephosphorylation of IRS proteins. This keeps IRS-PI3K pathway active for a longer time. (Morris et al., 2009). Leptin resistance, increased food intake and body weight have been observed in SH2B1 knockout mice. When SH2B1 expression is rescued in a neuron-specific manner in SH2B1 knockout mice, the obesity phenotype is attenuated (Ren et al., 2007). Consistent with this data, the upregulation of SH2B1 expression in the brain enhances leptin sensitivity (Ren et al., 2007).

Food clock:

The circadian rhythm produced by the rhythmic expression of proteins controlled by internal clocks plays a crucial role in regulating metabolism by creating an internal timing mechanism that organisms can adapt to the changes in the environment (Takahashi et al., 2017). One of the notable instances of clock-controlled rhythm is the secretion of hormones by endocrine glands (Gamble et al., 2014). Circadian clocks can also impact food-related behaviors by regulating meal timing and preventing food intake during the sleep phase (Armstrong et al., 1980).

The suprachiasmatic nucleus (SCN) of the hypothalamus is the central clock in the brain that controls secondary clocks in the central nervous system (CNS) and other organs (Bechtold et al., 2013). The detection of light by the retina is the primary regulator of SCN (Golombek et al., 2010). Furthermore, food availability also controls the circadian clocks in the brain. The

mealtime in a day is the food clock's primary regulator (Feillet et al., 2008). Changes in meal timing can result in metabolic problems (Bray et al., 2013). Therefore, studying the underlying mechanism of food clocks is essential to understand food-related behaviors and metabolic disorders.

Although homeostatic signals are the primary regulator of temporal control of energy intake, there is growing literature that demonstrates the circadian clock's role in energy intake and energy expenditure (Kennedy et al., 1953). Although orexinergic and anorexigenic signals regulate satiety and feeding, the timing of refeeding after fasting plays a role in the amount of food intake. Rats that the food is provided at the dark phase after fasting consume more food than the rats that the food is provided at the end of the light phase after fasting (Rivera-Estrada et al., 2018). Furthermore, the food preference of rats is different at different time points in the day. They prefer to consume more carbohydrates at dusk. In contrast, they prefer fat and protein at dawn (Leibowitz et al., 1988). Furthermore, genes that are crucial regulators of the circadian clock also play a crucial role in temporal food intake (Armstrong et al., 1980). Knocking out genes, including Cry2, Cry1, Rev-Erba, and Per2, changes the nocturnal food intake pattern in mice (Kettner et al., 2015).

In addition to studies focusing on the effects of genes on the food clock, the impact of several brain regions on the food clock is also investigated. The suprachiasmatic nucleus (SCN) is one of the hypothalamic nuclei playing a role in food intake. Since SCN sends axonal projections into different regions in the hypothalamus that are orchestrating the food intake, it is evident that SCN impacts food intake (Guzmán-Ruiz et al., 2014). Furthermore, the ablation of neurons in SCN results in changes in food intake's daily timing without affecting food consumption and the number of meals. However, the effect of the SCN on meal timing is proposed to secondary to its effect on the sleep-wake pattern (Stoynev et al., 1982), since the activity of SCN is regulated mainly by light perceived by the retina and not by the timing of the meal (Castillo et al., 2004). Another nucleus that has a role in scheduled feeding is DMH. NPY neurons in the DMH play a role in the meal's timing during the night (Verwey et al., 2011). Furthermore, several clock proteins' expression is synchronized with scheduled feeding in the DMH (Davidson et al., 2009). The activity of the neuronal population in DMH increases during and before scheduled feeding, and the ablation of DMH neurons results in decreasing food anticipation. However, there are some controversial findings of DMH's role in food anticipatory activity (FAA) (Yu et al., 2014). The activity patterns of neurons in the VMH also change during scheduled feeding (Feillet et al., 2008).

However, the ablation of neurons in VMH does not impact the FAA (Challet et al., 1997). In the brain stem, daily oscillations of the expression of genes regulating the circadian clock in NTS and parabrachial nucleus have been identified (Juárez et al., 2012). Furthermore, the activity of neurons in the NTS and the parabrachial nucleus is increased during and after scheduled feeding (Angeles-Castellanos et al., 2005).

The expression of crucial peptides and hormones that regulate food intake are also controlled by the daily food intake pattern. NPY mRNA expression reaches a maximum level at dusk and dawn (Jhanwar-Uniyal et al., 1990). AgRP mRNA level is also highest at the beginning of the activity phase while POMC expression does not change throughout the day, POMC neurons are more active at the end of the active phase, and α-MSH expression reaches a maximum level at dawn (Lu et al., 2002). mRNA expression of Pro-Melanin Concentrating Hormone (PMCH) that is cleaved to produce MCH peptide and mRNA expression of Hypocretin Neuropeptide Precursor (HCRT) that is cleaved to produce orexin peptide also increases during the night (Akiyama et al., 2004). Furthermore, the concentration of hormones, including glucocorticoids, glucagon, and ghrelin expression, increases just before mealtime, while secretion of leptin and insulin increase after a meal (Challet et al., 2015).

In addition to the role of the level of hormones and peptides in meal timing, nutrient levels of several molecules in the bloodstream, including ketone bodies, glucose, and fatty acids, play a role in the meal's timing. In addition to the role of glucose in regulating the activity of neuronal populations in Arc, DMH, LH, VMH, and NTS (Fioramonti et al., 2017), the concentration of glucose in circulation shows oscillations through the day (Grosbellet et al., 2015). Rapid changes in glucose levels affect the circadian clock by regulating the expression of the Per2 gene in the hypothalamus (Oosterman et al., 2016). Furthermore, high fat diet results in the disruption of circadian clocks in peripheral organs by diminishing the chromatin recruitment of BMAL1 and CLOCK genes (Eckel-Mahan et al., 2013). An increase in fatty acid during HFD also impacts the circadian clock via PPARβ/δ (Challet et al., 2013).

Binge Eating:

One of the most common eating disorders is binge eating disorder (BED) (Kessler et al., 2013). Although the incidence rate of binge eating is very high, the underlying molecular and neuronal mechanism of BED is not well studied (Schienle et al., 2009). Individuals with BED display an increase in impulsive and compulsive behaviors and changes in reward valuation (Carrard et

al., 2012). Impulsivity observed in the patients with BED explains the underlying mechanism of decreased control during binge-eating periods (Galanti et al., 2007). Furthermore, changes in reward valuation can result in increasing focus on food during binge-eating episodes (Schag et al., 2013). Several other cognitive functions are impaired in patients with BED, including problem-solving, scheduling a task, and work-related memory (Monica et al., 2010). Furthermore, there is an increase in attention towards cues related to food in individuals with BED (Schmitz et al., 2014). The decision-making process is also impaired in obese patients with BED that they are more prone to making riskier decision compared to non-BED obese patients (Svaldi et al., 2010)

Furthermore, changes in the brain structure of patients with BED have been investigated to comprehend the underlying mechanism of cognitive impairments observed in individuals with BED. Showing pictures containing high calories food results in increased activity in centroparietal cortical regions of obese women compared to obese women without BED (Svaldi et al., 2010). Furthermore, in the resting state, obese women with BED showed higher frontal beta electroencephalography activity than obese women without BED (Tammela et al., 2010). When obese women with BED are exposed to food, an increase in cerebral and frontal cortices blood circulation has been observed (Karhunen et al., 2000). Furthermore, increased activation in the striatum and insular cortex has been observed in patients with BED. These regions' activity is decreased by sibutramine treatment, although binge eating episodes continued with the same frequency (Balodis et al., 2012). Additionally, activity in the ventromedial prefrontal region decreased in response to food stimuli in patients with BED compared to individuals without BED (Balodis et al., 2013). Although activity changes in several regions in the brain are associated with binge eating, further studies are needed to reveal whether activity changes in these regions cause binge eating disorder.

Genetics of Binge eating:

In addition to brain regions involved in binge eating disorder, identifying the genetic cause of binge eating is essential to the underlying molecular mechanism of binge eating. Familial BED is prevalent, and the inheritance rate of BED is between 40% and 60%, suggesting a genetic contribution to the development of binge eating (Javaras et al., 2008). Copy number variations in Taq1A have been associated with decreasing D2-like receptor density and increased reward sensitivity observed in patients with BED (Jonsson et al., 1999). Furthermore, polymorphisms in DRD2 (rs6277) and DAT1(9-repeat allele) are more common in obese

individuals with BED than obese individuals without BED (Davis et al., 2009). G allele in position 118 of μ-opioid-receptor-encoding gene is more common in obese BED individuals than obese individuals without BED (Davis et al., 2009). Although these studies are valuable to understand the molecular mechanism of BED, since the sample size of these studies is small, replication studies are needed.

In addition to polymorphisms associated with BED, changes in the expression level of several genes in dopamine and opioid systems, also associated with BED (Avena and Bocarsly, 2012). While the dopamine system plays a role in regulating food-seeking behaviors, the opioid system affects reward valuation in BED (Castro and Berridge, 2014). Changes in dopamine release and a decrease in dopamine D2-like receptor in nucleus accumbens is associated with increased impulsivity in BED patients (Belin et al., 2008). Furthermore, the expression of dopamine D2-like receptor in the striatum is decreased in animal models of binge eating that also reduce the dopamine system's effect (Avena et al., 2010). Mice treated with a high-fat diet display binge eating like phenotype when D2 receptors in the striatum are down-regulated (Johnson and Kenny, 2010). Furthermore, the GABAergic system in nucleus accumbens is a key to regulate impulsive behaviors in rats that are associated with BED (Caprioli et al., 2014). By using new techniques in neuroscience, including DREADD, optogenetic, iDISCO and viral tracing methods etc., it will be plausible to understand the role of these regions in the regulation of BED.

Chapter 2: GSBS Neurons in the DMH Suppress Food Intake in Response to Hyperphagia

Significance Statement:

Several studies have been conducted to reveal neuronal populations that are regulating food-related behaviors. However, it is known that there are still some other novel neuronal populations controlling food-related behaviors. Therefore, we focused on identifying novel neuronal populations modulating food intake and body weight. In this article, we report that DMH^{GSBS} neurons are activated by increased food intake and activating them results in decreasing food intake and body weight while inhibiting them leads to increased body weight and food intake in different settings. In addition to its basic science importance, these findings could have therapeutic applications as they suggest that pharmacologic activation of GSBS neurons could potentially reduce weight in settings of obesity and binge like eating.

Introduction

Obesity and its comorbidities, type 2 diabetes mellitus and cardiovascular diseases are global health problems affecting 400 million people worldwide (Pontzer et al., 2012) and is a substantial economic burden (Mokdad et al., 2003). Obesity develops as a consequence of positive energy balance, which in human is typically a result of increased food intake. Among mammals, food intake is regulated by an array of sensory and interoceptive signals that regulate the activity of key sets of neurons that control appetite (Schwartz et al., 2000).

Leptin is a key metabolic signal that relays information from adipose tissue to the brain to regulate energy balance (Friedman et al., 1998). Leptin deficient ob/ob mice develop extreme hyperphagia and obesity ((Zhang et al., 1994) and these abnormalities are corrected by leptin treatment (Halaas et al., 1995). After secretion from adipose tissue, leptin acts primarily on the long isoform of Leptin receptor (Leprb) (Bates et al., 2003), which is expressed in several brain regions in particular the brainstem and hypothalamus (Scott et al., 2009, Flak et al., 2014, Fulton et al., 2006, Hayes et al., 2010, Hommel et al., 2006). Deletion of Leprb in Agouti-related protein (AgRP) expressing neurons in the hypothalamus leads to increased adiposity similar to that of ob mice though other populations also contribute to its effects (Xu et al., 2018, Jais et al., 2020, Zhang et al.,2016).

In this report, we set out to compile an inventory of leptin responsive neurons by identifying and then testing the function of neurons identified by using Phospho-Trap, an unbiased profiling method. Phospo-trap enables the identification of molecular markers for neurons whose state of activation has changed in response to a defined stimulus (16). We applied Phospho-Trap to the hypothalamus and brainstem in ob mice before and after leptin treatment, as well as comparing ob to wild-type mice, and identified a set of previously known leptin responsive populations. We also found that, as assessed by cFos expression, GSBS neurons in the dorsal medial hypothalamus (DMH) are active in ob mice and suppressed by leptin treatment. Because these neurons are active in ob mice, which are extremely hyperphagic, we initially expected that chemogenetic activation of GSBS neurons in the DMH would increase food intake while decreasing their activity would diminish it. Surprisingly, we found the opposite effect of GSBS activation on the food intake of ob mice, as it led to decreased food intake while GSBS inhibition in these animals increased food intake. Similarly, GSBS activation in mice during scheduled feeding, which is also associated with hyperphagia, also decreased food intake and body weight. Finally, we found that pair feeding of ob mice to a leptin treated cohort reduced the cFos expression in DMH^{Gsbs} neurons as did limiting the available food during scheduled feeding. We conclude that rather than being directly regulated by leptin, these neurons are instead activated by hyperphagia and that they act to restrain excessive food intake and limit binges of eating.

Results

Identification of Markers for Leptin Regulated Neurons

We set out to identify neural populations in the brainstem and hypothalamus that are either activated or inhibited by leptin treatment of ob mice using Phospho-Trap, an unbiased transcriptomic method to molecularly profile neurons based on a change in activity (16). This method takes advantage of the fact that neuronal activation results in a cascade of signaling events culminating in the phosphorylation of the S6 ribosomal protein (pS6). These phosphorylated ribosomes can then be immunoprecipitated from mouse brain homogenates using a pS6 specific antibody, thereby enriching mRNAs selectively expressed in the activated neuronal population, or depleting RNAs from neurons with reduced activity. After polysome immunoprecipitation, RNA extraction and sequencing, RNAs enriched relative to total RNA have been shown to mark activated neurons while those that are depleted mark inhibited neurons (Knight et al., 2014, Knight et al., 2012). We applied this method to identify markers for neurons

whose state of activation was changed in ob mice relative to wild type animals and in ob mice after 14 days of leptin treatment. After polysome precipitation with an anti-pS6 antibody, the number of reads in the immunoprecipitated RNA (IP) for each gene was compared between ob mice treated with leptin for 14 days vs. pbs. The enrichment for each gene was calculated as the number of reads in the immunoprecipitated RNA (IP) (Fig. 1 A and B). As expected, we found enrichment for activity-related gene, c-fos, in the brainstem and hypothalamic samples from mice treated with leptin (Fig. 7). Consistent with previous studies we also found enrichment of hemoglobin alpha, adult chain 1 (HBA-A1) (Fig. 1A). HBA-A1 is expressed in the red blood cells in the brain as a result of constitutively increased mTOR activity in reticulocytes giving rise to enrichment of HBA-A1 mRNA after phosphotrap (Knight et al., 2014). As seen previously, the enrichment of this RNA likely reflected increased blood contamination in this sample.

We next studied the time course of enrichment or depletion of the gene we identified by precipitating polysomes from the brainstem and hypothalamus of ob mice treated with leptin for 2 days, 4 days and 7 days and analyzing the abundance of specific mRNAs using qPCR. Genes that were enriched or depleted in the precipitated polysomes were then validated in histologic analyses of pS6 expression.

Previously Identified Leptin Responsive Populations

We found enrichment for several genes previously shown to mark leptin activated neurons including the Pro-opiomelanocortin (POMC) transcript which was enriched after leptin treatment (Fig. 1D). POMC neurons are located in arcuate nucleus and leptin treatment of ob mice is known to increase their activity (Cowley et al., 2001). We further confirmed that leptin treatment increased pS6 in POMC neurons using in situ hybridization (ISH) and immunohistochemistry (IHC) (Fig. 2A). In contrast, AgRP mRNA was enriched in precipitated polysomes from ob mice relative to wild type and depleted after 14 days of leptin treatment consistent with the fact that AgRP neurons are known to be highly active in ob mice and suppressed by leptin treatment (van den Top et al., 2004) (Fig. 1E). We did however find that AgRP mRNA was transiently enriched after 2-day leptin treatment while mice were recovering from surgery, presumably in response to a short term decrease of feeding during the immediate post-operative period (Fig. 1E).

We also identified other leptin regulated neural populations in the brainstem including neurons expressing Tryptophan Hydroxylase 2 (TPH2) mRNA which was significantly enriched after leptin treatment of ob/ob mice (Fig. 1F). TPH2 is the rate limiting enzyme

in serotonin production and is primarily expressed in the Dorsal Raphe Nucleus (Bonkale et al., 2008). We confirmed that leptin treatment increased pS6 in TPH2 expressing neurons in Dorsal Raphe histologically by in situ hybridization (Fig. 2B). RNA-seq analysis and qPCR results also showed that Neuropeptide W (NPW) transcripts were enriched after leptin treatment (Fig. 1G). NPW mRNA has previously been shown to be enriched in LepRb neurons in the brainstem (Allison et al., 2015).

We also identified two new populations not previously associated with leptin treatment. RNA-seq analysis followed by qPCR confirmation revealed that Corticotrophin Releasing Hormone (CRH) transcript is enriched after leptin treatment (Fig. 1H). We confirmed the activation of CRH neurons by showing increased pS6 expression in CRH neurons in the Inferior Colliculus after leptin treatment of ob mice (Fig. 2C). While leptin has not been shown to regulate CRH neurons in the Inferior Colliculus previously, CRH neurons in the hypothalamus are known to reduce of food intake as part of a stress response (Herman *et al., 2016*). We also found that the RNA for G-substrate (Gsbs) was enriched in the hypothalamus of ob mice relative to wild type mice. This neural population has not been previously shown to be leptin regulated and we studied it further.

Functional Studies of Gsbs Neurons in DMH

The initial RNA-seq analysis and subsequent confirmation using qPCR after polysome precipitation showed that the RNA for Gsbs transcript is significantly enriched in ob hypothalamic samples relative to wild type and that this transcript is depleted from polysomes from ob/ob hypothalamus after leptin treatment (Fig. 1C). This enrichment was not associated with an altered expression of Gsbs mRNA in the hypothalamus of ob mice relative to leptin treated ob mice or wt mice suggesting that the activity of these neurons is increased in ob hypothalamus (Fig. 8). Gsbs is expressed at many sites in the brain, but in the hypothalamus, it is expressed mainly in the compact region of the DMH (Endo et al., 2012). Consistent with the data generated using phospho-trap, we found high levels of pS6 immunoreactivity in DMH^{Gsbs} neurons at baseline in ob mice and that pS6 expression in these neurons was suppressed by leptin (Fig. 2D).

To study the function of the DMH^{Gsbs} neurons, we obtained a GSBS-cre mouse line which we confirmed co-expressed cre and Gsbs using ISH for Cre together with IHC for GSBS (Fig. 9A). We first evaluated the effect of activating or inhibiting DMH^{Gsbs} neurons in wild type mice using chemogenetics with activating or inhibitory Dreadds. We injected the cre inducible viral constructs for

inhibition (AAV8-hSyn-DIO-hM4D(Gi)-mCherry) and activation (AAV8-hSyn-DIO-hM3D(Gq)-mCherry) into the DMH of Gsbs-Cre mice (50 nl, AP: −1.80 mm, DV: −5.1 mm, ML: ±0.3 mm). Signal transduction from these receptors can be activated by administration of clozapine-N-oxide (CNO) and we compared the effect of CNO to saline in animals expressing the chemogenetic vs. control constructs. Three weeks after stereotactic injections into the DMH, mice were habituated to IP injections for 10 days. As expected, after injection of AAV8-hSyn-DIO-hM3D(Gq)-mCherry into GSBS-cre mice, c-fos was expressed in mCherry expressing GSBS neurons 1 hour after CNO treatment (Fig. 9B). To determine the feeding response following DMH^{Gsbs} neurons' activation, mice were injected IP with vehicle or CNO (1 mg/kg) one hour before the onset of the dark cycle (1900 h).

Chemogenetic activation of DMH^{Gsbs} neurons in wild type mice by CNO acutely decreased food intake during the dark cycle relative to the vehicle control with ~ 33% decrease in consumption at 4 hours ($p < 0.0001$, Fig. 3A). Chronic activation of these neurons was associated with significant weight loss after two and four days of treatment ($p < 0.01$ and $p < 0.0001$ respectively, Fig. 3A). The phosphotrap data and histologic analyses of pS6 in these neurons suggested that these neurons are not active in wild type mice and consistent with this, acute inhibition of DMH^{Gsbs} neurons by CNO did not show an effect on food intake relative to the vehicle control (Fig. 3B). Similarly, chronic inhibition of DMH^{Gsbs} neurons by CNO did not show a significant effect on body weight relative to the vehicle control at 6 days of treatment (Fig. 3D). As an additional control, we injected CNO into animals that received injections of a control virus expressing GFP in Gsbs neurons in the DMH and failed to observe a change in food intake (Fig. 3 A and B).

Functional Studies of DMH^{Gsbs} Neurons in ob Mice

We hypothesized that inhibition of Gsbs neurons did not affect food intake in wild type mice because they show low activity at baseline in fed, wild type animals. In contrast, DMHGsbs neurons are active in ob/ob mice (Fig. 2D). We thus tested whether inhibition of DMH^{Gsbs} neurons would modulate food intake and body weight in ob/ob mice. To investigate this, we crossed ob/ob mice to Gsbs-cre mice (Gsbs-Cre::Ob/Ob) followed by injection of the inhibitory AAV8-hSyn-DIO-hM4D(Gi)-mCherry or activating AAV8-hSyn-DIO-hM3D(Gq)-mCherry Dreadds into the DMH. Inhibition of DMH^{Gsbs} neurons in ob mice with CNO significantly increased acute food intake by 62% at 2 hours ($p < 0.05$), 36% at 4 hours ($p < 0.01$) and 22% at 24 hours ($p < 0.01$, Fig. 4A). Similarly, chronic inhibition of Gsbs neurons in ob mice with daily injections of CNO resulted in a significant and progressive increase of body weight

after 4, 8 and 12 days of treatment relative to saline ($p < 0.05$, $p < 0.01$ and $p < 0.01$ respectively, Fig. 4C). In contrast, activation of DMH^{Gsbs} neurons after CNO injections into mice that received the activating AAV8-hSyn-DIO-hM3D(Gq)-mCherry Dreadd showed a small but significant decrease of food intake of 8% at 24 hours ($p < 0.05$, Fig. 4B) that was also associated with decreased body weight at day 12 ($p < 0.05$, Fig. 4D). Overall, these results are consistent with the results seen after modulation of Gsbs neurons in wild type mice and show that DMH^{Gsbs} neurons can bi-directionally regulate food intake and body weight in ob/ob animals. As described below, we next evaluated the function of Gsbs neurons in mice during scheduled feeding a protocol that, similar to ob mice, is associated with an increase of food intake during a short time interval (i.e. a binge of eating).

Functional Analysis of DMH^{Gsbs} Neurons During Scheduled Feeding

The DMH has been shown to play a prominent role in mediating the biologic response to scheduled feeding (Gooley et al., 2006). In a scheduled feeding paradigm, animals are provided with food during a three-hour window during the light phase rather than being fed ad libitum during the dark phase. After a 1-2 week period during which the animals learn the new feeding schedule, animals then show a marked increase in the rate of food intake now consuming their total daily intake during the three-hour window (Fig. 5A). In addition, the mice show a marked increase in locomotor activity just prior to the onset of this window known as Food Anticipatory Activity (FAA) as well as other physiologic responses (Fig. 5 B and C).

To test the role of DMH^{Gsbs} neurons in the adaptation to scheduled feeding, we injected the inhibitory AAV8-hSyn-DIO-hM4D(Gi)-mCherry or the activating AAV8-hSyn-DIO-hM3D(Gq)-mCherry into the DMH of different groups of Gsbs-cre mice. Each day for 12 days, CNO (1mg/kg) or vehicle was injected four hours prior to providing food (i.e.; at 8:00 during the light phase) intraperitoneally. Inhibition of DMH^{Gsbs} neurons during scheduled feeding by CNO led to a rapid, and highly significant increase of between 16- 44% of daily food intake relative to the vehicle control between day 3 and day 9 during the three-hour feeding window (p-value < 0.0001, Fig. 5A), while activation of DMH^{Gsbs} neurons by CNO led to a significant decrease of between 19-33% daily food intake between day 1 and day 12 (p-value < 0.0001, Fig. 5A). Scheduled feeding is generally associated with weight loss until the animals learn that they need to consume their food during the three-hour window. In these studies, where we modulated the activity of these neurons before they had regained their weight, inhibition of DMH^{Gsbs} neurons led to a more rapid regain of body weight of 3% by 12 days (p-

value < 0.01), while activation of DMH^{Gsbs} neurons resulted in 4% slower weight regain with animals not returning to the weight of controls until 12 days (p-value < 0.001, Fig. 10). Modulating the activity of DMH^{Gsbs} neurons did not show any effect on any of the other physiologic responses to scheduled feeding including food anticipatory activity (Fig. 5 B and C), oxygen consumption (Fig. 5D) or core temperature (Fig. 5E).

Gsbs Activity is Regulated by Increased Food Consumption, not by Leptin

Leptin treatment of ob mice reduces food intake and we had initially expected that inhibiting the activity of a leptin activated population such as the DMH^{Gsbs} neurons would also reduce food intake. However, as shown above, we instead found that inhibiting DMH^{Gsbs} neurons increased food intake in both ob mice and during scheduled feeding, while activating them decreased food intake in these settings as well as in wild type mice. These findings thus appeared paradoxical leading us to consider the possibility that these neurons may not in fact be directly regulated by leptin. Consistent with an indirect effect of leptin on these neurons, IHC revealed that DMH^{Gsbs} neurons do not express the leptin receptor (Fig. 6A). Fluorescent dual immunohistochemistry results showed that LepRb neurons in DMH do not co-localize with GSBS neurons and rather that there is a separate population of LepRb neurons by using LepRb-Ires-Cre::tdTOM mice that express the tdTomato fluorescent protein specifically in LepRb cells (Fig. 6A).

To further assess whether these neurons are directly regulated by leptin, we compared cFos expression in DMH^{Gsbs} neurons in leptin treated ob mice to ob mice pair-fed to the leptin treated group. DMH^{Gsbs} neurons in ob mice showed high levels of cFos expression and pair-feeding led to a similar decrease in cFos and pS6 expression as did leptin treatment (Fig. 2D, 6B and 11). In aggregate, these data show that the activity of these neurons, assessed using cFos and pS6 expression, is increased during binges of eating and diminished by a reduction of food consumption. Thus, the data further suggest that the effect of leptin is secondary to its appetite suppressing effect.

To further address this, we next analyzed the effect of reducing food intake during scheduled feeding on cFos expression in animals after they had fully acclimated to this protocol. In a standard scheduled feeding study, by day 12, animals consume their total daily intake (~ 4 g of food) during the 3-hour window. We generated three cohorts of mice who had fully acclimated to the new feeding schedule, and then tested the effect of limiting the food that was

available during this same 3-hour window over the course of several days. Group 1 continued to receive 4 gr, the amount they had been consuming ad libitum, during the three-hour feeding period. Group 2 received 2gr during the three-hour window (still an increased rate of consumption) and Group 3 received 1 gr of food which is similar to the normal rate of consumption of wild type mice during the dark phase within 3 hours. Consistent with the possibility that these neurons are activated by significantly increased food consumption, we found that while DMH^{Gsbs} neurons in group 1 and 2 continued to express cFos, cFos was no longer induced in DMH^{Gsbs} neurons in group 3 (Fig. 6C). These results showed that DMH^{Gsbs} neurons are activated during periods of intense, supraphysiologic binges of food intake.

Discussion

Leptin has pleiotropic effects on a number of behaviors and physiologic responses including food intake, sexual activity, locomotion, glucose metabolism, the reward value of food intake, reproductive capacity and others (Scott et al., 2009, Flak et al., 2014, Fulton et al., 2006, Hayes et al., 2010, Hommel et al., 2006). While numerous distinct neural populations have been shown to regulate one or more of these responses, it is unclear to what extent these known populations account for all of leptin's effects (Scott et al., 2009, Flak et al., 2014, Fulton et al., 2006, Hayes et al., 2010, Hommel et al., 2006). We addressed this using phosphotrap (Knight et al., 2012), to create an inventory of leptin responsive neurons and potentially identify additional neural populations in the brainstem and hypothalamus that mediate some portion of leptin's effects. In addition, because phospho-trap identifies any neurons whose activity has changed after leptin treatment, it can also identify indirect (i.e.; downstream) targets of leptin action.

Several neuronal populations were identified after leptin treatment of ob mice including neurons in the compact DMH expressing GSBS, an inhibitor of protein phosphatase 1 (Endo et al., 1999, Hall et al., 1999, Hutchinson et al., 2011). GSBS neurons are more active in ob mice relative to wild type mice and their activity is suppressed by leptin treatment. However, this reduced activity is also observed during pair feeding which, together with the finding that Gsbs neurons do not express LepRb, indicates that leptin's effects on these neurons is indirect and secondary to its appetite suppressing effects. Consistent with this, the activity of these neurons is increased during periods of intense hyperphagia after scheduled feeding and reduced when the available food is limited.

These neurons act to limit the amount of food that is consumed during periods of marked overconsumption as shown by the effect of chemogenetic inhibition to increase the food intake of ob mice. This effect is opposite to what would be expected for a leptin suppressed population. Similarly, inhibiting GSBS neurons also increased the food intake and body weight mice during scheduled feeding, another circumstance during which mice consume greatly increased amounts of nutrient. In contrast, activating GSBS neurons in ob mice, chow-fed wild type mice and during scheduled feeding reduced food intake and body weight. In aggregate, these data suggest that DMH^{Gsbs} neurons are activated in response to increased food consumption i.e.; a binge of eating and in turn reduce food intake. The finding that DMH^{Gsbs} neural inhibition further increased the food intake and weight of ob mice indicates that even when leptin is absent there are neural pathways that nonetheless act to restrain hyperphagia. Gsbs inhibits protein phosphatase 1 whose substrates include the S6 ribosomal protein (Hutchinson et al., 2011). Since phospho-trap depends on the correlation of S6 phosphorylation and neural activity, the identification of these neurons could have been a result of this catalytic activity rather than an effect of changing neural activity. However, we confirmed that the enrichment of Gsbs is indeed a result of a change of neural activity by also showing increased cFos in this DMH population in ob mice and that leptin treatment of ob mice reduces cFos expression.

Numerous sensory and interoceptive signals regulate food intake and body weight in addition to leptin, including a set of endocrine and neural satiety signals from the gastrointestinal tract (Friedman et al., 2019). These signals including CCK, GLP1 and others are processed largely by specific populations of neurons in the nodose ganglion, area postrema and nucleus of the solitary tract (NTS) and relayed to other feeding centers (Alhadeff et al., 2017). Recent studies have also shown that vagal inputs to the nodose ganglion and NTS can also reduce feeding. For example, neurons expressing GPR65 and projecting into NTS regulate the entry of food into the intestine (Williams et al., 2016). Furthermore, vagal gut afferents have been shown to regulate meal size via a negative-feedback mechanism and have been implicated in brain reward (Han et al., 2018). Our finding that Gsbs neurons are activated when food intake is acutely increased and act to reduce appetite raises the possibility that they are downstream targets of the brainstem centers that process these gut derived signals. Indeed, the characteristics of DMH^{Gsbs}, which are activated by feeding and decrease food intake, resemble those of several populations of vagal afferent neurons projecting to NTS including those expressing CCK1R, GLP1R, VGLUT2 and GPR65 (Williams et al., 2016). Consistent with this possibility, it has been shown that

several regions in the brainstem including NTS, parabrachial nucleus and periaqueductal gray innervate the DMH (Thompson et al., 1998). In addition, leucine sensing NTS neurons also regulate food intake by sending projections to the DMH (Tsang et al., 2020). Several brain regions in the hypothalamus also innervate the DMH including preoptic (POA), paraventricular and arcuate nuclei (Thompson et al., 1998). For example, GAD2 neurons in the POA regulate body temperature by sending projections into DMH neurons innervating the rostral raphe pallidus (Tan et al., 2016). AgRP neurons in the arcuate nucleus also innervate the DMH and control heart rate (Shi et al., 2017). Based on this, we hypothesize that DMH^{Gsbs} are an indirect downstream target of vagal afferent neurons projecting into the NTS though there may also be additional functional inputs from other hypothalamic regions and elsewhere. Studies defining the neural inputs and outputs of DMH^{Gsbs} neurons and their functions can be performed to address this.

In addition to regulating food intake during scheduled feeding (Knight et al., 2012), the DMH plays an important role to regulate the hyperactivity of mice just prior to animals receiving food during a scheduled feeding paradigm. Specific lesions in the DMH region also blunt the increase in body temperature, locomotor activity and wakefulness that are observed in unlesioned mice just before food presentation during scheduled feeding (Gooley et al., 2006) Leprb neurons in anterior DMH also modulate body temperature and locomotor activity while Leprb neurons in posterior DMH regulates food intake (Rezai-Zadeh et al., 2014). However, the modulation of the activity of the Gsbs neurons does not alter any of these other responses and appears to be limited to an effect on food intake.

While we did not identify many novel populations of leptin responsive neurons, these studies also further validate the utility of phospho-trap. These included neurons expressing AgRP (Aponte et al., 2011, Krashes et al., 2011, Atasoy et al., 2012, Betley et al., 2015) and POMC in the hypothalamus (Aponte et al., 2011, Zhan et al., 2013, Atasoy et al., 2012) in the arcuate nucleus. We also found enrichment of NPW mRNA in the brainstem. Previous studies of the transcriptome of brainstem identified a subpopulation of LepRb neurons also expressing NPW though the effect of leptin was not evaluated (Allison et al., 2015). NPW mRNA is also expressed in the dorsal raphe nucleus, periaqueductal gray matter and ventral tegmental area and our data suggest that leptin regulates their activity (Motoike et al., 2016, Naso et al., 2014). We also found that activity of TPH2 neurons are regulated by leptin. While some studies have suggested that these neurons are not leptin responsive, recent studies have suggested that TPH2 neurons in Dorsal Raphe can regulate the reward value of

food (Voigt et al., 2015). Our data are consistent with an effect of leptin on these neurons though our results do not establish whether this is direct or indirect. We also found that the activity of CRH neurons in the inferior colliculus is regulated by leptin. While this neural population has not previously been shown to be leptin responsive, similar to leptin, CRH suppresses food intake ((Okamato et al., 2018, Bazhan et al., 2013). These data raise the possibility that CRH neurons in inferior colliculus could play a role in mediating some of the effects of leptin.

However, some populations of known leptin responsive neurons were not identified including those expressing SF1 and Neurotensin (Dhillon et al., 2006, Leinninger et al., 2011). The basis for this is not clear though we did not evaluate the effects of acute leptin treatment and it is possible that increased S6 phosphorylation is not durable in these neurons over longer periods of time. In addition, some SF1 and Neurotensin neurons in the hypothalamus do not express LepRb (Dhillon et al., 2006, Leinninger et al., 2011) and it is possible that leptin could elicit different effects on different subpopulations (i.e. some could be activated while others might be inhibited, canceling out an observable effect). Thus, while these results suggest the possibility that most of the leptin responsive neurons in the hypothalamus and brainstem have already been identified, we cannot however exclude the possibility that, similar to Sf1 and neurotensin neurons, additional novel populations of leptin responsive neurons were missed.

In summary, we identified a novel neural population in DMH that controls food intake and body weight as part of a counter regulatory response to markedly increased food consumption. These neurons normally limit the food intake and body weight of ob mice, as well as mice who binge eat during scheduled feeding, revealing that there are neural mechanisms in hyperphagic that in fact restrict their intake. Furthermore, the finding that DMH^{Gsbs} neurons are activated during periods of intense food intake and in turn limit consumption raises the possibility that defects in this response lead to binge eating and could thus be of clinical relevance.

Chapter 3: Materials and Methods

Animal Treatment

All experiments were approved by The Rockefeller University Institutional Animal Care and Use Committees and performed following the National Institutes of Health guidelines. Male mice were used for behavioral studies (>8 weeks). Mice were housed in a 12 hr light-dark cycle with ad libitum access to water and food, except otherwise specified in text.

Obrb-cre (B6.129(Cg)-Leprtm2(cre)Rck/J, Jackson laboratory stock number: 008320) was used to express cre in lerpb neurons. Ppp1r17(GSBS)-cre (Tg(Ppp1r17-cre) NL146Gsat/Mmucd) mice used to express cre in GSBS neurons. ROSA-loxSTOPlox-tdTomato (Jackson Laboratories Stock number: 007909, B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) was used to express the reporter gene in the presence of Cre recombinase by using lox-STOP-lox sequence.

Viral Vectors

All viruses used in these studies were obtained from Addgene. AAV8-hSyn-DIO-mCherry (control virus), AAV8-hSyn-DIO-hM3D(Gq)-mCherry (activation) or AAV8-hSyn-DIO-hM4D(Gi)-mCherry (inhibition) were used, for chemogenetic studies.

Stereotaxic Surgery

Mice age 8-12 weeks were anesthetized by using 2% isoflurane. Paxinos mouse brain atlas were used to identify coordinates. GSBS-Cre mice were injected with 50 nl AAV8-hSyn-DIO-mCherry (control virus) or AAV8-hSyn-DIO-hM3D(Gq)-mCherry (activation) or AAV8-hSyn-DIO-hM4D(Gi)-mCherry (inhibition) virus in the DMH, bilaterally (coordinates AP: −1.80 mm, DV: −5.1 mm, ML: ±0.3). All listed DV coordinates are relative to bregma. 10 minutes after injection, needle is slowly retracted. Suturing or surgical clips are used to close the skin.

Immunohistochemistry

After anesthetizing mice with isoflurane, mice were transcardially perfused with PBS followed by 10% formalin. Dissected brains are post-fixed in 10% formalin at 4°C overnight. Brain slices at 40-50 µm were obtained by using a vibratome. Free floating sections are washed with PBS for three times and incubation is performed in blocking buffer (PBS, 0.1% Triton-X, 2% goat serum, 3% BSA) 1 hour at room temperature to block sections. Next, sections are incubated at 4°C overnight with primary antibodies in block solution. After

overnight incubation, PBS, 0.1% Triton-X was used to wash sections three times for 15 minutes followed by incubation in Alexa Fluor conjugated secondary antibodies at 1:1000 dilution at room temperature for 2 hours. Lastly, sections are washed in three times in PBS, 0.1% Triton-X for 15 minutes and mounted on slides. Secondary antibodies were Alexa Fluor conjugated (Life Technologies). All images were captured using confocal microscopy (Zeiss or Leica).

Primary antibodies used were: rabbit anti- PPP1R17 (1:300, Sigma-Aldrich HPA047819), rabbit anti-cfos (1:1000, Santa Cruz, sc52), goat anti-cfos (1:1000, Santa Cruz, sc52G), Phospho-S6 Ser244, Ser247 (1:1000, Thermo Fisher scientific, 44-923G), chicken anti-GFP (1:1000, Abcam, ab13970).

RNA-Seq and Taqman Array Analysis

TopHat and Cufflinks apps were used to analyze RNA-seq results. Musmusculus assembly mm10 was used to annotate alignments. Genes that have FPKM>2 are depicted in the RNAseq graph. Taqman probes were used to validate RNA-seq results. QuantiTect Reverse Transcription Kit was used to obtain complementary DNA (cDNA). Taqman Gene Expression Master Mix is used to quantify the abundance of the genes. Transcript abundance was normalized to rpL27. Differential fold enrichment values were calculated by ΔΔCt method. P value is calculated by using unpaired two-tailed t test.

Awake Chemogenetic Studies to Measure Thermogenesis

Mice that were injected with DREADD (hM3D(Gq) or hM4D(Gi)) or control virus into DMH were injected with 1mg/kg CNO or 0.9% saline and single housed. Core body temperature was measured in the home cage during the animal's light phase by using an anal probe (Braintree Scientific). P value is calculated by using unpaired two-tailed t test.

Chemogenetic Studies using Metabolic Home Cages

Mice that were injected with DREADD (hM3D(Gq), hM4D(Gi)) or control virus into DMH were placed in metabolic cages and single housed for one week to allow them to habituate to social isolation. Metabolic paramaters were recorded automatically in metabolic cages with an automated home cage phenotyping system (TSE-Systems). Locomotor activity and oxygen consumption of mice were monitored during metabolic phenotyping. Custom software for metabolic cages analyzes the beam breaks to record locomotor activity. Oxygen consumption is measured by an indirect gas calorimetry module that is a part of TSE-Systems. After

habituation, locomotor activity is recorded for 12 days during scheduled feeding. CNO is injected intraperitoneally four hours before the food presence for 12 days. Oxygen consumption is recorded during light phase of mice 1 hour after CNO injection for 3 hours. All data were collected and processed with Phenomaster software. For food intake and body weight, p value is calculated by two-way RM Anova testing. For FAA and oxygen consumption, p value is calculated by using unpaired two-tailed t test.

Food Intake and Body Weight Assay for Chemogenetic Studies

Mice were single housed and placed in home cages during food intake assay. For acute food intake measurement, CNO is injected intraperitoneally just before the dark phase and measurements of food intake were performed at 1, 2 and 4hr post i.p. injection of CNO for wt mice experiments and 1, 2, 4 and 24hr after post i.p. injection of CNO for ob mice experiments. For body weight and food intake measurement at 24hr, CNO is injected twice per day at a concentration of 1mg/kg. P value is calculated by using unpaired two-tailed t test.

Animals: Diet and leptin normalization

Wild-type and ob/ob C57BL/6J mice were purchased from Jackson laboratories (Bar Harbor, ME). Micro-osmotic pump (model 2002; Durect, Cupertino, CA) were used to dispense either leptin (150 ng/h in PBS) or vehicle (PBS). Pumps were implanted to 10 weeks old mice subcutaneously and mice were single housed. Recombinant murine leptin was purchased from Amylin Pharmaceuticals (San Diego, CA).

Fluorescent In Situ Hybridization with IHC

Anti-sense digoxigenin-labeled riboprobe were synthesized for cre (953 base pair), gsbs (958 base pair), pomc (915 base pair), tph2 (631 base pair) and crh (934 base pair). To quench endogenous peroxidase activity, 40 μm free-floating brains sections were treated with 3% H2O2 for 1h at room temperature followed by incubation in 0.20% acetic anhydride for 30 minutes and incubated in 1% Triton-X for 30 min each. For prehybridization, free floating brain slices was incubated in hybridization buffer at 62°C (50% formamide, 5x SSC, 5x Denhardts, 250 ug/ml baker's yeast RNA, 500 ug/ml ssDNA) for 1h. Hybridization is carried out at at 62°C with riboprobe, overnight. Free floating slices were washed once in 5x SSC and two times with 0.2x SSC at 62°C. slices were incubated in the primary antibody with riboprobe. Sections were washed with 0.2x SSC and buffer B1 (0.1 M Tris pH 7.5, 0.15 M NaCl) briefly following by blocking in TNB (1% blocking reagent in B1, Roche

#1096176) for 1h at room temperature. Anti-digoxigenin-POD antibody (1:100, Roche #11207733910) was carried out overnight at 4°C. To combine immunohistochemistry with FISH, sections are incubated with a secondary antibody conjugated to Alexa 488/594 for 1h at room temperature before riboprobe was detected. TSA Plus Fluorescence System (Perkin Elmer, #NEL744) was used to develop riboprobe according to the manufacturer's instructions.

Phosphotrap experiment:

After loading 150 ul protein A Dynabeads (Invitrogen 10002D) with 4ug of Phospho-S6 Ser244, Ser247 (Thermo Fisher scientific, 44-923G) in Buffer A (150mM KCl, 10mM HEPES, 1% NP40, 5mM MgCl2 and 0.05% IgG-free BSA), Beads loaded with pS6 antibody were washed in Buffer A for three times. Following to sacrificing mice by cervical dislocation, 15-20 hypothalamus and brainstem was dissected in Buffer B containing 4mM NaHCO3, 1xHBSS, 100ug/mL cycloheximide, 2.5mM HEPES and 35mM glucose on ice and pooled, separately. Hypotahalami and brainstems are transferred to separate glass homogenizers (Kimble Kontes 20). Buffer C (protease and phosphatase inhibitor cocktails, 150mM KCl, 10mM HEPES, pH 7.4, 100nM calyculin A, 2mM DTT, 100U/mL RNasin, 5mM MgCl2, 100ug/mL cycloheximide) was used to resuspend hypothalami and brainstems and Speed homogenizer (Glas-Col) was used to homogenize samples at 4ºC. Homogenized samples were transferred to Eppendorf tubes and centrifuge at 2000x g for 10 mins at 4ºC to clarify samples. The supernatant was transferred into a new eppendorf tube containing 90ul of 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC, Avanti Polar Lipids: 100mg/0.69mL) and 90ul of 10% NP40. After mixing the solution, it was centrifuged at 17000x g at 4ºC for 10 minutes. 20 ul of the supernatant is collected in another tube containing 350 ul of buffer RLT (QIAGEN, RNeasy Micro kit, 74004) and kept at -80ºC for purification as input RNA. Remaining supernatant was collected into a new eppendorf tube and kept on ice for 10 minutes for immunoprecipitation. Buffer D (10mM HEPES, pH 7.4, 350mM KCl, 5mM MgCl2, 2mM DTT, 1% NP40, 100U/mL RNasin and 100ug/mL cycloheximide) was used to wash the beads for 4 times. After the second wash, beads were collected in a new tube and incubated on ice for 10 minutes followed by a final wash. 350ul buffer RLT was added into the tube containing beads to elute RNA. Magnets were used to separate beads from RNA. To perform RNA sequencing (RNA-seq) analysis, cDNA was obtained by using the SMARTer Ultralow Input RNA for Illumina Sequencing Kit and then sequenced by using an Illumina HiSeq 2000.

Chapter 4: Summary and Conclusion

Several studies have been conducted to understand the neuronal populations' role in the regulation of energy metabolism. These studies revealed that abnormalities in this regulation can result in obesity and other metabolic problems. Although several neuronal populations that are regulating food-related behaviors have been identified, it is known that there are still some other novel neuronal populations that are regulating food-related behaviors. Therefore, we focused on identifying novel neuronal populations regulating food intake and body weight. Since leptin is one of the well-known regulators of food intake and body weight and controls the activity of different neuronal populations in the brain, we decided to reveal novel neuronal populations regulated by leptin. To create an inventory of which neuronal populations are regulated by leptin, we used ob mice as our model mice. Ob mice do not express leptin and become massively obese, and leptin treatment of ob mice results in decreasing body weight and food intake. By using a novel profiling methodology, Phospho-Trap, we identify molecular markers for neurons that were either activated or inhibited after leptin treatment of ob mice. The Phospho-Trap method utilizes the finding that phosphorylation of the ribosomal S6 protein correlates with neuronal activity. Therefore, there is more phosphorylated RpS6 in activated neurons compared to inactive neurons. By immuno-precipitating polysomes using a phospho-specific antibody to ribosomal protein S6, the molecular profile of activated or inhibited neurons by different nutritional signals can be obtained. Therefore, while mRNAs enriched relative to controls mark neurons that were activated, RNAs that are depleted mark neurons that were inactivated.

Since leptin is a nutritional signal, we used this method to identify novel neural populations regulated by leptin. After reconfirming leptin’s effects on known neural populations including those expressing AGRP and POMC, we focused on a novel neuronal population appeared in our RNA-seq analysis located in the dorsomedial hypothalamus that expresses GSBS protein, a substrate of Protein Kinase G. These neurons are activated in ob mice, and leptin treatment of ob mice results in decreased their activity. Since ob mice are hyperphagic, and leptin treatment leads to a decrease in food intake, we hypothesized that activating these neurons will increase food intake and body weight. However, when we activate these neurons by Dreadd, we found that this resulted in decreased food intake. Conversely, inhibiting these neurons using chemogenetics increased food intake. This finding was perplexing until we performed follow up experiments showing that GSBS neurons are activated by increased food intake to decrease food intake and that fasting ob mice result in decreased activity

of GSBS neurons. Showing some neuronal populations suppress food intake, although ob mice have a massively increased appetite. We further found that activating GSBS neurons suppress binge like eating that has been observed in scheduled feeding. Several studies by other groups have identified novel neuronal populations regulated by inputs from the gut via vagal signaling through the vagus to the brainstem to regulate food intake. Therefore, we hypothesize that vagal signaling can be one of the regulators of the activity of GSBS neurons in DMH. In addition to its basic science importance, these findings could have therapeutic applications as they suggest that pharmacologic activation of GSBS neurons could potentially reduce weight in settings of obesity and binge like eating.

Future Directions

Previous work in the central regulation of energy balance had limited success in translating its results to effective treatments for eating disorders. We showed that GSBS neurons are one of the regulators of binge like eating. Studies spanning a few decades have established the importance of dopamine and serotonin system in ingestion of a large amount of food in a short timeframe, binge eating. However, the pathophysiology of binge eating is not well understood, and the effective treatments for this condition are limited. Identifying the molecular profile of Gsbs neurons in the DMH region will be crucial to understand how GSBS neurons are regulating binge eating. vTRAP methodology developed by our lab can be used to examine the molecular profile of Gsbs neurons in DMH specifically. Furthermore, examining where Gsbs neurons project and where they are getting inputs is crucial to understand the neural networks of Gsbs neurons better. Based on the molecular profile of Gsbs neurons and their projection sites, it will be plausible to test the functional importance of neuropeptides and receptors expressed by Gsbs neurons in binge-like eating and food related behaviors. This approach will allow us to determine whether Gsbs neurons can be targeted pharmacologically.

Furthermore, Gsbs is first described as a substrate of Protein kinase G (PKG) and highly expressed in the cerebellar purkinje cells (Schlichter et al., 1978). It is one of the components of NO-cGMP-PKG cascade. In this pathway, Nitric oxide synthase 1 (NOS1) produces Nitric oxide from l-arginine. Released Nitric oxide from neurons diffuses into nearby cells that bind its receptor, soluble guanylyl cyclase, and activate it. Activated soluble guanylyl cyclase increases the amount of cGMP, which binds and increases the activity of protein kinase G. Protein kinase G (PKG) phosphorylates Gsbs to activate it. Activated Gsbs phosphorylates protein phosphatase 1 (PP1) to inhibit it. This

cascade is vital for LTD in cerebellar purkinje cells. Furthermore, PP1 dephosphorylates RpS6 (Endo et al., 2012). In this cascade, nitric oxide is implicated in feeding behaviors. Intraperitoneal (IP) and intracerebroventricular (ICV) infusion of L-NAME, nitric oxide inhibitors, lead to weight loss in ob/ob and db/db mice (Morley et al., 1994). Icv administration of leptin with L-arginine blunts the anorexic effect of leptin (Calapai et al., 1998). In addition to Nitric oxide, another molecule in this pathway, protein kinase G (PKG), is also implicated in food-related behavior in different organisms. The natural variant in PKG gene in drosophila leads to a highly active form of PKG that results in the foraging behavior in drosophila larvae. While the larvae having a more active form of PKG moves more between food patches, the larvae having a less active PKG form move less between food patches. Furthermore, this difference in locomotor activity does not exist in the absence of food, suggesting PKG's role in food-seeking behavior (Sokolowski et al., 2001). In addition to drosophila, an increase in PKG activity in honey bees' brains leads to the transition from worker to forager (Ben-Sharar et al., 2005). Although modulation of PKG activity is crucial for foraging behavior in different organisms, the neural circuit and underlying mechanism are not known. Since different elements of this cascade are implicated in food-related behavior, investigating this cascade's role in regulating GSBS neurons and binge like eating can improve the understanding how food related behaviors are regulated.

Furthermore, identifying additional loci in the CNS that are activated by binge eating and identify the specific neuronal populations in premotor areas linked to binge eating will be a key to understand the circuit logic underlying reasons of binge-like eating. To identify the specific loci in the brain activated by binge eating, an unbiased whole-brain activity mapping approach iDISCO+ can be used. Retrograde tracing pseudorabies virus (PRV) can also be used to reveal premotor areas involved in binge-like eating and hyperphagia.

The basic neurobiological mechanisms through which binge eating and hyperphagia are regulated are incompletely understood. Identifying these neurons, how they coordinate food intake, and functionally dissecting the neural circuitry are fundamental to understanding how the brain responds to overeating. This also has significant implications for human health. Understanding the molecular mechanisms and the pathways through which the CNS prevents or activates binge eating will allow us to develop targeted pharmacological approaches to prevent overeating, ultimately decrease body weight, and prevent binge eating.

Chapter 5: Figures

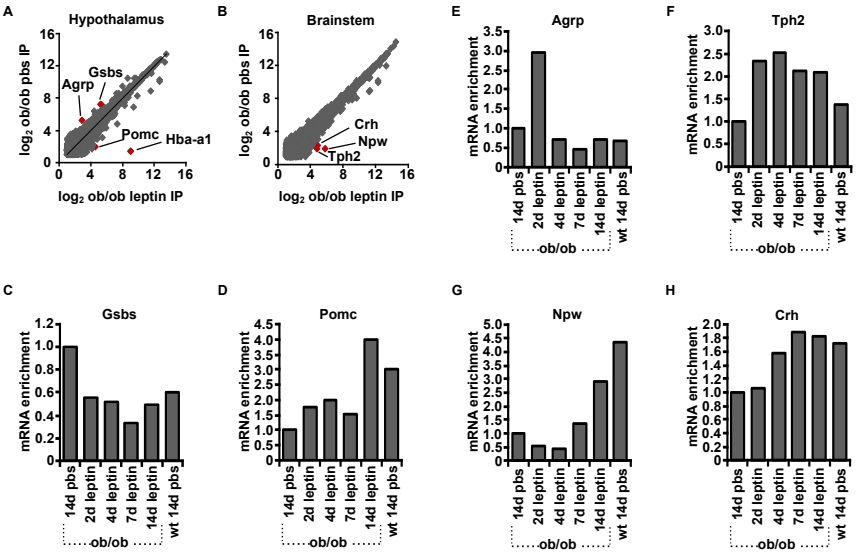

Figure 1. Differential enrichment of genes after leptin treatment. Phosphotrap was performed on the samples indicated below and the RNA abundance determined by the number of RNA sequence reads, was plotted in pair-wise comparisons showing: A. Differential enrichment of genes in pS6 immunoprecipitates determined by Rna-seq in 14 day leptin treatment of ob/ob mice vs PBS in A. Hypothalamus and B. Brainstem. Taqman assays were then performed for the on pS6 precipitated polysomes after 14 day PBS treatment of ob/ob mice, after the number of days of leptin treatment (2, 4, 7and 14 days) of ob mice and after 14 day PBS treatment of wild type mice. The genes which are shown are those whose level of enrichment had changed in the comparison between 14 days of leptin vs. PBS (the specific genes are shown in Panels A and B: C. Gsbs. D. POMC E. AGRP F. Tph2 G. Npw H. CRH. Data are expressed as the ratio of fold enrichment (IP/input) for each group of mice divided by the fold enrichment (Ip/input) for ob/ob mice treated with PBS for 14 days

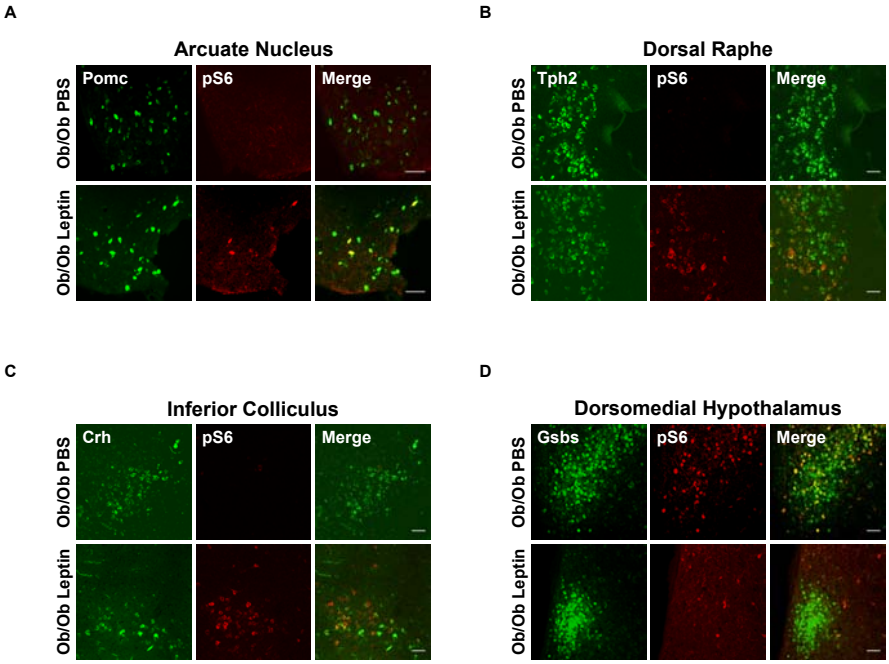

Figure 2. pS6 expression after leptin treatment of ob mice. Dual in situ hybridization and immunohistochemistry in hypothalamus and brain stem was performed for the marker genes and pS6 (244 and 247) as indicated in sections from ob mice treated with leptin (14 days) vs. PBS.A. POMC. ISH for Pomc and IHC for pS6 in Arcuate Nucleus. Pomc neurons in Arc are activated by 14 days leptin treatment of ob/ob mice B. Tph2. ISH for Tph2 and IHC for pS6 in Dorsal Raphe. Tph2 neurons in Dorsal Raphe are activated by 14 days leptin treatment of ob/ob mice C. CRH. ISH for Crh and and IHC for pS6 in Inferior Colliculus Crh neurons in Inferior colliculus are activated by 14 days leptin treatment of ob/ob mice D. Gsbs. ISH for Gsbs and IHC for pS6 in Dorsomedial Hypothalamus Gsbs neurons in DMH are inhibited by 14 days leptin treatment of ob/ob mice. All scale bars, 50 μm

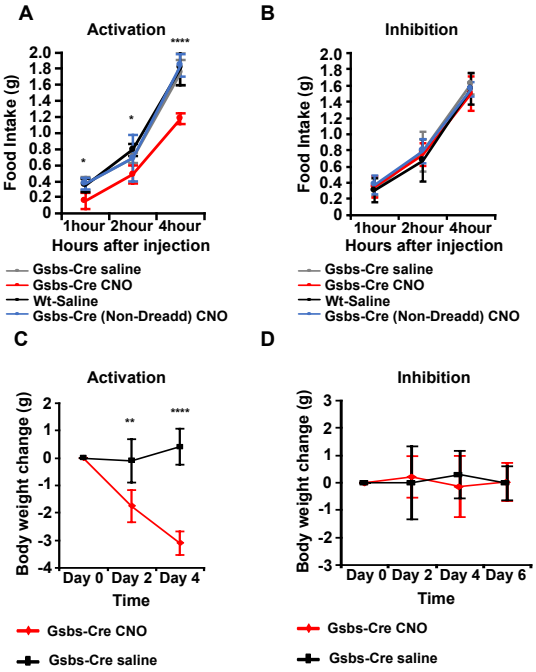

Figure 3. Effect of chemogenetic modulation of DMH^{Gsbs} neurons on food intake and body weight in wild type mice. A. Chemogenetic activation of DMH^{Gsbs} neurons using AAV8-hSyn-DIO-hM3D(Gq)-mCherry. DREADD induced activation of DMH^{Gsbs} neurons significantly decreases food intake over the course of 4 hours. B. Chemogenetic inhibition of DMH^{Gsbs} neurons using AAV8-hSyn-DIO-hM4D(Gi)-mCherry. DREADD induced inhibition of DMH^{Gsbs} neurons does not affect food intake over the course of 4 hours. C. Body weight of Gsbs-Cre mice are significantly reduced after chronic activation of DMH^{Gsbs} neurons by i.p injection of CNO 2 times per day. D. Body weight of Gsbs-Cre mice does not significantly change after chronic inhibition of DMH^{Gsbs} neurons by i.p injection of CNO 2 times per day. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ using two-tailed unpaired t test. n=5-6 mice. All error bars are mean ±SD

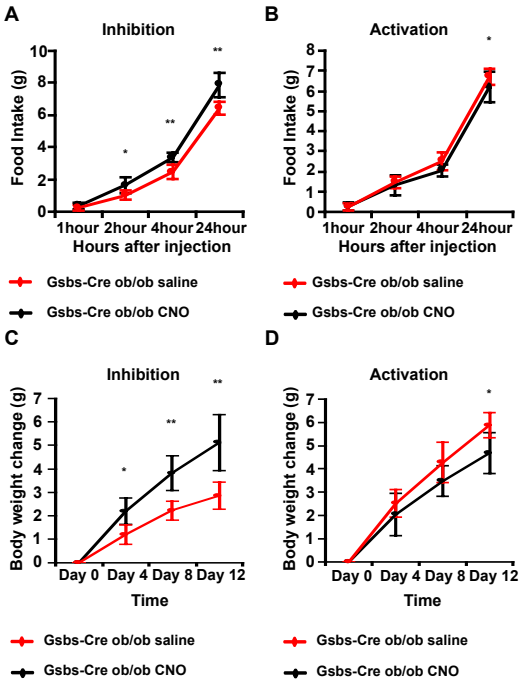

Figure 4. Effect of chemogenetic modulation of DMH^{Gsbs} neurons on food intake and body weight in ob/ob mice. A. Chemogenetic inhibition of DMH^{Gsbs} neurons in ob/ob mice. DREADD induced inhibition of DMH^{Gsbs} neurons significantly increases food intake over the course of 24 hours. B.Chemogenetic activation of DMH^{Gsbs} neurons in ob/ob mice. DREADD induced activation of DMH^{Gsbs} neurons significantly decreases food intake over the course of 24 hours. C. Body weights of ob/ob mice are significantly increased after chronic inhibition of DMH^{Gsbs} neurons by i.p injection of CNO 2 times per day. D. Body weights of ob/ob mice are significantly reduced after chronic activation of DMH^{Gsbs} neurons by i.p injection of CNO 2 times per day at the end of 12 days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ using two-tailed unpaired t test. n=5 mice. All error bars are mean ±SD

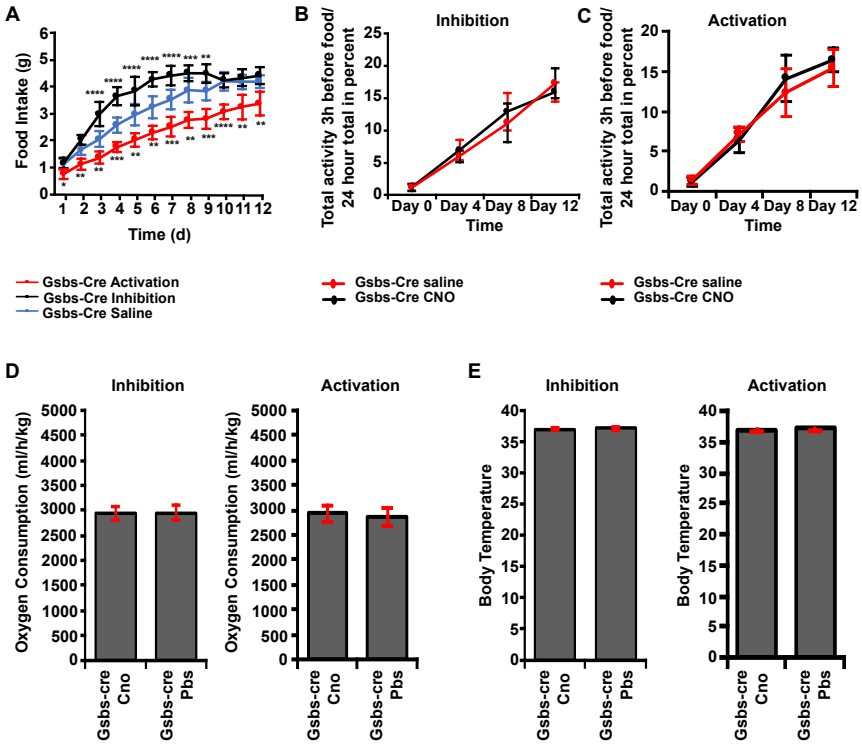

Figure 5. Effect of chemogenetic modulation of DMH^{Gsbs} neurons on food intake, body weight and FAA during scheduled feding. A. DREADD induced activation (Treatment: p< 0.0001) and inhibition (Treatment: p<0.0001) of DMH^{Gsbs} neurons significantly alters food intake during scheduled feeding after i.p injection of CNO 4 hours before food presentation. Two-way RM ANOVA comparing treated and control groups B. DREADD induced inhibition of DMH^{Gsbs} neurons does not affect food anticipatory activity. C. DREADD induced activation of DMH^{Gsbs} neurons does not affect food anticipatory activity. D. DREADD induced activation or inhibition does not alter oxygen consumption during scheduled feeding. Two-tailed unpaired t test comparing treated (GSBS-cre CNO) and control group (GSBS-cre PBS) E. DREADD induced activation or inhibition does not alter body temperature during scheduled feeding. Two-tailed unpaired t test comparing treated (GSBS-cre CNO) and control group (GSBS-cre PBS) n=8 mice. * p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. All error bars are mean ±SD

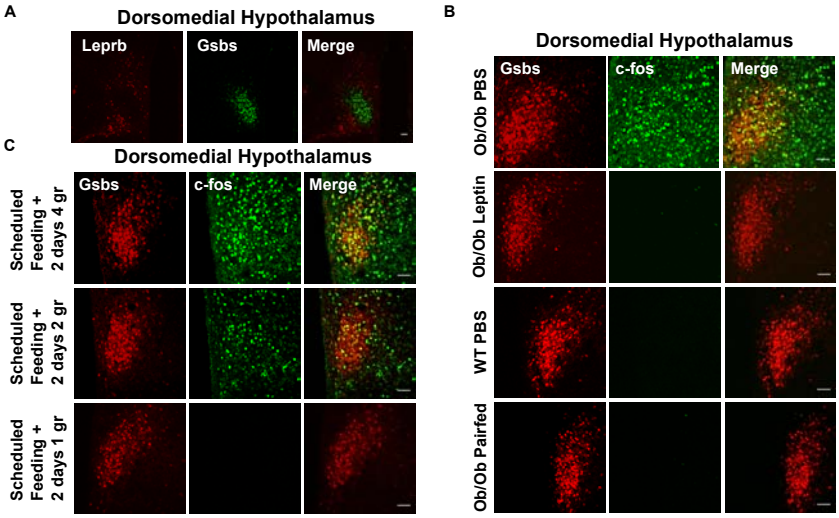

Figure 6. cFos expression in Gsbs Neurons in ob mice and during scheduled feeding. A. IHC for Gsbs and TdTomato in ObRb TdTomato mice. DMH^{Gsbs} neurons do not express Leprb. B. cFos expression DMH^{GSBS} neurons in ob/ob mice is reduced by 14 days pair-feeding. C. cFos expression in DMH^{GSBS} neurons in scheduled feeding during the 3-hour feeding window in response to different amounts of chow. Food is provided to mice between CT4-CT7. Upper panel, 4 grams of food, the amount normally consumed during the three-hour feeding window. Middle Panel, 2 grams of food, the amount mice consume in 3 hours after a fast and Lower panel, 1 g, the amount consumed during the dark phase within 3 hours. All scale bars, 50 μm.

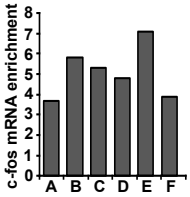

Figure 7. c-fos mRNA enrichment in pS6immunoprecipitates determined by taqman A: ob/ob mice treated with PBS for 14 days B: ob/ob mice treated with leptin for 2 days C: ob/ob mice treated with leptin for 4 days D: ob/ob mice treated with leptin for 7 days E: ob/ob mice treated with leptin for 14 days F: Wt mice treated with Pbs for 14 days Data are expressed as the ratio of fold enrichment (IP/input) for each group of mice

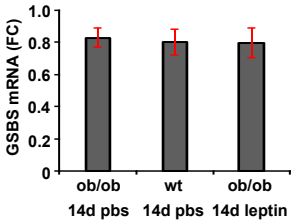

Figure 8. GSBS mRNA expression in hypothalamus is determined by Taqman assays. ΔΔCT analysis is used to calculate the fold change of GSBS mRNA expression in hypothalamus of ob mice treated with leptin for 14 days, ob mice treated with PBS for 14 days and Wt mice treated with PBS 14 days. n=3 mice. All error bars are mean ±SD

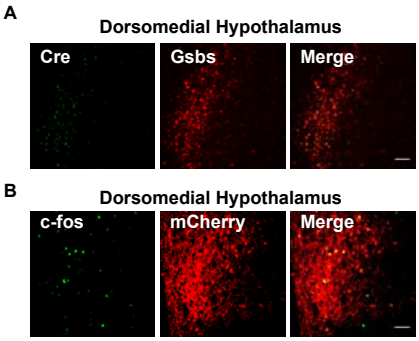

Figure 9. Validation of GSBS-cre mice A. Cre is expressed in DMH^{Gsbs} neurons of Gsbs-Cre mice. B. IHC for c-fos and mCherry in DMH. AAV8-hSyn-DIO-hM3D(Gq)-mCherry is injected into DMH of GSBS-cre mice. 1 hour after i.p. CNO injection, c-fos is detected in mCherry expressing neurons in DMH of GSBS-cre mice.

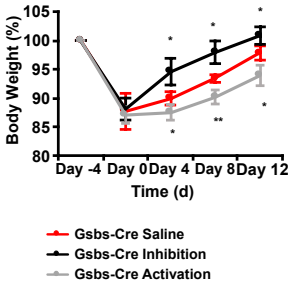

Figure 10. Body weight changes after activating and inhibiting DMHGSBS neurons during scheduled feeding. DREADD induced activation (Treatment: p-value<0.001) and inhibition (Treatment: p-value<0.01) of DMH^{Gsbs} neurons significantly alter food intake during scheduled feeding by i.p injection of CNO 4 hours before food presentation. Two-way RM ANOVA comparing treated and control groups n=5 mice. Scheduled feeding starts at Day 0. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ All error bars are mean ±SD

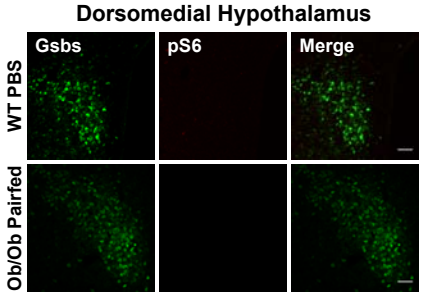

Figure 11. pS6 expression in DMH^{Gsbs} neurons in ob/ob mice is reduced by 14 day pair-feeding. ISH for Gsbs and IHC for pS6 in the Dorsomedial Hypothalamus

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