# mGluR5 functionally interacts with estrogen receptors and is required in the female ventromedial hypothalamus for the regulation of glucose and lipid homeostasis

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**Abstract**

The metabolic syndrome, characterized by dysregulated glycemic control and lipid metabolism, disproportionately affects postmenopausal women and is a major public health concern. The central mechanisms governing peripheral glucose and lipid homeostasis and the role of estrogens in these systems are poorly understood. Previous investigations demonstrated that brain-derived neurotrophic factor (BDNF) in the ventromedial hypothalamus (VMH) is a critical regulator of energy and glucose metabolism. We found that metabotropic glutamate receptor subtype 5 (mGluR5) was markedly reduced in the VMH of mutant mice with global BDNF depletion. Thus, we hypothesize that mGluR5 may be acting within this nucleus to mediate energy and glucose balance. We show that mGluR5 in steroidogenic factor-1 $(SF1)^{+}$ neurons, which are exclusive to the VMH, serves an essential and sex-specific role in glycemic control and lipid metabolism. Accordingly, female but not male mice with mGluR5 deletion in $SF1^{+}$ neurons ($mGluR5^{2L/2L:SF1\text{-}cre}$) exhibit severe glucose intolerance, insulin resistance and triglyceride accumulation in white adipose tissue accompanied by adipocyte hypertrophy. Reduced excitability of $SF1^{+}$ neurons and diminished sympathetic tone was observed exclusively in $mGluR5^{2L/2L:SF1\text{-}cre}$ females and underlie these metabolic alterations. Moreover, sex-specific effects of mGluR5 are explained by its necessity in mediating the beneficial effects of estrogen receptor-α (ERα) signaling on glucose balance control in females. Collectively, these findings inform a novel and critical synergism between mGluR5 and estrogen receptors in the control of VMH neuronal activity, glucose homeostasis and lipid metabolism. These results are significant as

central mechanisms predisposing estrogen deficient postmenopausal women to increased risk of metabolic symptoms remain elusive.

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**List of Commonly Used Abbreviations**

**2-DG,** 2-deoxyglucose

**Arc,** arcuate nucleus

**AgRP,** agouti-related protein

**AMPA,** α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid

**ATGL,** adipose triglyceride lipase

**BAT,** brown adipose tissue

**BDNF,** brain-derived neurotropic factor

**CCK,** cholecystokinin

**CNS,** central nervous system

**CREB,** calcium responsive element binding protein

**DIO,** diet-induced obesity

**DMH,** dorsomedial hypothalamus

**E2,** 17β-estradiol

**ER,** estrogen receptor

**G-6-Pase,** glucose-6-phosphatase

**GABA,** γ-amino-butyric acid

**GPCR,** G-protein coupled receptor

**GE and GI,** Glucose-excited and glucose-inhibited

**HFD,** high fat diet

**HSL,** hormone sensitive lipase

**ICV,** intracerebroventricular

**$K_{ATP,}$** ATP-sensitive potassium channels

**LH,** lateral hypothalamus

**LTD,** long-term depression

**LTP,** long-term potentiation

**MAPK,** mitogen-activated protein kinase

**MCH,** melanin-concentrating hormone

**MC4R,** melanocortin receptor 4

**mGluR#,** metabotropic glutamate receptor subtype #

**NMDA,** N-methyl-D-aspartic acid

**NPY,** neuropeptide Y

**OVX,** ovariectomized

**$p75^{NTR}$,** pan-neurotrophin receptor 75

**PEPCK,** phosphoenolpyruvate carboxykinase

**PI3K,** phosphoinositide-3-kinase

**PLC,** phospholipase C

**POMC,** pro-opiomelanocortin

**PVN,** paraventricular nucleus of the hypothalamus

**SC,** standard chow

**SNS**, sympathetic nervous system

**Trk,** tropomyosin-related kinase

**WAT,** white adipose tissue

**VMH,** ventromedial hypothalamus

# Chapter 1: Introduction

Energy, glucose and lipid homeostasis are tightly controlled by the central nervous system (CNS), and disruptions in this equilibrium leads to obesity and metabolic diseases such as type 2 diabetes and insulin resistance. Insight as to how the brain modulates energy, glucose and lipid control is paramount for developing effective therapies to treat these prevalent and debilitating metabolic diseases. Within the brain, the hypothalamus is a critical region that integrates signals conveying energy availability from the periphery such as glucose, leptin, insulin, gut peptides and hormones. The responsiveness of the hypothalamus to these signals mediates necessary changes in food intake, energy expenditure and glucose and lipid metabolism. These physiological effects are mediated, in part, through activation of the sympathetic nervous system (SNS) and outputs onto metabolic peripheral tissues, including the liver, white adipose tissue and skeletal muscle. Within the hypothalamus, the ventromedial region (VMH) is a sexually dimorphic region known to critically regulate these metabolic processes.

Brain-derived neurotrophic factor (BDNF), a neurotrophic protein that promotes neuronal survival and synaptic plasticity, is a required factor within the VMH for the regulation of energy and glucose homeostasis, although the mechanisms of action are not entirely understood. Our research, presented in Chapter 3, identified metabotropic glutamate receptor subtype 5 (mGluR5) as a novel factor that may be acting downstream of BDNF to facilitate energy and glucose balance control. Considering the known roles mGluR5 plays influencing neuronal functioning and synaptic plasticity in other brain regions, we examined whether mGluR5 is a requisite factor within a subset of VMH neurons expressing steroidogenic factor-1 (SF1) in energy, glucose and lipid homeostasis. We discovered that mGluR5 action in SF1 neurons is critical for glycemic control and

lipid balance, exclusively in female mice. Therefore, we investigated the role of estrogenic signaling and specific estrogen receptors in the VMH as a mechanism by which mGluR5 facilitates these metabolic effects. In this chapter, we review the important and relevant hypothalamic circuits, as well as the role of BDNF, mGluR5 and estrogenic signaling pathways in the modulation of synaptic activity and energy, glucose and lipid homeostasis.

## Chapter 1.1 Hypothalamic control of energy and glucose homeostasis

Perturbed hypothalamic responses to peripheral nutritional cues can precipitate obesity, diabetes, insulin resistance and other metabolic-related diseases. Several hypothalamic nuclei play important regulatory roles in these homeostatic behavioral and physiological modifications, including the arcuate nucleus (Arc), dorsomedial hypothalamus (DMH), paraventricular nucleus (PVN), lateral hypothalamus (LH) and ventromedial hypothalamus (VMH). The convergence of signals within the hypothalamus and activation of discrete populations of cells within these nuclei is important for the maintenance of homeostatic control of energy, glucose and lipid balance. Further, the connectivity of hypothalamic nuclei to the brainstem and periphery is critical for the autonomic control of physiological and behavioral responses.

### *Arcuate Nucleus*

The arcuate nucleus (Arc) is a well-studied population of heterogeneous cells located in the mediobasal region of the hypothalamus. It is considered a primary nutrient-sensing nucleus based on its close proximity to the third ventricle and median

eminence, both structures with little to no blood brain barrier. The Arc contains two cell populations that directly receive peripheral metabolic signals from the bloodstream and that have opposing effects on feeding behavior and energy balance and glycemic control.

Anorexigenic neurons within the Arc co-express pro-opiomelanocortin (POMC) and cocaine-amphetamine-related transcript (CART). Activation of POMC neurons leads to the secretion of melanocortin peptides, including adrenocorticotropic hormone (ACTH) and α, β and γ-melanocyte stimulating hormone (MSH), which are derived from post-translational modifications of POMC. These POMC-derived peptides activate five G protein-coupled melanocortin receptors (MC1-5R) to potently decrease food intake and increase energy expenditure [1, 2]. MC3R and MC4R are broadly expressed in the CNS and MC3R is restricted to neurons in the Arc [3]. The role of MC4R specifically as a critical regulator of energy and glucose homeostasis has been well established. Accordingly, central administration of MC4R agonists promotes satiety and increase energy expenditure, leading to weight loss. Conversely, MC4R antagonists increase food intake and decrease energy expenditure, leading to body weight gain [4, 5].

The Arc also contains orexigenic neurons that co-express agouti-related protein (AgRP) and neuropeptide Y (NPY), and increase feeding, reduce energy expenditure and promote body weight gain [6, 7]. Indeed, selective ablation of AgRP neurons leads to decreased feeding and starvation in mice [8]. AgRP was first discovered as an inverse agonist for MC3R and MC4R, suggesting that, similar to POMC neurons, this cell population regulates energy and glucose balance through the melanocortin signaling system. However, deletion of AgRP induced anorexia in $A^y$ mice, a model in which the melanocortin receptors are already inhibited [9], suggests that AgRP neurons regulate

feeding behaviors independently of melanocortin signaling. Later studies expanded on this finding by showing that AgRP/NPY neurons exert tonic GABAergic inhibition onto POMC neurons [10]. Accordingly, simultaneous photoactivation of POMC and AgRP neurons results in a rapid increase in feeding [11], indicating that GABAergic control of POMC neurons by AgRP neurons is an important mechanism regulating energy balance in the Arc. POMC/CART and AgRP/NPY neurons collectively project to several other hypothalamic nuclei including the PVN, ventromedial, dorsomedial and lateral hypothalamus, and to regions outside the hypothalamus [12].

Several important signaling mechanisms controlling glucose balance in AgRP and POMC neurons have also been identified. For example, selectively re-expressing the leptin receptor in the Arc or within POMC neurons robustly restores glucose tolerance and insulin sensitivity in leptin receptor-deficient (*db/db*) mice with modest effects on body weight [13, 14]. Insulin action within the Arc is also important for glucose balance, as mice lacking leptin and insulin receptors specifically in POMC neurons display systemic insulin resistance, an effect that is distinct from the deletion of either receptor alone [15]. Insulin also signals in AgRP neurons through the PI3K pathway and $K_{ATP}$ channels to impact hepatic glucose production [16]. These studies collectively demonstrate that leptin and insulin signaling through POMC and AgRP neurons in the Arc is important for the maintenance of glucose homeostasis.

In total, the critical role of the Arc in mediating energy and glucose homeostasis is highlighted by the location of these antagonistic neuronal cell types next to the blood brain barrier, their functional interactions with each other and the melanocortin system, and their projections to other important brain regions that mediate metabolic balance.

***Dorsomedial hypothalamus***

The importance of the dorsomedial hypothalamus (DMH) in regulating energy and glucose balance was discovered when targeted lesions to this region resulted in hyperphagia and altered feeding responses to exogenous glucose and insulin [17, 18]. It has since been linked to a wide variety of metabolic functions including ingestive behavior, body weight, thermogenesis, circadian rhythms, cardiovascular function and energy expenditure [19-22], partially through its high connectivity with other hypothalamic nuclei including the Arc, PVN, VMH, LH and dorsal vagal complex (DVC) in the brainstem [23]. However, the molecular mechanisms mediating effects on energy and glucose homeostasis have been relatively understudied compared to other hypothalamic nuclei. Like the Arc, NPY is also expressed in the DMH, and NPY knockdown in this region improved insulin sensitivity, glucose tolerance and prevented high fat diet (HFD)-induced hyperglycemia and hyperinsulinemia [24], indicating an important role of NPY DMH in the regulation of glucose homeostasis. The DMH also acts as an important relay center for the central actions of leptin on thyrotropin-releasing hormone (TRH)-expressing PVN neurons, which play a role in glucose balance control [25]. Taken together, these findings indicate factors expressed within the DMH, such as NPY, and the complex connectivity of the DMH with other brain regions is important for energy and glucose balance control.

***Paraventricular nucleus***

The paraventricular nucleus (PVN) lies above the third ventricle, where it perceives and responds to a variety of signals related to energy status. Accordingly, the

PVN contains glucose-sensing neurons that modify their activity in response to changes in peripheral glucose [26]. The PVN shares reciprocal intra-hypothalamic connectivity with the DMH, VMH and LH and is a major integration site for leptin and melanocortin signaling from AgRP/NPY and POMC neurons from the Arc [1]. The PVN regulates peripheral lipid mobilization through direct neuronal projections to peripheral WAT, and PVN lesions lead to increased adiposity [27]. Further, PVN efferent fibers extend to autonomic centers of the brainstem to control metabolic functions in peripheral tissues, such as pancreatic insulin secretion, lipid accumulation in adipocytes, thermogenesis and glucose uptake [28-30].

The PVN is also an important site of AMP-activated protein kinase (AMPK) activity. AMPK is an important signal of energy status, as low energy reserves increases cellular AMPK activity, leading to fatty acid oxidation and the inhibition of lipogenesis and gluconeogenesis [31]. In the PVN and VMH, AMPK activity is increased by insulin-induced hypoglycemia and reduced by peripheral and intracerebroventricular (ICV) glucose injections [32, 33]. Thus, the PVN is also involved in the counterregulatory response to hypoglycemia. Collectively, these studies demonstrate that the PVN plays a role in central and peripheral functions impacting energy, glucose and lipid homeostasis.

***Lateral hypothalamus***

The lateral hypothalamus (LH) is classically considered a “feeding center,” as lesions in this region suppress food and water intake [34, 35] and electrical activation elicits voracious feeding in animal models [36]. The LH is also sensitive to glucose levels and contains glucose-sensing neurons [26]. The complex and coordinated

regulation of energy and glucose balance by the LH is further exemplified by its enrichment of both glutamatergic [37] and GABAergic [38] neuronal markers and the expression of two important neuropeptides involved in feeding and metabolic regulation: orexin/hypocretin and melanin-concentrating hormone (MCH) [39].

Orexin/hypocretin-releasing neurons are exclusively expressed in the LH and are inhibited by glucose [40]. These neurons regulate feeding behaviors, as injections of this peptide into the LH increase feeding, while chemical antagonism decreases food consumption [41, 42]. Additionally, studies in orexin-knockout mice reveal an age-related development of glucose intolerance and insulin resistance in both non-obese male mice and mildly obese female mice on a standard chow (SC) diet, indicating that these neurons regulate glucose homeostasis by body weight-independent mechanisms [43].

MCH-producing cells are also glucose-sensing and predominantly expressed in the LH [44]. Genetic studies in rodents revealed that overexpression of MCH results in hyperphagia and obesity, and mice lacking MCH are hypophagic and lean [45, 46]. However, opposite of orexin neurons, MCH neurons in the LH are excited by glucose [40]. Importantly, MCH neurons critically regulate peripheral glucose metabolism. A previous study demonstrated that the excitatory response to glucose by MCH neurons is mediated by potassium-sensitive ATP ($K_{ATP}$) channels and impairing the glucose-sensing capabilities of these neurons leads to severely impaired responses to a glucose challenge, with no changes in food intake or body weight [47]. Thus, MCH neurons in the LH are important regulators of glycemic balance. The collective literature highlights both the required role of the LH in regulating feeding behaviors and glucose metabolism.

***Ventromedial hypothalamus***

The ventromedial hypothalamus (VMH), the focus of this thesis, is located adjacent to the third ventricle and superior to the Arc. This elliptical-shaped nucleus is surrounded by a dense layer of fibers and consists mostly of glutamatergic neurons that form reciprocal connections with other VMH neurons. Despite the fibrous barrier, the VMH also sends and receives projections from several surrounding hypothalamic nuclei, including the Arc, LH and PVN, as well as far reaching brain regions like the amygdala [48]. The ventrolateral portion of the VMH sends excitatory projections to the Arc, where they synapse onto and regulate POMC neuronal excitability in an energy status-dependent manner [49]. The input and output connections of the VMH are critically important in the regulation of energy, glucose and lipid balance, and several additional VMH-mediated behaviors.

The VMH is historically considered a "satiety center" as early anatomical studies demonstrated that lesions to this nucleus resulted in hyperphagia and severe obesity [34, 50] and electrical stimulation reduced food intake [51]. Although robust and ravenous feeding was the most obvious effect, hyperglycemia was also a consequence of VMH lesions. It is now appreciated that the VMH also exerts control over glucose homeostasis independent of its role regulating feeding behaviors. The VMH is a key glucose sensing region, as intravenous glucose administration increases VMH neuronal activity [52]. Additionally, injection of the glucose analog, 2-deoxyglucose (2-DG), into the VMH increases plasma glucose levels [53], demonstrating that the activity of VMH neurons in response to sensed glucose influences peripheral physiological processes required to

maintain glycemic control. However, the molecular mechanisms underlying the body weight-independent effects on glucose homeostasis are poorly understood.

Two types of VMH neurons sense changes in glucose levels: those that increase (glucose-excited; GE) or decrease (glucose-inhibited; GI) their action potential firing in response to rising glucose levels. Similar to the glucose-sensing mechanisms of pancreatic β-cells, GE neurons utilize $K_{ATP}$ channels to regulate VMH neuronal excitability [54]. Accordingly, the concentration-response curve for $K_{ATP}$ channel currents and action potential frequency of VMH GE neurons reveals a linear-like relationship for glucose concentration and neuronal activity [55]. In contrast, GI neurons react to sensed glucose in a completely distinct manner. GI neurons respond to decreases in glucose through activation of an AMPK-dependent pathway, leading to nitric oxide (NO) production, closure of chloride channels and neuronal hyperpolarization [56]. While identification of GE and GI neurons is accessible by electrophysiological analysis, molecular markers for these cell types have not been developed.

In addition to glucose, many secreted peptides also act within the VMH to regulate neuronal activity and glycemic control. Notably, both leptin and insulin play critical roles in the endocrine feedback loop between the VMH and peripheral energy stores. For example, several studies have shown that hyperinsulinemia and adipocyte hypertrophy in diabetic humans and animal models is reversed by leptin replacement with menial effects on food intake and body weights [57-59], highlighting the importance of leptin in the regulation of glucose and lipid balance. Further, ICV injection of leptin reduces serum insulin levels and increases insulin sensitivity in diabetic rats before changes in body weight are observed [60]. In the VMH, leptin directly activates SF1

neurons [61], and VMH specific-deletion of the leptin receptor leads to increased adiposity, hepatic steatosis, dyslipidemia and hyperleptinemia even when these mutant mice were weight-matched to controls [62].

Insulin action in the VMH is also critical for glucose homeostasis. VMH-specific injections of insulin decreased glucagon secretion by pancreatic-α cells [63] and hepatic glucose production through a reduction in glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK) levels [64]. These studies demonstrated that insulin action in the VMH regulates peripheral pancreatic and hepatic metabolic processes. Further, deletion of the insulin receptor in SF1 neurons activated PI3K and reduced firing frequency in these cells through activation of $K_{ATP}$ channels, preventing these mice from diet induced leptin resistance, weight gain, adiposity and impaired glucose tolerance [65]. Thus, insulin action in the VMH modulates peripheral metabolic function through regulation of neuronal activity.

Not only is the VMH a key region regulating glucose metabolism, it also plays a chief role mediating counterregulatory responses to glucoprivation. Previous studies demonstrated that rats with chemical VMH lesions exhibited impaired glucagon, epinephrine and norepinephrine responses to hypoglycemia [66]. These hormones normally facilitate hepatic glucose production as a mechanism to restore adequate glucose levels. In contrast, infusion of 2-DG into the VMH resulted in a rapid increase in plasma glucose, glucagon, epinephrine and norepinephrine [53].

The VMH coordinates and regulates energy and glucose balance via control of the SNS. Indeed, electrical stimulation of the VMH activates the SNS, leading to increased lipolysis in peripheral tissues [67], while rats with VMH lesions exhibit reduced SNS

activity as measured by fat mobilization, reduced sympathetic tone onto peripheral tissues, reduced thermogenesis in BAT and reduced catecholamine turnover [68-72]. Therefore, the connectivity of the VMH to the SNS is an important mechanism by which counterregulatory responses to glucoprivation are generated and energy, glucose, and lipid homeostasis is achieved. However, the cellular mechanisms by which discrete populations of VMH cells control these peripheral processes remain elusive.

In addition to leptin and insulin action, the VMH is also a critical site of brain-derived neurotrophic factor (BDNF) action, as selective deletion of this neurotrophin from the VMH results in hyperphagia, obesity, and metabolic dysfunction [73]. How BDNF regulates energy and glucose balance in the brain and in the VMH will be discussed in the next section.

The VMH is comprised of various cell types with differential peptide expression and function (Figure 1.1). Several peptide expression patterns have been elucidated including cannabinoid receptor 1 (CB1), pituitary adenylate cyclase activating polypeptide (PACAP), BDNF and cerebellin 1 (Cbln1) which are broadly expressed [74], and estrogen receptor α (ERα) and progesterone receptor which are limited to the ventrolateral portion of the VMH [75].

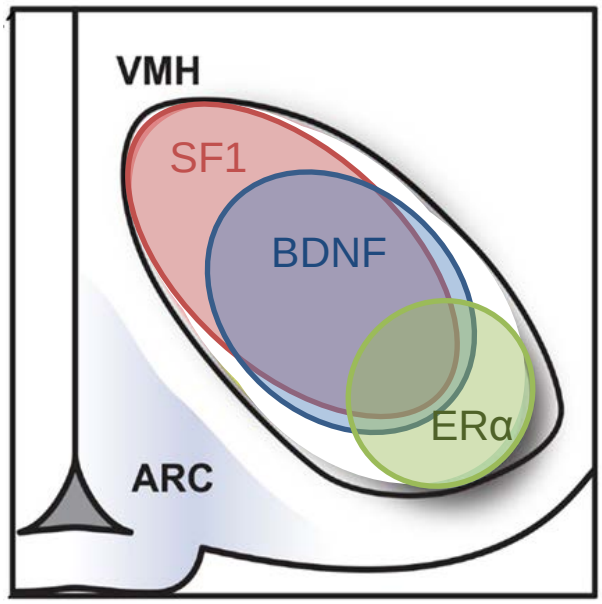


**Figure 1.1 Pattern of gene expressions in the VMH.** Schematic diagram showing the pattern of important genes expressed in the VMH. ARC, arcuate nucleus; VMH, ventromedial hypothalamus; BDNF, brain-derived neurotrophic factor; ERα, estrogen receptor-α; SF1, steroidogenic factor-1. Figure modified from Choi *et al.* (2013) [76].

Of note, it is well established that the VMH is a sexually dimorphic nucleus, and gene expression patterns vary between females and males. For example, ERα expression is significantly higher in female VMH compared to males [77-79]. ERβ, although expressed less abundantly than ERα, is also increased in female VMH compared to males, particularly during postnatal development [80]. Further, sexually dimorphic differences in VMH size, synaptic structural organization and cellular makeup have also been reported [81-83]. As expected, these sexual dimorphic differences confer different VMH-mediated functions and behaviors in females versus males, such as reproduction and social behaviors [84]. Although genetic manipulation of estrogen receptors in the VMH are associated with some sexually dimorphic differences in energy, glucose and lipid balance, additional VMH cellular targets regulating these physiologically processes in coordination with estrogenic signaling remain elusive.

***Steroidogenic factor 1***

Of the many VMH-enriched genes, steroidogenic factor 1 (SF1; encoded by *NR5A1*) is the only one expressed exclusively in the VMH. SF1 is a transcription factor that is required for the differentiation and organization of the VMH during development as well as several physiological processes in the mature brain, including energy, glucose and lipid homeostasis [74, 85, 86]. The generation of germline SF1 knockout mice resulted in neonatal lethality due to adrenal insufficiency [85]. However when wild-type adrenal glands were transplanted into these knockout mice, the mice survived but developed severe obesity, indicating a critical role of SF1 in the regulation of energy balance. To circumvent adrenal issues, a new model was developed by crossing floxed SF1 mice with mice expressing cre recombinase under the CamKII promoter, thus deleting SF1 post-embryonically. These mice also display metabolic symptoms, confirming SF1's role in modulating energy and glucose homeostasis [87].

Because SF1 cells are exclusive to the VMH, the generation of SF1-cre transgenic lines greatly advanced the understanding of the genes functioning within the VMH. Using this approach, studies showed that deletion of the leptin receptor in SF1 neurons resulted in increased weight gain and decreased energy expenditure [61]. Furthermore, SF1-specific insulin receptor knockout mice showed improved glucose metabolism and resistance to diet-induced obesity (DIO) [65]. The deletion of several other genes such as p110α (a subunit of PI3K), FOXO1, SOCS3 and SIRT1 also resulted in dysregulated energy, glucose and lipid balance control [76], confirming the importance of SF1 neurons and molecules they express in mediating these metabolic effects. The cumulative data provide inarguable evidence of the essential role of the VMH and in $SF1^{+}$ neurons in the

whole-body regulation of energy balance, glycemic control and lipid metabolism. However, there are still many unresolved questions regarding how and what biological factors are involved in the development of obesity and diabetes. This is particularly important for postmenopausal women at increased risk for metabolic disease. Further investigations of cellular mechanisms facilitating differences in energy, glucose and lipid balance between women and men are necessary for the development of targeted therapeutic approaches to treat the debilitating effects of metabolic diseases.

## Chapter 1.2 Neurotrophins and the role of BDNF in energy, glucose and lipid homeostasis

Neurotrophins are a family of growth factors that promote the survival, differentiation and function of neurons. They are secreted in the central and peripheral nervous system and are critical for neuronal maintenance, particularly during development, but also in the mature brain. The family encompasses four structurally similar members: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT3) and neurotrophin-4 (NT4). Mature neurotrophins exist as noncovalently bound homodimers that interact with four receptors, $p75^{NTR,}$ TrkA, TrkB and TrkC to mediate downstream signaling pathways [88]. Relevant to this thesis, I will focus on BDNF and its signaling through TrkB and, to a lesser extent, $p75^{NTR}$, to mediate energy, glucose and lipid balance control. The physiological importance of NGF, NT3 and NT4 can be explored elsewhere [89].

***BDNF structure, function and signaling***

BDNF was first discovered as a promoting factor of neuronal survival [90], and its multifaceted role in mediating neuronal growth, differentiation, survival and synaptic plasticity has since been extensively studied. BDNF is a highly conserved neurotrophic factor expressed in limiting amounts in the developing and mature brain. *Bdnf* maps to chromosome 11p14.1 in humans and chromosome 2qE3 in mice and is comprised of nine exons spanning 52.3 kb. In both humans and mice, *Bdnf* appears to be comprised of at least eight homologous exons, each driven by a separate promoter, a common coding sequence and a 3' UTR exon [91]. *Bdnf* transcription is complexly controlled in specific tissues and by a diverse array of stimuli through the activation of these different promoters [92]. However, the functional importance of the many promoters of *Bdnf* that all encode the same mature BDNF protein is incompletely understood. Pertinent to this thesis, the regulation of BDNF expression by stimuli related to energy and glucose balance will be discussed in a later section.

Neurotrophins, including BDNF, are produced as pro-isoforms that are cleaved to release mature ligands that activate one or more of the tropomyosin-related kinase (Trk) receptors. Binding of ligands to Trk receptors results in dimerization, transphosphorylation and activation of several downstream signaling pathways. BDNF exists in the proBDNF and mature form, and the physiological functions of proBDNF have been controversial. However, recent studies suggest that proBDNF is indeed produced and released by neurons and, in the hippocampus, is able to promote long-term depression (LTD) through the activation of $p75^{NTR}$ receptor [93, 94]. Mature BDNF binding to full-length TrkB leads to activation of the signaling molecules phospholipase

C-γ (PLC-γ), mitogen-activated protein kinase (MAPK) and PI3K. These molecular pathways promote neuronal differentiation, survival and synaptic plasticity [95]. Although most physiological functions of BDNF are attributed to its association with full-length TrkB, a truncated TrkB receptor (TrkB.T1) is also centrally expressed. TrkB.T1 was originally considered a negative modulator of BDNF and TrkB receptor expression and function [96, 97], but recent studies have identified its role in astrocytes as a modulator of calcium signaling and thus, a potential regulator of neuronal activity [98]. Less is known about the physiological significance of mature BDNF binding to $p75^{NTR}$. In general, studies have shown ligand binding to $p75^{NTR}$ activates signal transduction pathways, including nuclear factor-κB (NF-κB), Jun kinase and sphingo-myelin hydrolysis. Further, $p75^{NTR}$ activation can lead to programmed cell apoptosis [99, 100], a mechanism very distinct from the classically protective effects of BDNF binding to TrkB.

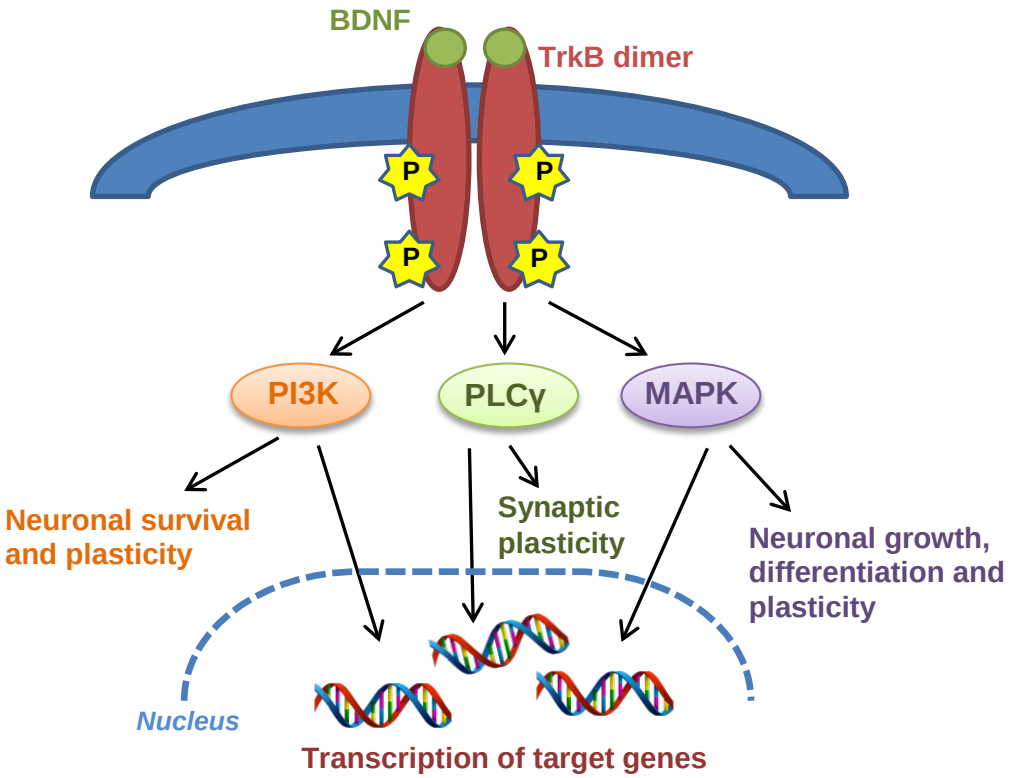


**Figure 1.2 TrkB receptors mediate the biological responses of BDNF through distinct signal transduction pathways.** BDNF binding to its cognate receptor, TrkB, induces receptor dimerization and transphosphorylation which activate PLCγ, PI3K and MAPK signaling cascades. These signaling pathways facilitate synaptic plasticity, survival, growth and differentiation of neurons as well as the transcription of several target genes.

***Central BDNF/TrkB signaling regulates energy and glucose homeostasis***

As mentioned previously, energy and glucose homeostasis is a delicate balance between caloric intake, energy expenditure and glucose utilization, which is crucial for survival. Regulation of energy and glucose homeostasis is influenced by short-term metabolic signals, such as glucose and insulin levels, and long-term energy storage signals, such as leptin secretion from adipose stores. These signals converge in the brain where they are processed in order to mount an appropriate behavioral or physiological response. BDNF is one such factor that facilitates the responsiveness of the brain to

peripheral energy cues, and disruptions in BDNF signaling and function result in metabolic imbalances, such as obesity and diabetes.

BDNF was first implicated in the control of feeding behavior and body weight regulation by early rodent studies showing that chronic ICV delivery of BDNF decreased body weight gain [102, 103]. Moreover, the generation of heterozygous BDNF +/- mutant mice revealed a hyperphagic and obese phenotype [104], and mutant mice carrying hypomorphic TrkB alleles, which express only 25% of normal TrkB levels, are also hyperphagic [105]. BDNF mutant mice with global BDNF depletion ($BDNF^{2L/2L:Ck\text{-}cre}$) are hyperphagic, obese and develop severe metabolic symptoms such as leptin and insulin resistance, dyslipidemia and hyperglycemia. These mice are also hyperactive and hyperaggressive [106]. Of note, several metabolic deficits induced by diminished BDNF function are more severe in female $BDNF^{2L/2L:Ck\text{-}cre}$ mutants compared to male BDNF mutant mice. Specifically, female $BDNF^{2L/2L:Ck\text{-}cre}$ mutants gained more weight (150% compared to 80% for males), had increased abdominal fat pads (10-fold increase compared to 2- to 3-fold for males) and more pronounced changes in bone microarchitecture [106, 107]. These studies raise the possibility that BDNF may be facilitating sex-specific effects on metabolic function, but literature confirming this notion is lacking.

Importantly, several investigations indicate that BDNF regulates glycemic control in rodent models independent of changes in food intake and body weight. For example, BDNF treatment in leptin receptor-deficient (*db/db)* mice decreased nonfasted glucose levels, whereas pair-feeding *db/db* mice to levels of BDNF-treated mice had no effect on glucose levels [108]. Similar results were observed in a DIO rodent model, where BDNF

administration reduced food intake and improved glucose tolerance in DIO mice, but had no effect on glucose tolerance when DIO mice were pair-fed to levels of BDNF-treated mice [109]. These studies suggest that at least some of the anti-diabetic effects of BDNF are independent of its ability to reduce food intake. Mechanistically, it was demonstrated that central BDNF administration reduces hepatic glucose production through the inhibition of the gluconeogenic genes *G-6-Pase* and *PEPCK*. However, BDNF administration does not improve glucose uptake in skeletal muscle or BAT in insulin-deficient mice [110], indicating an interplay between BDNF and insulin on glycemic control in peripheral tissues. In total, these and other studies confirm that BDNF function is important for the whole-body regulation of energy, glucose and lipid homeostasis.

***Clinical consequences of impaired BDNF/TrkB function in humans***

BDNF functioning has substantial clinical implications, as genetic mutations and disruptions in BDNF/TrkB signaling in humans are associated with energy and glucose balance dysregulation. For example, an association meta-analysis of nearly 250,000 individuals identified *Bdnf* as a top gene loci linked to obesity susceptibility in humans [111]. Moreover, an 8-year-old female with monoallelic BDNF expression due to a de novo chromosomal inversion and an individual with the Y722C de novo missense mutation in *TrkB* both presented with clinical signs of hyperphagia and obesity [112, 113]. Further evidence comes from investigations of individuals afflicted with Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome. WAGR patients with large truncations in chromosome 11, which encompasses the BDNF gene, were obese by the age of 10, whereas only 20% of WAGR patients with intact

BDNF alleles developed adolescent obesity [114]. In addition, the prevalent BdnfVal66Met polymorphism, which impedes normal BDNF secretion and signaling [115], is associated with higher body mass index (BMI) in humans [111, 116-119].

To a lesser extent, BDNF dysfunction in humans has been associated with impaired glucose metabolism. For example, in a study of patients with Type 2 Diabetes (T2D), serum BDNF levels were significantly lower in male and female T2D patients compared to controls, independent of BMI. Further, in T2D patients, BDNF levels were significantly higher in females than in males [120]. The significance of estrogen modulation on BDNF expression will be discussed in a later section. An additional study found that plasma BDNF levels were inversely associated with fasting plasma glucose levels. Interestingly, this study also measured the amount of cerebral BDNF release (defined as the jugular to arterial concentration difference of BDNF) and found that in the hyperglycemic state, reduced levels of BDNF are produced and released from the brain into the bloodstream [121].

Collectively, these clinical investigations highlight the necessity of a thorough understanding of the role BDNF plays in energy and glucose regulation so that therapies to treat obesity and associated metabolic disturbances such as T2D can be developed.

***BDNF action within the VMH***

An interesting study revealed that BDNF action on TrkB differentially regulates energy and glucose homeostasis in the brain and periphery. Specifically, it was demonstrated that central BDNF administration reduced food intake and body weight, whereas BDNF action on TrkB in the periphery induced increased feeding, body weight

gain, adiposity and hyperleptinemia [122]. Thus, subsequent studies focused on how BDNF regulates energy and glucose in discrete sub-regions in the brain. Accordingly, BDNF expression within the hypothalamus is most abundant in the VMH. To determine whether this nucleus is a required substrate for the appetite-suppressing effects of BDNF, Unger *et al.*, (2007) selectively deleted *Bdnf* in the adult VMH of mice. This selective depletion of BDNF produced hyperphagia, obesity, insulin resistance and hyperlipidemia [73], demonstrating that the VMH is an important site of BDNF action. Additional evidence comes from studies showing that BDNF infusion into the VMH of male rats reduced food intake and decreased body weights under SC conditions [123]. Furthermore, BDNF and TrkB expression in the VMH is dynamically regulated by energy status. Indeed, prolonged fasting reduces BDNF expression whereas glucose administration increases BDNF and TrkB transcript content in this region [73] [105].

BDNF action in the VMH also influences glycemic control via mechanisms independent of reduced food intake. A previous study reported that chronic BDNF administration to the VMH reduced food intake and impaired glucose intolerance in DIO mice, and pair-feeding DIO mice to levels consumed by BDNF-treated mice did not improve glucose tolerance [124]. Thus, BDNF's antidiabetic effect on glucose balance is not due to reduced food intake. Further, BDNF microinjection into the VMH also lowered fasting blood glucose levels in an insulin-deficient diabetic mouse model independent of changes in feeding behaviors [110].

Mechanisms mediating BDNF's satiety and antidiabetic effects in the VMH remain unclear. However, it was reported that $BDNF^{2L/2L:Ck\text{-}cre}$ mice exhibit reduced spontaneous excitatory postsynaptic currents (sEPSCs) in VMH cells, indicating that

impaired BDNF function is associated with reduced excitatory drive onto anorexigenic VMH cells [125]. Thus, hypoactivity of VMH neurons may be driving the metabolic alterations in $BDNF^{2L/2L:Ck\text{-}cre}$ mice. Work described in this thesis investigated the role of metabotropic glutamate receptor subtype 5 (mGluR5) as a downstream effector of BDNF in the VMH. Specifically, this thesis investigates the possibility that perturbed function of mGluR5 in the VMH reduces the excitability of these anorexigenic neurons, ultimately regulating energy, glucose and lipid homeostasis.

## Chapter 1.3 Metabotropic glutamate receptors and regulation of synaptic activity by mGluR5

Transcriptional analysis of cells laser-captured from the VMH of mice with global central deletion of *Bdnf* and controls performed previously in the Rios laboratory indicated that mGluR5 is significantly downregulated in the BDNF mutant VMH. mGluR5 is abundantly expressed in this hypothalamic region, where it localizes to both neurons and astrocytes that surround synaptic complexes [126]. Considering its reported roles in the regulation of excitatory synaptic plasticity in multiple brain regions [127-129], we hypothesized that mGluR5 serves similar functions in the VMH to mediate energy and glucose balance control downstream of BDNF.

Metabotropic glutamate receptors (mGluRs) are a sub-family of G-protein coupled receptors (GPCRs) that are widely expressed throughout the nervous system, where they are functionally implicated in neural development [130] and act as important regulators of synaptic plasticity in the mature brain [131]. mGluRs modulate synaptic activity by localizing at and around synapses, where they bind glutamate and transmit

signals to an array of downstream signaling partners. As expected, mGluR dysfunction is associated with a wide variety of CNS diseases.

Structurally, GPCRs are seven trans-membrane bound proteins that undergo a conformational change when activated by extracellular ligands, which then promotes several intracellular signaling cascades. The G protein portion of the receptor is composed of a heterotrimeric complex of α, β and γ subunits. When activated, the α-subunit of the G protein exchanges the binding of guanosine 5-diphosphatae (GDP) for guanosine 5-triphosphate (GTP). This exchange activates the subunits and allows them to modulate the function of various nearby effector molecules such as enzymes, ion channels, receptors and transcription factors. When the bound GTP is hydrolyzed back to GDP, the G protein reassembles into an inactive heterotrimer unit [132].

Eight known splice variants of the mGluR family have been identified, each with distinct expression patterns and functions throughout the CNS. These variants are subdivided into three groups based on sequence homology and ligand selectivity. Group 1 mGluRs include mGluR1 and mGluR5, which are expressed in neurons, astrocytes and taste buds. mGluR1 and mGluR5 are predominantly postsynaptically localized and serve excitatory functions via coupling to $G_q/G_{11}$ pathways. Group 2 mGluRs include mGluR2 and mGluR3 and are also widely expressed in neurons and astrocytes, but are predominantly presynaptically localized and serve inhibitory functions through coupling with $G_{i/o}$ pathways. Less is known about group 3 mGluRs 4, 6, 7 and 8, which are expressed both pre- and post-synaptically in the CNS and couple to similar inhibitory signaling cascades as group 2 mGluRs [133, 134]. The complexity of synaptic functions

modulated by mGluRs is increased by alternative splicing, regulation by phosphorylation and protein-protein interactions.

***Group 1 mGluR regulation of synaptic plasticity and physiology***

Group 1 mGluRs couple to $G_q/G_{11}$ and activate PLC, resulting in the generation of 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). This canonical pathway mobilizes calcium from intracellular stores and activates PKC, often leading to cell depolarization and neuronal excitability. However, there are some reported exceptions. For example, group 1 mGluRs can act presynaptically to regulate neurotransmitter release [135]. Group 1 mGluRs influence a wide range of downstream signaling pathways in context-specific manners [132, 136]. Notably, previous studies have demonstrated that group 1 mGluRs regulate synaptic plasticity through short- and long-term (LTP and LTD) mechanisms in several brain regions, including the hippocampus and cortex [127-129]. While mGluR1 and mGluR5 are similar in some functional aspects, they can regulate neuronal activity differently even within the same cell population. For example, in CA1 pyramidal cells of the hippocampus, mGluR1 activation increases somatic calcium transients and induces depolarization, whereas mGluR5 activation in these cells inhibits $K^+$ currents and potentiates NMDA receptor currents [137]. The focus of this thesis will pertain to mGluR5 exclusively.

Accordingly, several mGluR5-mediated mechanisms regulate synaptic plasticity, physiology and transmission in both neurons and astrocytes [138-141]. In neurons, mGluR5 activation mobilizes intracellular $Ca^{2+}$, leading to the initiation of local transcription and the translation of several proteins involved in excitatory synaptic

plasticity [142, 143]. In astrocytes, activation of mGluR5 increases also intracellular $Ca^{2+}$, leading to the release of gliotransmitters, modulation of glutamate uptake and other mechanisms that regulate neuronal circuitry and synaptic activity [141, 144-147].

mGluR5 also functionally interacts with NMDA receptors to form a positive-feedback loop in several brain regions. As such, recordings in striatal slices revealed that application of mGluR5 agonists resulted in a dose-dependent potentiation of NMDA-induced membrane depolarization, an effect that was mimicked by a PKC activator, thus suggesting that mGluR5 positively modulates NMDA responses via PKC activation [148]. Moreover, excitatory synaptic transmission in hippocampal neurons required co-stimulation of mGluR5 and NMDA receptors and downstream mGluR5-mediated $IP_3$ activation and $Ca^{2+}$ release [149]. In the subthalamic nucleus (STN), mGluR5 activation directly depolarized, increased the firing frequency and increased burst-firing activity of STN neurons and potentiated NMDA-evoked currents [150]. Lastly, mGluR5 agonist treatment increased NMDA currents while mGluR5 antagonists decreased NMDA currents in cortical slices [151].

mGluR5 activity alters the morphology and density of dendritic spines in several brain regions. For example, direct activation of mGluR5 via injection of its agonist into the nucleus accumbens decreased the density of dendritic spines [152]. Further, systemic treatment with a negative allosteric modulator of mGluR5 increased dendritic spine density in pyramidal neurons of the rat medial prefrontal cortex [153]. These studies suggest that mGluR5 positively regulates dendritic spine density, consistent with its role as a mediator of excitatory synaptic transmission. mGluR5 also regulates the intrinsic excitability of neurons via regulation of $K^+$ and $Ca^{2+}$ currents [137, 140, 154], which will

be discussed in detail in later sections. Above all, the collective literature demonstrates that mGluR5 is a critical player in mechanisms mediating synaptic function and dendritic spine morphology.

Lastly, like most GPCRs, mGluR5 has several known binding partners that influence its expression and signaling (Figure 1.3). Several studies have shown that Homer, a postsynaptic scaffolding protein, is an integral part of the mGluR5 signaling complex [155-157]. Additionally, Homer-mGluR5 interactions are required for activation of MAPK/ERK and PI3K signaling in the striatum and hippocampus, respectively [158, 159]. Shank, a family of postsynaptic proteins that link NMDA receptors to PSD-95 complexes at the synapse [160], also regulates mGluR5 localization and signaling. Shank clusters mGluR5 at the cell surface and mediates Homer-mGluR5 interactions with PSD-95/guanylate kinase-associated protein (GKAP), potentially influencing the synergistic signaling properties of mGluR5 and NMDA receptors simultaneously [161]. Therefore, the abundance and expression of these scaffolding proteins may play important roles in the signaling capabilities of mGluR5.

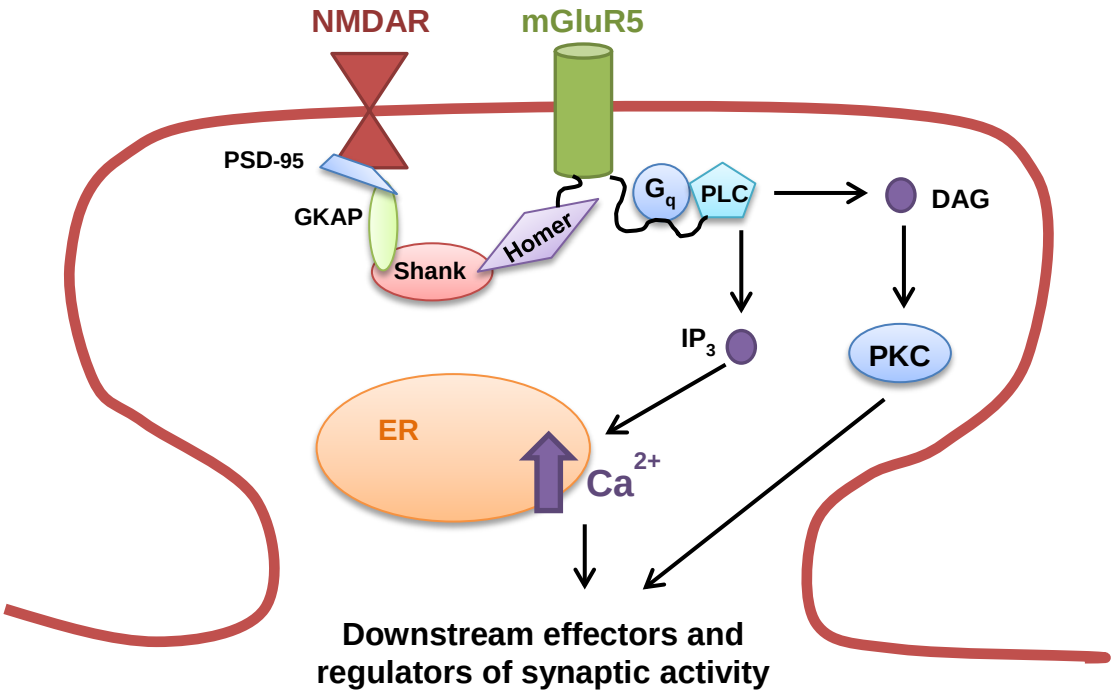


**Figure 1.3 mGluR5 interactions with signaling molecules and scaffolding proteins.** mGluR5 activation by glutamate initiates $G_q$ protein signaling that regulates phospholipase C (PLC) function. Activation of PLC results in the release of the second messengers 1,2-Diacylglycerol (DAG) and $IP_3$. DAG is the physiological activator of protein kinase C (PKC), which in turn activates various downstream intracellular signaling cascades. $IP_3$ binds to intracellular receptors on the endoplasmic reticulum (ER) membrane initiating $Ca^{2+}$ release from intracellular stores into the cytoplasm, generating complex $Ca^{2+}$ concentration-dependent signals, including depolarization and neuronal excitation. mGluR5 also interacts with the ionotropic glutamatergic *N*-methyl-D-aspartate (NMDA) receptors via several scaffolding proteins. For example, Shank interacts with guanylate kinase-associated protein (GKAP), which is associated with NMDA receptor-PSD95 complexes. Shank also interacts with Homer proteins to form multimers that link Shank to mGluR5.

### *mGluR5 dysfunction and neurological disease*

Considering the vast roles that mGluR5 plays in the CNS regulating synaptic activity, it is not surprising that its dysregulation is associated with several neurological disorders [162]. Fragile X Syndrome (FXS), the most commonly inherited form of mental retardation in humans, is caused by a lack of the fragile X mental retardation

protein (FMRP) which regulates mGluR5-dependent protein synthesis. Aberrant and exaggerated protein synthesis by mGluR5 in the absence of FMRP is believed to contribute to the developmental delay and autistic phenotype in FXS patients as well as the structural spine abnormalities in FMRP-knockout mice [163-167]. mGluR5 may also play a role in the pathogenesis of epilepsy. In support, administrations of group 1 mGluR agonists are pro-convulsive [168], and selective mGluR5 antagonists block seizures in rodent models of epilepsy, including an FXS model [169, 170]. Lastly, there is growing interest in the role of mGluR5 in schizophrenia. Several studies have demonstrated that mGluR potentiation of NMDA function may alleviate the cognitive defects associated with schizophrenia [148, 150, 151, 171]. Furthermore, anti-psychotic effects were observed after treatment with mGluR5 agonists and positive allosteric modulators (PAMs) in rat and mouse models of schizophrenia [172, 173].

mGluR5 functioning is also implicated in mood and anxiety disorders, although less is known about the molecular underpinnings in these diseases. Dozens of studies have investigated the role of mGluR5 antagonists as treatments for anxiety-like behaviors in animal models, and all but two studies demonstrated anxiolytic effects [174]. mGluR5 antagonists also show some efficacy in treating animal models of depression, but positive effects are limited and findings are inconclusive in human patients [173]. Investigations of mGluR5 and addiction have also yielded interesting results. mGluR5 is also associated with addiction. As such, Chiamulera *et al.,* (2001) showed that mice lacking the mGluR5 receptor failed to self-administer cocaine, despite normal dopamine levels in the nucleus accumbens following acute injection of the drug [175]. Follow up studies corroborated

that mGluR5 antagonists reduce self-administration of other addictive drugs such as nicotine and ethanol [176].

Notably, evidence linking mGluR5 to the regulation of metabolic function is scarce. One study found that in a baboon model of binge-eating behavior, mGluR5 antagonism decreased candy consumption without altering candy-seeking behavior [177], indicating that mGluR5 may impact the rewarding value of a reinforcing stimuli. With regard to feeding, mGluR5 has been scarcely cited in studies involving food intake. In one study, body weights were monitored for a limited period of 3 days and it was reported that global mGluR5 knockout mice weighed significantly less than control mice [178]. Additionally, a study investigated the effect of systemic injection of a mGluR5 antagonist and found decreased 24 hour food intake in rats [179]. However, these studies lack regional specificity as they examine whole-body effects of mGluR5 antagonism on feeding behavior and measure feeding behavior and body weights for limited amounts of time. Therefore, questions remain regarding the role of mGluR5 in energy and glucose balance control.

More recent studies have examined mGluR5 function specifically in the hypothalamus. For example, mGluR5 expression in the LH has been confirmed with immunostaining studies [126] and has been linked to the regulation of food intake. Accordingly, targeted administration of a group 1 mGluR agonist into the LH of satiated adult rats stimulated feeding, whereas antagonism of mGluR5 in the LH suppressed feeding [180]. Effects on glucose metabolism were not investigated in this study. Additionally, it was determined that mGluR5 upregulation increased the excitability of PVN sympathetic neurons through NMDAR-mediated mechanisms in a rat model of

hypertension [181], indicating that mGluR5 regulates excitatory synaptic activity within the PVN. These studies support the role of mGluR5 as a regulator of energy balance and neuronal excitability within the hypothalamus. However, the role of mGluR5 in the VMH has not been investigated.

***Sex-specific effects of central mGluR5 activity***

Several studies indicate sex-specific central effects of mGluR5. For example, expression levels of mGluR5 in the prefrontal cortex were elevated in adult males, but not females, exposed to a prenatal chronic mild stress paradigm [182]. Furthermore, activation of group 1 mGluRs induced anxiolytic behavior in female rats, but pro-conflict behavior in male rats in a conditioned conflict-based anxiety model [183], suggesting that mGluR5 may be involved in the increased susceptibility of women compared to men to develop anxiety disorders.

Sex hormones are often associated with sex-specific behaviors and physiology. Pertinent to this thesis, both estrogen receptors [184, 185] and mGluR5 [126, 186] are localized near dendritic spines and membrane-localized estrogen receptors functionally interact with mGluR5 in the striatum [187]. Thus, the interaction of mGluR5 and ERα is particularly important for sex-specific synaptic functioning in females. In support, previous studies demonstrated that ERα required mGluR5 for activation of CREB phosphorylation in female striatal neurons, likely through PLC and MAPK signaling pathways [188, 189]. Additionally, estradiol-induced changes in synaptic density in the nucleus accumbens in female mice were blocked by treatment with a mGluR5 antagonist, whereas mGluR5 antagonist treatment alone had no effect [190], suggesting that mGluR5

and estrogenic signaling coordinate to regulate dendritic spine density in females. Additional studies have linked these two important synaptic regulators to female-specific behaviors. For example, estradiol-induced spinogenesis in the Arc and female sexual lordosis responses were dependent upon group 1 mGluR activation [191]. Further, mGluR5 activation was necessary for the estradiol-mediated enhanced responses to cocaine, whereas direct mGluR5 activation was insufficient to mimic the female response to estradiol [192].

In total, these studies reveal an important and synergistic action of estradiol and mGluR5 in the regulation of synaptic physiology and dimorphic behaviors in females. However, the molecular details of their interactions in the VMH and whether they interact and coordinate the regulation of energy, glucose or lipid homeostasis have not yet been studied.

### Chapter 1.4 Estradiol-mediated effects on energy, glucose and lipid balance control

In premenopausal women, 17β-estradiol (E2) is the main circulating estrogen produced by the ovaries. It is generated by the aromatization of androstenedione to estrone, which is converted to E2. Estradiol is the main facilitator of menstrual cycles in women, and the loss of E2 production by the ovaries in postmenopausal women is associated with an increased risk of diabetes, insulin resistance, cardiovascular disease and osteoporosis [193, 194]. Estradiol plays a fundamental role in the physiology of several systems, notably the CNS, where it regulates energy homeostasis and glucose and lipid metabolism.

Since estrogens produced in the ovaries travels through the blood brain barrier to exert central effects on metabolism, it is often difficult to determine what discrete effects estradiol action is having in the brain versus the periphery. A previous study found that subcutaneous and ICV-delivery ameliorated the increased body weight, adiposity, glucose intolerance and insulin resistance induced by OVX mice fed a HFD. Mechanistically, peripherally administered E2 decreased the expression of TNFα, lipoprotein lipase, and fatty acid synthase in WAT, whereas centrally administered E2 increased HSL expression in WAT, decreased the expression of hepatic gluconeogenic enzymes and increased thermogenesis in BAT [195]. Therefore, estradiol may improve metabolic function through distinct mechanisms in the brain versus the periphery.

Estradiol signals through several estrogen receptors, including ERα, ERβ, G Protein-Coupled Estrogen Receptor 1 (GPER1) and membrane bound ER (mER) to exert its physiological functions. Early studies identified ERα and ERβ as classical, nuclear transcription factors [196]. In this pathway, ligand activated ERα and ERβ dissociate from their chaperone heat-shock proteins and bind as dimers to estrogen responsive elements (EREs), target gene promotors or cofactors (co-activators or co-suppressors) to control gene transcription [197]. ERα and ERβ acting as these classical nuclear transcription factors influence reproductive functions. In contrast, many of the actions of ERs influencing energy metabolism involve extranuclear ER expression, where estradiol activates membrane bound ERs to initiate rapid signaling events [198].

***Estradiol-induced regulation of energy balance***

The decline in circulating estrogens in postmenopausal women is associated with a higher risk for energy and glucose homeostasis dysregulation and increased subcutaneous intraabdominal body fat [199]. Similarly, ovariectomy in animal models results in increased adiposity [200-202] that is prevented by estrogen replacement [203]. The obese phenotype is mediated by both an increase in food intake as well as other metabolic changes. For example, reduced E2 synthesis by aromatase inactivation promotes obesity without hyperphagia by reducing energy expenditure in both female and male mice [204], and ERα deficiency in female and male mice results in reduced spontaneous activity and a shift to increased adiposity and decreased lean fat mass [205]. These studies show that decreased estradiol signaling through ERs throughout the whole body results in dysregulated energy balance.

Several studies suggest that the hypothalamus is a critical and central site of estrogenic signaling mediating energy balance. ERα is abundantly expressed in the VMH, Arc, PVN and brainstem [206, 207]. ERβ is also expressed in these hypothalamic nuclei, but at much lower levels relative to ERα. Thus, it is not surprising that many of the effects of E2 on energy balance are primarily mediated by ERα. Female and male mice with ERα gene deletions are obese [205, 208] and ERα deletion blocks the anti-obesity effects of E2 replacement [203]. Several metabolic effects of ERα deletion are more pronounced in females and will be discussed in future sections. In contrast, female and male ERβ deficient mice do not exhibit obesity [208], but show alterations in thermogenesis and expression of uncoupling protein-1 (UCP1) in brown adipose tissue [209].

Less is known about the recently discovered GPER1, which is a membrane-bound estrogen receptor mediating the rapid, non-genomic actions of estrogen. GPER1 is expressed broadly across the male and female VMH [210]. In the striatum and hippocampus, GPER1 is synaptically localized to dendritic spines [211-213], indicating estrogenic signaling through this receptor may regulate synaptic function. Although several lines of GPER-knockout mice have been generated, only one exhibits disrupted energy and glucose metabolism. Notably, a sexually dimorphic effect of GPER1 deletion on energy homeostasis was reported in these mice. As such, female GPER1-knockout mice were less sensitive to the satiety effects of cholecystokinin (CCK) and leptin, independent of body weight, whereas these effects were preserved in male GPER1-knockout mice [214]. GPER1 is also required for the neuroprotective effects of ERα in dopaminergic neurons [215], suggesting that GPER1 and ERα may coordinate the modulation of estrogenic signaling pathways.

Within the VMH, estradiol action on specific ERs, particularly ERα, depolarizes neurons and alters electrophysiological properties [216], which may have profound effects on energy, glucose and lipid homeostasis. Indeed, deletion of ERα in the VMH by small hairpin RNA knockdown or through the use of SF1-cre mice results in reduced responsiveness to E2-induced weight loss, increased visceral fat accumulation and reductions in energy expenditure with no changes in food intake in female mice [217, 218]. Thus, it appears that ERα in the VMH modulates neuronal activity and lipid accumulation independent of alterations in food intake.

ERα expression in the Arc is also associated with energy balance regulation. Accordingly, POMC neurons express ERα, and ERα knockout mice exhibit decreased

levels of POMC mRNA [219] and hyperphagia without changes in energy expenditure or lipid imbalance [217]. Estradiol also regulates POMC neuronal excitability, and E2 administration increases the number of excitatory synapses and miniature excitatory postsynaptic currents in POMC neurons [220]. Further studies elucidated that these E2-mediated effects on POMC neurons are dependent upon MC4R, as E2-induced hypophagia was absent when MC4R antagonists were co-applied [221]. These studies indicate that estradiol acts in the Arc predominantly through POMC neurons, ERα and melanocortinergic signaling pathways to regulate feeding control.

Energy balance is also mediated by estrogenic signaling through ERs in the brainstem, including the nucleus tractus solitarius (NTS) and dorsal vagal complex (DVC) [222]. Previous studies demonstrated that estradiol administration in wild-type mice suppressed food intake by potentiating CCK-induced satiety and increasing neuronal activity of ERα-expressing cells in the NTS [223, 224]. Interestingly, the estradiol-induced potentiation of CCK satiety signals is absent in mice lacking ERα [224]. Therefore, estradiol signaling through ERα, particularly in the brainstem, is another mechanism by which energy balance is regulated. The cumulative evidence indicates that estrogenic signaling in the hypothalamus and brainstem is necessary for many aspects of energy homeostasis. However, the underlying mechanisms remain to be fully elucidated.

***Estradiol-induced regulation of glucose balance***

Estrogens play paramount roles in regulating glucose homeostasis and insulin sensitivity, particularly in women. As such, abdominal adipocytes from premenopausal

women are more insulin sensitive than age- and BMI-matched men [225, 226], potentially through increased expression of genes involved in glucose and lipid metabolism such as the glucose transporters (GLUT1 and GLUT4) and the lipogenic enzymes fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) [225]. Accordingly, the risk of T2D sharply increases once women reach menopause [227], and E2 replacement therapy is associated with increased insulin action in postmenopausal women [228]. Interestingly, men with mutations leading to deficient estrogen secretion [229, 230], and men [231] and women [232] with ERα deficiency develop hyperglycemia, glucose intolerance and insulin resistance, indicating that estrogen signaling is important for glucose homeostasis in both sexes.

Animal models have demonstrated a role for both ERα and ERβ influencing glucose homeostasis. Female and male ERα knockout mice show severe insulin resistance, glucose intolerance and adipocyte hypertrophy [205, 233, 234]. In contrast, ERβ knockdown in female and male mice has no effect on body weight or fat distribution [232]. However, female ERβ knockout mice are protected from diet-induced insulin resistance and glucose intolerance, indicating that ERβ plays a role in glycemic control specifically in female mice under HFD conditions. It remains unclear whether ERβ is regulating glucose metabolism through ERα-dependent mechanisms.

Similar to the VMH, ERα is found in much higher abundance than ERβ in skeletal muscle. Whether ERα regulates GLUT4 expression in skeletal muscle, as reported by early studies, remains a controversial topic. A previous study showed that ERα knockout mice exhibit reduced GLUT4 levels and insulin resistance [234]. However, later investigations attributed the insulin resistance in ERα knockout mice to proinflammatory

signaling on insulin action and normalized GLUT4 levels [235]. Further, in humans with insulin resistance, obesity or T2D, there are no observed changes in GLUT4 mRNA or protein expression [236]. Therefore, the cumulative evidence suggests that in the absence of ERα, other transcription factors may compensate to regulate GLUT4 levels.

In the liver, ERα and ERβ are both expressed, although their function here is not as well understood. A previous study of global ERα knockout female mice showed decreased insulin suppression of hepatic glucose production, but no insulin resistance in skeletal muscle [234]. Additionally, in HFD-fed and leptin-resistant mice, estradiol suppressed lipogenic gene expression, triglyceride accumulation and liver steatosis, effects that were recapitulated with ERα, but not ERβ, agonists. These results suggest that estradiol signaling, most likely through ERα, is important for the regulation of hepatic insulin sensitivity and glucose homeostasis.

Estrogens may also contribute to glucose imbalance and metabolic symptoms through interactions with leptin and adiponectin. In support, serum leptin levels are higher in premenopausal women than in postmenopausal women and men [237]. Estradiol administration also increases leptin receptor gene expression and secretion via ER-dependent transcriptional mechanisms [238]. In addition, estrogen deficiency causes central leptin insensitivity and an increase in hypothalamic NPY in rats [239]. Adiponectin is an insulin-sensitive hormone that, in contrast to leptin, is inversely correlated with BMI and visceral adiposity [240, 241]. A previous study demonstrated that E2 treatment in OVX mice significantly decreased the ratio of leptin to adiponectin levels, indicating a potential protective mechanism of estrogen through adiponectin secretion in OVX mice [242]. Further, E2 treatment suppresses adiponectin mRNA and

protein expression in adipocytes, and this effect is blocked by treatment with an estrogen antagonist [243]. Taken together, these studies highlight the role of estrogen in preventing glucose imbalances and metabolic symptoms through the regulation of leptin and adiponectin.

***Estrogen regulation of adiposity in peripheral tissues***

Sexual dimorphisms in body fat distribution have been well-established. Women tend to accumulate more gluteal/femoral and subcutaneous depots of fat, whereas men tend to have less fat mass and a more central/intraabdominal distribution [244, 245]. Following menopause, fat distribution in women shifts to a phenotype more similar to men [246], an effect that is ameliorated by estrogen replacement therapy [247]. The regional distribution of fat tissue in humans has substantial clinical consequences, as accumulation of intraabdominal adipose tissue (male-pattern obesity) is associated with an increased risk of mortality by T2D, hyperlipidemia and cardiovascular disease. Subcutaneous adipose tissue is less associated with metabolic disturbances due to its functional ability to expand and allow for storage of excess caloric intake [248]. These, and many other studies, highlight the protective role that estrogens play in the regulation of adiposity and fat distribution.

In addition to ovarian production, estrogens are also synthesized in adipocytes by the aromatization of androgens, an effect that is proportional to the amount of total body adiposity in both females and males [249, 250]. Estrogens regulate WAT by controlling the synthesis and breakdown of fatty acids and triglycerides. Accordingly, estradiol administration in OVX female mice decreased adipocyte size by reducing fatty acid

uptake and lipogenesis. Further, these effects on adiposity were mediated by catecholamine-stimulated lipolysis in WAT [251], indicating that estrogen exerts its regulatory effects on adiposity in part due to facilitating SNS outflow.

With regard to specific ER distributions, ERα is expressed in adipose tissue [252], and reduced ERα expression and function are also associated with several aspects of the metabolic syndrome in humans and animal models [205, 231, 253, 254]. Studies also suggest that ERβ may have anti-lipogenic properties in adipocytes. For example, ERβ induces WAT accumulation in female mice on HFD by increasing peroxisome proliferator-activated receptor-gamma (PPARγ) signaling [255]. PPARγ is a receptor found in adipose tissue that promotes fatty acid storage [256]. The collective studies suggest that although ERα may play a predominant role, both ERα and ERβ contribute to maintenance of lipid balance and metabolism in female WAT.

Estradiol also promotes the activity of proteins and enzymes in fat tissue that are important for energy metabolism. Lipoprotein lipase (LPL) is a key regulating enzyme that breaks down plasma triglycerides into free fatty acids and glycerol, and estradiol is a major transcriptional suppressor of fasting LPL activity in women [257, 258]. Lipin 1 (LPIN1) expression is also associated with obesity [259] and is downregulated by E2 administration [260]. Therefore, expression of LPL and LPIN1 are two mechanisms by which estradiol balance lipogenesis and lipolysis in adipose tissue, particularly in women.

In total, the role estrogens play in regulating energy, glucose and lipid metabolism cannot be understated. They play a variety of functions in CNS, as well as in peripheral metabolic tissues. Dysregulated or dysfunctional estrogenic signaling has severe consequences, as postmenopausal women are at increased risk for metabolic and

cardiovascular disease. The widespread use of estrogen and progesterone replacement as a therapy for postmenopausal symptoms turned out to have dangerous side effects, as it was linked to uterine and breast cancer and cardiovascular events [261]. The need for improved therapeutic actions for postmenopausal women, and those suffering from estrogen deficiencies remains an unmet public health concern.

# Chapter 2: Methods and Materials

### *Animals*

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Tufts University and conducted in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals guidelines. Standard gene targeting techniques were used to generate mutant floxed mGluR5 mice. Genomic DNA for mGluR5 was cloned from a phage library of 129 SVJ mouse genomic DNA fragments. A cassette containing a neomycin resistance (*neo*) gene, flanked by loxP sites, under the control of the phosphoglycerol kinase (PGK) promoter was introduced into the intron 720 base pairs downstream of exon 7. R1 embryonic stem cells were electroporated with the linearized targeting construct, maintained under G418 positive selection and screened by Southern blot analysis for homologous recombination. Chimeric animals produced by injection of these cells into C57BL/6 blastocysts were bred with C57BL/6 mice, and germ-line transmission of the mutation was assessed by PCR and Southern blot. The *neo* cassette was removed in mice by crossing with transgenic mice with Cre recombinase under control of the protamine promoter to produce the floxed mice [262]. Experiments described herein used female and male age-matched littermate control ($mGluR5^{2L/2L}$) and mutant ($mGluR5^{2L/2L:SF1\text{-}Cre}$) mice to reduce genetic background differences. For the electrophysiology experiments, mice containing the floxed mGluR5 and SF1-cre alleles were crossed to mice with mice with cre-dependent expression of tdTomato (Stock No: 007914; B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG\text{-}tdTomato)Hze}$/J) obtained from The Jackson Laboratory.

***Western blot analysis***

Western blot analysis of samples was conducted using standard methods previously described [73, 125]. Blot images were acquired using a Fuji film LAS-4000 Image reader and densitometry performed using Quantity One analysis software (BioRad, Hercules, CA). The following primary antibodies were used: rabbit anti-mGluR5 (1:1000, Millipore #AB5675), rabbit anti-ERα (D8H8) (1:1000, Cell Signaling #8644), rabbit anti-ERβ (1:500, Abcam #ab3576) and mouse anti-β tubulin (1:10,000 Sigma, #T4026).

***Food intake and body weight measurements***

Mice were individually housed with unrestricted access to water and a pre-measured amount of food. Body weight and food intake measurements were monitored beginning at 6 weeks of age and measured weekly at the same time of day. The amount of grams of food consumed each week was then used to calculate caloric intake (kcal). Animals were fed a standard chow diet (SC; Harlan Teklad 2918; 3.1 kcal/g with 18% from fat) for 20 weeks, or at 8 weeks of age, switched to a 58% high fat diet with sucrose (HFD; Research Diets D12331; 5.56 kcal/g) for up to 20 weeks of age.

***Indirect calorimetry***

Energy expenditure was assessed by indirect calorimetry using OxyletPro Physiocage metabolic chambers, a LE405 Gas Analyzer to sample air from individual cages, and Metabolism v2.2 software to integrate gas samples and compute energy expenditure (Panlab/Harvard Apparatus). Mice on a standard chow diet were housed in

individual metabolic assessment chambers containing unrestricted access to food and water. The flow rate of fresh air into each cage was set according to individual animal's body weight (e.g. 25 g mouse would set a flow rate of 0.25 L/min). Each cage was sampled every 15 min for 3 min increments. Data was discarded for the first day animals were in the chamber. Measurements for the remaining 3 test days were analyzed, and the data, averaged across days, are reported as average volume of oxygen uptake (VO2) and carbon dioxide production (VCO2).

***Locomotor activity***

Mice were individually housed in their home cages (standard 15x24 cm plastic cages) and transported to the testing room (12-h light/dark cycle) for 6 days of acclimation. Home cages were surrounded by the Smart Frame Activity System photobeam frame. During testing, locomotor activity, as measured by beam breaks, was recorded continuously using MotorMontitor software (Hamilton/Kinder). The first day of data collection was discarded. The remaining 5 days of data were binned in hourly increments and averaged across test days.

***Body temperature measurements***

Core body temperature was measured using the MicroTherma 2T Hand Held Thermometer (Braintree Scientific, Braintree MA) fitted with a mouse rectal probe (part #RET3, 3/4" L, 0.28 dia, .065 tip). Surface temperature of interscapular brown adipose tissue and the posterior subcutaneous depot of white adipose tissue adjacent to the hind legs (perirenal) was measured using Flir Tools and Flir One Thermal Imaging Camera

Attachment for iOS (FLIR Systems, Wilsonville, OR). All measurements were conducted 3 to 5 hours after lights on and were recorded when the temperature readout stabilized for >5 seconds.

***Glucose and insulin tolerance tests***

Mice were fasted overnight for 16 hours and blood droplets were obtained by slicing a lateral nick in the distal portion of the tail with a scalpel. Blood glucose values (mg/dL) were measured using a Freestyle Blood Glucose Monitoring System (Abbot Diabetes Care Inc.; Alameda, CA). Following a baseline (0) measurement, 1.5 g/kg of D-Glucose was administered intraperitoneally (i.p.). Bloods samples were collected to measure circulating blood glucose at 15, 30, 60 and 120 min post-injection. The GTT was performed at 20 weeks of age. For the insulin tolerance test (ITT), animals were fasted for 6 hours followed by the baseline (0) blood glucose measurement, similar to the GTT. Then, mice were injected with 0.85 U/kg Insulin (Human-R Insulin (rDNA) U100, Lily) administered i.p. Subsequent blood samples were collected at 15, 30, 60, 90 and 120 min post-insulin injection. The ITT was performed when mice were at least 12 weeks of age.

***c-fos immunofluorescence***

Mice were deeply anesthetized and transcardially perfused with 30 ml of cold saline followed by 30 ml of 4% paraformaldehyde (PFA), pH 7.2. Brains were immediately removed, post-fixed in 4% PFA for 4 hours at 4$^{o}$C, cryoprotected in a 30% sucrose solution and frozen in mounting media until 30 μm-thick coronal sections were

obtained using a Leica CM1900 Cryostat. Free floating sections were blocked using 5% normal donkey serum (NDS, 017-000-121, Jackson ImmunoResearch Laboratories, Inc.), 1% bovine serum albumin (BSA, Thermo Fisher Scientific BP1600-100) and 0.4% Triton X-100 for 1 hr at RT. Tissue was then incubated overnight at 4°C with a goat anti- c-fos primary antibody (sc-52-G, Santa Cruz, polyclonal goat anti- c-fos antibody, 1:200) diluted in the donkey blocking solution. Sections were then washed in 1x PBS and incubated for 1 hour at RT with the donkey anti-goat Cy3 secondary antibody (705-175-148, Jackson ImmunoResearch Laboratories, Inc., 1:500) diluted in the donkey blocking solution. A Nikon A1R microscope configured for confocal microscopy was used to image brain sections.

***Ovariectomy and silastic capsule implantation***

8- to 12-week old female mGluR5$^{2L/2L:SF1\text{-}cre}$ and mGluR5$^{2L/2L}$ littermate controls were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and received a bilateral ovariectomy, followed by subcutaneous implants of silastic capsules (Silastic© laboratory tubing, 1.575 ID, 3.175 mm OD, 14mm L) filled with 17β-estradiol (E2, 2 ug/ul dissolved in sesame oil), the ERα agonist 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT, 2 ug/ul, Tocris, Bristol, U.K. #1426 dissolved in DMSO/sesame oil), ERβ agonist diarylpropionitrile (DPN, 2 ug/ul, Tocris, Bristol, U.K. #1494 dissolved in DMSO/sesame oil), GPER1 receptor agonist G-1 (10 ug/ul, Tocris, Bristol, U.K. #3577 dissolved in DMSO/sesame oil), or vehicle (sesame oil or DMSO/sesame oil). Mice were allowed to recover for 1 week and body weights were measured weekly. After five weeks, mice were subjected to a GTT and two days later, mice were sacrificed

3 to 5 hours after lights on after deep anesthesia for trunk serum, brain and tissue collection.

***Tissue collection and measurements***

Animals received *ad libitum* access to food and water and were euthanized under basal feeding conditions 3 to 5 hours after lights on. Mice were anesthetized using isofluorane and, following cervical dislocation, tissues were dissected, flash frozen in liquid nitrogen and stored at -80°C. Tissues collected included liver, perigonadal WAT, brain and serum. Triglyceride concentrations in liver, WAT and serum samples were determined by the Vanderbilt Hormone Assay and Analytical Services Core (Vanderbilt University, Nashville, TN). Norepinephrine content in serum samples was analyzed using HPLC methods by the Neurochemistry Core Laboratory at the Vanderbilt Brain Institute (Vanderbilt University School of Medicine, Nashville, TN).

***Histology of adipose tissue***

Perigonadal white adipose tissue samples were extracted and fixed in 10% neutral buffered formalin. Paraffin embedding and H&E staining was performed by the Tufts Histology Core. The adipose tissue from each subject was sectioned and mounted on four slides with three samples per slide. Four images were analyzed per animal. Images acquired on the Zeiss Axioplan 2 microscope at 10x magnification were captured with a Retiga 1300B camera using Q Capture Suite Plus (version 3.1.3.10; 2012) software. After import into Adobe Photoshop, image contrast was autocorrected and inverted before upload to analysis software. Using a modified adipocyte analysis pipeline from

the Rodeheffer laboratory [263] coded for the free software Cell Profiler (version 2.2.0), data output for individual adipocyte radius and area was recorded in Excel and analyzed using Prism GraphPad.

***Current-clamp recordings***

The number of action potentials generated in response to a series of 500-ms current injections from 20 to 300 pA in 20-pA steps was measured in $SF1^{+}$ neurons from female and male $mGluR5^{+/+:SF1\text{-}cre:TdTomato}$ (control) and $mGluR5^{2L/2L:SF1\text{-}cre:TdTomato}$ (mutant) mice in the current-clamp configuration. The intracellular recording solution contained (in mM): 130 K-gluconate, 10 KCl, 4 NaCl, 10 HEPES, 0.1 EGTA, 2 Mg-ATP, and 0.3 Na-GTP (pH = 7.25, 280–290 mosM). The firing rate of $SF1^{+}$ neurons was measured in response to a 1-s, 100-pA step pulse. Throughout the experiment, series resistance and whole cell capacitance were continually monitored and compensated. Recordings were excluded from data analysis if the series resistance increased by more than 20%. Ohm's law was used to calculate input resistance in response to a −100-pA current injection. Input-output curves were fit with a Boltzmann equation: $f(W) = (\text{Max}/\{1 + \exp((I - I_{50})/k)\} + \text{Max})$, where $I$ is the current injected, Max is the maximum response, $k$ is a slope factor, and $I_{50}$ is the current injection amplitude that elicits 50% of Max. In this set of experiments, the Max value was determined as the number of spikes fired in $mGluR5^{+/+:SF1\text{-}cre:TdTomato}$ and $mGluR5^{2L/2L:SF1\text{-}cre:TdTomato}$ mice in response to a 300-pA current injection to prevent adverse effects of a larger current injection on neuronal viability. Data was acquired using an Axopatch 200B (Axon Instruments) and PowerLab hardware and software (ADInstruments). Data analysis was

performed using either LabChart Pro (ADInstruments) or MiniAnalysis software (Synaptosoft).

***Statistical analysis***

Analytical software used to perform the statistical analysis was performed by GraphPad Prism. Repeated measures ANOVA, using Bonferroni's method to adjust for multiple comparisons, was performed to analyze weekly food intake and body weight measurements as well as the time course data for the glucose and insulin tolerance tests. Two-way ANOVAs were used to analyze comparative female and male data including energy expenditure, locomotor activity, thermoregulation, fasted insulin, triglyceride contents, serum norepinephrine and area under the curve for glucose and insulin tolerance tests. Student's unpaired t-tests were performed to analyze comparisons of two groups including mGluR5 protein expression in $BDNF^{2L/2L:Ck\text{-}cre}$ and $mGluR5^{2L/2L:SF1\text{-}cre}$ experimental mice, fatty acid profile, adipocyte size, c-fos immunoreactivity and protein expression of ERα, ERβ and GPER1. Comparisons were determined by be statistically significant when $p < 0.05$. All values are depicted as mean ± SEM.

# Chapter 3: Results

**mGluR5 expression is reduced in the VMH of BDNF$^{2L/2L:Ck\text{-}cre}$ mutant mice and is regulated by energy status**

BDNF$^{2L/2LCk\text{-}cre}$ mice have global central depletion of BDNF and exhibit excessive feeding, glucose intolerance, dramatic obesity and reduced excitatory drive onto the VMH [106, 125]. Selective deletion of BDNF in the VMH also leads to hyperphagia, hyperglycemia and hyperinsulinemia, indicating an important role of BDNF in this nucleus. Because mGluR5 is highly expressed in the VMH and has an integral role regulating excitatory synaptic plasticity in other brain regions [126, 138-140], we asked whether alterations in its function in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mutant mice might play a part. As a first step to test this idea, we measured protein levels of mGluR5 in VMH of BDNF$^{2L/2LCk\text{-}cre}$ mutant and control mice. We found that mGluR5 expression levels were significantly decreased in the VMH of female and male mutants compared to sex-matched controls (Figure 3.1 A-C).

To further test the possibility that mGluR5 might act as a downstream effector of BDNF in the VMH, we investigated whether its expression is dynamically regulated by energy status as it is the case for BDNF and TrkB [73, 107]. We found that mGluR5 protein levels in VMH of fasted wild-type female and male mice were reduced to 41% and 30%, respectively, compared to sex-matched fed control mice (Figure 3.1 D-F). These results suggest that mGluR5 may be acting downstream of BDNF in the VMH to mediate energy and glucose homeostasis.

**mGluR5 in SF1 neurons is not required for the regulation of energy balance**

Next, we interrogated the necessity of mGluR5 expression in the VMH for the control of metabolic function. To achieve this, we deleted it from $SF1^{+}$ neurons, which in the brain are exclusive to the VMH, play critical roles in the regulation of energy and glucose balance [87, 264] and express mGluR5 (Figure 3.2 A). Mutant $mGluR5^{2L/2L:SF1\text{-}Cre}$ and control $mGluR5^{2L/2L}$ mice were generated by crossing floxed mGluR5 mice with mice expressing cre recombinase under the direction of the SF1 promoter (SF1-cre mice) [61]. We confirmed mGluR5 depletion in $mGluR5^{2L/2L:SF1\text{-}Cre}$ mice by Western blot analysis of VMH tissue punches. As shown in Figure 3.2 B, mGluR5 expression in mutant VMH was significantly decreased and only 43% and 24% of female and male controls, respectively. Two-way ANOVA analysis revealed a significant main effect of genotype ($p < 0.001$) and sex ($p = 0.03$) on mGluR5 expression in the VMH. The increased levels of mGluR5 in male $mGluR5^{2L/2L}$ compared to female $mGluR5^{2L/2L}$ control mice suggest that this receptor might be present in limiting amounts in female VMH or that mGluR5 is expressed at higher levels in males for a reason not relevant for females. Further, the incomplete knockdown of mGluR5 in the VMH is expected considering that a heterogeneous population of cells resides in this region and that $SF1^{-}$ cells also express this receptor.

Female and male $mGluR5^{2L/2L:SF1\text{-}Cre}$ mice administered a SC diet exhibited normal food intake and body weights compared to littermate controls. Similarly, feeding behavior and weights of male and female mutants were comparable to those of sex-matched controls when challenged under HFD conditions for 12 weeks (5.56 kcal/g)

(Figure 3.3 A and B) indicating that mGluR5 in SF1 neurons is not a critical food intake regulator.

Alterations in energy expenditure were assessed by indirect calorimetry, measurements of body temperature and home cage locomotor activity. Female and male mutant mice exhibited no differences in O2 input or CO2 output compared to littermate control mice (Figure 3.3 C), indicating normal basal metabolic rates. Locomotor activity measurements revealed a significant main effect of sex in number of basic movements ($p = 0.0007$) and distance traveled ($p = 0.01$). However, female and male mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice exhibited similar numbers of basic movements and distance traveled as sex-matched controls (Figure 3.3 D). Thermoregulation, measured by both core body temperature and as a ratio of brown adipose tissue (BAT) to white adipose tissue (WAT) temperature was also normal in female and male mutant mice (Figure 3.3 E and F). In total, these findings indicate that mGluR5 expression in SF1 neurons is not required for the regulation of energy balance.

**mGluR5 deletion in VMH elicits female-specific impairments in glucose homeostasis and insulin sensitivity**

The VMH is a key glucose-sensing region of the brain that responds to peripheral cues to control glycemic balance [265, 266]. Therefore, we investigated whether deleting mGluR5 in SF1 neurons impacted glucose homeostasis. Fasting levels of glucose were not significantly altered in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice compared to control females. However, female mutants exhibited severely impaired responses to a glucose challenge compared to female control mice (Figure 3.4 A and B). Accordingly, female

mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants exhibited glucose levels 17% and 44% higher than control littermates at 15 and 30 minutes post-bolus injection of glucose, respectively. A significant main effect of time ($p < 0.0001$), genotype ($p = 0.02$), subjects (matching) ($p = 0.0001$) and an interaction ($p = 0.0055$) were observed by two-way repeated measures ANOVA analysis (Figure 3.4 A). In far contrast, responses of male mGluR5$^{2L/2L:SF1\text{-}cre}$ mice to a glucose challenge were indistinguishable from those of male mGluR5$^{2L/2L}$ controls (Figure 3.4 B).

To assess whether decreased insulin sensitivity was associated with decreased glycemic control, we performed insulin tolerance tests (ITT). Consistent with our previous findings in the glucose tolerance test, mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant females, but not males, exhibited insulin resistance as indicated by elevated levels blood glucose following an insulin challenge relative to their respective control mGluR5$^{2L/2L}$ mice (Figure 3.5 A and B). Accordingly, 90 minutes post-injection of insulin, glucose levels of female controls were 50% of baseline levels, whereas glucose levels in female mutants were significantly higher and 73% of their baseline levels. Female mGluR5$^{2L/2L:SF1\text{-}Cre}$ mice also exhibited elevated fasted levels of serum insulin compared to female controls ($p = 0.04$) as well as control males ($p = 0.02$) and mutant males ($p = 0.03$), further indicating reduced insulin sensitivity as consequence of mGluR5 depletion in the female VMH. In contrast, fasted serum levels of insulin were indistinguishable between male mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant and control mice (Figure 3.5 C). Our collective findings demonstrate a required and sex-specific role of mGluR5 in SF1 neurons regulating glucose homeostasis.

**Female mGluR5$^{2L/2L:SF1\text{-}Cre}$ mice exhibit alterations in lipid homeostasis**

The VMH has been implicated in the regulation of peripheral lipid metabolism, in part, by modulating SNS output to adipose tissues [67, 267, 268]. However, the detailed cellular mechanisms are poorly understood. We investigated whether mGluR5 function in SF1$^{+}$ neurons played a required role in this process. For this, we measured triglyceride (TG) content in serum, WAT and liver of mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutants and mGluR5$^{2L/2L}$ controls under fed conditions. Female, but not male, mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutants exhibited a significant increase in TG content in WAT compared to sex-matched controls (718.4 +/- 105.29 ug/mg versus 405.8 +/- 31.86 ug/mg; $p = 0.01$) (Figure 3.6 A). Indeed, two-way ANOVA analysis indicated a significant effect of genotype ($p = 0.02$) and a significant interaction between genotype and sex ($p = 0.03$). Liver and serum TG concentrations were not significantly different between genotypes in female or male mice, but two-way ANOVA analysis revealed a significant main effect of sex in serum ($p = 0.04$) and liver ($p = 0.02$) TG content (Figure 3.6A). Further examination of fatty acid profiles revealed significant increases in several fatty acid chains, specifically 14:0 ($p = 0.04$) and 16:1 ($p = 0.04$) in female mutant WAT tissue (Figure 3.6 B), indicating impaired lipid balance in WAT. No differences in fatty acid chain concentrations were observed in tissue collected from male mGluR5$^{2L/2L:SF1\text{-}cre}$ mice (*data not shown*).

White adipose tissue is the major energy reservoir in the body, and its purpose is to synthesize and store TG in periods of positive energy and hydrolyze TG to generate free fatty acids for use by other metabolic organs during periods of energy deprivation. Obesity-related diseases such as diabetes and cardiovascular disease are often characterized by adipocyte hypertrophy that occurs when TG breakdown (lipolysis) is

decreased, leading to TG accumulation [269]. To determine whether adipocyte hypertrophy accompanied the TG accumulation observed in WAT from female mutant mice, we examined the histological profile of WAT tissue in female mGluR5$^{2L/2L:SF1\text{-}Cre}$ and mGluR5$^{2L/2L}$ mice. We found that in the fed state, perigonadal adipocytes from female mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutants were significantly larger compared to adipocytes from control mice (1899 +/- 44.45 um$^2$ versus 1477 +/- 32.35 um$^2$; $p < 0.0001$) (Figure 3.6 C and E). Adipocyte size was similar in male mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutants compared to controls (Figure 3.6 D). The cumulative results indicate mGluR5 plays a requisite role in female VMH regulating lipid metabolism.

**mGluR5 deletion in SF1 neurons decreases sympathetic outflow to the periphery**

The VMH regulates glucose and lipid metabolism in peripheral tissues through direct projections to autonomic centers of the hindbrain that regulate sympathetic output in the periphery [270]. To investigate whether mGluR5 deletion in SF1$^+$ neurons might result in decreased sympathetic tone, triggering glucose intolerance and disrupted lipid homeostasis, we measured plasma norepinephrine levels as a measure of sympathetic output. Indeed, plasma norepinephrine levels were significantly lower in female mGluR5$^{2L/2L:SF1\text{-}Cre}$ mice compared to mGluR5$^{2L/2L}$ females (750.17 +/- 49.34 pg/ul versus 1096.9 +/- 99.88 pg/ul; $p = 0.008$) (Figure 3.7 A), indicating decreased sympathetic tone. In contrast, no alterations in plasma norepinephrine levels were observed in mGluR5$^{2L/2L:SF1\text{-}Cre}$ male mice (Figure 3.7 B). In total, these results suggest that VMH mGluR5 is required for the regulation of sympathetic output to peripheral metabolic tissues in female mice.

**Neuronal activity is decreased in the VMH of female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants**

Considering the important roles ascribed to mGluR5 in the regulation of excitatory synaptic plasticity, we asked whether the metabolic alterations and reduced sympathetic output observed exclusively in mGluR5$^{2L/2L:SF1\text{-}cre}$ females might be related to modifications in VMH neuronal activity. To test the responsiveness of VMH neurons to peripheral glucose, we administered a bolus of glucose similar to that applied during a GTT to female mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice. After 30 minutes, when circulating glucose levels are highest, mice were sacrificed and their brains were processed for c-fos immunoreactivity as a marker of neuronal activation. Female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibited a significant and dramatic decrease in c-fos$^{+}$ cells in response to glucose administration compared to female controls (38 +/- 10.02 cells versus 179.8 +/- 44.74 cells; $p$ = 0.03) (Figure 7 A and B), indicating blunted activity of VMH cells to glucose administration.

mGluR5 influences neuronal membrane excitability via regulation of $K^{+}$ ion channels and $Ca^{2+}$ currents in other brain regions [271-273]. Thus, we investigated whether the intrinsic excitability of VMH neurons was influenced by a deficit in mGluR5 expression. Our preliminary data indicates that the response to increasing depolarizing current injections was decreased in SF1$^{+}$ neurons from mGluR5$^{2L/2L:SF1\text{-}cre}$ females compared to female mGluR5$^{2L/2L}$ controls and unaffected in male mutants compared to sex-matched controls (Figure 3.8 C and F). No significant differences were observed in resting membrane potential or input resistance (Figure 3.8 D and E). These preliminary findings support our claim that the intrinsic excitability of female mutant SF1 neurons is reduced.

The collective data indicate that mGluR5 depletion reduces the excitability of $SF1^{+}$ neurons exclusively in females, informing a mechanism underlying the sex-specific metabolic alterations observed in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mutants.

**$mGluR5^{2L/2L:SF1\text{-}cre}$ female mutants show deficits in estradiol signaling**

The hypothalamus is an important sex hormone-responsive center of the brain. Accordingly, estrogen signals through ERα, ERβ and GPER1 receptors within this region to regulate feeding behavior, energy expenditure, lipogenesis and glucose metabolism, and these effects are for the most part, female-specific [220, 251, 274]. Indeed wild-type OVX mice exhibit excessive weight gain, glucose intolerance and dyslipidemia, which can be reversed by hormone replacement therapy [275, 276]. Previous reports have shown mGluR5 functionally interacts with estrogen receptors to facilitate synaptic plasticity in other brain regions [188, 190, 192]. Therefore, we asked whether alterations in estrogenic signaling might explain the observed female-specific effects of depleting mGluR5 in the VMH. To test whether VMH mGluR5 deficits impede the beneficial effects of estrogen signaling on glycemic control, we measured the effect of chronic (5 weeks) systemic administration of 17β-estradiol (E2) or vehicle (sesame oil) to female OVX $mGluR5^{2L/2L}$ and $mGluR5^{2L/2L:SF1\text{-}cre}$ mice. Control and mutant OVX mice treated with vehicle gained significantly more weight compared to OVX mice treated with E2, indicating that mGluR5 in $SF1^{+}$ neurons is not required for estradiol-mediated body weight control (Figure 3.9 A). However, glucose tolerance testing revealed that, whereas E2 treatment significantly improved glucose balance in control OVX females ($p = 0.02$), it had no effect in mutant OVX mice. Accordingly, analysis of the area under the curve

(AUC) revealed OVX mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibited similar levels of glucose intolerance as control and mutant OVX mice treated with vehicle, despite weighing significantly less (Figure 3.9 B). A significant interaction of genotype and treatment was observed by two-way ANOVA analysis ($p$ = 0.0005). These results are consistent with our previous findings that there are no alterations in body weight control but severely impaired responses to a glucose challenge in our female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice, confirming that mGluR5 does not function in SF1 neurons to regulate energy balance.

We next sought to identify specific estrogen receptors that might functionally interact with mGluR5 to regulate glucose and lipid metabolism. First, we examined whether altered expression levels ERα and ERβ in mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice were associated with decreased responsiveness to the protective effects of estrogen on glycemic control. We found that female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibited a 30% decrease in ERα protein expression in the VMH compared to control female mice, whereas there were no alterations in male mGluR5$^{2L/2L:SF1\text{-}cre}$ compared to sex-matched controls (Figure 3.10 A and B). ERβ expression was similar in mutant and control VMH from both sexes (Figure 3.10 C). Western blot analyses of GPER1 levels are ongoing.

To directly test whether the inability of estradiol administration to protect OVX mutant mice from glucose intolerance was caused by functional interactions of mGluR5 with specific estrogen receptors, we implanted OVX and control mice with capsules containing selective agonists for ERα (4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol; PPT), ERβ (diarylpropionitrile; DPN), GPER1 (G1) or vehicle. After chronic treatment, we found that PPT, DPN and G1 had no impact on cumulative body weight gain. However, chronic treatment with PPT significantly improved glucose

tolerance in OVX control mice ($p$ = 0.03), but had a markedly reduced effect in OVX mutant mice, indicating that mGluR5 and ERα are functionally coupled in the regulation of glucose tolerance in female mice (Figure 3.11 A). No effects of DPN or G1 were observed in body weight changes or GTT (Figure 3.11 B and C).

In total, these findings illustrate that mGluR5 is a critical facilitator of estrogenic signaling in the VMH. Further, mGluR5 signaling in conjunction with specific estrogen receptors is perturbed in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice and this interaction within the VMH is imperative for the protective effects of estradiol on glucose balance control.

**Chapter 3.1 Figures**

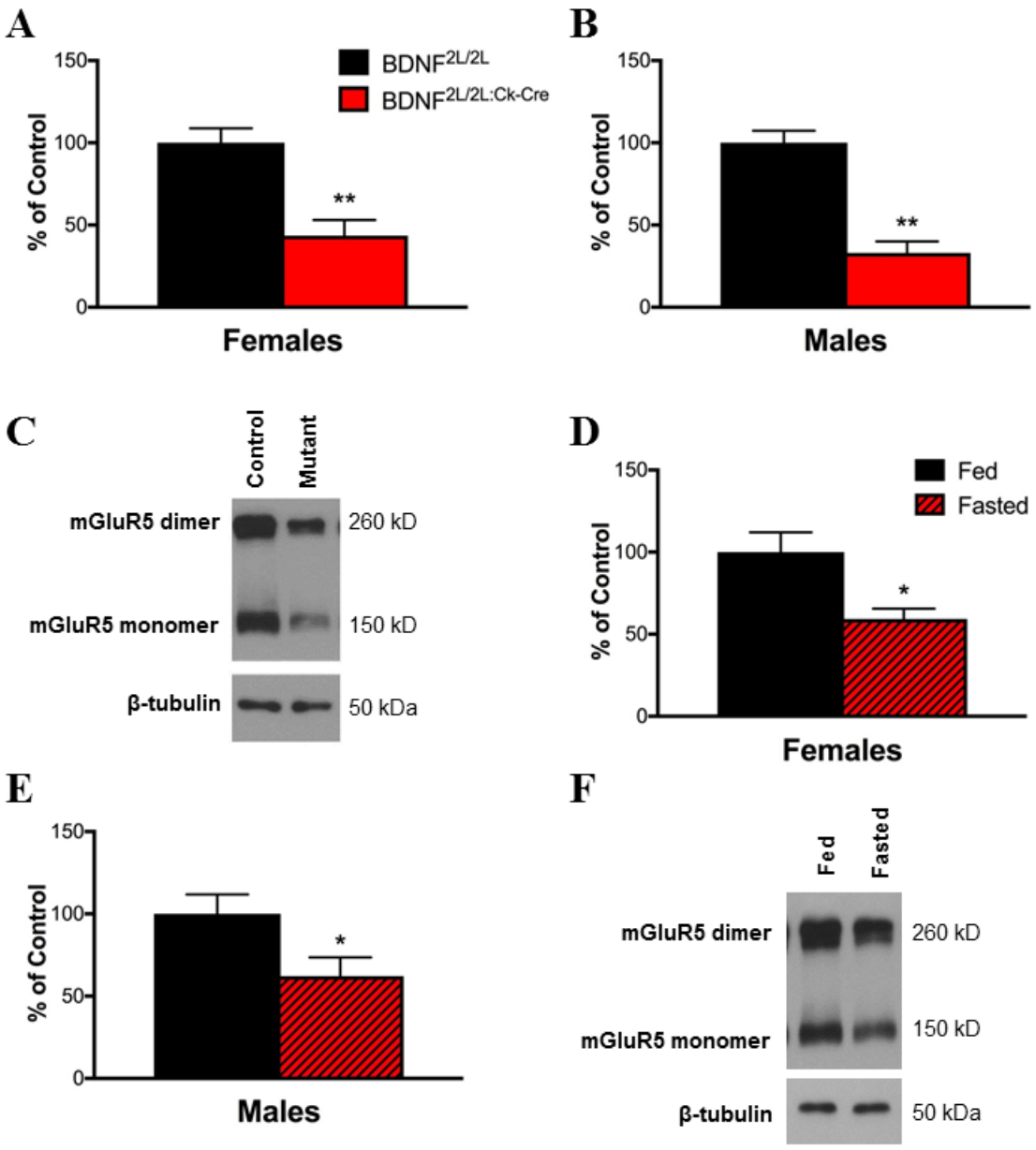


**Figure 3.1 mGluR5 expression is decreased in the VMH of BDNF mutant mice and regulated by energy status.** mGluR5 protein expression is reduced in female **(A)** and male **(B)** $BDNF^{2L/2L}$ control and $BDNF^{2L/2L:Ck\text{-}Cre}$ mutant mice (n= 4-5 per group; * = $p < 0.05$). **(C)** Representative western blot image of mGluR5 VMH content in control and $BDNF^{2L/2L:Ck\text{-}Cre}$ mutant mice. mGluR5 protein expression in the VMH of fed and fasted female **(D)** (n= 8-9; ** = $p < 0.01$) and male **(E)** (n= 6-8; * = $p < 0.05$) $BDNF^{2L/2L}$ control mice. **(F)** Representative western blot image of mGluR5 VMH content in fed and fasted wild-type mice.

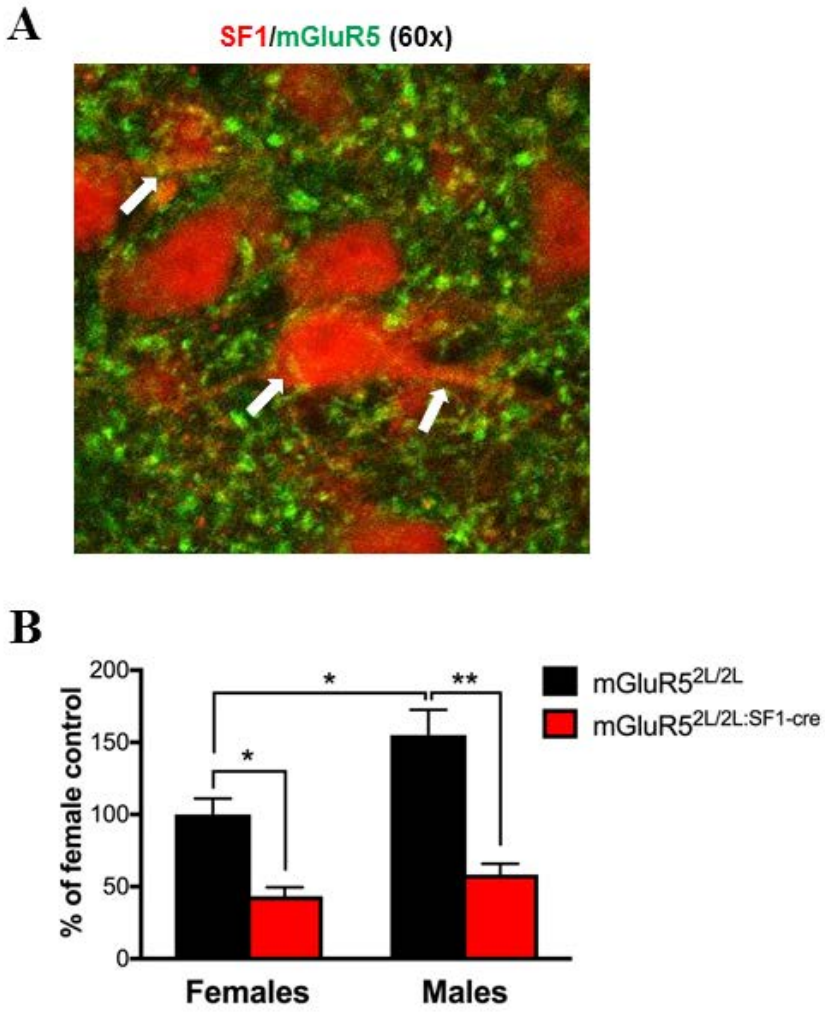


**Figure 3.2 Expression of mGluR5 in SF1 neurons of the VMH and confirmation of knockdown in mGluR5$^{2L/2L:SF1\text{-}Cre}$ mice. (A)** Immunolabeling of mGluR5 (green) in TdTomato-tagged SF1 neurons (red) in the VMH demonstrating extensive co-localization of mGluR5 and SF1 (arrows) in the VMH. **(B)** Western blot analysis of mGluR5 content in VMH of female (n= 5-9; * = $p$ <0.05) and male (n= 3-6; * = $p$ < 0.05; ** = $p$ < 0.01) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice.

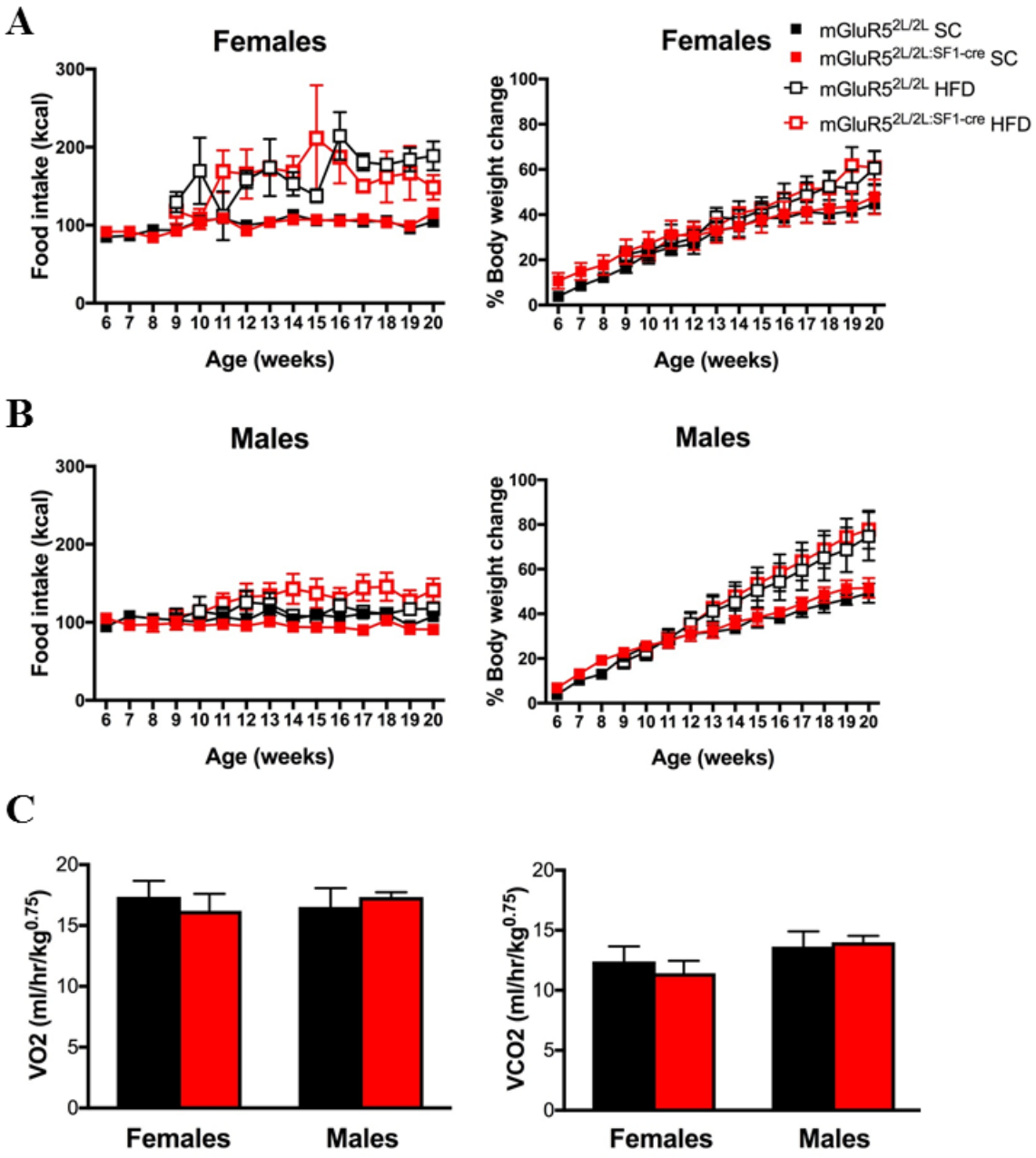


**Figure 3.3 Mice with mGluR5 depletion in VMH SF1 neurons exhibit normal food consumption, body weight, energy expenditure, locomotor activity and thermoregulation.** Weekly food consumption (*left*) and cumulative body weight gain (*right*) for individually housed **(A)** female (n= 5-8) and **(B)** male (n= 6) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1-Cre}$ mutant mice on a standard chow (SC) or high fat diet (HFD). **(C)** Energy expenditure expressed as average volume of $O_2$ consumption (*left*) and volume of $CO_2$ consumption (*right*) for age- and weight-matched female and male mice on a standard chow diet (n= 4-6 per group)**.**

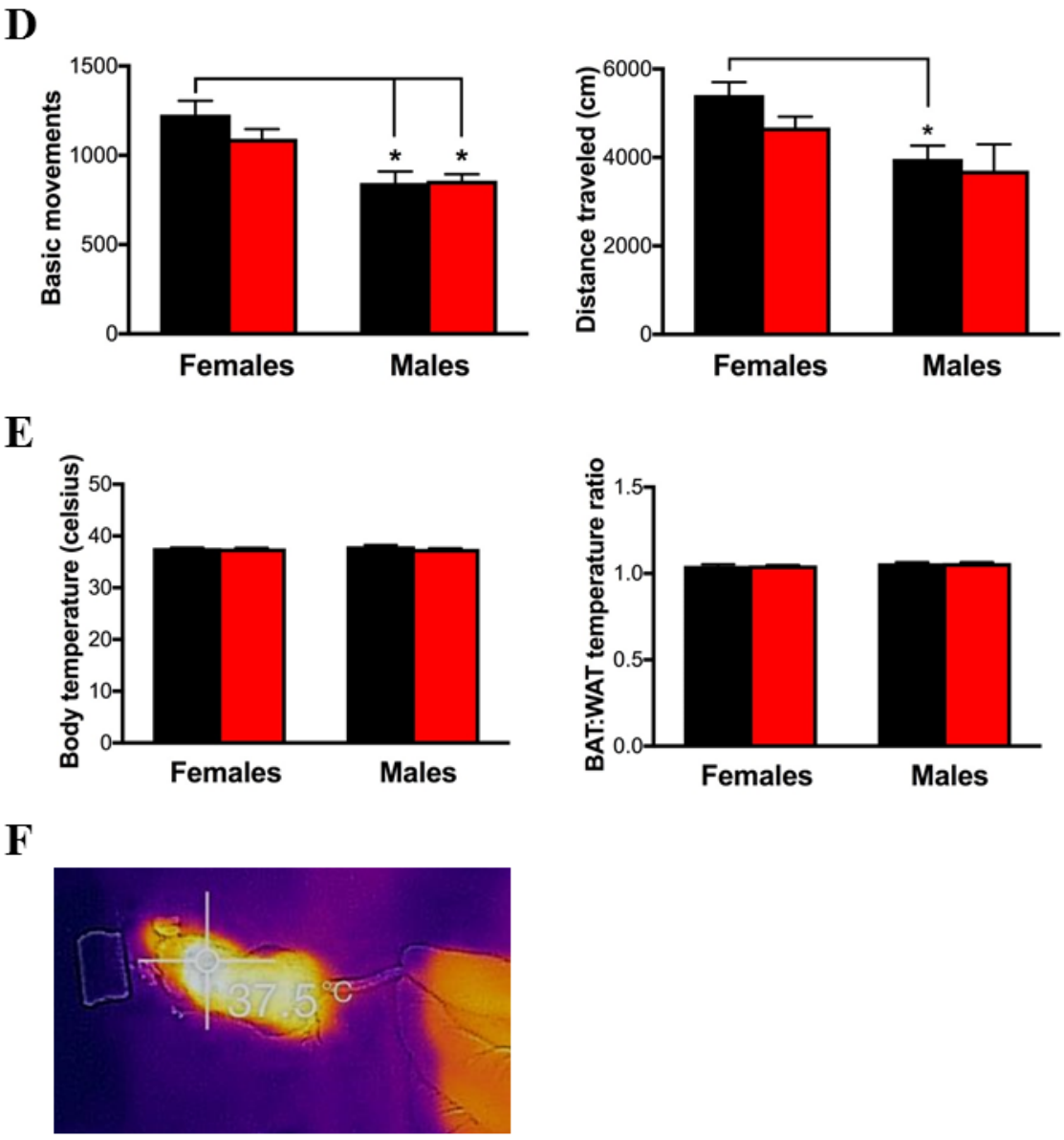


**Figure 3.3 (con't) Mice with mGluR5 depletion in VMH SF1 neurons exhibit normal food consumption, body weight, energy expenditure, locomotor activity and thermoregulation. (D)** Homecage locomotor activity expressed as average daily basic movements (photobeam breaks) (*left*) and distance traveled (*right*) for age- and weight-matched female (n= 12-16) and male mice (n= 6) on a standard chow diet (* = $p$ <0.05). **(E)** Core body temperature (*left*) and temperature ratio of intrascapular brown adipose tissue (BAT) and perirenal white adipose tissue (WAT) depots (*right*) in female (n= 8-11) and male (n= 6-8) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice on a standard chow diet. **(F)** Representative image of BAT temperature reading.

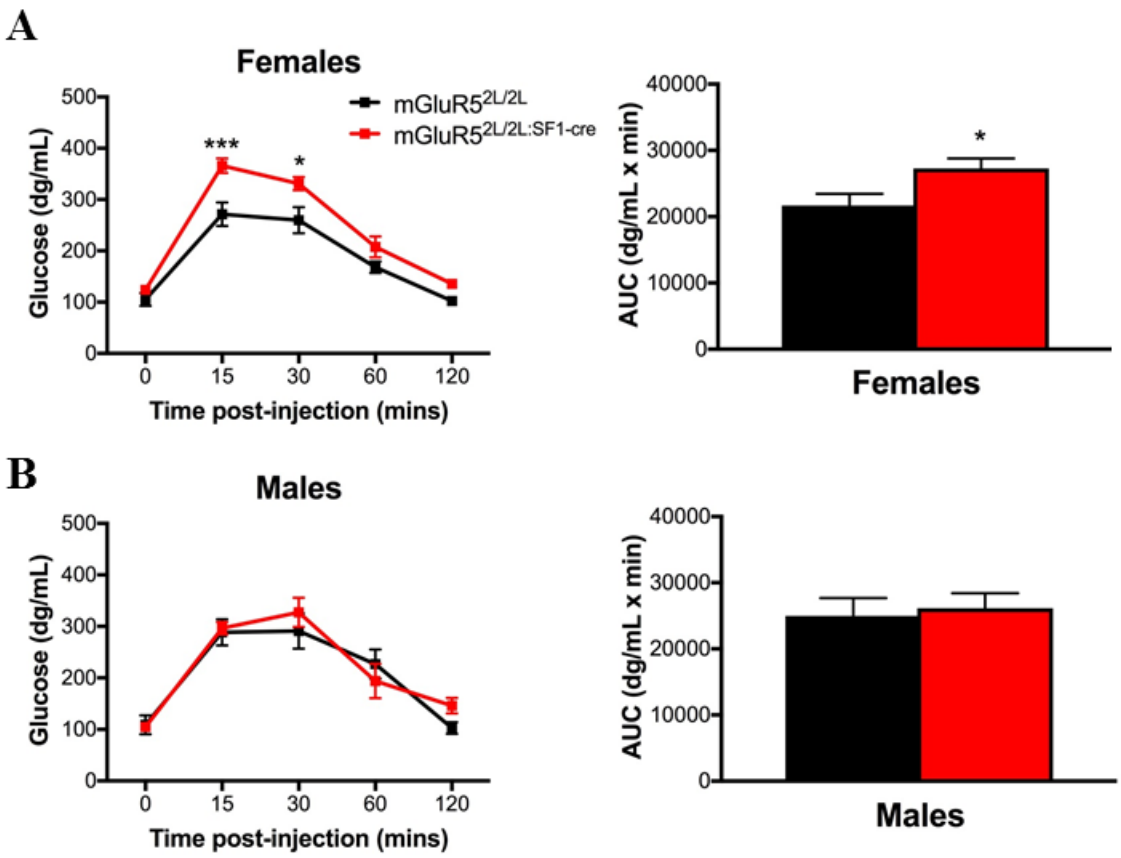


**Figure 3.4 Female mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice exhibit severe deficits in glucose homeostasis.** Time course (*left*) and area under the curve (AUC) (*right*) for glucose tolerance tests in **(A)** female (n= 6-8; *** = $p$ <0.001, * = $p$ <0.05) and **(B)** male (n= 3-4) mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mice on a standard chow diet.

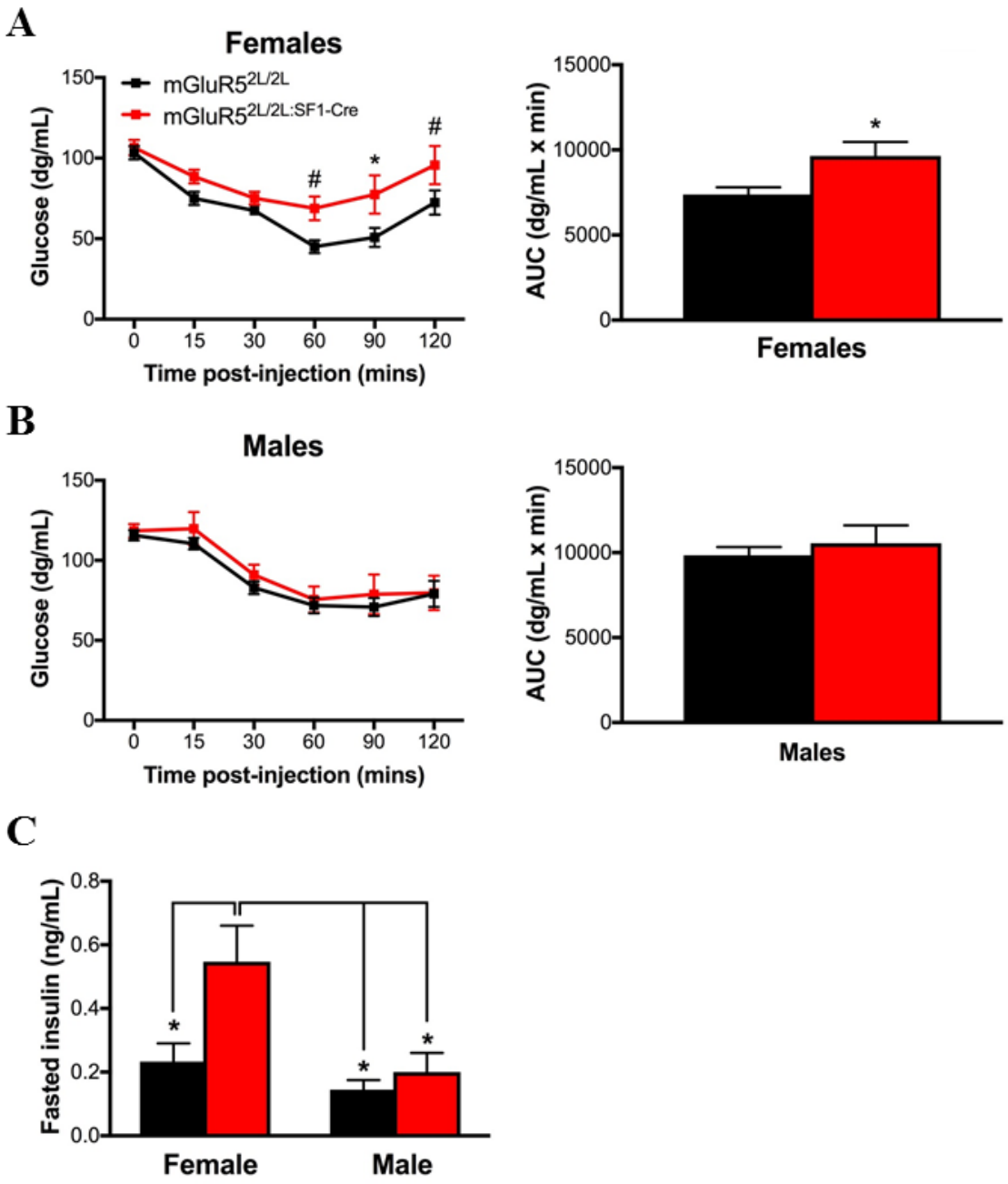


**Figure 3.5 Female mGluR5$^{2L/2L:SF1-Cre}$ mutant mice are insulin resistant.** Time course (*left*) and area under the curve (AUC) (*right*) for insulin tolerance tests following a 6 hr fast at baseline, at 15, 30, 60, 90 and 120-minutes following an i.p. injection of 0.85 U/kg insulin for **(A)** female (n= 9-10; * = $p$ <0.05, # = $p$ <0.08) and **(B)** male (n= 8-9) mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ and mice on a chow diet. **(C)** Fasted insulin levels exhibited by female (n= 11-13; * = $p$ <0.05) and male (n= 8-9) mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ and mice on a standard chow diet.

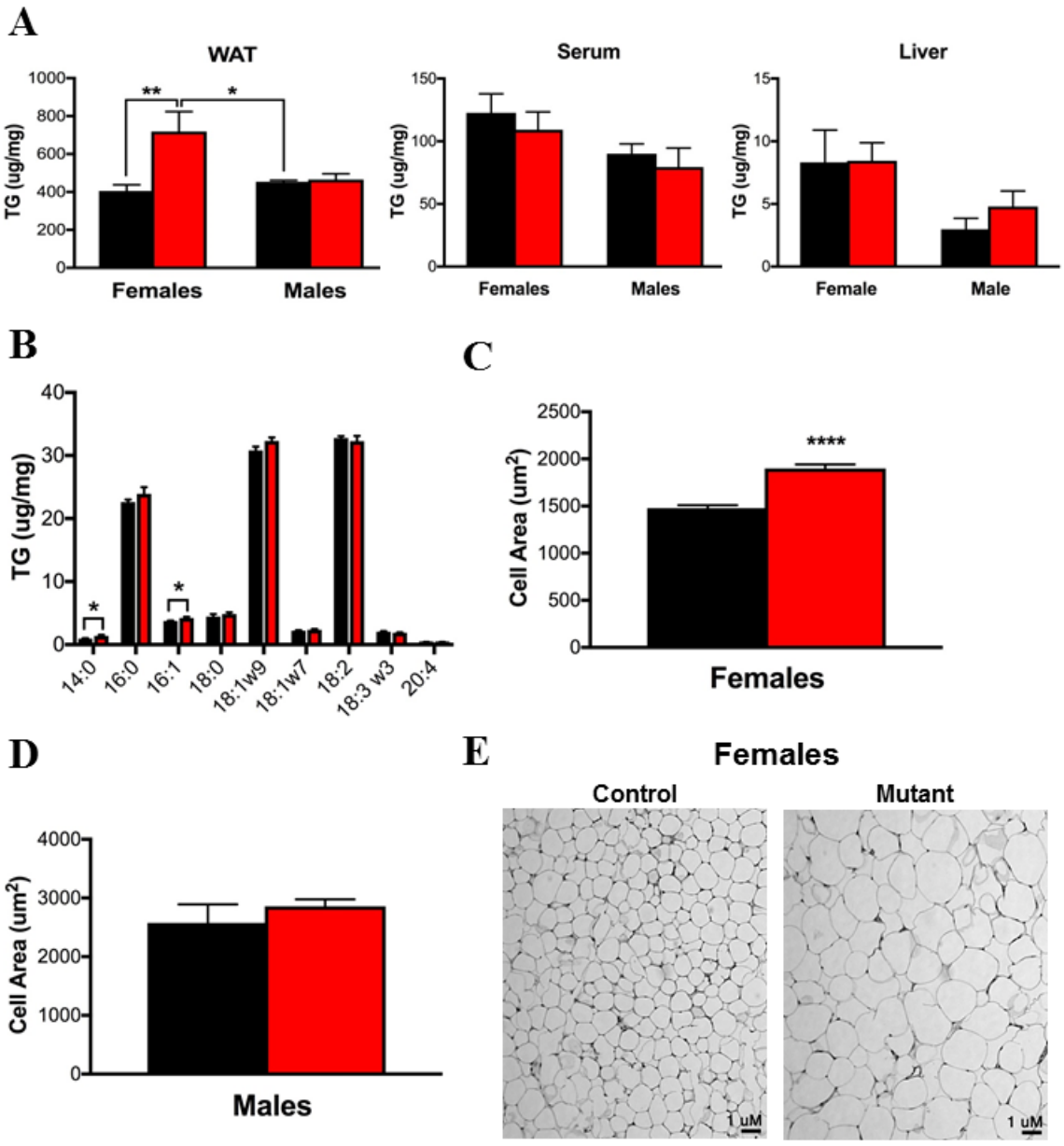


**Figure 3.6 Female mGluR5$^{2L/2L:SF1-Cre}$ mutant mice exhibit alterations in lipid balance and white adipocyte size.** **(A)** Measurement of triglyceride (TG) content in perigonadal white adipose tissue (WAT), serum and liver of female (n= 6; ** = $p$ <0.01, * = $p$ <0.05) and male (n=5) mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ mice fed a standard chow diet. **(B)** Fatty acid profile of WAT from female mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ mice in the fed state (n = 6; * = $p$ <0.05). Quantification of mean cell area of white adipocytes from **(C)** female and **(D)** male mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ mice (n= 6; **** = $p$ < 0.0001). **(E)** Representative WAT histology images from female mGluR5$^{2L/2L}$ (control) and mGluR5$^{2L/2L:SF1-Cre}$ (mutant) mice.

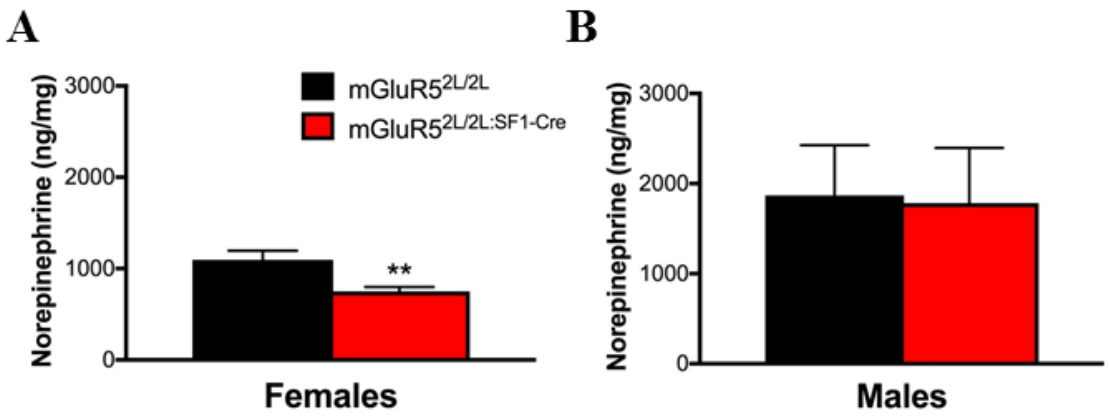


**Figure 3.7 Female mGluR5$^{2L/2L:SF1-Cre}$ mutant mice exhibit reduced sympathetic tone.** Norepinephrine content in serum of **(A)** female and **(B)** male mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1-Cre}$ on a chow diet (n = 6-8 per group; ** = $p < 0.01$).

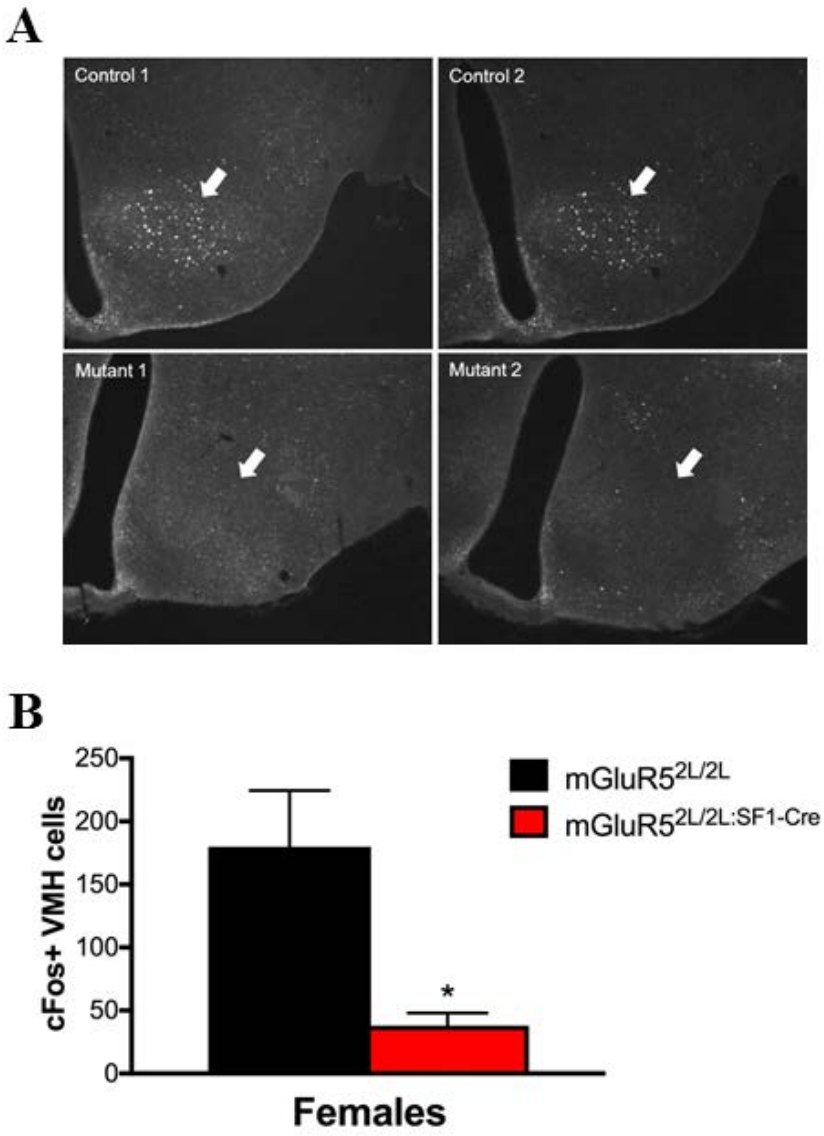


**Figure 3.8: Neuronal activity is decreased in the VMH of female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants.** **(A)** Representative images of neuronal activation as marked by c-fos$^{+}$ immunoreactivity in VMH (arrows) of female mGluR5$^{2L/2L}$ control (*top panel*) and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant (*bottom panel*) mice and **(B)** quantification of c-fos$^{+}$ cells in female mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice 30-minutes after glucose administration (n= 4-5; * = $p$ <0.05).

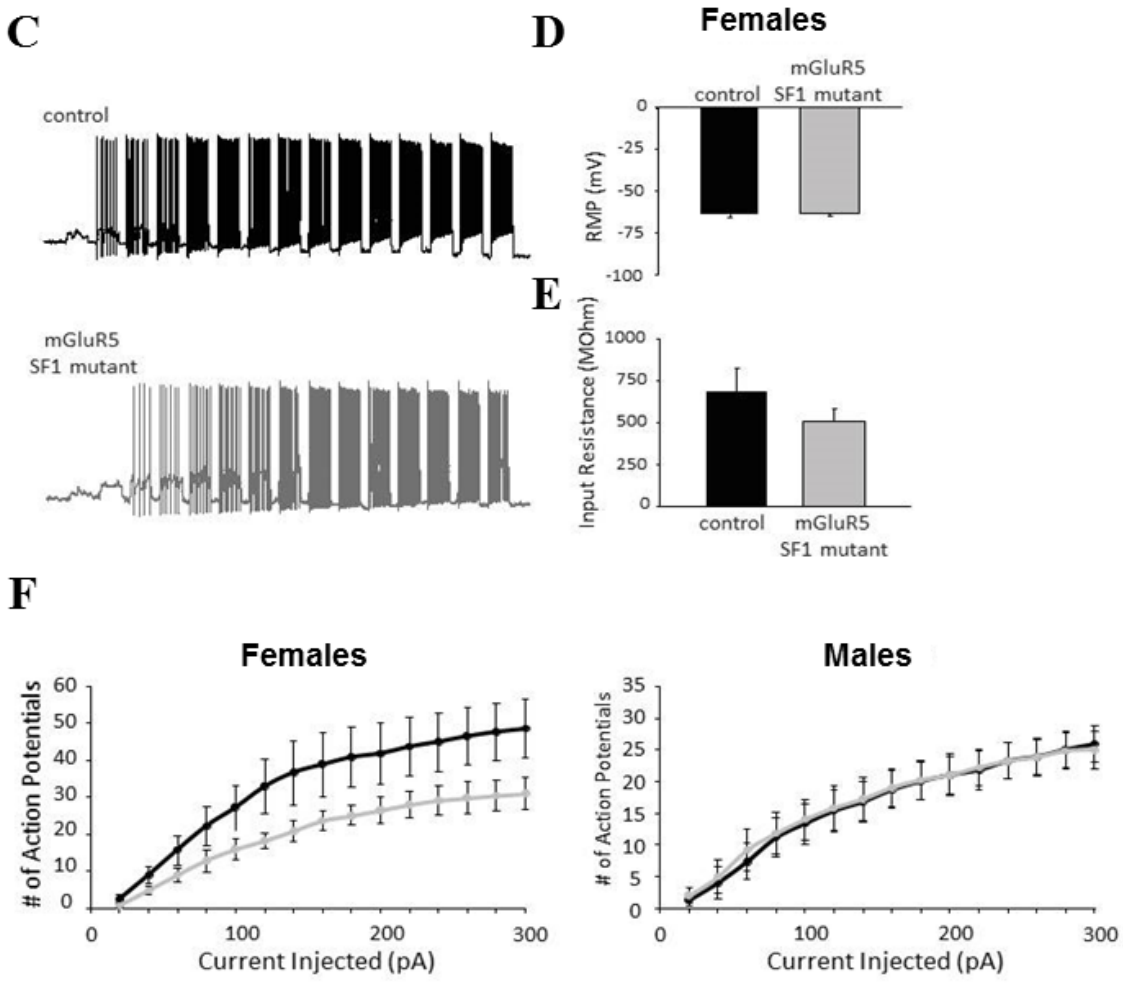


**Figure 3.8 (con't) Neuronal activity is decreased in the VMH of female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants.** **(C)** Representative traces of action potentials fired by female mGluR5$^{2L/2L}$ control (black) and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant (gray line) mice. **(D)** Resting membrane potential (RMP) and **(E)** input resistance of female mGluR5$^{2L/2L}$ control (black) and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant (gray) mice. **(F)** Average number of action potentials fired in response to increasing current injections in female (*left*) and male (*right*) mGluR5$^{2L/2L}$ control (black line) and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant (gray line) mice.

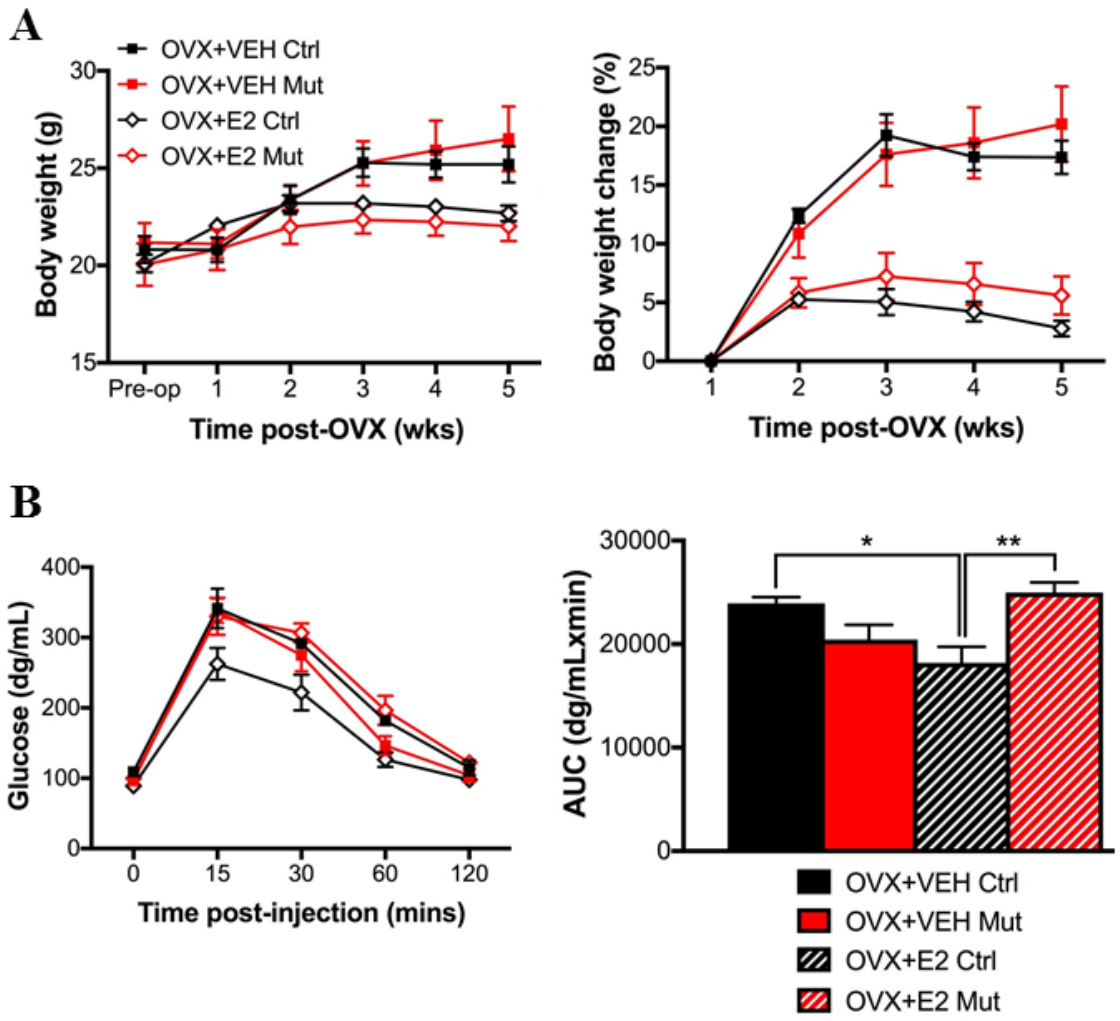


**Figure 3.9 mGluR5$^{2L/2L:SF1\text{-}cre}$ female mutants show deficits in estradiol-mediated regulation of glucose homeostasis.** **(A)** Body weight (*left*) and cumulative body weight gain (*right*) in female ovariectomized (OVX) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice implanted with silastic capsules containing vehicle (sesame oil) or 17β-estradiol (E2) (n= 6-7). **(B)** Time course (*left*) and area under the curve (AUC) (*right*) for glucose tolerance tests in female OVX mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice implanted with silastic capsules containing vehicle (sesame oil) or 17β-estradiol (E2) (n= 6-7; ** = $p$ <0.01, * = $p$ <0.05).

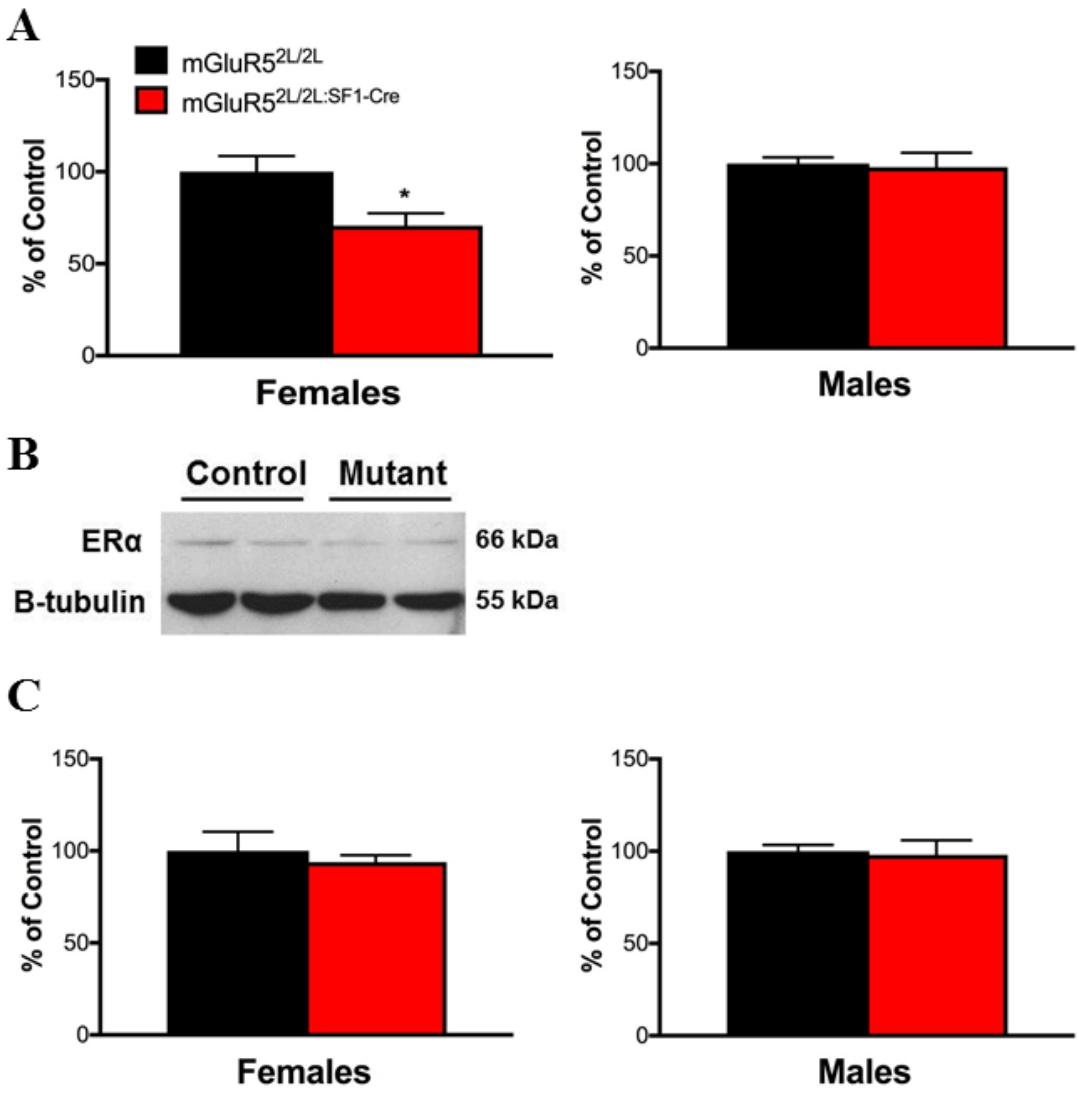


**Figure 3.10 ERα expression is reduced in the VMH of female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants. (A)** ERα protein expression in VMH of female (*left*; n=11-12; * = $p$ <0.05) and male (*right*; n= 4-5) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice. **(B)** Representative western blot image of ER**α** VMH content in female control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice. **(C)** ERβ protein expression in VMH of female and male mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice (n= 4-5 per group).

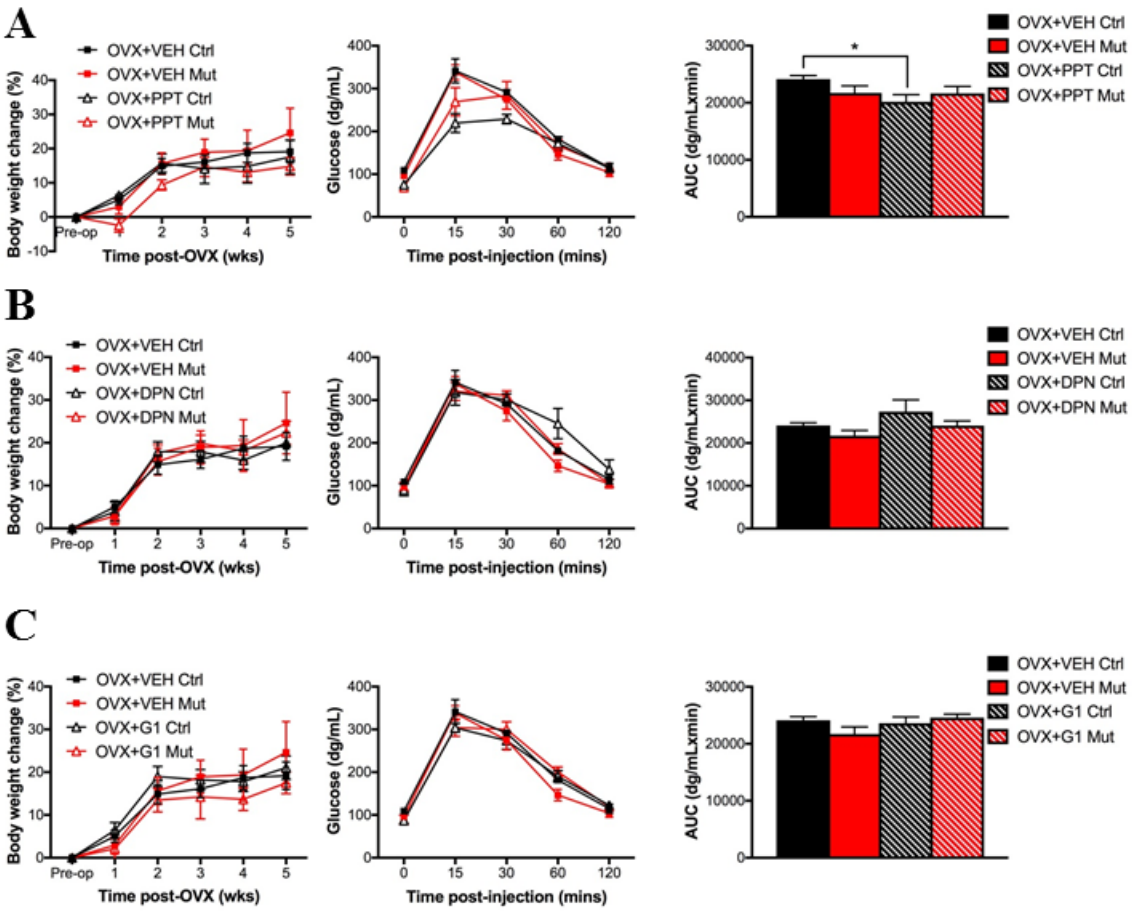


**Figure 3.11 mGluR5 functionally interacts with ERα to regulate glucose tolerance in female mice.** **(A)** Cumulative body weight gain (*left*), glucose tolerance testing (GTT) (*middle*) and area under the curve (AUC) (*right*) in female ovariectomized (OVX) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice implanted with silastic capsules containing vehicle or the ERα agonist PPT (n= 5; * = $p$ <0.05). **(B)** Cumulative body weight gain (*left*) GTT (*middle*) and AUC (*right*) in female ovariectomized (OVX) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice implanted with silastic capsules containing vehicle or the ERβ agonist DPN (n= 5). **(C)** Cumulative body weight gain (*left*) GTT (*middle*) and AUC (*right*) in female ovariectomized (OVX) mGluR5$^{2L/2L}$ control and mGluR5$^{2L/2L:SF1\text{-}Cre}$ mutant mice implanted with silastic capsules containing vehicle or the GPER1 agonist G1 (n= 5)

# Chapter 4: Discussion

The thesis research described herein identifies a critical and sex-specific role of mGluR5 in $SF1^{+}$ neurons of the VMH regulating neuronal activity, sympathetic output and glucose and lipid metabolism in female mice. Further, the collective results indicate that mGluR5 functionally interacts with selective estrogen receptors in the VMH, such as ERα, to mediate the effects of estradiol signaling on glycemic and lipid metabolism control. These studies are significant as they inform novel central mechanisms underlying the protective effects of estrogen against metabolic disease and the reported increased risk for insulin resistance and diabetes in postmenopausal women.

***mGluR5 is not required in SF1 neurons for energy balance control***

We conducted a comprehensive analysis of body weight, food intake, energy expenditure, thermoregulation and locomotor activity in $mGluR5^{2L/2L:SF1\text{-}cre}$ mice and found that mGluR5 is not required for the regulation of energy balance in females or males. This is in contrast with $BDNF^{2L/2L:Ck\text{-}cre}$ mutant mice, which in addition to exhibiting reduced mGluR5 expression in the VMH, are also profoundly obese and hyperphagic [73, 106]. These findings suggest that BDNF acts through mGluR5-independent mechanisms or that $SF1^{+}$ cells are not the critical cellular substrate for mGluR5 in the VMH for the regulation of feeding behavior and body weight. The latter finding is consistent with other genetic manipulations of SF1 neurons that do not show alterations in feeding behaviors under SC conditions [217, 277-279].

The normal energy balance exhibited by $mGluR5^{2L/2L:SF1\text{-}cre}$ mutant mice may also be explained by the heterogeneity of VMH cells [280], many of which are $SF1^{-}$ and have intact mGluR5 function. Moreover, mutant $mGluR5^{2L/2L:SF1\text{-}cre}$ mice exhibit an

incomplete knockdown of mGluR5 in the VMH, suggesting that residual expression of mGluR5 in these other cell types, including astrocytes, may be responsible for energy balance regulation. Alternatively, because mGluR5 is deleted during early development in our mGluR5$^{2L/2L:SF1\text{-}cre}$ mice, compensatory mechanisms might be playing a protective effect against severe deficits in energy homeostasis. Future experiments using an inducible line of SF1-cre mice will assess how mGluR5 deletion in mature SF1$^{+}$ neurons impacts energy and glucose balance control.

***mGluR5 action in SF1 neurons is required for glycemic control***

Glucose metabolism is predominantly controlled by glucose uptake by muscle and glucose uptake, storage and production in the liver. Insulin-dependent and insulin-independent mechanisms, as well as an array of additional physiological factors likely contribute to the maintenance of glucose levels that is crucial for survival.

In the fed state, increased glucose released into the bloodstream is sensed by pancreatic β cells, which respond by secreting insulin. In skeletal muscle, activation of insulin receptors by insulin leads to an increase in the surface expression of the glutamate transporter, VGLUT4, allowing for glucose uptake into the tissue to be used for energy. Insulin further activates signaling cascades to indirectly increase hepatic glycolysis (breakdown of glucose for ATP production), hepatic glycogenesis (storage of glucose as glycogen) and lipogenesis (fatty acid and triglyceride formation from glucose) in adipose tissue. In the fasted state, pancreatic α cells release glucagon, which acts on the liver to increase glycogenolysis (breakdown of glycogen to produce glucose), increase

gluconeogenesis (synthesis of glucose) and increase lipolysis in adipose tissue stores. The proper functioning of these metabolic processes is imperative for glycemic control.

We assessed glucose homeostasis by performing glucose tolerance tests (GTT) and found that female, but not male, mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants were severely glucose intolerant compared to sex-and age-matched controls, despite normal energy balance. Furthermore, female, but not male mGluR5$^{2L/2L:SF1\text{-}cre}$ mice were insulin resistant as indicated by elevated levels of circulating glucose during an ITT and decreased fasting levels of insulin, suggesting that insulin-mediated glucose uptake and utilization by peripheral metabolic tissues might be impaired. These findings indicate that mGluR5 action in SF1 neurons is an essential regulator of glucose homeostasis and insulin sensitivity, independent of energy balance control. In support, body weight-independent actions of SF1 neurons on glycemic control have been reported previously, as mice lacking suppressor of cytokine signaling-3 (SOCS3) in SF1 neurons showed improved glucose homeostasis despite no change in body weight [277].

Insulin-dependent mechanisms in the VMH may be driving the glucose intolerance in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. Previous studies demonstrate that insulin acts on VMH receptors to regulate sympathetic outflow onto peripheral tissues to increase glucose uptake and lipolysis in adipose tissue, inhibit gluconeogenesis in liver and promote glucose uptake in skeletal muscles [281, 282]. In SF1 neurons, insulin activates the PI3K pathway, which binds and opens $K_{ATP}$ channels to reduce neuronal firing. Further, mice with knockdown of the insulin receptor in SF1 neurons display normal levels of food intake and body weight under standard chow and are protected from diet induced weight gain, adiposity, leptin resistance and glucose intolerance [65].

Body weight-independent effects of insulin on glycemic control were also described by a previous study showing a reduction in insulin receptors in the VMH of rats led to glucose intolerance with no change in body weight [283]. Whether mGluR5 deletion in $SF1^{+}$ neurons alters insulin signaling or receptor expression is unclear.

Interestingly, a role for mGluR5 in the control of endocrine function in the pancreas has recently emerged. mGluR5 is expressed in pancreatic α- and β- cells and pharmacological activation of group 1 mGluRs enhances glucose-stimulated insulin secretion from β-cells [284]. mGluR5 functions at the cell membrane and intracellularly to permit glucose-stimulated increases in intracellular $Ca^{2+}$, leading to insulin secretion. In support, mice lacking mGluR5 receptors and wild-type treated with mGluR5 antagonists showed an attenuated insulin response to glucose [285]. It is well established that both pancreatic β cells and GE VMH neurons sense and respond to glucose via $K_{ATP}$ channels. Therefore, by similar mechanisms, it is possible that deletion of mGluR5 in $SF1^{+}$ neurons is decreasing the $Ca^{2+}$-stimulated release of peptides by these neurons when in response to glucose. Decreased glucose excitability of SF1 neurons would affect local neuronal circuits and reduce SNS activation and outflow to peripheral metabolic tissues, leading to decreased glucose uptake or uninhibited hepatic glucose production in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice.

Additionally, insulin resistance was observed exclusively in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice, suggesting insulin signaling facilitation by sex hormones and estrogen receptors. Indeed, several studies have demonstrated that estradiol signaling through ERα is protective against glucose dysregulation and insulin resistance [218, 286]. At the whole-body level, systemic administration of the ERα agonist PPT increased

insulin-stimulated glucose uptake in skeletal muscle [287], and insulin administration failed to suppress endogenous glucose production in ERα knockout female mice fed a HFD [288], highlighting a role for ERα in the peripheral control of metabolism. Although these studies do not pinpoint ERα activation in the VMH, it does not preclude that activation of VMH ERα may also facilitate these metabolic effects.

VMH- and SF1-specific deletion of ERα are associated with metabolic perturbations including obesity, hyperphagia, glucose intolerance and reduced energy expenditure [217, 218], but there is little mention of impaired insulin-dependent mechanisms. Our results show female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit a reduction in ERα in the VMH and are not responsive to the protective effects of systemic ERα agonist administration against glucose intolerance. Therefore, it is plausible that ERα-mediated effects on insulin signaling in the periphery or centrally are impaired in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice and contributing to their metabolic dysfunction. Further investigations of the regulation of insulin action and secretion by estradiol and mGluR5 in the VMH specifically will greatly advance our understanding of central mechanisms mediating glucose homeostasis in females.

Glucose homeostasis is also critically balanced by SNS outputs onto peripheral metabolic tissues. The SNS receives information about energy status from regions within the brain, leading to activity-dependent changes in the release of several factors that impact metabolic function. Moreover, stimulation of the VMH is associated with enhanced glucose utilization in skeletal muscle and BAT via sympathetic innervation [289, 290], indicating the VMH in the management of sympathetic outflow to peripheral tissues. Female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice are glucose intolerant and exhibit decreased

plasma levels of norepinephrine, suggesting that sympathetic outflow to peripheral tissues mediating glycemic control is disrupted in these mice.

Notably, SNS facilitation of glycemic control is imperative for the counterregulatory response to hypoglycemia. When low blood glucose levels are detected, several counterregulatory mechanisms occur: pancreatic β-cell insulin secretion decreases, pancreatic α-cell glucagon secretion increases and SNS activation leads to an increase in cortisol, norepinephrine and epinephrine release from the adrenal glands [291]. It is possible that female mGluR$^{2L/2L:SF1\text{-}cre}$ mice exhibit alterations in the counterregulatory response to insulin-induced hypoglycemia, as indicated by the elevated glucose levels at 90 and 120 minutes post-insulin injection compared to female controls. This finding might indicate a lack of an appropriate sympathetic response, and reduced norepinephrine release may be driving decreased glucose uptake by muscle or unchecked hepatic glucose production in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. In total, impairments in the counterregulatory would explain the persistently high plasma glucose levels in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice during the GTT and the decreased sensitivity to insulin in the ITT.

Leptin also plays a primary role in the central regulation of glucose homeostasis, independent of actions on food intake, energy expenditure or body weight [292]. Leptin action in the VMH depolarizes and increases the firing rate of SF1$^{+}$ neurons, and mice lacking leptin receptors in SF1$^{+}$ neurons display increased adiposity, hyperleptinemia and resistance to DIO, with no alterations in food intake [61]. Similarly, female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit alterations in lipid metabolism independent of food intake. Gonadal hormones, including estradiol, are critical regulators of leptin sensitivity.

In support, female OVX wild-type mice are leptin insensitive, independent of body weight, an effect that is reversed by peripheral estradiol replacement [293]. Female OVX mGluR5$^{2L/2L:SF1\text{-}cre}$ mice are insensitive to the protective effects of peripheral estradiol replacement on glucose intolerance, indicating impaired estrogenic signaling. Therefore, these mice may also exhibit leptin resistance. Moreover, leptin action in the VMH also plays a pivotal role in sympathetic activation and catecholamine secretion. Accordingly, leptin injections into the VMH, but not the Arc, PVN or DMH, significantly increased plasma norepinephrine and epinephrine levels [294]. Further, leptin injection into the VMH increased glucose utilization in skeletal muscle of mice [295]. As a consequence of mGluR5 deletion in SF1$^{+}$ cells, female mice display VMH hypoactivity and decreased plasma norepinephrine. It is possible that in these mice, leptin is not able to properly activate VMH neurons, leading to decreased sympathetic outflow and peripheral metabolic dysfunction. Whether SF1$^{+}$ neurons with mGluR5 deletion show attenuated responses to leptin and whether estradiol differentially regulates responsivity to leptin in mGluR5$^{2L/2L:SF1\text{-}cre}$ requires additional electrophysiological analysis.

***mGluR5 regulates lipid metabolism in white adipose tissue***

Dysregulated glycemic control in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants was coupled with an increase in triglyceride content, altered fatty acid profiles and hypertrophy of WAT, indicative of aberrant lipid homeostasis. Decreased sympathetic output may also explain these results. Indeed, SF1$^{+}$ efferents make close contacts with catecholaminergic nerve fibers in the brainstem [270], and these sympathetic terminals extend into the periphery to form neuron-adipose junctions with adipocytes [296]. Local stimulation of

sympathetic fibers increases lipolysis, and decreased sympathetic output is associated with hypertrophy of gonadal WAT [278], consistent with the phenotype of our female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice.

In WAT, lipolysis drives the breakdown of triglycerides and release of its by-products, glycerol and nonesterified fatty acids, to be used as energy by other organs. The rate of lipolysis in response to energy status is tightly controlled by numerous hormone and biochemical signals, which modulate the activity of lipolytic enzymes and proteins. The dysfunction of these lipolytic signals can lead to lipid accumulation and an increased risk for obesity, insulin resistance, cardiovascular disease and inflammation [297]. Thus, an explanation for the adipocyte hypertrophy observed in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants could be alterations of key lipolytic proteins such as HSL, ATGL, perilipin A, LPL or Lipin1 in WAT. Notably, HSL and ATGL account for over 90% of lipolytic activity in WAT [298].

HSL is the major rate-limiting enzyme in lipolysis, and phosphorylation of HSL is important for activity-dependent changes in adipocyte lipolysis. The phosphorylation of specific resides is also important for regulation of lipolytic activity. Several studies have identified PKA-mediated phosphorylation of serines 563, 649, 650, 659, 660 activate HSL, leading to increased lipolysis [299-301]. However, AMPK-mediated phosphorylation of HSL at serine 565 significantly reduced epinephrine-stimulated lipolysis through inhibition of PKA-mediated phosphorylation at serine 563 and 660 [302, 303]. Thus, phosphorylation of HSL and lipolytic activity is a complex process influenced by several different effectors.

Notably, a previous study of conditional deletion of the cannabinoid receptor 1 (CB1) in SF1 neurons reported an increase in sympathetic drive and decreased adiposity, an effect associated with phosphorylation of HSL at serines 563 and 660 [304]. This finding links $SF1^{+}$ projections with sympathetic tone and lipolytic activity in WAT, a pathway that may be impaired in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice that exhibit decreased sympathetic tone and hypertrophy of WAT. Whether phosphorylation of HSL at activating sites such as serine 563 and 660 or at inactivating sites such as serine 565 is altered in WAT from female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice will be determined in future studies.

In addition, estradiol is linked to the regulation of several factors involved in lipolysis, but detailed mechanisms of estradiol signaling in the hypothalamus regulating lipolysis remain unclear. A direct effect of estradiol on adipocytes was demonstrated *in vitro*, as estradiol administration to subcutaneous abdominal adipocytes from women increased HSL expression and lipolytic activity [305]. However, the influence of estrogenic and mGluR5 signaling in $SF1^{+}$ neurons mediating lipolytic protein expression in WAT requires additional experiments.

Another mechanism that may be contributing to the observed adipocyte hypertrophy in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mutants is alterations in the subtypes of adrenergic receptors expressed in mutant WAT tissue. β-adrenergic receptors (β-ARs) positively regulate lipolysis via activation of adenylyl cyclase and cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase (PKA), leading to the phosphorylation of HSL and perilipin A, wheras α-ARs inhibit cAMP production [306-308]. In support, studies in human and animal models suggest that an increased ratio of α-AR/β-AR is associated with obesity and lipid overload [309]. Estradiol also interacts with these

adrenergic receptors to influence adiposity. In support, estradiol treatment increased beta-adrenergic receptor expression in cultured adipocytes through ERalpha [310]. Whether the ratio of adrenergic receptors is imbalanced in WAT from female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice is unclear. Regardless of mechanism, the dysregulated lipid homeostasis evident in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutants is indicative of a paramount role of mGluR5 in VMH regulating these physiological processes in the periphery**.**

Lipid metabolism is critically important for an organism's physiological wellbeing, as adipocyte functioning not only affects fat tissue, but several other metabolic processes. As mentioned previously, dysregulated lipid metabolism is linked to an increased risk of obesity, insulin resistance, cardiovascular disease and inflammation [29]. The danger of dysregulated lipolysis is exemplified by the fact that mutations in HSL in humans are associated with dyslipidemia, hepatic steatosis, insulin resistance and diabetes [311]. Therefore, it is possible that the triglyceride accumulation and adipocyte hypertrophy exhibited by female mGluR5$^{2L/2L:SF1\text{-}cre}$ is having negative consequences on a myriad of metabolic functions.

***mGluR5 and estradiol functionally signal through ERα to facilitate glucose homeostasis***

Because mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant females, but not males, exhibit such drastic impairments in glucose and lipid metabolism, we investigated whether the sex-specific effects of mGluR5 may be mediated through functional interactions with estrogen signaling. Previous studies support this proposal, as membrane-localized estrogen receptors are known to interact with mGluR5 to mediate excitatory synaptic plasticity in

other brain regions [187]. To determine the effect of estradiol on glucose tolerance, we OVX female control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice and subcutaneously implanted silastic capsules containing vehicle (sesame oil) or 17β-estradiol. We found that chronic estradiol treatment attenuated body weight gain and glucose intolerance in OVX control mice but reduced body weight gain with no amelioration of glucose intolerance in OVX mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice. Therefore, we conclude that mGluR5 functionally interacts with estrogenic signaling pathways in the VMH to facilitate glycemic control, and not body weight regulation, in female mice.

We then extended our investigation to determine whether mGluR5 signaling through specific estrogen receptors were responsible for glycemic control. We found that chronic treatment with the selective ERα agonist PPT failed to protect female mGluR5$^{2L/2L:SF1\text{-}cre}$ mutant mice against glucose intolerance. In support, we found no effect on glucose tolerance or body weight in OVX control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice treated with the ERβ agonist DPN or the GPER1 agonist G1.

Selective deletion of ERα in SF1 neurons in female mice results in increased adiposity and impaired glucose tolerance due to reduced sympathetic activity to metabolic tissues [217], demonstrating that ERα function in these neurons are essential for many metabolic functions, including glucose and lipid metabolism. The phenotype of SF1-specific ERα knockdown mice is very reminiscent of our female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice, which also exhibit a significant decrease in VMH ERα. It is therefore possible that mGluR5 and ERα interact in the VMH and synergize downstream signaling pathways to mediate glucose and lipid control in females.

How mGluR5 deletion in $SF1^+$ neurons leads to a decrease in VMH ERα expression remains unclear. It is possible that signaling pathways activated downstream of mGluR5 may regulate ERα expression and protein stability. For example, the cAMP/PKA pathway is a major contributor to activity-dependent synaptic plasticity in the brain [312]. Previous studies have shown that mGluR5 stimulation potentiates cAMP accumulation in the striatum and cortex [313, 314]. Additionally, mGluR5 activated PKA in several brain regions including the cortex and amygdala [315, 316]. Importantly, a previous study showed that stimulation of PKA-dependent pathways stabilized ERα and prevented its degradation in pituitary cells [317]. Further, ERα transcription is activated by cAMP signaling [318]. Therefore, it is possible that a reduction in cAMP/PKA signaling as a result of mGluR5 deletion in $SF1^+$ neurons may lead to a downregulation of ERα transcription and protein degradation in these VMH cells.

The functional interaction between mGluR5 and ERα may also be influenced by scaffolding proteins. In support, a previous study demonstrated that ERα required mGluR5 for activation of CREB phosphorylation in female striatal neurons, an interaction that was dependent upon the caveolin-1 (CAV1) scaffolding protein [188]. Thus, the absence of mGluR5 might lead to destabilization, removal and endocytosis of ERα from the cell surface. In support, mGluR5 association with and trafficking to the plasma membrane is mediated by CAV1-dependent endocytosis [319], and CAV1 knockdown in the arcuate nucleus attenuated sexual receptivity in female rats through a reduction in membrane bound ERα expression [320]. Experiments aimed at elucidating the details of mGluR5 and ERα interactions and the downstream pathways involved are ongoing.

***mGluR5 deletion in SF1 neurons decreases VMH activity***

How might the discrete manipulation of deleting mGluR5 in $SF1^{+}$ neurons lead to severe glucose intolerance, insulin resistance, impaired lipid metabolism and decreased sympathetic tone exclusively in $mGluR5^{2L/2L:SF1\text{-}cre}$ mutant females? Our results suggest that hypoactivity of SF1 neurons synapsing onto autonomic-regulating centers resulting in decreases in sympathetic outflow may be a mechanism by which glucose and lipid metabolism is perturbed in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice.

However, it is also possible that parasympathetic system (PNS) outflow is altered in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice. During hyperglycemia, activation of the PNS results in decreased hepatic glucose release and increased insulin secretion. Mechanistically, this occurs by innervation of the liver and pancreas by vagal nerve fibers [321, 322]. Although evidence of $SF1^{+}$ neurons contacting vagal nerves is lacking, the Arc and PVN send projections to the nucleus of the solitary tract (NTS) which project to vagal afferent fibers in the nodose ganglion [323]. Thus, hypoactivity of VMH neurons projecting to the PVN and Arc may reduce the excitatory drive onto vagal nerves, leading to decreased PNS activity, increased hepatic glucose release and decreased insulin secretion during a hyperglycemic state.

As mentioned previously, glucose sensing neurons in the VMH are extremely important for the counterregulatory response to hypoglycemia and for the secretion of pancreatic insulin in response to increased glucose levels [324, 325]. Therefore, we assessed the effect of glucose administration on VMH neuronal activation by c-fos immunolabeling. We found that female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice exhibited a significant and dramatic decrease in $c\text{-}fos^{+}$ cells in response to glucose administration compared to

controls. These results support our hypothesis that the metabolic alterations observed in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice are associated with reduced excitability of SF1$^{+}$ neurons. In support, mice with whole-body knockout of uncoupling protein 2 (UCP2) displayed reduced c-fos$^{+}$ immunolabeling in the VMH after peripheral administration of glucose. These mice also exhibited glucose intolerance due to reduced insulin sensitivity in peripheral organs. Further, the glucose intolerance and neuronal hypoactivity in UCP2-knockout mice was restored by selective re-expression of UCP2 in SF1$^{+}$ neurons [326], highlighting the important role of SF1 neurons in the regulation of neuronal activity and concomitant glycemic control.

Several mGluR5-dependent mechanisms of estradiol action on synaptic plasticity and physiology have been proposed. For example, coupling of mGluR5 to membrane bound ERα induced MAPK-dependent CREB phosphorylation, a known regulator of excitatory synaptic plasticity and dendritic spine formation, in female striatal neurons [188]. Moreover, estradiol-initiated increases in dendritic spine density in the nucleus accumbens are dependent upon mGluR5 activation [190, 320]. Therefore, it is possible that female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit decreased phosphorylated CREB expression and alterations in dendritic spine density or morphology. Further, several candidate proteins that influence synaptic plasticity and are associated with mGluR5 and estradiol such as FMRP, MeCP2 and GSK3β may be dysregulated in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice and will be discussed in the following section. In total, the aforementioned studies and our experimental results suggest mGluR5 depletion in SF1$^{+}$ cells may impair estradiol-mediated synaptic modifications in VMH neural circuits mediating glycemic and lipid control.

In addition to effects on excitatory synaptic plasticity and physiology, mGluR5-estrogen receptor interactions in female $SF1^{+}$ neurons may influence the intrinsic excitability of VMH cells. Both estrogen and mGluR5 influence neuronal excitability through regulation of $K^{+}$ ion channels and $Ca^{2+}$ currents in other brain regions [327, 328]. Consistent with decreased membrane excitability in the VMH, we found that female, but not male, $mGluR5^{2L/2L:SF1\text{-}cre}$ mice displayed decreased action potential firing in response to increasing depolarizing current injection.

Glucose-sensing neurons in the VMH modulate their firing rate in response to raising glucose levels. Mechanistically, this occurs as glucose closes ATP-sensitive $K^{+}$ channels ($K_{ATP}$) on GE neurons and opens chloride channels on GI neurons [265]. $K_{ATP}$ channels in GE neurons of the hypothalamus and in pancreatic β-cells are essential for the maintenance of glucose homeostasis [54]. Importantly, both mGluR5 and estrogen regulate $K_{ATP}$ functioning in other brain regions. For example, mGluR5 stimulation generated $K_{ATP}$ currents in rat subthalamic nucleus neurons [329], and estradiol mediated the excitability of gonadotropin-releasing hormone (GnRH) neurons through $K_{ATP}$ channels [330]. Thus, the hypoactivity of VMH cells in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mutants may be mediated by $K_{ATP}$ channels. An electrophysiological analysis of the firing properties of VMH neurons is warranted to determine the molecular mechanisms underlying the decreased intrinsic excitability in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mutant mice.

***The potential role of BDNF in regulating mGluR5 and body weight-independent metabolic effects in mGluR5$^{2L/2L:SF1\text{-}cre}$ mice***

BDNF may regulate mGluR5 at the transcriptional level via downstream signaling pathways. As mentioned previously, BDNF binding to TrkB activates several intracellular signaling cascades including MAPK, PI3K and PLC-γ [331]. Activation of the MAPK pathway mediated by BDNF/TrkB signaling leads to the phosphorylation of serine 133 of CREB in neurons [332]. Phosphorylated CREB then recruits the necessary protein to initiate transcription in CREB target genes [333]. Interestingly, mGluR5 contains a functional promoter site for CREB binding [334]. Additionally, BDNF activates an additional transcription factor, Elk-1, in cultured cortical neurons, leading to an increase in gene transcriptions involved in Alzheimer's disease [335]. Similar to CREB, mGluR5 also contains a binding site for Elk-1 transciptional activation within its promoter regions [334]. Therefore, the loss of BDNF in mutant BDNF$^{2L/2L:Ck\text{-}cre}$ may reduce MAPK signaling and phosphorylated CREB and Elk-1 activation, leading to decreased transcriptional activity and translation of mGluR5.

Few studies have investigated an interaction between BDNF and mGluR5. A previous study found that the multidomain scaffolding protein, Preso1, was required for the BDNF-facilitated increase in mGluR5 association with Homer [336], suggesting that BDNF may indirectly regulate mGluR5 stability by altering the dynamics of mGluR5's binding partners. In rat C6 glioma cells, treatment with an mGluR5 agonist increased BDNF expression, an effect dependent upon PKC signaling [337]. Further, increased glutamate release and neuronal excitability induced by BDNF/TrkB signaling was dependent upon mGluR5 activation in entorhinal cortex cells [338]. These studies

suggest that BDNF and mGluR5 may reciprocally regulate each other by feedback mechanisms at the level of transcription, translation or protein stability. In total, the limited literature available highlights the critical need to elucidate the interactions between BDNF and mGluR5, particularly in the control of energy, glucose and lipid metabolism.

Our observation that mGluR5 was significantly downregulated in the VMH of female and male $BDNF^{2L/2L:Ck\text{-}cre}$ mutant mice suggested that mGluR5 may act as a downstream effector of BDNF in this region in the regulation of energy, glucose and lipid homeostasis. Furthermore, we determined that female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice were severely glucose intolerant, despite normal energy balance. As discussed previously, several metabolic deficits induced by diminished BDNF function are more severe in female $BDNF^{2L/2L:Ck\text{-}cre}$ mutants compared to male BDNF mutant mice, including as body weight gain, increased adiposity and bone cytoarchitecture [106, 107]. Table 4.1 outlines the key phenotypic differences in mice with global BDNF knockout ($BDNF^{2L/2L:Ck\text{-}cre}$ mice, mice with AAV-mediated BDNF knockdown in the VMH and mice with mGluR5 deletion in SF1 neurons ($mGluR5^{2L/2L:SF1\text{-}cre}$). Further investigations of female $BDNF^{2L/2::Ck\text{-}cre}$ mice and female mice with VMH-specific deletion of BDNF remain warranted. Additionally, it is well established that BDNF exerts body weight-independent effects on glucose homeostasis [108-110]. Therefore, we hypothesize that some metabolic effects of BDNF may require functional mGluR5-estrogenic signaling in female mice. Future studies will investigate this hypothesis by administering BDNF to the VMH of male and female $mGluR5^{2L/2L}$ and $mGluR5^{2L/2L:SF1\text{-}cre}$ mice (naïve or gonadectomiced and delivered vehicle or 17β-estradiol) and monitoring the effects on

energy (food intake and body weight), glucose (GTT and ITTs) and lipid (WAT histology) homeostasis.

**Table 4.1 Comparison of phenotypes in BDNF$^{2L/2L:Ck\text{-}cre}$ mice, mice with AAV-mediated BDNF knockdown in the VMH and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice**

| | BDNF$^{2L/2L:Ck\text{-}cre}$ | AAV-mediated BDNF knockdown in VMH | mGluR5$^{2L/2L:SF1\text{-}cre}$ |
|---|---|---|---|
| **Males** | - Hyperphagic<br>- Obese (80% of age-matched controls; 55g at 30 wks of age)<br>- Hyperglycemic (215 mg/dl)<br>- Hyperinsulinemic (3 ng/ml)<br>- Hyperleptinemic (80 ng/ml)<br>- Hyperaggressive<br>- Increased anxiety-like behavior | - Hyperphagic<br>- Obese (52% of age-matched controls; 38g at 16 wks of age)<br>- Hyperglycemic (130 mg/dl)<br>- Hyperinsulinemic (15 ng/ml)<br>- Hyperleptinemic (14 ng/ml) | Normal |
| **Females** | - Hyperphagic<br>- Obese (150% of age-matched controls; 65g at 30 wks of age) | Not tested | - Normal food intake and body weight<br>- Glucose intolerant<br>- Insulin resistant<br>- Triglyceride accumulation<br>- Hypertrophy of WAT<br>- Decreased SNS output<br>- Decreased intrinsic excitability<br>- Decreased neuronal activity in response to glucose<br>- Decreased responsivity to estrogen- and ERα-mediated glucose control |

## Chapter 4.1 Future Studies and Perspectives

### *Are other metabolic perturbations in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice dependent upon estrogenic signaling?*

We have shown that regulation of glucose tolerance is impaired in female mice lacking mGluR5 in SF1$^{+}$ neurons. Further, we show that estrogenic signaling facilitates glucose homeostasis in these mice through the ERα receptor, as estradiol and ERα treatment in OVX mGluR5$^{2L/2L:SF1\text{-}cre}$ mice failed to protect against glucose intolerance. However, it has not been determined whether there are additional estradiol-dependent metabolic impairments in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. For these tests, OVX control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice implanted with silastic capsules containing vehicle (sesame oil), 17β-estradiol or the ERα agonist PPT will be used. To test whether cell surface expression of ERα is mediating any observed metabolic effects, a membrane impermeable form of estradiol (E2-BSA) can also be administered.

To assess insulin sensitivity, we will measure fasting serum levels using an insulin immunoassay kit and perform ITTs in these mice. We will also determine whether the counterregulatory response to hypoglycemia is impaired in female mGluR5$^{2L/2L:SF1\text{-}cre}$ by measuring serum levels of glucagon, epinephrine and cortisol after a prolonged fast. Lipid homeostasis will be investigated by measuring triglyceride content in serum, BAT, WAT, liver and skeletal muscle. Further, we will measure serum leptin and adiponectin levels, as aberrant lipid accumulation could alter secretion of these adipokines and contribute to glucose intolerance. Lastly, we will utilize high performance liquid chromatography (HPLC) to measure serum levels of norepinephrine as readout of sympathetic outflow. Together, these experiments will reveal whether, in

addition to glucose intolerance, other phenotypes of female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice are dependent upon estradiol signaling and mGluR5-estrogenic signaling through ERα.

We will also determine whether the functional interaction between mGluR5 and ERα is due to a physical binding of the two proteins. To test this, VMH lysates will be immunoprecipated for mGluR5 and co-immunoblotted for ERα. These experiments will inform whether mGluR5 and ERα physically interact and whether this binding is disrupted as a result of mGluR5 deletion from SF1 neurons in female mice.

***Is glycemic control impaired in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice by insulin-dependent or insulin-independent mechanisms?***

The molecular mechanisms and signaling pathways governing the glucose intolerance exhibited by female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice is unclear. Insulin-dependent actions such as insulin secretion, insulin-induced glucose uptake by skeletal muscle and inhibition of hepatic glucose production are possible mechanisms driving glucose intolerance in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. To investigate insulin release, serum insulin will be measured at various time points during a glucose tolerance test. Because female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit decreased fasting levels of insulin, we predict that during a GTT, decreased release of insulin is reducing glucose clearance from the bloodstream. We will measure glucose uptake by skeletal muscle after a glucose challenge using commercially available glucose uptake assays, which measures labeled 2-DG content in tissue. Further, we will assess hepatic glucose production by measuring expression levels of gluconeogenic proteins such as G6Pase and PEPCK with Western blot analysis.

We will also investigate whether insulin-independent mechanisms, such as leptin action, are responsible for the glucose intolerance observed in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. To achieve this, we will administer leptin peripherally and/or specifically in the VMH and measure blood glucose levels in male and female control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice.

These collective experiments will reveal whether insulin-dependent or insulin-independent mechanisms are driving the glucose intolerance evident in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. It is also possible that these effects are facilitated by sympathetic outflow, as female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit decreased sympathetic tone. To address the effects of SNS outflow, these experiments will be repeated in mice administered central SNS blockers (for example, rilmenidine or moxonidine) or selective β-adrenergic blockers.

***Is lipolysis altered in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice?***

Although no body weight differences were observed in female control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice, it does not preclude the possibility that there are alterations in the ratio of fat to lean muscle tissue. Increased adiposity was reported in female mice with SF1-specific knockdown of ERα [217]. Since female mGluR5$^{2L/2L}$ also exhibit decreased VMH expression of ERα, they may exhibit increased fat mass and decreased lean mass, resulting in normalized body weights. It is thus important to measure body densitometry of female and male control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice by quantitative magnetic resonance spectroscopy. Additionally, this experiment will be repeated in OVX control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice implanted with capsules containing vehicle or 17β-

estradiol to determine whether mGluR5-estrogenic signaling mediates adiposity in these mice.

Further, female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice exhibit triglyceride accumulation and hypertrophy of WAT, indicative of aberrant lipolysis in this metabolic tissue. However, the lipolytic mechanisms driving this imbalance have not been determined. Ongoing Western blot experiments are measuring expression levels of key proteins and enzymes involved in lipolysis including activating phosphoHSLser563 and 660, inactivating phosphoHSLser565, ATGL, perilipin A, LPL and Lipin1 in WAT collected from female control and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice.

***What are the cellular mechanisms underlying decreased intrinsic excitability in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice?***

We investigated the electrophysiological properties of SF1$^{+}$ neurons in mGluR5$^{+/+:SF1\text{-}cre:TdTomato}$ (control) and mGluR5$^{2L/2L:SF1\text{-}cre:TdTomato}$ (mutant) mice. We determined that the intrinsic excitability of SF1 neurons is decreased in female, but not male, mGluR5$^{2L/2L:SF1\text{-}cre:TdTomato}$ mice. This effect may be mediated by mGluR5 and estrogen action in the VMH, which are known regulators of $K^{+}$ and $Ca^{2+}$ currents in other brain regions [329, 330]. To determine whether estradiol-mediated effects on intrinsic excitability are involved, future studies will focus on OVX mGluR5$^{+/+:SF1\text{-}cre:TdTomato}$ and mGluR5$^{2L/2L:SF1\text{-}cre:TdTomato}$ mice implanted with silastic capsules containing vehicle or 17β-estradiol.

As discussed previously, dysregulation of $K_{ATP}$ channels are a candidate mechanism driving the reduced excitability of SF1$^{+}$ neurons in female mGluR5$^{2L/2L:SF1\text{-}cre}$

because of the important roles they play in activating GE VMH neurons, influence on glucose homeostasis and regulation by both mGluR5 and estrogenic signaling. To test the functional role of $K_{ATP}$ channels, we will measure the evoked response of $SF1^+$ neurons in the presence of the $K_{ATP}$ channel opener, diazoxide. We predict that diaxoxide administration will have an attenuated effect on $SF1^+$ neuronal activity in VMH slices from $mGluR5^{2L/2L:SF1\text{-}cre:TdTomato}$ mutant mice compared to $mGluR5^{+/+:SF1\text{-}cre:TdTomato}$ control mice.

Intrinsic excitability may also be mediated by voltage-gated $K^+$ (Kv) channels. Whether mGluR5- or estrogen-dependent regulation of Kv channels influence glucose or lipid homeostasis has not been studied. A previous study demonstrated that mGluR5 regulates the intrinsic excitability of parvalbumin cells through the downregulation of voltage-gated Kv1 channels in the hippocampus [273]. To measure whether mGluR5 deficits in $SF1^+$ neurons in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice are also associated with a reduction in Kv channels, we can co-immunolabel for $SF1^+$ neurons and various Kv subunits. Moreover, it was published that estrogen had an inhibitory effect on the outward $K^+$ currents ($I_A$) in PVN neurons [339]. Thus, we can measure the electrophysiological properties of $I_A$ in the presence or absence of a Kv channel blocker (for example, 4-AP or DTx-1) in OVX $mGluR5^{+/+:SF1\text{-}cre:TdTomato}$ and $mGluR5^{2L/2L:SF1\text{-}cre:TdTomato}$ mice treated with vehicle or 17β-estradiol.

***Do female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice exhibit alterations in excitatory synaptic plasticity?***

In addition to effects on intrinsic excitability, we hypothesize that mGluR5-estrogenic signaling in female $SF1^+$ may be impairing excitatory synaptic activity.

Ongoing electrophysiological experiments are measuring AMPA and NMDA currents and spontaneous EPSC frequency and amplitude in female and male mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice. We also plan to elucidate interactions between NMDA receptors and mGluR5 and determine whether their positive coupling is dependent upon estrogenic signaling. Previous studies have demonstrated that mGluR5 interacts with NMDA receptors and potentiates their activity [148-150]. If NMDA receptor currents are altered in female mGluR5$^{2L/2L:SF1\text{-}cre}$ mice, we will first use biotinylation and Western blot analysis to determine whether there are reductions in the surface expression of NMDA receptors in female mGluR5$^{2L/2L:SF1\text{-}cre}$ as a consequence of mGluR5 deletion in SF1$^{+}$ neurons. We will also examine whether PLC activation and release of $Ca^{2+}$ from intracellular stores is required for mGluR5-mediated effects on NMDA currents by administration of the PLC inhibitor U73122 and administration of thapsigargin, which depletes intracellular $Ca^{2+}$ stores in neurons. The involvement of PKC, another canonical signaling player activated downstream of NMDA receptors can be assessed electrophysiologically using the PKC inhibitor, chelerythrine. However, it is also possible that mGluR5 itself does not impact AMPA or NMDA currents. In support, Kotecha *et al.*, (2003) found that excitatory synaptic transmission in hippocampal neurons required co-stimulation of mGluR5 and NMDA, as mGluR5 administration alone was insufficient to enhance NMDA and AMPA currents [149]. It is also possible that mGluR5 in SF1$^{+}$ neurons may influence inhibitory GABAergic signaling in the VMH. A thorough electrophysiological analysis is warranted to elucidate whether female mGluR5$^{2L/2L:SF1\text{-}cre}$ exhibit disruptions in excitatory synaptic activity.

mGluR5-estrogenic interactions in $SF1^{+}$ neurons may also affect synaptic plasticity and physiology through alterations in FMRP, MeCP2 and GSK3β. FMRP, as previously mentioned, is a key regulator of synaptic plasticity that is absent in Fragile X Syndrome. Phosphorylation of FMRP mediates changes in local protein translation in dendritic spines, and deletion of FMRP in an FXS mouse model resulted in defective dendritic spine shape and stabilization [340]. A previous study also demonstrated that mGluR5 activation in the nucleus accumbens led to a reduction in phosphoFMRP and increased transcription and translation of synaptic scaffolding proteins [341]. Therefore, mGluR5 deficits in the VMH of $mGluR5^{2L/2L:SF1\text{-}cre}$ mice could lead to increased phosphoFMRP, reduced local translation of synaptic proteins and compromised dendritic spine formation and stability. Expression levels of phosphoFMRP and FMRP in the VMH of male and female $mGluR5^{2L/2L}$ and $mGluR5^{2L/2L:SF1\text{-}cre}$ mice (naïve or gonadectomized and delivered vehicle or 17β-estradiol) will be determined by Western blot analysis. We will also evaluate dendritic spine density and morphology in female and male $mGluR5^{+/+:SF1\text{-}cre:TdTomato}$ $mGluR5^{2L/2L:SF1\text{-}cre:TdTomato}$ mice using biocytin filling and confocal microscopy analysis.

Dysregulation of MeCP2 by mGluR5 or estrogenic signaling is an additional mechanism that may be impairing synaptic function in female $mGluR5^{2L/2L:SF1\text{-}cre}$ mice. MeCP2 binds methylated DNA to repress or activate transcriptional activity [342]. The importance of this protein is exemplified by the fact that mutations in the *MeCP2* gene cause Rett syndrome (RTT), an autistic-like developmental disorder [343]. Phosphorylation of MeCP2 is important for neuronal morphology, synapse formation and synaptic plasticity [344]. Importantly, MeCP2 function is associated with metabolic

symptoms, and its deletion in mice results in hyperphagia, increased adiposity and hyperleptinemia [345]. Relevant to our research, MeCP2 phosphorylation regulates synaptic plasticity and physiology through mGluR5 [346], and ERα is one of MeCP2's transcriptional targets in the brain [347]. Therefore, it is possible that deficits in mGluR5 in female $SF1^{+}$ neurons is leading to altered synaptic functioning and morphology, reduced MeCP2-mediated ERα transcription and alterations in glucose and lipid homeostasis. Expression levels of phosphoMeCP2 and MeCP2 in the VMH of male and female $mGluR5^{2L/2L}$ and $mGluR5^{2L/2L:SF1\text{-}cre}$ mice (naïve or gonadectomized and delivered vehicle or 17β-estradiol) will be determined by Western blot analysis.

Lastly, an investigation of GSK3β in $mGluR5^{2L/2L:SF1\text{-}cre}$ mice is warranted due to its association with metabolic diseases such as insulin resistance and diabetes in humans [348]. *In vitro* studies have suggested that GSK3β influences glucose homeostasis through an upregulation of hepatic gluconeogenesis and phosphorylated CREB-mediated increases in PEPCK expression [349]. Notably, a recent study overexpressed GSK3β in skeletal muscle, which resulted in mice that were obese, insulin resistant and glucose intolerant [350], indicating a critical role of GSK3β in the regulation of metabolic control. Further, GSK3β is a known regulator of synaptic plasticity, including NMDA receptor-dependent LTP and LTD [351]. Both mGluR5 and estradiol negatively regulate GSK3β expression [352, 353]. Therefore, deficits in mGluR5-estrogenic function and signaling in female $SF1^{+}$ neurons could lead to a loss of inhibition of GSK3β expression, impaired synaptic functioning and dysregulated glucose and lipid homeostasis. Future experiments will measure GSK3β in the VMH and peripheral metabolic tissues of male

and female mGluR5$^{2L/2L}$ and mGluR5$^{2L/2L:SF1\text{-}cre}$ mice (naïve or gonadectomized and delivered vehicle or 17β-estradiol) utilizing Western blot analysis.

***Does BDNF regulate mGluR5 transcription in hypothalamic neurons?***

As previously discussed, transcription factors downstream of BDNF/TrkB signaling, such as CREB and Elk-1 may regulate mGluR5 transcription, providing an explanation as to why mutant BDNF$^{2L/2L:Ck\text{-}cre}$ mice exhibit reduced mGluR5 expression in the VMH. To test this hypothesis, we propose utilizing hypothalamic cell lines (for example, the gonadotropin lines GT1-7) or a neuroblastoma cell line (SMS-KCN) and cloning mGluR5 promoter constructs into the luciferase reporter system. We can then perform luciferase assays on these cell lines with acute and chronic treatment with BDNF to determine whether BDNF increases mGluR5 transcription. To investigate whether any of the signaling cascades activated by BDNF/TrkB is involved in the activation of mGluR5 transcription in response to BDNF, we will study the effect of specific inhibitors of these pathways (U0126 for MAPK, LY294002 for PI3K, and U73122 PLCγ) in luciferase assays. To determine whether phosphorylated CREB and Elk-1 is increased with BDNF application, we can immunolabel these transcription factors in VMH sections from mGluR5$^{+/+:SF1\text{-}cre:TdTomato}$ and mGluR5$^{2L/2L:SF1\text{-}cre:TdTomato}$ mice. These *in vitro* studies will quantitatively reveal whether BDNF application is regulating mGluR5 transcription and by which signaling pathway mechanisms.

## Chapter 4.2 Conclusion and Proposed Model

The collective results of this study demonstrate a novel and required role of mGluR5 regulating SF1 neuronal activity and metabolic function exclusively in female mice by interacting with specific estrogen receptors. Not only do these results inform pathological mechanisms that may underlie the metabolic disturbances brought on by estrogen deficits in postmenopausal women, they also provide insight as to inherent physiological differences between females and males in the regulation of glucose and lipid metabolism. The recent NIH mandate to study both sexes in biological research was necessary because disease mechanisms driving dimorphic differences in women have been less established than those in men [354]. Elucidating the mechanistic functions of central mGluR5 action and in VMH circuitry is a significant advancement towards these efforts and may lead to improved therapeutic treatments of metabolic disorders in women.

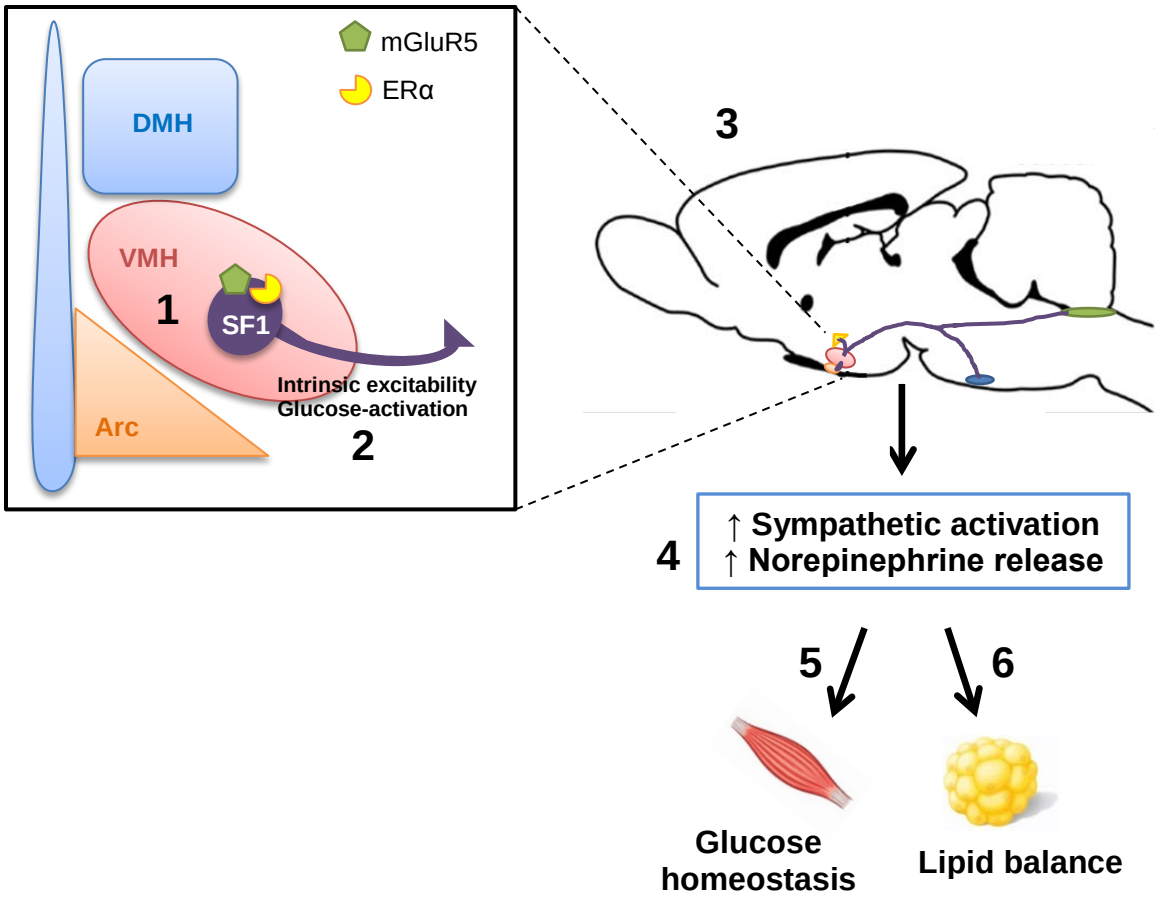


**Figure 4.1 A model of mGluR5 regulation of peripheral glucose and lipid control through modulation of SF1 neuronal excitability and sympathetic outflow.** In our proposed model, mGluR5 functionally interacts with estrogen receptors in the female VMH, including ERalpha **(1)**, to facilitate the intrinsic excitability and glucose-reactivity of SF1 neurons **(2)**. SF1 neurons send projections to nearby hypothalamic regions such as the arcuate nucleus (Arc; *orange*) and the paraventricular nucleus (PVN; *yellow*) as well as to areas that control sympathetic nervous system outflow such as the rostral ventrolateral medulla (RVLM; *blue*) and the nucleus of the solitary tract in the hindbrain (NTS; *green*) **(3)** [270]. Sympathetic activation leads to the release of norepinephrine **(4)**, which acts in the periphery to promote glucose homeostasis **(5)** in skeletal muscle and liver and lipid balance **(6)** in adipose tissues.

# Chapter 5: Bibliography

1. Cone, R.D., *Anatomy and regulation of the central melanocortin system.* Nat Neurosci, 2005. **8**(5): p. 571-8.
2. Mizuno, T.M., et al., *Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin.* Diabetes, 1998. **47**(2): p. 294-7.
3. Jegou, S., I. Boutelet, and H. Vaudry, *Melanocortin-3 receptor mRNA expression in pro-opiomelanocortin neurones of the rat arcuate nucleus.* J Neuroendocrinol, 2000. **12**(6): p. 501-5.
4. Garfield, A.S., et al., *Role of central melanocortin pathways in energy homeostasis.* Trends Endocrinol Metab, 2009. **20**(5): p. 203-15.
5. Huszar, D., et al., *Targeted disruption of the melanocortin-4 receptor results in obesity in mice.* Cell, 1997. **88**(1): p. 131-41.
6. Fan, W., et al., *Role of melanocortinergic neurons in feeding and the agouti obesity syndrome.* Nature, 1997. **385**(6612): p. 165-8.
7. Clark, J.T., et al., *Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats.* Endocrinology, 1984. **115**(1): p. 427-9.
8. Bewick, G.A., et al., *Post-embryonic ablation of AgRP neurons in mice leads to a lean, hypophagic phenotype.* Faseb j, 2005. **19**(12): p. 1680-2.
9. Wu, Q., et al., *Starvation after AgRP neuron ablation is independent of melanocortin signaling.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2687-92.
10. Broberger, C. and T. Hokfelt, *Hypothalamic and vagal neuropeptide circuitries regulating food intake.* Physiol Behav, 2001. **74**(4-5): p. 669-82.
11. Atasoy, D., et al., *Deconstruction of a neural circuit for hunger.* Nature, 2012. **488**(7410): p. 172-7.
12. Broberger, C., et al., *The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 15043-8.
13. Coppari, R., et al., *The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity.* Cell Metab, 2005. **1**(1): p. 63-72.
14. Huo, L., et al., *Leptin-dependent control of glucose balance and locomotor activity by POMC neurons.* Cell Metab, 2009. **9**(6): p. 537-47.
15. Hill, J.W., et al., *Direct insulin and leptin action on pro-opiomelanocortin neurons is required for normal glucose homeostasis and fertility.* Cell Metab, 2010. **11**(4): p. 286-97.
16. Lam, C.K., M. Chari, and T.K. Lam, *CNS regulation of glucose homeostasis.* Physiology (Bethesda), 2009. **24**: p. 159-70.
17. Bernardis, L.L. and L.L. Bellinger, *The dorsomedial hypothalamic nucleus revisited: 1986 update.* Brain Res, 1987. **434**(3): p. 321-81.
18. Bellinger, L.L. and L.L. Bernardis, *The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: lessons learned from lesioning studies.* Physiol Behav, 2002. **76**(3): p. 431-42.
19. Bernardis, L.L. and L.L. Bellinger, *The dorsomedial hypothalamic nucleus revisited: 1998 update.* Proc Soc Exp Biol Med, 1998. **218**(4): p. 284-306.

20. Bernardis, L.L. and L.L. Bellinger, *Brown (BAT) and white (WAT) adipose tissue in high-fat junk food (HFJF) and chow-fed rats with dorsomedial hypothalamic lesions (DMNL rats).* Behav Brain Res, 1991. **43**(2): p. 191-5.
21. Buijs, R.M., et al., *Suprachiasmatic nucleus lesion increases corticosterone secretion.* Am J Physiol, 1993. **264**(6 Pt 2): p. R1186-92.
22. Gallo, R.V., *Effect of electrical stimulation of the dorsomedial hypothalamic nucleus on pulsatile LH release in ovariectomized rats.* Neuroendocrinology, 1981. **32**(3): p. 134-8.
23. Luiten, P.G. and P. Room, *Interrelations between lateral, dorsomedial and ventromedial hypothalamic nuclei in the rat. An HRP study.* Brain Res, 1980. **190**(2): p. 321-32.
24. Chao, P.T., et al., *Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity.* Cell Metab, 2011. **13**(5): p. 573-83.
25. Mihaly, E., et al., *Hypothalamic dorsomedial nucleus neurons innervate thyrotropin-releasing hormone-synthesizing neurons in the paraventricular nucleus.* Brain Res, 2001. **891**(1-2): p. 20-31.
26. Dunn-Meynell, A.A., N.E. Rawson, and B.E. Levin, *Distribution and phenotype of neurons containing the ATP-sensitive K+ channel in rat brain.* Brain Res, 1998. **814**(1-2): p. 41-54.
27. Foster, M.T., C.K. Song, and T.J. Bartness, *Hypothalamic paraventricular nucleus lesion involvement in the sympathetic control of lipid mobilization.* Obesity (Silver Spring), 2010. **18**(4): p. 682-9.
28. Marino, J.S., Y. Xu, and J.W. Hill, *Central insulin and leptin-mediated autonomic control of glucose homeostasis.* Trends Endocrinol Metab, 2011. **22**(7): p. 275-85.
29. Stanley, S., et al., *Identification of neuronal subpopulations that project from hypothalamus to both liver and adipose tissue polysynaptically.* Proc Natl Acad Sci U S A, 2010. **107**(15): p. 7024-9.
30. Geerling, J.C., et al., *Paraventricular hypothalamic nucleus: axonal projections to the brainstem.* J Comp Neurol, 2010. **518**(9): p. 1460-99.
31. Hardie, D.G. and K. Sakamoto, *AMPK: a key sensor of fuel and energy status in skeletal muscle.* Physiology (Bethesda), 2006. **21**: p. 48-60.
32. Han, S.M., et al., *Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats.* Diabetologia, 2005. **48**(10): p. 2170-8.
33. Minokoshi, Y., et al., *AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus.* Nature, 2004. **428**(6982): p. 569-74.
34. Anand, B.K. and J.R. Brobeck, *Localization of a "feeding center" in the hypothalamus of the rat.* Proc Soc Exp Biol Med, 1951. **77**(2): p. 323-4.
35. Montemurro, D.G. and J.A. Stevenson, *Adipsia produced by hypothalamic lesions in the rat.* Can J Biochem Physiol, 1957. **35**(1): p. 31-7.
36. Delgado, J.M. and B.K. Anand, *Increase of food intake induced by electrical stimulation of the lateral hypothalamus.* Am J Physiol, 1953. **172**(1): p. 162-8.

37. Ziegler, D.R., W.E. Cullinan, and J.P. Herman, *Distribution of vesicular glutamate transporter mRNA in rat hypothalamus.* J Comp Neurol, 2002. **448**(3): p. 217-29.
38. Karnani, M.M., et al., *Lateral hypothalamic GAD65 neurons are spontaneously firing and distinct from orexin- and melanin-concentrating hormone neurons.* J Physiol, 2013. **591**(4): p. 933-53.
39. Luiten, P.G., G.J. ter Horst, and A.B. Steffens, *The hypothalamus, intrinsic connections and outflow pathways to the endocrine system in relation to the control of feeding and metabolism.* Prog Neurobiol, 1987. **28**(1): p. 1-54.
40. Burdakov, D., S.M. Luckman, and A. Verkhratsky, *Glucose-sensing neurons of the hypothalamus.* Philos Trans R Soc Lond B Biol Sci, 2005. **360**(1464): p. 2227-35.
41. Sakurai, T., *Orexins and orexin receptors: implication in feeding behavior.* Regul Pept, 1999. **85**(1): p. 25-30.
42. Haynes, A.C., et al., *Anorectic, thermogenic and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice.* Regul Pept, 2002. **104**(1-3): p. 153-9.
43. Tsuneki, H., et al., *Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice.* Diabetologia, 2008. **51**(4): p. 657-67.
44. Elias, C.F., et al., *Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area.* J Comp Neurol, 1998. **402**(4): p. 442-59.
45. Ludwig, D.S., et al., *Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance.* J Clin Invest, 2001. **107**(3): p. 379-86.
46. Shimada, M., et al., *Mice lacking melanin-concentrating hormone are hypophagic and lean.* Nature, 1998. **396**(6712): p. 670-4.
47. Kong, D., et al., *Glucose Stimulation of Hypothalamic MCH Neurons Involves K(ATP) Channels, is Modulated by UCP2, and Regulates Peripheral Glucose Homeostasis.* Cell Metab, 2010. **2**(15): p. 545-52.
48. King, B.M., *The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight.* Physiol Behav, 2006. **87**(2): p. 221-44.
49. Sternson, S.M., G.M. Shepherd, and J.M. Friedman, *Topographic mapping of VMH --> arcuate nucleus microcircuits and their reorganization by fasting.* Nat Neurosci, 2005. **8**(10): p. 1356-63.
50. Penicaud, L., C. Larue-Achagiotis, and J. Le Magnen, *Endocrine basis for weight gain after fasting or VMH lesion in rats.* Am J Physiol, 1983. **245**(3): p. E246-52.
51. Valenstein, E.S., V.C. Cox, and J.W. Kakolewski, *Modification of motivated behavior elicited by electrical stimulation of the hypothalamus.* Science, 1968. **159**(3819): p. 1119-21.
52. Oomura, Y., et al., *RECIPROCAL ACTIVITIES OF THE VENTROMEDIAL AND LATERAL HYPOTHALAMIC AREAS OF CATS.* Science, 1964. **143**(3605): p. 484-5.
53. Borg, W.P., et al., *Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release.* Diabetes, 1995. **44**(2): p. 180-4.

54. Miki, T., et al., *ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis.* Nat Neurosci, 2001. **4**(5): p. 507-12.
55. Wang, R., et al., *The regulation of glucose-excited neurons in the hypothalamic arcuate nucleus by glucose and feeding-relevant peptides.* Diabetes, 2004. **53**(8): p. 1959-65.
56. Murphy, B.A., et al., *AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons.* Am J Physiol Cell Physiol, 2009. **297**(3): p. C750-8.
57. Ebihara, K., et al., *Efficacy and safety of leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy.* J Clin Endocrinol Metab, 2007. **92**(2): p. 532-41.
58. Petersen, K.F., et al., *Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy.* J Clin Invest, 2002. **109**(10): p. 1345-50.
59. Shi, Z.Q., et al., *Intracerebroventricular administration of leptin markedly enhances insulin sensitivity and systemic glucose utilization in conscious rats.* Metabolism, 1998. **47**(10): p. 1274-80.
60. Hidaka, S., et al., *Chronic central leptin infusion restores hyperglycemia independent of food intake and insulin level in streptozotocin-induced diabetic rats.* Faseb j, 2002. **16**(6): p. 509-18.
61. Dhillon, H., et al., *Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis.* Neuron, 2006. **49**(2): p. 191-203.
62. Bingham, N.C., et al., *Selective Loss of Leptin Receptors in the Ventromedial Hypothalamic Nucleus Results in Increased Adiposity and a Metabolic Syndrome.* Endocrinology, 2008. **149**(5): p. 2138-48.
63. Paranjape, S.A., et al., *Influence of insulin in the ventromedial hypothalamus on pancreatic glucagon secretion in vivo.* Diabetes, 2010. **59**(6): p. 1521-7.
64. Pocai, A., et al., *Hypothalamic K(ATP) channels control hepatic glucose production.* Nature, 2005. **434**(7036): p. 1026-31.
65. Klockener, T., et al., *High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons.* Nat Neurosci, 2011. **14**(7): p. 911-8.
66. Borg, W.P., et al., *Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia.* J Clin Invest, 1994. **93**(4): p. 1677-82.
67. Shimazu, T., *Central nervous system regulation of liver and adipose tissue metabolism.* Diabetologia, 1981. **20 Suppl**: p. 343-56.
68. Bray, G.A. and Y. Nishizawa, *Ventromedial hypothalamus modulates fat mobilisation during fasting.* Nature, 1978. **274**(5674): p. 900-2.
69. Niijima, A., F. Rohner-Jeanrenaud, and B. Jeanrenaud, *Role of ventromedial hypothalamus on sympathetic efferents of brown adipose tissue.* Am J Physiol, 1984. **247**(4 Pt 2): p. R650-4.
70. Yoshimatsu, H., et al., *Effects of hypothalamic lesion on pancreatic autonomic nerve activity in the rat.* Brain Res, 1984. **303**(1): p. 147-52.
71. Hogan, S., J. Himms-Hagen, and D.V. Coscina, *Lack of diet-induced thermogenesis in brown adipose tissue of obese medial hypothalamic-lesioned rats.* Physiol Behav, 1985. **35**(2): p. 287-94.

72. Yoshida, T. and G.A. Bray, *Catecholamine turnover in rats with ventromedial hypothalamic lesions.* Am J Physiol, 1984. **246**(4 Pt 2): p. R558-65.
73. Unger, T.J., et al., *Selective deletion of Bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity.* J Neurosci, 2007. **27**(52): p. 14265-74.
74. Segal, J.P., et al., *Use of laser-capture microdissection for the identification of marker genes for the ventromedial hypothalamic nucleus.* J Neurosci, 2005. **25**(16): p. 4181-8.
75. Musatov, S., et al., *RNAi-mediated silencing of estrogen receptor {alpha} in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors.* Proc Natl Acad Sci U S A, 2006. **103**(27): p. 10456-60.
76. Choi, Y.H., et al., *Revisiting the Ventral Medial Nucleus of the Hypothalamus: The Roles of SF-1 Neurons in Energy Homeostasis.* Front Neurosci, 2013. **7**.
77. Brown, T.J., et al., *Sex differences in estrogen receptor and progestin receptor induction in the guinea pig hypothalamus and preoptic area.* Brain Res, 1996. **725**(1): p. 37-48.
78. Lauber, A.H., et al., *Estrogen receptor messenger RNA expression in rat hypothalamus as a function of genetic sex and estrogen dose.* Endocrinology, 1991. **129**(6): p. 3180-6.
79. Scott, C.J., et al., *The distribution of cells containing estrogen receptor-alpha (ERalpha) and ERbeta messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: comparison of males and females.* Endocrinology, 2000. **141**(8): p. 2951-62.
80. Ikeda, Y., et al., *Sexually dimorphic and estrogen-dependent expression of estrogen receptor beta in the ventromedial hypothalamus during rat postnatal development.* Endocrinology, 2003. **144**(11): p. 5098-104.
81. Matsumoto, A. and Y. Arai, *Sex difference in volume of the ventromedial nucleus of the hypothalamus in the rat.* Endocrinol Jpn, 1983. **30**(3): p. 277-80.
82. Matsumoto, A. and Y. Arai, *Male-female difference in synaptic organization of the ventromedial nucleus of the hypothalamus in the rat.* Neuroendocrinology, 1986. **42**(3): p. 232-6.
83. Xu, X., et al., *Modular genetic control of sexually dimorphic behaviors.* Cell, 2012. **148**(3): p. 596-607.
84. Flanagan-Cato, L.M., *Sex differences in the neural circuit that mediates female sexual receptivity.* Front Neuroendocrinol, 2011. **32**(2): p. 124-36.
85. Majdic, G., et al., *Knockout mice lacking steroidogenic factor 1 are a novel genetic model of hypothalamic obesity.* Endocrinology, 2002. **143**(2): p. 607-14.
86. Kurrasch, D.M., et al., *The neonatal ventromedial hypothalamus transcriptome reveals novel markers with spatially distinct patterning.* J Neurosci, 2007. **27**(50): p. 13624-34.
87. Kim, K.W., et al., *SF-1 in the ventral medial hypothalamic nucleus: a key regulator of homeostasis.* Mol Cell Endocrinol, 2011. **336**(1-2): p. 219-23.
88. Radziejewski, C., et al., *Dimeric structure and conformational stability of brain-derived neurotrophic factor and neurotrophin-3.* Biochemistry, 1992. **31**(18): p. 4431-6.

89. (Eds.), G.R.L.a.B.D.C., *Neurotrophic Factors*. Handbook of Experimental Pharmacology. Vol. 220. 2014: Springer Link.
90. Barde, Y.A., D. Edgar, and H. Thoenen, *Purification of a new neurotrophic factor from mammalian brain.* Embo j, 1982. **1**(5): p. 549-53.
91. Timmusk, T., et al., *Multiple promoters direct tissue-specific expression of the rat BDNF gene.* Neuron, 1993. **10**(3): p. 475-89.
92. West, A.E., *Activity-Dependent Regulation of Brain-derived neurotrophic factor Transcription*, in *Transcriptional Regulation by Neuronal Activity: To the Nucleus and Back*, S.M. Dudek, Editor. 2008, Springer US: Boston, MA. p. 155-173.
93. Woo, N.H., et al., *Activation of p75NTR by proBDNF facilitates hippocampal long-term depression.* Nat Neurosci, 2005. **8**(8): p. 1069-77.
94. Rosch, H., et al., *The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus.* Proc Natl Acad Sci U S A, 2005. **102**(20): p. 7362-7.
95. Patapoutian, A. and L.F. Reichardt, *Trk receptors: mediators of neurotrophin action.* Curr Opin Neurobiol, 2001. **11**(3): p. 272-80.
96. Eide, F.F., et al., *Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling.* J Neurosci, 1996. **16**(10): p. 3123-9.
97. Haapasalo, A., et al., *Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival.* Biochem Biophys Res Commun, 2001. **280**(5): p. 1352-8.
98. Rose, C.R., et al., *Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells.* Nature, 2003. **426**(6962): p. 74-8.
99. Casaccia-Bonnefil, P., et al., *Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75.* Nature, 1996. **383**(6602): p. 716-9.
100. Dechant, G. and Y.A. Barde, *The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system.* Nat Neurosci, 2002. **5**(11): p. 1131-6.
101. Duman, R.S. and B. Voleti, *Signaling pathways underlying the pathophysiology and treatment of depression: novel mechanisms for rapid-acting agents.* Trends Neurosci, 2012. **35**(1): p. 47-56.
102. Lapchak, P.A. and F. Hefti, *BDNF and NGF treatment in lesioned rats: effects on cholinergic function and weight gain.* Neuroreport, 1992. **3**(5): p. 405-8.
103. Pelleymounter, M.A., M.J. Cullen, and C.L. Wellman, *Characteristics of BDNF-induced weight loss.* Exp Neurol, 1995. **131**(2): p. 229-38.
104. Lyons, W.E., et al., *Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15239-44.
105. Xu, B., et al., *Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor.* Nat Neurosci, 2003. **6**(7): p. 736-42.
106. Rios, M., et al., *Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity.* Mol Endocrinol, 2001. **15**(10): p. 1748-57.

107. Camerino, C., et al., *Central depletion of brain-derived neurotrophic factor in mice results in high bone mass and metabolic phenotype.* Endocrinology, 2012. **153**(11): p. 5394-405.
108. Tonra, J.R., et al., *Brain-derived neurotrophic factor improves blood glucose control and alleviates fasting hyperglycemia in C57BLKS-Lepr(db)/lepr(db) mice.* Diabetes, 1999. **48**(3): p. 588-94.
109. Nakagawa, T., et al., *Anti-obesity and anti-diabetic effects of brain-derived neurotrophic factor in rodent models of leptin resistance.* Int J Obes Relat Metab Disord, 2003. **27**(5): p. 557-65.
110. Meek, T.H., et al., *BDNF Action in the Brain Attenuates Diabetic Hyperglycemia via Insulin-Independent Inhibition of Hepatic Glucose Production.* Diabetes, 2013. **62**(5): p. 1512-8.
111. Speliotes, E.K., et al., *Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index.* Nat Genet, 2010. **42**(11): p. 937-48.
112. Gray, J., et al., *Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene.* Diabetes, 2006. **55**(12): p. 3366-71.
113. Yeo, G.S., et al., *A de novo mutation affecting human TrkB associated with severe obesity and developmental delay.* Nat Neurosci, 2004. **7**(11): p. 1187-9.
114. Han, J.C., et al., *Brain-derived neurotrophic factor and obesity in the WAGR syndrome.* N Engl J Med, 2008. **359**(9): p. 918-27.
115. Chen, Z.Y., et al., *Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior.* Science, 2006. **314**(5796): p. 140-3.
116. Beckers, S., et al., *Association of the BDNF Val66Met variation with obesity in women.* Mol Genet Metab, 2008. **95**(1-2): p. 110-2.
117. Skledar, M., et al., *Association between brain-derived neurotrophic factor Val66Met and obesity in children and adolescents.* Prog Neuropsychopharmacol Biol Psychiatry, 2012. **36**(1): p. 136-40.
118. Thorleifsson, G., et al., *Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity.* Nat Genet, 2009. **41**(1): p. 18-24.
119. Shimizu, E., K. Hashimoto, and M. Iyo, *Ethnic difference of the BDNF 196G/A (val66met) polymorphism frequencies: the possibility to explain ethnic mental traits.* Am J Med Genet B Neuropsychiatr Genet, 2004. **126b**(1): p. 122-3.
120. Fujinami, A., et al., *Serum brain-derived neurotrophic factor in patients with type 2 diabetes mellitus: Relationship to glucose metabolism and biomarkers of insulin resistance.* Clin Biochem, 2008. **41**(10-11): p. 812-7.
121. Krabbe, K.S., et al., *Brain-derived neurotrophic factor (BDNF) and type 2 diabetes.* Diabetologia, 2007. **50**(2): p. 431-438.
122. Lin, J.C., et al., *Appetite enhancement and weight gain by peripheral administration of TrkB agonists in non-human primates.* PLoS One, 2008. **3**(4): p. e1900.
123. Wang, C., et al., *Brain-derived neurotrophic factor in the ventromedial nucleus of the hypothalamus reduces energy intake.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(3): p. R1037-45.

124. Godar, R., et al., *Reduction of high fat diet-induced obesity after chronic administration of brain-derived neurotrophic factor in the hypothalamic ventromedial nucleus.* Neuroscience, 2011. **194**: p. 36-52.
125. Cordeira, J.W., et al., *Hypothalamic dysfunction of the thrombospondin receptor alpha2delta-1 underlies the overeating and obesity triggered by brain-derived neurotrophic factor deficiency.* J Neurosci, 2014. **34**(2): p. 554-65.
126. van den Pol, A.N., C. Romano, and P. Ghosh, *Metabotropic glutamate receptor mGluR5 subcellular distribution and developmental expression in hypothalamus.* J Comp Neurol, 1995. **362**(1): p. 134-50.
127. Anwyl, R., *Metabotropic glutamate receptors: electrophysiological properties and role in plasticity.* Brain Res Brain Res Rev, 1999. **29**(1): p. 83-120.
128. Bellone, C., C. Luscher, and M. Mameli, *Mechanisms of synaptic depression triggered by metabotropic glutamate receptors.* Cell Mol Life Sci, 2008. **65**(18): p. 2913-23.
129. Kullmann, D.M. and K. Lamsa, *Roles of distinct glutamate receptors in induction of anti-Hebbian long-term potentiation.* J Physiol, 2008. **586**(6): p. 1481-6.
130. Zirpel, L., et al., *Developmental changes in metabotropic glutamate receptor-mediated calcium homeostasis.* J Comp Neurol, 2000. **421**(1): p. 95-106.
131. Kumar, V., et al., *Activation of intracellular metabotropic glutamate receptor 5 in striatal neurons leads to up-regulation of genes associated with sustained synaptic transmission including Arc/Arg3.1 protein.* J Biol Chem, 2012. **287**(8): p. 5412-25.
132. Niswender, C.M. and P.J. Conn, *Metabotropic glutamate receptors: physiology, pharmacology, and disease.* Annu Rev Pharmacol Toxicol, 2010. **50**: p. 295-322.
133. Pin, J.P., T. Galvez, and L. Prezeau, *Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors.* Pharmacol Ther, 2003. **98**(3): p. 325-54.
134. Hermans, E. and R.A. Challiss, *Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors.* Biochem J, 2001. **359**(Pt 3): p. 465-84.
135. Pinheiro, P.S. and C. Mulle, *Presynaptic glutamate receptors: physiological functions and mechanisms of action.* Nat Rev Neurosci, 2008. **9**(6): p. 423-36.
136. Li, X.M., et al., *JNK1 contributes to metabotropic glutamate receptor-dependent long-term depression and short-term synaptic plasticity in the mice area hippocampal CA1.* Eur J Neurosci, 2007. **25**(2): p. 391-6.
137. Mannaioni, G., et al., *Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function.* J Neurosci, 2001. **21**(16): p. 5925-34.
138. Purgert, C.A., et al., *Intracellular mGluR5 can mediate synaptic plasticity in the hippocampus.* J Neurosci, 2014. **34**(13): p. 4589-98.
139. Sepulveda-Orengo, M.T., et al., *Fear extinction induces mGluR5-mediated synaptic and intrinsic plasticity in infralimbic neurons.* J Neurosci, 2013. **33**(17): p. 7184-93.
140. Sourdet, V., et al., *Long-term enhancement of neuronal excitability and temporal fidelity mediated by metabotropic glutamate receptor subtype 5.* J Neurosci, 2003. **23**(32): p. 10238-48.

141. Panatier, A. and R. Robitaille, *Astrocytic mGluR5 and the tripartite synapse.* Neuroscience, 2016. **323**: p. 29-34.
142. Wilkerson, J.R., et al., *A role for dendritic mGluR5-mediated local translation of Arc/Arg3.1 in MEF2-dependent synapse elimination.* Cell Rep, 2014. **7**(5): p. 1589-600.
143. Kumar, V., et al., *Activation of Intracellular Metabotropic Glutamate Receptor 5 in Striatal Neurons Leads to Up-regulation of Genes Associated with Sustained Synaptic Transmission Including Arc/Arg3.1 Protein.* J Biol Chem, 2012. **287**(8): p. 5412-25.
144. Porter, J.T. and K.D. McCarthy, *GFAP-positive hippocampal astrocytes in situ respond to glutamatergic neuroligands with increases in [Ca2+]i.* Glia, 1995. **13**(2): p. 101-12.
145. Porter, J.T. and K.D. McCarthy, *Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals.* J Neurosci, 1996. **16**(16): p. 5073-81.
146. Pasti, L., et al., *Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ.* J Neurosci, 1997. **17**(20): p. 7817-30.
147. D'Ascenzo, M., et al., *mGluR5 stimulates gliotransmission in the nucleus accumbens.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1995-2000.
148. Pisani, A., et al., *Enhancement of NMDA responses by group I metabotropic glutamate receptor activation in striatal neurones.* Br J Pharmacol, 1997. **120**(6): p. 1007-14.
149. Kotecha, S.A., et al., *Co-stimulation of mGluR5 and N-methyl-D-aspartate receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons.* J Biol Chem, 2003. **278**(30): p. 27742-9.
150. Awad, H., et al., *Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus.* J Neurosci, 2000. **20**(21): p. 7871-9.
151. Attucci, S., et al., *Activation of type 5 metabotropic glutamate receptors enhances NMDA responses in mice cortical wedges.* Br J Pharmacol, 2001. **132**(4): p. 799-806.
152. Gross, K.S., et al., *Opposite Effects of mGluR1a and mGluR5 Activation on Nucleus Accumbens Medium Spiny Neuron Dendritic Spine Density.* PLoS One, 2016. **11**(9): p. e0162755.
153. LaCrosse, A.L., et al., *mGluR5 Positive and Negative Allosteric Modulators Differentially Affect Dendritic Spine Density and Morphology in the Prefrontal Cortex.* CNS Neurol Disord Drug Targets, 2015. **14**(4): p. 476-85.
154. Fontanez-Nuin, D.E., et al., *Memory for fear extinction requires mGluR5-mediated activation of infralimbic neurons.* Cereb Cortex, 2011. **21**(3): p. 727-35.
155. Duncan, R.S., S.Y. Hwang, and P. Koulen, *Effects of Vesl/Homer proteins on intracellular signaling.* Exp Biol Med (Maywood), 2005. **230**(8): p. 527-35.
156. Ronesi, J.A., et al., *Disrupted Homer scaffolds mediate abnormal mGluR5 function in a mouse model of fragile X syndrome.* Nat Neurosci, 2012. **15**(3): p. 431-40, s1.

157. Ronesi, J.A. and K.M. Huber, *Homer interactions are necessary for metabotropic glutamate receptor-induced long-term depression and translational activation.* J Neurosci, 2008. **28**(2): p. 543-7.
158. Rong, R., et al., *PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis.* Nat Neurosci, 2003. **6**(11): p. 1153-61.
159. Mao, L., et al., *The scaffold protein Homer1b/c links metabotropic glutamate receptor 5 to extracellular signal-regulated protein kinase cascades in neurons.* J Neurosci, 2005. **25**(10): p. 2741-52.
160. Naisbitt, S., et al., *Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin.* Neuron, 1999. **23**(3): p. 569-82.
161. Tu, J.C., et al., *Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins.* Neuron, 1999. **23**(3): p. 583-92.
162. Piers, T.M., et al., *Translational Concepts of mGluR5 in Synaptic Diseases of the Brain.* Front Pharmacol, 2012. **3**: p. 199.
163. Sokol, D.K., et al., *Autism, Alzheimer disease, and fragile X: APP, FMRP, and mGluR5 are molecular links.* Neurology, 2011. **76**(15): p. 1344-52.
164. Bear, M.F., et al., *Fragile X: translation in action.* Neuropsychopharmacology, 2008. **33**(1): p. 84-7.
165. Dolen, G. and M.F. Bear, *Role for metabotropic glutamate receptor 5 (mGluR5) in the pathogenesis of fragile X syndrome.* J Physiol, 2008. **586**(6): p. 1503-8.
166. Dolen, G., et al., *Correction of fragile X syndrome in mice.* Neuron, 2007. **56**(6): p. 955-62.
167. Pop, A.S., et al., *Rescue of dendritic spine phenotype in Fmr1 KO mice with the mGluR5 antagonist AFQ056/Mavoglurant.* Psychopharmacology (Berl), 2014. **231**(6): p. 1227-35.
168. Conn, P.J. and J.P. Pin, *Pharmacology and functions of metabotropic glutamate receptors.* Annu Rev Pharmacol Toxicol, 1997. **37**: p. 205-37.
169. Chapman, A.G., et al., *Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893).* Neuropharmacology, 2000. **39**(9): p. 1567-74.
170. Yan, Q.J., et al., *Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP.* Neuropharmacology, 2005. **49**(7): p. 1053-66.
171. Rosenbrock, H., et al., *Functional interaction of metabotropic glutamate receptor 5 and NMDA-receptor by a metabotropic glutamate receptor 5 positive allosteric modulator.* Eur J Pharmacol, 2010. **639**(1-3): p. 40-6.
172. Conn, P.J., C.W. Lindsley, and C.K. Jones, *Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia.* Trends Pharmacol Sci, 2009. **30**(1): p. 25-31.
173. Krystal, J.H., et al., *Potential psychiatric applications of metabotropic glutamate receptor agonists and antagonists.* CNS Drugs, 2010. **24**(8): p. 669-93.
174. Riaza Bermudo-Soriano, C., et al., *New perspectives in glutamate and anxiety.* Pharmacol Biochem Behav, 2012. **100**(4): p. 752-74.

175. Chiamulera, C., et al., *Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice.* Nat Neurosci, 2001. **4**(9): p. 873-4.
176. Kalivas, P.W., *The glutamate homeostasis hypothesis of addiction.* Nat Rev Neurosci, 2009. **10**(8): p. 561-72.
177. Bisaga, A., W. Danysz, and R.W. Foltin, *Antagonism of glutamatergic NMDA and mGluR5 receptors decreases consumption of food in baboon model of binge-eating disorder.* Eur Neuropsychopharmacol, 2008. **18**(11): p. 794-802.
178. Bradbury, M.J., et al., *Metabotropic glutamate receptor mGlu5 is a mediator of appetite and energy balance in rats and mice.* J Pharmacol Exp Ther, 2005. **313**(1): p. 395-402.
179. Ploj, K., et al., *The metabotropic glutamate mGluR5 receptor agonist CHPG stimulates food intake.* Neuroreport, 2010. **21**(10): p. 704-8.
180. Charles, J.R., et al., *Activation of lateral hypothalamic mGlu1 and mGlu5 receptors elicits feeding in rats.* Neuropharmacology, 2014. **79**: p. 59-65.
181. Li, D.P., et al., *mGluR5 Upregulation increases excitability of hypothalamic presympathetic neurons through NMDA receptor trafficking in spontaneously hypertensive rats.* J Neurosci, 2014. **34**(12): p. 4309-17.
182. Wang, Y., et al., *Sex-specific effects of prenatal chronic mild stress on adult spatial learning capacity and regional glutamate receptor expression profiles.* Exp Neurol, 2016. **281**: p. 66-80.
183. De Jesús-Burgos, M.I., et al., *Amygdalar activation of group I metabotropic glutamate receptors produces anti- and pro-conflict effects depending upon animal sex in a sexually dimorphic conditioned conflict-based anxiety model.* Behavioural Brain Research, 2016. **302**: p. 200-212.
184. Milner, T.A., et al., *Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites.* J Comp Neurol, 2001. **429**(3): p. 355-71.
185. McEwen, B.S., *Invited review: Estrogens effects on the brain: multiple sites and molecular mechanisms.* J Appl Physiol (1985), 2001. **91**(6): p. 2785-801.
186. Mitrano, D.A. and Y. Smith, *Comparative analysis of the subcellular and subsynaptic localization of mGluR1a and mGluR5 metabotropic glutamate receptors in the shell and core of the nucleus accumbens in rat and monkey.* J Comp Neurol, 2007. **500**(4): p. 788-806.
187. Meitzen, J. and P.G. Mermelstein, *Estrogen receptors stimulate brain region specific metabotropic glutamate receptors to rapidly initiate signal transduction pathways.* J Chem Neuroanat, 2011. **42**(4): p. 236-41.
188. Grove-Strawser, D., M.I. Boulware, and P.G. Mermelstein, *Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons.* Neuroscience, 2010. **170**(4): p. 1045-55.
189. Boulware, M.I., et al., *Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein.* J Neurosci, 2005. **25**(20): p. 5066-78.
190. Peterson, B.M., P.G. Mermelstein, and R.L. Meisel, *Estradiol mediates dendritic spine plasticity in the nucleus accumbens core through activation of mGluR5.* Brain Struct Funct, 2015. **220**(4): p. 2415-22.

191. Christensen, A., P. Dewing, and P. Micevych, *Membrane-initiated estradiol signaling induces spinogenesis required for female sexual receptivity.* J Neurosci, 2011. **31**(48): p. 17583-9.
192. Martinez, L.A., et al., *Estradiol Facilitation of Cocaine Self-Administration in Female Rats Requires Activation of mGluR5.* eNeuro, 2016. **3**(5).
193. Keating, N.L., et al., *Use of hormone replacement therapy by postmenopausal women in the United States.* Ann Intern Med, 1999. **130**(7): p. 545-53.
194. Tchernof, A., et al., *Menopause, central body fatness, and insulin resistance: effects of hormone-replacement therapy.* Coron Artery Dis, 1998. **9**(8): p. 503-11.
195. Yonezawa, R., et al., *Central versus peripheral impact of estradiol on the impaired glucose metabolism in ovariectomized mice on a high-fat diet.* Am J Physiol Endocrinol Metab, 2012. **303**(4): p. E445-56.
196. O'Malley, B.W., *Mechanisms of action of steroid hormones.* N Engl J Med, 1971. **284**(7): p. 370-7.
197. Safe, S. and K. Kim, *Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways.* J Mol Endocrinol, 2008. **41**(5): p. 263-75.
198. Hammes, S.R. and E.R. Levin, *Extranuclear steroid receptors: nature and actions.* Endocr Rev, 2007. **28**(7): p. 726-41.
199. Carr, M.C., *The emergence of the metabolic syndrome with menopause.* J Clin Endocrinol Metab, 2003. **88**(6): p. 2404-11.
200. Blaustein, J.D. and G.N. Wade, *Ovarian influences on the meal patterns of female rats.* Physiol Behav, 1976. **17**(2): p. 201-8.
201. Drewett, R.F., *Sexual behaviour and sexual motivation in the female rat.* Nature, 1973. **242**(5398): p. 476-7.
202. Wallen, W.J., M.P. Belanger, and C. Wittnich, *Sex hormones and the selective estrogen receptor modulator tamoxifen modulate weekly body weights and food intakes in adolescent and adult rats.* J Nutr, 2001. **131**(9): p. 2351-7.
203. Geary, N., et al., *Deficits in E2-Dependent Control of Feeding, Weight Gain, and Cholecystokinin Satiation in ER-alpha Null Mice.* Endocrinology, 2001. **142**(11): p. 4751-4757.
204. Jones, M.E., et al., *Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12735-40.
205. Heine, P.A., et al., *Increased adipose tissue in male and female estrogen receptor-alpha knockout mice.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12729-34.
206. Simerly, R.B., et al., *Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study.* J Comp Neurol, 1990. **294**(1): p. 76-95.
207. Merchenthaler, I., et al., *Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses.* J Comp Neurol, 2004. **473**(2): p. 270-91.
208. Ohlsson, C., et al., *Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice.* Biochem Biophys Res Commun, 2000. **278**(3): p. 640-5.

209. Yepuru, M., et al., *Estrogen receptor-{beta}-selective ligands alleviate high-fat diet- and ovariectomy-induced obesity in mice.* J Biol Chem, 2010. **285**(41): p. 31292-303.
210. Hazell, G.G., et al., *Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues.* J Endocrinol, 2009. **202**(2): p. 223-36.
211. Akama, K.T., et al., *Post-synaptic density-95 (PSD-95) binding capacity of G-protein-coupled receptor 30 (GPR30), an estrogen receptor that can be identified in hippocampal dendritic spines.* J Biol Chem, 2013. **288**(9): p. 6438-50.
212. Almey, A., et al., *Estrogen Receptors Are Found in Glia and at Extranuclear Neuronal Sites in the Dorsal Striatum of Female Rats: Evidence for Cholinergic But Not Dopaminergic Colocalization.* Endocrinology, 2012. **153**(11): p. 5373-83.
213. Waters, E.M., et al., *G-Protein-Coupled Estrogen Receptor 1 Is Anatomically Positioned to Modulate Synaptic Plasticity in the Mouse Hippocampus.* J Neurosci, 2015. **35**(6): p. 2384-97.
214. Davis, K.E., et al., *Sexually dimorphic role of G protein-coupled estrogen receptor (GPER) in modulating energy homeostasis.* Horm Behav, 2014. **66**(1): p. 196-207.
215. Bourque, M., M. Morissette, and T. Di Paolo, *Neuroprotection in Parkinsonian-treated mice via estrogen receptor alpha activation requires G protein-coupled estrogen receptor 1.* Neuropharmacology, 2015. **95**: p. 343-52.
216. Minami, T., et al., *17 beta-estradiol depolarization of hypothalamic neurons is mediated by cyclic AMP.* Brain Res, 1990. **519**(1-2): p. 301-7.
217. Xu, Y., et al., *Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction.* Cell Metab, 2011. **14**(4): p. 453-65.
218. Musatov, S., et al., *Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2501-6.
219. Hirosawa, M., et al., *Ablation of estrogen receptor alpha (ERalpha) prevents upregulation of POMC by leptin and insulin.* Biochem Biophys Res Commun, 2008. **371**(2): p. 320-3.
220. Gao, Q., et al., *Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals.* Nat Med, 2007. **13**(1): p. 89-94.
221. Polidori, C. and N. Geary, *Estradiol treatment fails to affect the feeding responses to melanocortin-3/4 receptor agonism or antagonism in ovariectomized rats.* Peptides, 2002. **23**(9): p. 1697-700.
222. Schlenker, E.H. and S.N. Hansen, *Sex-specific densities of estrogen receptors alpha and beta in the subnuclei of the nucleus tractus solitarius, hypoglossal nucleus and dorsal vagal motor nucleus weanling rats.* Brain Res, 2006. **1123**(1): p. 89-100.
223. Asarian, L. and N. Geary, *Estradiol enhances cholecystokinin-dependent lipid-induced satiation and activates estrogen receptor-alpha-expressing cells in the nucleus tractus solitarius of ovariectomized rats.* Endocrinology, 2007. **148**(12): p. 5656-66.
224. Geary, N., *Estradiol, CCK and satiation.* Peptides, 2001. **22**(8): p. 1251-63.

225. Macotela, Y., et al., *Sex and depot differences in adipocyte insulin sensitivity and glucose metabolism.* Diabetes, 2009. **58**(4): p. 803-12.
226. Guerre-Millo, M., et al., *Increased insulin sensitivity and responsiveness of glucose metabolism in adipocytes from female versus male rats.* J Clin Invest, 1985. **76**(1): p. 109-16.
227. Mattsson, C. and T. Olsson, *Estrogens and glucocorticoid hormones in adipose tissue metabolism.* Curr Med Chem, 2007. **14**(27): p. 2918-24.
228. O'Sullivan, A.J. and K.K. Ho, *A comparison of the effects of oral and transdermal estrogen replacement on insulin sensitivity in postmenopausal women.* J Clin Endocrinol Metab, 1995. **80**(6): p. 1783-8.
229. Mauvais-Jarvis, F., *Estrogen and androgen receptors: regulators of fuel homeostasis and emerging targets for diabetes and obesity.* Trends Endocrinol Metab, 2011. **22**(1): p. 24-33.
230. Bilezikian, J.P., et al., *Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency.* N Engl J Med, 1998. **339**(9): p. 599-603.
231. Smith, E.P., et al., *Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man.* N Engl J Med, 1994. **331**(16): p. 1056-61.
232. Gallagher, C.J., et al., *Association of the estrogen receptor-alpha gene with the metabolic syndrome and its component traits in African-American families: the Insulin Resistance Atherosclerosis Family Study.* Diabetes, 2007. **56**(8): p. 2135-41.
233. Naaz, A., et al., *Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta).* Horm Metab Res, 2002. **34**(11-12): p. 758-63.
234. Bryzgalova, G., et al., *Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver.* Diabetologia, 2006. **49**(3): p. 588-97.
235. Ribas, V., et al., *Skeletal muscle action of estrogen receptor alpha is critical for the maintenance of mitochondrial function and metabolic homeostasis in females.* Sci Transl Med, 2016. **8**(334): p. 334ra54.
236. Fu, M.H., et al., *Exercise, sex, menstrual cycle phase, and 17beta-estradiol influence metabolism-related genes in human skeletal muscle.* Physiol Genomics, 2009. **40**(1): p. 34-47.
237. Shimizu, H., et al., *Estrogen increases in vivo leptin production in rats and human subjects.* J Endocrinol, 1997. **154**(2): p. 285-92.
238. Machinal, F., et al., *In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones.* Endocrinology, 1999. **140**(4): p. 1567-74.
239. Ainslie, D.A., et al., *Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y.* Int J Obes Relat Metab Disord, 2001. **25**(11): p. 1680-8.
240. Arita, Y., et al., *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. 1999.* Biochem Biophys Res Commun, 2012. **425**(3): p. 560-4.
241. Lihn, A.S., et al., *Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects.* Mol Cell Endocrinol, 2004. **219**(1-2): p. 9-15.

242. Kim, J.H., et al., *Tissue-selective estrogen complexes with bazedoxifene prevent metabolic dysfunction in female mice.* Mol Metab, 2014. **3**(2): p. 177-90.
243. Combs, T.P., et al., *Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin.* Diabetes, 2003. **52**(2): p. 268-76.
244. Bouchard, C., J.P. Despres, and P. Mauriege, *Genetic and nongenetic determinants of regional fat distribution.* Endocr Rev, 1993. **14**(1): p. 72-93.
245. Enzi, G., et al., *Subcutaneous and visceral fat distribution according to sex, age, and overweight, evaluated by computed tomography.* Am J Clin Nutr, 1986. **44**(6): p. 739-46.
246. Guthrie, J.R., et al., *The menopausal transition: a 9-year prospective population-based study. The Melbourne Women's Midlife Health Project.* Climacteric, 2004. **7**(4): p. 375-89.
247. Gambacciani, M., et al., *Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women.* J Clin Endocrinol Metab, 1997. **82**(2): p. 414-7.
248. Wajchenberg, B.L., *Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome.* Endocr Rev, 2000. **21**(6): p. 697-738.
249. Schneider, G., et al., *Increased estrogen production in obese men.* J Clin Endocrinol Metab, 1979. **48**(4): p. 633-8.
250. Tchernof, A., et al., *Relation of steroid hormones to glucose tolerance and plasma insulin levels in men. Importance of visceral adipose tissue.* Diabetes Care, 1995. **18**(3): p. 292-9.
251. D'Eon, T.M., et al., *Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways.* J Biol Chem, 2005. **280**(43): p. 35983-91.
252. Mizutani, T., et al., *Identification of estrogen receptor in human adipose tissue and adipocytes.* J Clin Endocrinol Metab, 1994. **78**(4): p. 950-4.
253. Okura, T., et al., *Association of polymorphisms in the estrogen receptor alpha gene with body fat distribution.* Int J Obes Relat Metab Disord, 2003. **27**(9): p. 1020-7.
254. Casazza, K., G.P. Page, and J.R. Fernandez, *The association between the rs2234693 and rs9340799 estrogen receptor alpha gene polymorphisms and risk factors for cardiovascular disease: a review.* Biol Res Nurs, 2010. **12**(1): p. 84-97.
255. Foryst-Ludwig, A., et al., *Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma.* PLoS Genet, 2008. **4**(6): p. e1000108.
256. Jones, J.R., et al., *Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance.* Proc Natl Acad Sci U S A, 2005. **102**(17): p. 6207-12.
257. Iverius, P.H. and J.D. Brunzell, *Relationship between lipoprotein lipase activity and plasma sex steroid level in obese women.* J Clin Invest, 1988. **82**(3): p. 1106-12.

258. Urabe, M., et al., *Effect of estrogen replacement therapy on hepatic triglyceride lipase, lipoprotein lipase and lipids including apolipoprotein E in climacteric and elderly women.* Endocr J, 1996. **43**(6): p. 737-42.
259. Miranda, M., et al., *Human subcutaneous adipose tissue LPIN1 expression in obesity, type 2 diabetes mellitus, and human immunodeficiency virus--associated lipodystrophy syndrome.* Metabolism, 2007. **56**(11): p. 1518-26.
260. Gonzalez, C.R., et al., *Regulation of lipin1 by nutritional status, adiponectin, sex and pituitary function in rat white adipose tissue.* Physiol Behav, 2012. **105**(3): p. 777-83.
261. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.* Jama, 2002. **288**(3): p. 321-33.
262. Xu, J., et al., *mGluR5 has a critical role in inhibitory learning.* J Neurosci, 2009. **29**(12): p. 3676-84.
263. Berry, R., et al., *Methods in Enzymology (MIE): Methods of Adipose Tissue Biology-: Chapter 7: Imaging of Adipose Tissue.* Methods in enzymology, 2014. **537**: p. 47-73.
264. Kim, K.W., et al., *Steroidogenic factor 1 directs programs regulating diet-induced thermogenesis and leptin action in the ventral medial hypothalamic nucleus.* Proc Natl Acad Sci U S A, 2011. **108**(26): p. 10673-8.
265. Song, Z., et al., *Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus.* Diabetes, 2001. **50**(12): p. 2673-81.
266. Kang, L., et al., *Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing.* Diabetes, 2006. **55**(2): p. 412-20.
267. Kumon, A., et al., *Mechanism of lipolysis induced by electrical stimulation of the hypothalamus in the rabbit.* J Lipid Res, 1976. **17**(6): p. 551-8.
268. Ruffin, M. and S. Nicolaidis, *Electrical stimulation of the ventromedial hypothalamus enhances both fat utilization and metabolic rate that precede and parallel the inhibition of feeding behavior.* Brain Res, 1999. **846**(1): p. 23-9.
269. Gregoire, F.M., C.M. Smas, and H.S. Sul, *Understanding adipocyte differentiation.* Physiol Rev, 1998. **78**(3): p. 783-809.
270. Lindberg, D., P. Chen, and C. Li, *Conditional viral tracing reveals that steroidogenic factor 1-positive neurons of the dorsomedial subdivision of the ventromedial hypothalamus project to autonomic centers of the hypothalamus and hindbrain.* J Comp Neurol, 2013. **521**(14): p. 3167-90.
271. Filippov, A.K., et al., *The scaffold protein NHERF2 determines the coupling of P2Y1 nucleotide and mGluR5 glutamate receptor to different ion channels in neurons.* J Neurosci, 2010. **30**(33): p. 11068-72.
272. Kammermeier, P.J., et al., *Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels.* J Neurosci, 2000. **20**(19): p. 7238-45.
273. Campanac, E., et al., *Enhanced intrinsic excitability in basket cells maintains excitatory-inhibitory balance in hippocampal circuits.* Neuron, 2013. **77**(4): p. 712-22.

274. Gao, H., et al., *Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3.* Mol Endocrinol, 2006. **20**(6): p. 1287-99.
275. Kamei, Y., et al., *Ovariectomy in mice decreases lipid metabolism-related gene expression in adipose tissue and skeletal muscle with increased body fat.* J Nutr Sci Vitaminol (Tokyo), 2005. **51**(2): p. 110-7.
276. Wang, Y., et al., *Administration of 17beta-estradiol to ovariectomized obese female mice reverses obesity-hypertension through an ACE2-dependent mechanism.* Am J Physiol Endocrinol Metab, 2015. **308**(12): p. E1066-75.
277. Zhang, R., et al., *Selective inactivation of Socs3 in SF1 neurons improves glucose homeostasis without affecting body weight.* Endocrinology, 2008. **149**(11): p. 5654-61.
278. Ramadori, G., et al., *SIRT1 deacetylase in POMC neurons is required for homeostatic defenses against diet-induced obesity.* Cell Metab, 2010. **12**(1): p. 78-87.
279. Tong, Q., et al., *Synaptic glutamate release by ventromedial hypothalamic neurons is part of the neurocircuitry that prevents hypoglycemia.* Cell Metab, 2007. **5**(5): p. 383-93.
280. McClellan, K.M., K.L. Parker, and S. Tobet, *Development of the ventromedial nucleus of the hypothalamus.* Front Neuroendocrinol, 2006. **27**(2): p. 193-209.
281. Scherer, T., et al., *Brain insulin controls adipose tissue lipolysis and lipogenesis.* Cell Metab, 2011. **13**(2): p. 183-94.
282. Obici, S., et al., *Hypothalamic insulin signaling is required for inhibition of glucose production.* Nat Med, 2002. **8**(12): p. 1376-82.
283. Paranjape, S.A., et al., *Chronic reduction of insulin receptors in the ventromedial hypothalamus produces glucose intolerance and islet dysfunction in the absence of weight gain.* Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E978-83.
284. Brice, N.L., et al., *Metabotropic glutamate and GABA(B) receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells.* Diabetologia, 2002. **45**(2): p. 242-52.
285. Storto, M., et al., *Insulin secretion is controlled by mGlu5 metabotropic glutamate receptors.* Mol Pharmacol, 2006. **69**(4): p. 1234-41.
286. Liu, J., et al., *Intrahypothalamic estradiol regulates glucose metabolism via the sympathetic nervous system in female rats.* Diabetes, 2013. **62**(2): p. 435-43.
287. Gorres, B.K., et al., *In vivo stimulation of oestrogen receptor alpha increases insulin-stimulated skeletal muscle glucose uptake.* J Physiol, 2011. **589**(Pt 8): p. 2041-54.
288. Zhu, L., et al., *Estrogen signaling prevents diet-induced hepatic insulin resistance in male mice with obesity.* Am J Physiol Endocrinol Metab, 2014. **306**(10): p. E1188-97.
289. Shimazu, T., et al., *Role of the hypothalamus in insulin-independent glucose uptake in peripheral tissues.* Brain Res Bull, 1991. **27**(3-4): p. 501-4.
290. Takahashi, A., et al., *Effects of ventromedial hypothalamic stimulation on glucose transport system in rat tissues.* Am J Physiol, 1992. **263**(6 Pt 2): p. R1228-34.

291. Sprague, J.E. and A.M. Arbeláez, *Glucose Counterregulatory Responses to Hypoglycemia.* Pediatr Endocrinol Rev, 2011. **9**(1): p. 463-75.
292. Denroche, H.C., F.K. Huynh, and T.J. Kieffer, *The role of leptin in glucose homeostasis.* J Diabetes Investig, 2012. **3**(2): p. 115-29.
293. Clegg, D.J., et al., *Gonadal hormones determine sensitivity to central leptin and insulin.* Diabetes, 2006. **55**(4): p. 978-87.
294. Satoh, N., et al., *Sympathetic activation of leptin via the ventromedial hypothalamus: leptin-induced increase in catecholamine secretion.* Diabetes, 1999. **48**(9): p. 1787-93.
295. Toda, C., et al., *Extracellular signal-regulated kinase in the ventromedial hypothalamus mediates leptin-induced glucose uptake in red-type skeletal muscle.* Diabetes, 2013. **62**(7): p. 2295-307.
296. Zeng, W., et al., *Sympathetic neuro-adipose connections mediate leptin-driven lipolysis.* Cell, 2015. **163**(1): p. 84-94.
297. Ahmadian, M., R.E. Duncan, and H.S. Sul, *Skinny on Fat Metabolism: Lipolysis and Fatty Acid Utilization.* Trends Endocrinol Metab, 2009. **20**(9): p. 424-8.
298. Zimmermann, R., et al., *Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase.* Science, 2004. **306**(5700): p. 1383-6.
299. Anthonsen, M.W., et al., *Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro.* J Biol Chem, 1998. **273**(1): p. 215-21.
300. Krintel, C., et al., *Ser649 and Ser650 are the major determinants of protein kinase A-mediated activation of human hormone-sensitive lipase against lipid substrates.* PLoS One, 2008. **3**(11): p. e3756.
301. Kim, S.J., et al., *AMPK Phosphorylates Desnutrin/ATGL and Hormone-Sensitive Lipase To Regulate Lipolysis and Fatty Acid Oxidation within Adipose Tissue.* Mol Cell Biol, 2016. **36**(14): p. 1961-76.
302. Anthony, N.M., M.P. Gaidhu, and R.B. Ceddia, *Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation.* Obesity (Silver Spring), 2009. **17**(7): p. 1312-7.
303. Sullivan, J.E., et al., *Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase.* FEBS Lett, 1994. **353**(1): p. 33-6.
304. Cardinal, P., et al., *CB(1) cannabinoid receptor in SF1-expressing neurons of the ventromedial hypothalamus determines metabolic responses to diet and leptin.* Mol Metab, 2014. **3**(7): p. 705-16.
305. Palin, S.L., et al., *17Beta-estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes.* Metabolism, 2003. **52**(4): p. 383-8.
306. Belfrage, P., et al., *Regulation of adipose tissue lipolysis: phosphorylation of hormones sensitive lipase in intact rat adipocytes.* FEBS Lett, 1980. **111**(1): p. 120-4.
307. Schimmel, R.J., C.A. Buhlinger, and R. Serio, *Activation of adenosine 3',5'-monophosphate-dependent protein kinase and its relationship to cyclic AMP and lipolysis in hamster adipose tissue.* J Lipid Res, 1980. **21**(2): p. 250-6.

308. Londos, C., R.C. Honnor, and G.S. Dhillon, *cAMP-dependent protein kinase and lipolysis in rat adipocytes. III. Multiple modes of insulin regulation of lipolysis and regulation of insulin responses by adenylate cyclase regulators.* J Biol Chem, 1985. **260**(28): p. 15139-45.
309. Lafontan, M. and M. Berlan, *Fat cell adrenergic receptors and the control of white and brown fat cell function.* J Lipid Res, 1993. **34**(7): p. 1057-91.
310. Monjo, M., E. Pujol, and P. Roca, *alpha2- to beta3-Adrenoceptor switch in 3T3-L1 preadipocytes and adipocytes: modulation by testosterone, 17beta-estradiol, and progesterone.* Am J Physiol Endocrinol Metab, 2005. **289**(1): p. E145-50.
311. Albert, J.S., et al., *Null mutation in hormone-sensitive lipase gene and risk of type 2 diabetes.* N Engl J Med, 2014. **370**(24): p. 2307-15.
312. Wei, F., et al., *Genetic elimination of behavioral sensitization in mice lacking calmodulin-stimulated adenylyl cyclases.* Neuron, 2002. **36**(4): p. 713-26.
313. Cartmell, J., et al., *mGluR-evoked augmentation of receptor-mediated cyclic AMP formation in neonatal and adult rat striatum.* Br J Pharmacol, 1997. **121**(7): p. 1263-8.
314. Schaffhauser, H., et al., *Involvement of a cyclic-AMP pathway in group I metabotropic glutamate receptor responses in neonatal rat cortex.* Eur J Pharmacol, 1997. **334**(2-3): p. 289-97.
315. Wang, H., et al., *Roles of calcium-stimulated adenylyl cyclase and calmodulin-dependent protein kinase IV in the regulation of FMRP by group I metabotropic glutamate receptors.* J Neurosci, 2008. **28**(17): p. 4385-97.
316. Li, Z., G. Ji, and V. Neugebauer, *Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior.* J Neurosci, 2011. **31**(3): p. 1114-27.
317. Tsai, H.W., et al., *Protein kinase A activation of estrogen receptor alpha transcription does not require proteasome activity and protects the receptor from ligand-mediated degradation.* Endocrinology, 2004. **145**(6): p. 2730-8.
318. Ince, B.A., M.M. Montano, and B.S. Katzenellenbogen, *Activation of transcriptionally inactive human estrogen receptors by cyclic adenosine 3',5'-monophosphate and ligands including antiestrogens.* Mol Endocrinol, 1994. **8**(10): p. 1397-406.
319. Francesconi, A., R. Kumari, and R.S. Zukin, *Regulation of group I metabotropic glutamate receptor trafficking and signaling by the caveolar/lipid raft pathway.* J Neurosci, 2009. **29**(11): p. 3590-602.
320. Christensen, A. and P. Micevych, *CAV1 siRNA reduces membrane estrogen receptor-alpha levels and attenuates sexual receptivity.* Endocrinology, 2012. **153**(8): p. 3872-7.
321. Holst, J.J., et al., *Nervous control of pancreatic endocrine secretion in pigs. I. Insulin and glucagon responses to electrical stimulation of the vagus nerves.* Acta Physiol Scand, 1981. **111**(1): p. 1-7.
322. Ahren, B. and G.J. Taborsky, Jr., *The mechanism of vagal nerve stimulation of glucagon and insulin secretion in the dog.* Endocrinology, 1986. **118**(4): p. 1551-7.

323. Morton, G.J., et al., *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-95.
324. Chan, O. and R. Sherwin, *Influence of VMH fuel sensing on hypoglycemic responses.* Trends Endocrinol Metab, 2013. **24**(12): p. 616-24.
325. Kim, J.D., et al., *Hypothalamic prolyl endopeptidase (PREP) regulates pancreatic insulin and glucagon secretion in mice.* Proc Natl Acad Sci U S A, 2014. **111**(32): p. 11876-81.
326. Toda, C., *UCP2 regulates mitochondrial fission and ventromedial nucleus control of glucose responsiveness.* 2016. **164**(5): p. 872-83.
327. Luthi, A., B.H. Gahwiler, and U. Gerber, *Potentiation of a metabotropic glutamatergic response following NMDA receptor activation in rat hippocampus.* Pflugers Arch, 1994. **427**(1-2): p. 197-202.
328. Kow, L.M., A. Easton, and D.W. Pfaff, *Acute estrogen potentiates excitatory responses of neurons in rat hypothalamic ventromedial nucleus.* Brain Res, 2005. **1043**(1-2): p. 124-31.
329. Shen, K.Z. and S.W. Johnson, *Group I mGluRs Evoke K-ATP Current by Intracellular Ca(2+) Mobilization in Rat Subthalamus Neurons.* J Pharmacol Exp Ther, 2013. **345**(1): p. 139-50.
330. Zhang, C., M.J. Kelly, and O.K. Ronnekleiv, *17 beta-estradiol rapidly increases ATP-sensitive potassium channel activity in gonadotropin-releasing hormone neurons [corrected] via a protein kinase signaling pathway.* Endocrinology, 2010. **151**(9): p. 4477-84.
331. Huang, E.J. and L.F. Reichardt, *Trk receptors: roles in neuronal signal transduction.* Annu Rev Biochem, 2003. **72**: p. 609-42.
332. Finkbeiner, S., et al., *CREB: a major mediator of neuronal neurotrophin responses.* Neuron, 1997. **19**(5): p. 1031-47.
333. Shaywitz, A.J. and M.E. Greenberg, *CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals.* Annu Rev Biochem, 1999. **68**: p. 821-61.
334. Corti, C., et al., *Gene structure of the human metabotropic glutamate receptor 5 and functional analysis of its multiple promoters in neuroblastoma and astroglioma cells.* J Biol Chem, 2003. **278**(35): p. 33105-19.
335. Tong, L., et al., *Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons.* J Neurosci, 2004. **24**(30): p. 6799-809.
336. Hu, J.H., et al., *Preso1 dynamically regulates group I metabotropic glutamate receptors.* Nat Neurosci, 2012. **15**(6): p. 836-44.
337. Viwatpinyo, K. and S. Chongthammakun, *Activation of group I metabotropic glutamate receptors leads to brain-derived neurotrophic factor expression in rat C6 cells.* Neurosci Lett, 2009. **467**(2): p. 127-30.
338. Gibon, J., P.A. Barker, and P. Séguéla, *Opposing presynaptic roles of BDNF and ProBDNF in the regulation of persistent activity in the entorhinal cortex.* Mol Brain, 2016. **9**.
339. Lee, K. and P.R. Boden, *Characterization of the inward current induced by metabotropic glutamate receptor stimulation in rat ventromedial hypothalamic neurones.* J Physiol, 1997. **504 ( Pt 3)**: p. 649-63.

340. Cruz-Martin, A., M. Crespo, and C. Portera-Cailliau, *Glutamate induces the elongation of early dendritic protrusions via mGluRs in wild type mice, but not in fragile X mice.* PLoS One, 2012. **7**(2): p. e32446.
341. Been, L.E., et al., *Metabotropic Glutamate Receptor and Fragile X Signaling in a Female Model of Escalated Aggression.* Biol Psychiatry, 2016. **79**(8): p. 685-92.
342. Nan, X., F.J. Campoy, and A. Bird, *MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin.* Cell, 1997. **88**(4): p. 471-81.
343. Amir, R.E., et al., *Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2.* Nat Genet, 1999. **23**(2): p. 185-8.
344. Cohen, S., et al., *Genome-wide activity-dependent MeCP2 phosphorylation regulates nervous system development and function.* Neuron, 2011. **72**(1): p. 72-85.
345. Fyffe, S.L., et al., *Deletion of Mecp2 in Sim1-expressing neurons reveals a critical role for MeCP2 in feeding behavior, aggression, and the response to stress.* Neuron, 2008. **59**(6): p. 947-58.
346. Zhong, X., H. Li, and Q. Chang, *MeCP2 phosphorylation is required for modulating synaptic scaling through mGluR5.* J Neurosci, 2012. **32**(37): p. 12841-7.
347. Wilson, M.E., J.M. Westberry, and A.K. Prewitt, *Dynamic Regulation of Estrogen Receptor-Alpha Gene Expression in the Brain: A Role for Promoter Methylation?* Front Neuroendocrinol, 2008. **29**(3): p. 375-85.
348. Henriksen, E.J. and B.B. Dokken, *Role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes.* Curr Drug Targets, 2006. **7**(11): p. 1435-41.
349. Fiol, C.J., et al., *A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression.* J Biol Chem, 1994. **269**(51): p. 32187-93.
350. Pearce, N.J., et al., *Development of glucose intolerance in male transgenic mice overexpressing human glycogen synthase kinase-3beta on a muscle-specific promoter.* Metabolism, 2004. **53**(10): p. 1322-30.
351. Peineau, S., et al., *The role of GSK-3 in synaptic plasticity.* Br J Pharmacol, 2008. **153**(Suppl 1): p. S428-37.
352. Liu, F., et al., *The inhibition of glycogen synthase kinase 3beta by a metabotropic glutamate receptor 5 mediated pathway confers neuroprotection to Abeta peptides.* J Neurochem, 2005. **95**(5): p. 1363-72.
353. Cimarosti, H., et al., *Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates GSK-3beta.* Neurochem Res, 2005. **30**(2): p. 191-9.
354. Collins, F.S. and L.A. Tabak, *Policy: NIH plans to enhance reproducibility.* Nature, 2014. **505**(7485): p. 612-3.