Adaptations of the Hypothalamic Arcuate Nucleus in Response to Exercise and Hunger

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The arcuate nucleus of the hypothalamus serves as a critical node for regulation of mammalian energy balance with the ability to sense the status of many organ systems and exert control over effector arms that potently regulate feeding behavior and energy expenditure. The goals of this dissertation were 1) To identify hypothalamic adaptations driven by exercise induced energy expenditure related to conferral of whole body health improvement and 2) To investigate synaptic adaptations that occur in AgRP/NPY neurons of the hypothalamus in response to hunger.

Chronic *ad libitum* feeding on high fat diet induces energy surplus, while exercise increases energy expenditure. To investigate the role of exercise to offset the effects of energy surplus, 8 week-old male C57BL/6J mice were provided with 1) normal chow, 2) with western style high fat diet, or 3) with high fat diet and voluntary running wheel exercise training for 12 weeks. Exercise training decreased high fat diet induced adiposity by increased energy expenditure, without impacting caloric consumption. Along with changes to body composition, mice that exercised exhibited improved glucose clearance and skeletal muscle insulin sensitivity paired with reduced liver lipid accumulation and less adipose tissue expansion than their sedentary counterparts. These peripheral adaptations occur in concordance with improved hypothalamic leptin sensitivity and reduced pro-opiomelanocortin neuronal apoptosis.

On the other hand, short term energy deficit induced by an overnight fast elicits activation of hypothalamic AgRP/NPY neurons to drive feeding behavior. Many hormonal and neuronal inputs contribute to the activation of AgRP/NPY neurons, including release of the excitatory neurotransmitter glutamate. Therefore, we investigated the influence of fasting on synaptic integration of glutamate by metabotropic glutamate receptor 1 (mGluR1) on AgRP/NPY neurons. Fasting enhances function of mGluR1 to increase excitability of AgRP/NPY neurons, while loss of mGluR1 function reduces AgRP/NPY neuronal firing and reduces feeding behavior.

Perturbation of hypothalamic action by intake/expenditure components of energy balance provides insight into mechanisms that can be leveraged for many disease states, including obesity and diabetes. Taken together, these discoveries elucidate adaptations of the arcuate nucleus hypothalamus to support its role as a critical node involved in maintenance of energy balance in the mammalian biological system.

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Abbreviations

3-MATIDA – α-amino-5-carboxy-3-methyl-2-thipheneacetic acid
5-HT – Serotonin
ACTH – Adrenocorticotropic hormone
AMPK – AMP activated protein kinase
α-MSH – Alpha melanocyte-stimulating hormone
aCSF – Artificial cerebrospinal fluid
AgRP – Agouti related peptide
AP – Area postrema
ARC – Arcuate nucleus of the hypothalamus
AVP – Arginine vasopressin
BDNF – Brain derived neurotropic factor
CNS - Central Nervous System
CPT1 – Carnitine palmitoyltransferase I
DIO – Diet-induced Obesity
DHPG - Dihydroxyphenylglycine
DMH – Dorsomedial hypothalamus
DMNX – Dorsal Motor Nucleus of the Vagus
EX – Exercise
ERK1/2 – Extracellular Regulated-Signal Kinase
GABA – Gamma-aminobutyric acid
GnRH – Gonadotropin-releasing hormone 1
GPCR – G-protein coupled receptor
HFD – High fat diet
IKK/β – Inhibitor of nuclear factor kappa-B kinase subunit beta
IPGTT – Intraperitoneal glucose tolerance test
IPITT – Intraperitoneal insulin tolerance test
mTOR– Mammalian Target of Rapamycin
NFκB – Nuclear factor kappa-light chain enhancer of activated B cells
NTS – Nucleus Tractus Solitarii of the Vagus
GLP-1 – Glucagon like Peptide 1
HDL-C – High-density lipoprotein cholesterol

HPA axis – Hypothalamic-Pituitary-Adrenal axis
HPT axis – Hypothalamic-Pituitary-Thyroid axis
IL6 – Interleukin 6
IRS – Insulin Receptor Substrate
LCFA - Long chain fatty acid
LDL-C – Low-density lipoprotein cholesterol
LepR – Leptin Receptor
MCR – Melanocortin receptor
MC4R – Melanocortin 4 Receptor
mGlurI – Metabotropic Glutamate Receptor I
mGlurII/III – Metabotropic Glutamate Receptor 2/3
mTOR – Mammalian Target of Rapamycin
NE - Norepinephrine
PKC – Protein Kinase C
PI3K – Phosphoinositide 3 Kinase
POMC – Pro-opiomelanocortin
RIP – Rat Insulin Promoter
STAT – Signal transducer and activator of transcription
PKA – Protein Kinase A
PVH – Paraventricular hypothalamus
PYY – Peptide YY
SON – Supraoptic nucleus
TH – Tyrosine Hydroxylase
TNFα – Tumor Necrosis Factor Alpha
TRH – Thyrotropin-releasing hormone
TrkB – Tyrosine Receptor Kinase B
TUNEL – Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
vGAT – Vesicular GABA transporter
VLDL – Very Low Density Lipoprotein
VMH – Ventromedial hypothalamus

Chapter One
Introduction

Obesity and diabetes arise from chronic energy surplus, resulting in adaptation and dysfunction across many organ systems. While primary adaptations may provide short term benefits, energy surplus increases chronic load across organ systems. Obesity and diabetes are risk factors and increase severity of costly diseases such as cardiovascular disease, hypertension[142], liver disease[125], acute pancreatitis[108], infection susceptibility[121], cancer[35], kidney disease[161], osteoarthritis[139], dementia and Alzheimer's[76], depression and anxiety[100].

Obesity and Diabetes: Dysfunction across Organ Systems

The consequences of chronic energy surplus distribute across many organ systems. While the influence of energy surplus can be partially offset by distribution of load across many organ systems in the short term, this also means that the damage over time is wide spread. Deterioration of function by the liver, pancreas, vasculature, heart, adipose, skeletal muscle and kidenys all contribute to the progression of obesity and diabetes.

Chronic energy surplus places substantial burden on the liver. Surplus free fatty acid increases hepatic TG synthesis and atherogenic very low density lipoproteins[6]. In addition, accumulation of fatty acyl CoA[156] indirectly inhibits insulin receptor substrate 1(IRS-1) – compounding insulin resistance and exacerbation of gluconeogenesis. While the liver typically acts as a short term reservoir to release energy as glucose during fasting, in the obese state the liver senses energy deficit despite energy surplus. This results in increased glucose output even when glucose levels are not low, which further elevates blood glucose levels.

For the pancreas under chronic hyperglycemia, apoptosis of β cells reduces the pool of insulin secreting cells. Of the β cells that do survive, glucose toxicity results in reduced gene

expression for insulin via a reduction in the promoter region for the transcription factors PDX-1 and MafA. Together, the "double jeopardy" that occurs in the pancreas drives overt diabetes[144]. Further, while small amounts of circulating lipids can enhance glucose stimulated insulin secretion, accumulation of lipids in islets impairs insulin secretion[16]. Obesity and diabetes thus combine to influence pancreatic maladaptation by induction of hyperinsulinemia and a subsequent loss of insulin secretion.

The vascular system provides a route of transit for energy substrates and hormones. In the case of obesity, high levels of circulating atherogenic very low density lipoproteins (VLDL) cause occlusion to the vasculature. Dynamic endothelial function gives way to arterial stiffness in the case of obesity, evidenced by decreased arterial compliance even in obese children[171]. Intracellular signal mediators also play a role in obesity and type 2 diabetes' contribution to vascular dysfunction. Chronic elevation of activity by protein kinase Akt and mammalian target of rapamycin (mTOR) induce vascular senescence and potentiate ischemic injury[180]. Further, lowered adiponectin leads vascular inflammation by reduced anti-inflammatory input. Obesity wreaks havoc on the cardiovascular system marked by systolic and diastolic dysfunction with increased risk of ventricular dysrhythmia[90]. Specifically within the heart a number of adaptations arise in response to obesity. Marked left ventricular hypertrophy and increased heart rate temporarily provide the bloodflow required to maintain function[89], but obesity and insulin resistance predict coronary heart disease.

Skeletal muscle consumes the majority of glucose relative to other organ systems, and has the capacity to rapidly remove large amounts of glucose from the bloodstream. For skeletal muscle, chronic surplus of FFA leads to downstream inhibition of IRS1 as occurs in liver. Loss

of insulin induced Glut4 translocation significantly impairs glucose uptake – resulting in muscle fatigue and compounding the energy surplus phenotype with physical inactivity[6].

Adipose undergoes prominent expansion from chronic energy surplus and obesity is marked by hypertrophy of white adipose cells. Intra-abdominal fat correlates highly with insulin resistance[18]. However, the role of adipose in the development of metabolic disease goes well beyond its role as a storage depot for lipids. The inflammation response to adipose is marked by macrophage accumulation inducible Nitric Oxide Synthase, Interleukin-6 (IL-6), and Tumor Necrosis Factor Alpha (TNF-α) expression[182]. Leptin levels correlate directly and positively with adiposity[76] to signal high levels of energy storage. Paradoxically, despite increased adiposity, obesity results in decreased adiponectin secretion[5].

Kidney disease develops in nearly a third of diabetic patients and is marked by enlargement of the kidneys and significantly elevated glomular filtration[184], often associated with macroalbuminuria. Generation of reactive oxygen species under hyperglycemic conditions contributes to pathogenesis[47]. Individuals who have co-morbid kidney disease and obesity have a significantly higher mortality rate than those with diabetes alone[1].

Altered Profile of Circulating Factors with Obesity and Diabetes

In order for organisms to house cooperative organ systems, a mode of communication between systems had to evolve. The most fundamental form of organ system communication occurs through circulation of factors via liquid transport such as blood or cerebrospinal fluid. This allows for transportation of energy substrates, hormones, and inflammatory signals. In the pathology of obesity and type 2 diabetes, the profile of these circulating factors is markedly altered.

The early stages of type 2 diabetes are marked by skeletal muscle insulin resistance, followed by hyperinsulinemia in response to poor glucose clearance. Over time, β-cell failure arises as these cells are unable to keep up with whole body demand[36]. Mild symptoms such as blurry vision, a headache or stomach ache can set in. The hyperglycemia underlying these symptoms can be resolved by insulin injection at early stages in diabetes[10]. If untreated, severe cases of hyperglycemia cause ketoacidosis and can lead to a deadly situation over the course of hours.

Leptin is a satiety signal in the body that communicates peripheral energy surplus. Increased circulating leptin levels occurs with the expansion of adiposity. As a signal, leptin notifies target cells of energy surplus. While diabetes does not directly influence leptin levels[105], obese patients have significantly higher free (bioactive) leptin levels than lean counterparts[157]. However, given that chronic high levels of leptin can result in leptin insensitivity, approaches to reduce circulating leptin levels may have therapeutic value[25].

As obesity and diabetes progress, the stimulation of Glucagon like Peptide-1 (GLP-1) and Peptide YY (PYY) by meal ingestion becomes blunted[105, 116]. While an empty stomach typically signal the release of ghrelin, food intake fails to suppress ghrelin secretion in obese individuals[42]. However, in diabetic individuals post-prandial suppression of ghrelin remains intact[151].

Obesity comes with a profound disturbance to stress hormone profile. Obese patients have higher levels of circulating norepinephrine[72]. In addition, poorer control of type 2 diabetes correlates with higher levels of norepinephrine[157]. Further, cortisol regulation is altered in obesity. Opposing effects maintain cortisol at similar levels between obese and lean subjects. In obese subjects, increased cortisol clearance by 5α-reductase leads to loss of negative feedback

control of the hypothalamic-pituitary-adrenal axis[4]. However, increased clearance is offset in women by reduced 5β-reductase activity or cortisol regeneration in men. In men, regeneration of cortisol occurs primarily in the liver to drive transcription of gluconeogenic enzymes while blockade of cortisol regeneration leads to hypoglycemia and improved insulin sensitivity.

Hormones work to transmit information across organs through circulation. In the cases of obesity and type 2 diabetes, chronic energy surplus distributes load across organs. As organ systems directly adapt to chronic energy surplus they also have to adapt to each other. Given the specificity of hormones to act only on individualized receptors via specific molecular mechanisms, the endocrine system is insufficient to coordinate the broad range of adaptations across organ systems.

Hypothalamic Integration of Energy Availability and Organ System Inputs

Central control of the periphery is a hallmark of the success of the metazoans which permitted the integration of organ system level inputs and command of coordinated responses[23]. The hypothalamus serves as the key integration center for inputs related to energy balance. While the blood-brain barrier (BBB) provides a formidable blockade to bloodborne signals for most of the brain, the ventral portion of the hypothalamus has unique access to circulating factors. The median eminence is open to portal vessels[145] and can control transport of molecules across the BBB into cerebrospinal fluid[8]. The arcuate nucleus (ARC) is highly exposed to cerebrospinal fluid – further evidencing an anatomical basis for the role as receiver of peripheral signals.

Action of energy surplus signals within the hypothalamus is critical for maintaining healthy energy balance between intake and expenditure. A decrease in ARC insulin receptor

causes hyperphagia and insulin resistance[129]. Chronic high fat diet results in apoptosis of hypothalamic neurons by activation of inflammatory pathways which results in resistance to insulin[111]. Direct injection of GLP1 to the ARC, but not other hypothalamic regions, results in reduced hepatic glucose production[147]. Peptide YY directly blocks fasting induced activation of the ARC[143]. Leptin acts directly on $POMC^{ARC}$ neurons but also indirectly reduces inhibitory tone by acting on GABAergic neurons[178]. Deletion of leptin receptor results in an obese phenotype comparable to leptin deficient *ob*/*ob* mice[31].

Signals of energy deficit are similarly reliant on direct hypothalamic action. Mice lacking the growth hormone secretagogue receptor exhibit blocked arcuate nucleus activation and subsequent food intake that typically occurs[200]. This effect is striking and consistent with the effects of diet-induced obesity to cause ghrelin resistance of arcuate neurons[20].

Relay of autonomic afferents exerts substantial influence over hypothalamic function. The locus coeruleus contains many neurons with norepinephrine (NE) precursor tyrosine hydroxylase (TH) that influence post-synaptic activity of the paraventricular hypothalamic nucleus by excitatory action. These neurons typically confer information about acute stress but also potentially function in a chronic fashion[198] and are primarily directed at vasopressin expressing neurons within the magnocellular division of the PVH[166].

Nutrient/Hormone Responses of Neuronal Populations

The ARC is comprised of heterogeneous populations of neurons that co-respond to nutrient and endocrine signals related to energy balance. Classic models of arcuate control of energy balance pit molecularly defined POMC neurons and AgRP/NPY neurons as yin and yang[197] in control of satiation/energy expenditure and hunger/energy conservation, respectively.

At the simplest level of sensing of energy balance, neurons within the ARC respond directly to glucose, lipid, and protein nutrients in the cerebrospinal fluid. A decrease in glucose levels can directly stimulate activation of a portion of Agouti-Related Peptide (AgRP) and Neuropeptide Y (NPY) neurons known as AgRP/NPY[118], while an increase in glucose levels can directly stimulate activation of pro-opiomelanocortin (POMC) neurons[32].

While neurons primarily use glucose for fuel, they also can oxidize lipids and serve as sensors of lipid levels. Reduction of carnitine palmitoyl transferase IA (CPT1A) activity by pharmacological inhibition or genetic knockdown of CPT1A in the medialbasal hypothalamus results in decreased food intake and glucose production. Disinhibited lipid flux prevents the accumulation of LCFA-CoA's, which serve as an input to lipid sensing at the onset of nutrient surplus. Inhibiting CPT1A causes accumulation of long chain fatty acid CoA's (LCFA-CoA) in the medialbasal hypothalamus and subsequently restores lipid sensing[137]. Well after the onset of impaired lipid sensing, at high levels of circulating fatty acids for multiple weeks, the accumulation of LCFA-CoA's contributes to ARC insulin insensitivity and inflammation that impair hypothalamic control of energy balance[138].

Leptin activates POMC neurons[37] and simultaneously hyperpolarizes AgRP/NPY neurons[11]. The acute effects of leptin[59] to depolarize POMC neurons depend upon phosphoinositide-3-kinase (PI3K) signaling and reduced input resistance. Conversely, insulin hyperpolarizes POMC and AgRP neurons via PI3K activation of tolbutamide sensitive potassium channels. Interestingly, the POMC neurons that respond to leptin and insulin are likely independent populations[183].

Hormones don't act in isolation to communicate peripheral status to neurons within the

ARC. Instead, multiple hormones can work in opposition or cooperatively on ARC neurons to relay information. For example, adiponectin and leptin synergistically activate POMC neurons while adiponectin inhibits AgRP/NPY neurons. The effect of adiponectin on POMC neurons is also dependent upon glucose levels whereby adiponectin with high glucose has inhibitory action, while in low glucose it has excitatory action[165]. This partially explains why chronic elevation of glucose levels suppresses satiety and expenditure as in the cases of diabetes and obesity. On the other hand, Peptide YY[143] and insulin[165] block ghrelin activation of neurons in the ARC. This demonstrates that hormones can transmit information about energy status to their target in synergy or opposition. The unique nature of the neuron allows it to integrate information from many sources and produce an output that is consistent with demand across the body.

Hypothalamic^{ARC} Output Controls the Periphery

Hypothalamic^{ARC} neurons have effector arms[199] that regulate circuit dynamics broadly across the central nervous system (Figure 1.1B) via direct projections to more than twenty brain regions[181] comprised of many cellular subpopulations Together, these circuits form networks capable of dynamic regulation of energy balance across organ systems.

The ARC exerts profound influence over energy intake. Chemogenetic[84] and optogenetic[164] activation of AgRP/NPY neurons results in voracious feeding behavior. While the hypothalamus only contains a few thousand AgRP/NPY neurons, activation of just 100 of these neurons is sufficient to detect a measurable change in feeding behavior. Ablation of AgRP/NPY neurons in adult mice results in starvation, while ablation in neonates has minimal effects on feeding[101]. Conversely, POMC^{ARC} neuron activation reduces chronic but not acute feeding behavior[193], while ablation of POMC^{ARC} neurons results in hyperphagia and obesity.

The ARC also controls energy expenditure. Ablation of POMC neurons results in reduced energy expenditure and locomotor behavior[193]. Likewise, genetic deletion of downstream target Melanocortin 4 Receptor (MC4R) results in reduced energy expenditure and obesity, while re-expression results in normalization of body mass[9]. Conversely, reduction in hypothalamic AgRP results in significantly increased heat production and VO2[103]. Consistent with this finding, central administration of AgRP reduces energy expenditure and bodyweight. Agonism of the NPY target Y5 receptor results in significantly reduced energy expenditure associated with decreased brown adipose tissue thermogenesis[65]. Chronic central administration of AgRP results in reduced energy expenditure[159]. Consistently, deletion of GABAergic transmission from AgRP neurons results in significantly increased energy expenditure[170].

Ablation of $POMC^{ARC}$ neurons results in glucose intolerance and increased circulating cholesterol levels (HDL-C and LDL-C). Insulin receptor activation in $POMC^{ARC}$ neurons results in significantly increased hepatic glucose production, while insulin receptor activation in AgRP/NPY neurons results in suppressed hepatic glucose production[98]. Some reports show that insulin depolarizes a subset of AgRP/NPY neurons[2, 30]. However, in a study that recorded from a much larger sample of AgRP/NPY neurons and biotin-marked the selected neurons, zero were found to be depolarized by insulin[68].

The canonical hypothalamic-pituitary-adrenal (HPA) axis serves as the controller of stress hormone levels throughout the body. Pituitary inputs originating from the parvocellular region of the PVH secrete corticotropin-releasing factor onto corticotropes[160]. This induces the pituitary release of adrenocorticotropic hormone into circulation and subsequently increases glucocorticoid output by the adrenal cortex. In addition, vasopressin (AVP) release from neurons

in the mangocellular region of the PVH induces phospholipase C mediated signaling in the pituitary that amplifies the effects of ACTH.

Central signaling in the canonical hypothalamic-pituitary-thyroid (HPT) axis originates with hypothalamic function. TRH neurons receive substantial innervation from arcuate POMC and AgRP/NPY neurons. Activity and production of pro-Thyrotropin Releasing Hormone in thyrotropin-releasing hormone (TRH) expressing neurons of the PVH is suppressed by AgRP and NPY, while it is activated by alpha-melanocyte stimulating hormone (α-MSH). During fasting, leptin levels typically drop and TRH activity becomes blunted[93] by enhanced antagonism of the function of α-MSH. Exogenous leptin administration can prevent fasting suppression of pro-TRH mRNA. Pharmacological ablation of the arcuate nucleus of the hypothalamus results in profound disruption to the HPT axis[92], which is consistent with reduced TRH administration by intracerebroventricular administration of AgRP and NPY[46].

Genetic deletion of AgRP and NPY results in complete inhibition of feeding behavior induced by ghrelin injection, suggesting that output by AgRP/NPY neurons is the key mediator in ghrelin's effect on hunger[28]. Interestingly, ghrelin can act directly on neurons in the ventral tegmental area[158, 74, 123] and can increase dopamine in the nucleus accumbens[69]. Taken together, these studies demonstrate that reward centers play an augmentative role for AgRP/NPY output.

Autonomic regulatory centers are under the influence of alpha-MSH target melanocortin receptors. POMC neurons are localized to both the ARC and the nucleus tractus solitarii (NTS). $POMC^{ARC}$ neurons project directly to autonomic centers such as the NTS, area postrema (AP), dorsal motor nucleus (DMNX), and around the central canal[197]. Interestingly, very few direct AgRP/NPY projections to autonomic control centers suggest little influence at the cell body

level[21], but projections to common downstream sites of the NTS suggests that they influence integration of satiety signals relayed by viscerosensory afferent nerves. Activation of the DMV and NTS results in enhanced parasympathetic induction of insulin secretion, while subdiaphragmatic vagotomy results in loss of pacemaker input to the pancreas for pulsatile secretion of insulin[22].

AgRP/NPY neuronal GABAergic transmission onto the parabrachial nucleus supports conferral of taste information and feeding behavior. Loss of this transmission results in starvation[186] which interestingly occurs independent of the melanocortin system[185]. Loss of AgRP and NPY gene expression results in mice that are indistinguishable from wild-type mice by measurement of bodyweight or leptin responsiveness, further suggesting that the function of AgRP/NPY neurons goes beyond their ability to release those peptides[136].

Intracerebroventricular orexin, which translates to the greek word appetite, significantly increases food intake[152]. Orexin expressing neurons of the lateral hypothalamus project to the locus coeruleus to increase arousal and locomotor activity[55]. Lateral hypothalamic orexin neurons receive terminal appositions from AgRP/NPY neurons as well as α-MSH immunoreactive fibers[41], suggesting that arcuate neurons contribute to relay circuitry connecting the central nervous system to whole body energy demand.

Arcuate Rat Insulin Promotor (RIP) neuron activation results in significantly increased whole body energy expenditure via brown adipose tissue thermogenesis, while loss of inhibitory transmission from these neurons by genetic deletion of vesicular GABA transporter (vGAT) results in obesity by impaired thermogenesis[80].

Kisspeptin neurons within the arcuate nucleus are essential for progression through

puberty and fertility. Kisspeptin neurons provide pulsatile excitatory input to gonadotropin-releasing hormone 1 (GnRH) neurons[124] by release of synaptic glutamate and neurokinin B. GnRH neurons control pulsatile secretion of luteinizing hormone[124]. Interestingly, Kisspeptin neurons link nutrition state and fertility by direct release of glutamate onto both AgRP/NPY neurons and POMC neurons[141]. The function of this release is mediated by metabotropic glutamate receptors whereby AgRP/NPY neurons are inhibited by mGlur2/3 and POMC neurons are excited by mGluR1[127].

The Effect of Energy Surplus on the Nervous System

In the obese state, the arcuate nucleus of the hypothalamus and peripheral organ systems become disjointed. Diet-induced obesity in rats exhibit increased AgRP mRNA[94] or at least a greater decrease of POMC than AgRP mRNA[43]. Obesity results in impaired glucose sensing by $POMC^{ARC}$ neurons[32]. Knockout of energy sensor AMP-activated protein kinase (AMPK) in AgRP and POMC neurons impairs typical neuronal response to glucose levels and results in a lean phenotype or an obese phenotype, respectively[30]. Diminished function of glucose sensors results in dysregulation of arcuate effector arms and compounds the progression of obesity by promoting energy intake and impairing expenditure.

Increases in blood lipid levels corresponds with increased lipid level measurements taken from the third ventricle by catheterization. In food restricted mice compared to mice fed *ad libitum*, central administration of oleic acid results in decreased AgRP and NPY mRNA[114]. While high circulating lipid levels typically induce a negative feedback loop to reduce food intake, hypothalamic lipid sensing becomes rapidly impaired after feeding on a high fat diet. After just three days of overfeeding, decreased medialbasal hypothalamic malonyl-CoA levels

paired with increased CPT1A activity prevents intracellular accumulation of LCFA-CoA's in response to high circulating lipids. Consistently, inhibition of hypothalamic CPT1A or hypothalamic beta-oxidation restores lipid sensing via accumulation of LCFA-CoAs, resulting in decreased food intake and glucose production[137]. Taken together these studies demonstrate that hypothalamic lipid detection is a critical component of the hypothalamic role as sensor and regulator of whole body energy balance.

Diet-induced obesity causes leptin resistance[44] via reduced hypothalamic access and impaired intracellular signaling[40]. Reduced access is attributable to impaired ERK-gated signaling in tanycytes that transport leptin across the blood-brain barrier[8] resulting in altered blood to CSF leptin ratio[27]. Consistently, in the early phase of obesity, peripheral injection of leptin can activate ARC Signal Transducer and Activator of Transcription 3 (STAT3) signaling, but eventually peripheral injection becomes ineffective to activate ARC STAT3. In mice lacking leptin receptors (*db/db*), STAT signaling is markedly impaired[50]. Modeling high leptin levels observed in obesity by chronic administration of leptin results in reduced leptin receptor mRNA and protein levels[107]. However, even with elevated levels of leptin observed with obesity, poor site of action accessibility paradoxically sensitizes the ARC to leptin such that fasting induced activation of AgRP/NPY neurons and phosphorylation of STAT3 is substantially blunted in diet-induced obese (DIO) mice but not leptin deficient *ob*/*ob* mice[12]. In healthy mice, electrophysiological recordings in artificial cerebrospinal fluid (aCSF) reveal that fasting enhances firing rate and reduces resting membrane potential compared to fed status[167, 99]. In *ex vivo* recordings conducted outside of the physiological milieu, AgRP/NPY neuronal firing rate of fasted obese mice is significantly elevated compared to fed obese mice but the degree of firing

rate enhancement is larger for fed mice compared with fasted[11]. Interestingly, loss of bodyweight restores leptin signaling in $POMC^{ARC}$ and AgRP/NPY neurons[43]. Taken together, these studies demonstrate that the ARC sensing of energy balance by leptin is markedly impaired in obesity and type 2 diabetes which disrupts coordination of hypothalamic effector arms.

Obesity blunts the action of insulin to suppress NPY mRNA[150] and induce K-ATP channel activation in neurons that become hyperpolarized by decreases in glucose[160], such as $POMC^{ARC}$ neurons[66]. Central administration of insulin via an intracerebroventricular cannula results in less PI3K activation in long-term high fat fed rats compared to low fat fed rats, which is attributable to inflammation resulting from increased palmitoyl and stearoyl-CoA's that is blocked via IKKβ inhibitor[138]. Abundance of circulating palmitic acid, but not oleic acid, results in increased membrane Protein Kinase CΘ (PKCΘ) and blunts insulin-induced phosphorylation of AKT. Genetic deletion of PKCΘ prevented impairment of insulin signaling induced by administration of palmitic acid[13]. Modeling insulin resistance on AgRP/NPY neurons by deletion of insulin receptor results in a loss of suppression of hepatic glucose production – which would further compound the high glucose levels and progression of diabetes even without changes in food intake or bodyweight[81]. In summary, hypothalamic insulin action is important for maintaining whole body energy balance despite not being as direct in terms of substrate utilization as peripheral tissues.

DIO models exhibit ghrelin resistance marked by blunted ghrelin-induced activation of AgRP/NPY neurons, diminished ghrelin induced enhancement of AgRP/NPY transcriptional activation, and suppressed ghrelin induced feeding behavior. However, central administration of NPY still results in feeding behavior suggesting that downstream pathways are still intact[20].

Ghrelin induced activation of feeding behavior is also blunted in the pre-obese and obese agouti mouse model, while these mice exhibit enhanced melanocortin independent sensitivity to the anorectic effects of Peptide YY[107].

Genetically obese mice (C57BL/6J*ob/ob)* have significantly reduced brain weight, cortical volumes. Additionally, these mice exhibit decreased cross-sectional area of the ventromedial hypothalamus and dorsal motor nucleus of the vagus[14]. The autonomic nervous system of an obese individual is marked by increased sympathetic activity but decreased parasympathetic activity at the heart[71]. Many dysfunctional cardiovascular adaptations driven by obesity are reversed by weight loss via reduced sympathetic activity and cardiac hypertrophy[89]. Consistent with these findings, heart rate variability analysis reveals that obese patients who lose weight exhibit improved autonomic balance[90].

Diet induced obesity results in a chronic low grade inflammatory response in the central nervous system that is attributable to increased recruitment of macrophages originating from bone marrow[24]. Disruption of NF-κB and IKK/β results in protection from obesity, while their enhancement results in increased SOCS3 inhibition of leptin and insulin signaling[195]. Interestingly, cerebrospinal fluid borne IL-6 is negatively correlated with obesity and leptin levels[163], suggesting an obesity induced deficiency in the production of IL-6 in the brain. Loss of bodyweight reverses IL-6 and IL-10 deficiencies in human cerebrospinal fluid[175].

Exercise as Medicine for the Central Nervous System

Exercise exerts profound effects on the function of an organism's circulating hormones. Acute exercise increases circulating ghrelin levels[3], acyl-ghrelin levels, and glucagon levels [104]. On the other hand, acute exercise decreases insulin[179] and growth hormone levels[104]. Chronic

aerobic exercise training lowers circulating insulin levels, reversing the hyperinsulinemia driven by hyperglycemia[201]. Chronic exercise training also decreases circulating leptin levels[25]. Exercise training decreases circulating and hypothalamic catecholamine levels in a manner highly correlated with decreased fat mass[87].

Exercise also improves target-site function such as hypothalamic insulin sensitivity by inducing IRS2 expression[133] and phosphorylation of AKT as well as leptin signaling through JAK2/STAT3[133, 196]. While high-fat diet results in hypothalamic insulin and leptin insensitivity partially by activation of IKKβ/NF-κB, exercise promotes hypothalamic IL-6 and IL-10 inhibition of IKKβ/NF-κB. Alteration to pro-inflammatory signals that suppress IKKβ/NF-κB are necessary for exercise induced improvement in insulin/leptin function[146]. Consistent with the role of exercise to improve inflammatory mediated neuronal function, exercise reduces high fat diet induced microglial activation[192].

One week of exercise ameliorates the elevation of hypothalamic NPY mRNA associated with diabetes progression in a streptozotocin rat model of type I diabetes[153]. Reduced NPY mRNA also occurs with three[134] or eight weeks of voluntary wheel running in WT mice. Paradoxically, low NPY and AgRP gene expression typical of MC4R knockout mice becomes normalized by the same exercise program, but is offset by a decrease in orexin mRNA[56] which shows functional enhancement of the melanocortin system coupled with maintenance of energy balance across multiple networks. Acute exercise increases levels of hypothalamic POMC mRNA immediately after exercise. POMC mRNA is still elevated for at least three hours after cessation of activity[70]. In MC4R knockout mice, chronic exercise offsets the massive increases in leptin and development of obesity compared to sedentary controls despite reduced POMC levels

– suggesting healthy energy balance largely relies on normalized balance between AgRP/NPY and POMC neuronal output relative to hormonal inputs.

During exercise, glucose utilization and glucose production become markedly increased, while blood glucose levels remain relatively close to baseline[179]. Exercise normalizes blood lipid profile by decreasing LDL, cholesterol, and triglyceride levels while also increasing HDL levels [199], which may help restore hypothalamic lipid sensing in impaired conditions. While acute exercise increases skeletal muscle energy sensor AMPK activity, one hour of acute exercise does not influence AMPK activity or phosphorylation of AMPK target Acetyl CoA Carboxylase in the hypothalamus[3].

An acute bout of aerobic[54] or resistance[189] exercise training transiently increases brain derived neurotropic factor levels (BDNF). While much attention has been paid to the benefits of BDNF for cognitive performance [177], the role of BDNF to influence metabolic diseases also indicates potential benefits. Intracerebroventricular administration of brain derived neurotropic factor (BDNF) increases pancreatic insulin content and brown adipose tissue uncoupling protein mRNA, resulting in decreased blood glucose levels[128]. Interestingly, less than 20% of AgRP/NPY or POMC neuron cell bodies are immunoreactive for the BDNF receptor Tyrosine Receptor Kinase (TrkB). The population of BDNF expressing arcuate neurons is independent of leptin receptor (LepR) expressing neurons. However, leptin indirectly activates BDNF expressing VMH neurons to promote leptin sensitivity and satiety through the arcuate nucleus. Consistently, deletion of BDNF impairs leptin arcuate sensitivity, reduces POMC projections to the VMH, and results in hyperphagia with obesity[97].

Chapter 1 Figures

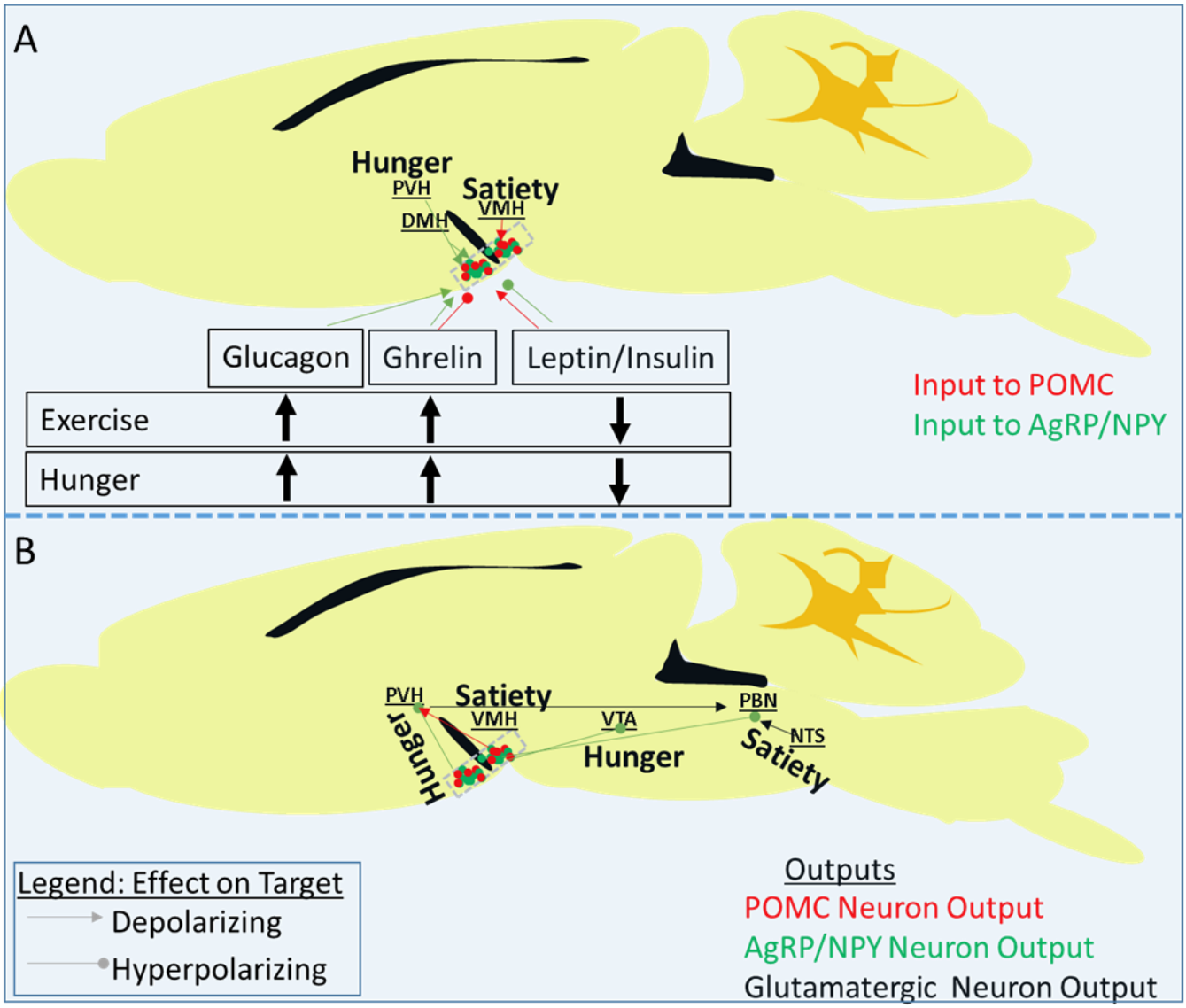


Figure 1.1: Inputs to and outputs of the arcuate nucleus integrate whole body energy balance. (A) Diagram of circulating and neuronal inputs to the arcuate nucleus. Glucagon and ghrelin increase in response to acute exercise[200] and have a depolarizing effect on AgRP/NPY neurons[68]. On the other hand, acute exercise decreases insulin levels[200] and chronic exercise decreases leptin levels[25]. Subsets of neurons within the PVH and DMH release excitatory glutamate onto arcuate AgRP/NPY neurons[85], while subsets of VMH neurons in release glutamate onto POMC neurons. **(B)**Output from POMC neurons increase satiety by output onto subsets of neurons in the PVH and VMH. Output from AgRP/NPY neurons antagonize satiety at the PVH, VTA, and PBN. The NTS relays physiological satiety from the periphery to the PBN, while disinhibition of the VTA during feeding may facilitate reward coding[25].

Statement of the Problem

Diseases of chronic energy surplus such as obesity and diabetes impact over 80 million Americans. As diseases driven by energy surplus trend upwards across the population, so do the burden of direct and indirect medical costs. Diseases of energy surplus present additional complexity for treatment of costly co-morbid diseases such as heart disease, chronic obstructive pulmonary disease, Alzheimer's disease, arthritis and many more. Therefore, a focus on the underpinnings of energy surplus may yield benefits across many diseases.

Mammals have enormous energy storage capacity that evolved under the selective pressure of low nutrient availability. However, rampant chronic energy surplus has emerged from a sedentary western society with highly available energy dense foods. While it is clear that energy surplus has negative health consequences, the mechanistic influence of normalized energy balance on positive health benefits via CNS control is incompletely explored. In a healthy individual, competing neuronal circuits influence satiety and hunger. Chronic energy surplus compounds detrimental effects by altering controllers of energy intake and expenditure towards thriftiness – starving in the midst of plenty. This dissertation will address adaptations of the arcuate nucleus in response to energy surplus, and test two approaches towards improved energy balance by exercise and restriction of energy intake.

Chapter Two:
Voluntary exercise improves hypothalamic and metabolic function in obese mice

Abstract:

Diseases of energy surplus such as diabetes and obesity have enormous financial and quality of life cost. One of the best known approaches to combat energy surplus is with increased energy expenditure. Therefore, we set out to determine the effects of 12 weeks running wheel exercise paired with ad libitum access to western style high fat diet. Voluntary wheel running improved body composition by reduction in adiposity across many depots ($P < .05$) without altering caloric consumption ($P > .05$). In addition, whole body glucose tolerance and insulin sensitivity is improved ($P < .05$). While these adaptations have been previously well explored, we demonstrate that exercise offsets damage of high fat diet induced apoptosis in POMC neurons and prevents hypothalamic leptin insensitivity. These adaptations occur in concordance with improvement to peripheral organ systems such as reduced liver steatosis, improved white adipose tissue morphology, and improved skeletal muscle insulin sensitivity. Taken together, this study identifies hypothalamic adaptations that may underlie the long-term benefits derived from exercise.

Laing BT, Do K, Matsubara T, et al. Voluntary exercise improves hypothalamic and metabolic function in obese mice. *The Journal of endocrinology*. 2016;229:109-122.

Introduction:

Obesity is reaching epidemic proportions in North America, affecting American society with increased morbidity and mortality as well as economic cost[82]. Obesity is usually associated with defects of energy intake and energy expenditure, which are tightly controlled by the CNS [139]. The CNS controls the important aspects of metabolism, particularly in the hypothalamus, where neurons directly respond to physiological changes such as hunger and satiety by secreted cytokines or hormones. Distinct nuclei within the hypothalamus such as arcuate (ARC), the paraventricular nucleus, the ventromedial hypothalamus (VMH), the dorsomedial hypothalamus (DMH), and the lateral hypothalamus share neuronal interconnections to maintain body homeostasis[120]. Although many neurons in the hypothalamus regulate metabolic functions, pro-opiomelanocortin (POMC)- expressing neurons, located in the ARC area, are key regulators of energy metabolism. Genetic ablation of POMC neurons causes increased food intake and reduced energy expenditure, leading to characteristics of the obese phenotype such as increased body weight and adiposity[53, 195]. Conversely, activation of POMC neurons suppresses food intake, increases energy expenditure, and induces the characteristics of the lean phenotype, such as decreased body weight and adiposity[193]. This indicates that POMC neurons play critical roles in body weight regulation. HFD affects POMC neurons in the ARC evidenced by increased caspase 3 immunoreactivity and decreased POMC mRNA[113]. Because overnutrition and high-fat diet (HFD) induce hypothalamic dysfunction, this may compound consequences of obesity and insulin resistance often leading to type 2 diabetes [195]. This suggests that POMC neurons are the target and part of the mechanism of HFD-induced obesity and diabetes[96].

Exercise therapy is a proven and effective clinical intervention for treating obesity and related diseases, such as hyperlipidemia and type 2 diabetes mellitus[173]. Exercise stimulates glucose uptake by skeletal muscle from the blood[51], decreases fat content from the adipose tissue[73], and prevents fat accumulation in the liver[67]. Besides the effects of exercise on peripheral tissues, voluntary exercise also improves brain function. For instance, it enhances learning and memory ability associated with the hippocampal area of the brain to prevent cognitive dysfunction and Alzheimer's[172, 49, 45].

Historical studies have demonstrated the effects of exercise on the CNS that uses neurotransmitters and trophic factors involved in energy homeostasis, such as norepinephrine, γ-amino butyric acid (GABA), serotonin (5-HT) [38], and brain-derived neurotrophic factor (BDNF) [126]. Furthermore, 40-day voluntary running wheel training significantly increases neuropeptide Y gene expression in Sprague–Dawley male rat ARC nucleus and DMH[95]. Recently, it has been reported that hypothalamic melanocortin receptor (MCR) expression has been associated with the exercise activity and nonexercise activity thermogenesis[154]. Although we have gained a better understanding that hypothalamic MCR signaling is highly regulated by the products of Pomc-expressing neurons[7], little is known about how exercise improves metabolic function via hypothalamic POMC-expressing neurons. In light of this gap, we sought to investigate the effects of voluntary exercise training on whole-body metabolic parameters and hypothalamic POMC neuron function in the diet-induced obese mice.

Methods:

Experimental animals

Eight-week-old C57BL6 male mice (n = 45) from Jackson lab (The Jackson Laboratory, Bar Harbor, ME, USA) were housed under controlled temperature and lighting conditions of 20–22° and 12-h light:12-h darkness cycle. Once the experimental protocol was initiated, all mice were divided into three groups: chow group (control; n = 15 with regular diet containing 26% protein, 14% fat, and 60% carbohydrate), HFD group (n = 15, 16% protein, 58% fat, and 26% carbohydrate, Research Diets D12331; Research Diets, Inc., New Brunswick, NJ, USA), and HFD with exercise training (HFD + EX; n = 15, 16% protein, 58% fat, and 26% carbohydrate, Research Diets D12331) and voluntary running wheel (TSE PhenoMaster System, Bad Homburg, Germany) for 12 weeks. For the study of voluntary wheel running, age-matched animals in the HFD + EX group were placed in cages equipped with running wheels for mice (TSE PhenoMaster), whereas animals in the control group and HFD group were housed in cages without running wheels for 12 weeks. Each cage accommodated one mouse. All aspects of animal care and experimentation were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committees of East Carolina University (Greenville, NC, USA).

Energy intake, energy expenditure, and body composition

Food intake was measured over a 5- to 7-day period, and the data were combined, averaged, and analyzed. Fresh pellets of food were provided every day to avoid temperature-dependent spoilage to the HFD group, and cages were changed every time that food weight was

measured. Any residual bits of food in the bedding were included in measurements. Cumulative food intake data were obtained by adding all intake measurements during the study. Fat and lean body mass were assessed using Echo MRI (Echo Medical Systems, Houston, MA, USA). Energy expenditure was measured by assessing oxygen consumption and carbon dioxide production using an indirect calorimetry with Comprehensive Lab Animal Monitoring System (CLAMS; TSE PhenoMaster). Mice were acclimated in the CLAMS chambers for 72h before data collection, and had free access to food and water for the duration of the studies.

Glucose tolerance test and insulin tolerance test

Two weeks before the last day of the experiment, an intraperitoneal glucose tolerance test (IPGTT) and an intraperitoneal insulin tolerance test (IPITT) were performed. After an overnight fast, IPGTT was performed by intraperitoneal injection of a 20% glucose solution (1g/kg). Blood samples were collected before and 15, 30, 60, 90, and 120 min after the injection. For IPITT, after a 4-h fast, an intraperitoneal injection of 1IU/kg human rapid insulin (Eli Lilly) was administered to the HFD-treated mice and 0.5U/kg human rapid insulin was administered to the chow diet-treated mice. Blood samples were collected before and 15, 30, 60, 90, and 120min after the injection. For the IPITT, the response of blood glucose levels was expressed as a percentage of the values before insulin injections.

Morphological analysis of the liver and white adipose tissue

Serial sections (5μM thickness) were taken from the post-fixed liver and epididymal fat, followed by hematoxylin and eosin (H&E) staining as described previously[63]. The stained sections were photographed digitally using an optical microscope (Leica DM6000, Germany), and the images were transferred to the computer medium.

Immunohistochemistry

For fluorescence detection of POMC, coronal brain sections from 20-week-old mice in three groups were generated, and immunohistochemistry was performed as described previously [62]. Briefly, brain sections were incubated with antibody to POMC (Phoenix Pharmaceuticals, Burlingame, CA, USA) and further incubated with fluorescent-labeled secondary antibodies. POMC-positive neurons throughout the mediobasal hypothalamus were counted using ImageJ software (NIH, Bethesda, MD, USA). Three serial sections were analyzed in each mouse (n=3).

Leptin-induced signal transducer and activator of transcription 3 phosphorylation

Mice were injected with leptin (A.F. Parlow National Hormone and Peptide Program, Torrance, CA, USA) intraperitoneally (3mg/kg) and killed 30 min later. The brain sections were evaluated for phosphorylatedSTAT3 (pSTAT3) in hypothalamus neurons as described previously [155]. Briefly, brain sections were incubated with an anti-pSTAT3 antibody (Cell Signaling), followed by an anti-fluoresces-conjugated rabbit antibody, pSTAT3 was then visualized under an optical microscope (Leica DM6000). All pSTAT3- immunoreactive ARC neurons were counted using ImageJ software (NIH). Cells within the median eminence were excluded from these analyses. Three serial sections were analyzed in each mouse (n = 3).

Proliferative assay and tunnel assay

An endogenous proliferative marker Ki67 was used to determine the neuronal proliferation. Briefly, Ki67 antibody (Abcam) was used for a single immunolabeling study in brain sections among the three groups, followed by an anti-fluoresces-conjugated rabbit antibody. Ki67-positive cells throughout the mediobasal hypothalamus were counted using ImageJ software (NIH). Three serial sections were analyzed in each mouse (n = 3).

A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was used to identify double-stranded DNA fragmentation. Briefly, coronal brain sections were washed in PBS, transferred to blocking solution for 2h, and then incubated in primary POMC antibody (Phoenix Pharmaceuticals) overnight. The next day, after washing, the sections were transferred to secondary antibody for 2h in light-deprived conditions. After being washed in PBS, samples were incubated at 4°C in permeability solution (PBS, 0.1% Triton-X, 0.1% sodium citrate) for 2min, and then incubated with TUNEL assay solution (In Situ Cell Death Detection Kit, Fluorescein, Sigma-Aldrich) for 1h at 37°. After being washed in PBS, all sections were mounted on slides with Vectashield antifade reagent. Negative and positive controls for the TUNEL assay were confirmed by staining the sections in the same manner without primary antibody (negative control) or pretreated with DNAse I (positive control). Positive cells were counted in the ARC from slides (n=3) of each group.

Statistical Analysis

Data are expressed as mean ± standard error. Differences between groups were compared for statistical significance by ANOVA or two-tailed Student's T-Test; $P < .05$ denoted significance.

Results:

Long-term voluntary exercise training lowers body weight gain and adiposity induced by HFD

To determine the effects of long-term voluntary running wheel exercise training on body weight regulation and adiposity, 45C57BL6 male mice were divided into three groups (control, HFD, and HFD+EX) for 12 weeks. Figures 1A and B show the average daily locomotion activity and running distance, respectively. Although HFD groups show lowered locomotion activity compared with the control group, the HFD+EX group shows significantly increased daily running distance, indicating increased total daily physical activity in the HFD+EX group. Next, we measured the body weight before and after the study, as shown in Figs. 1C and D; there was no difference in body weight among the three groups at the beginning of the study. However, the HFD group significantly increased body weight compared with the control group (41.1 ± 0.4 vs 28.9 ± 1.1 g) after 12 weeks, and voluntary exercise training significantly lowered HFD-induced body weight gain (36.3 ± 1.7 vs 41.1 ± 0.4 g) at the end of the study.

Echo MRI data revealed that 12 weeks of HFD increased total fat mass significantly compared with the control group (Fig. 1E; 8.1 ± 1.9 vs 2.5 ± 0.4 g). Data also indicated that 12-week of voluntary running wheel exercise training significantly reduced total fat mass (6.1 ± 1.7 vs 8.1 ± 1.9 g) compared with the HFD group. There was no significant difference in total lean mass between the HFD groups (Fig. 1F).

By the end of the study, some regional fat pads were harvested and fat contents were weighed. Fat contents such as epididymal fat content (Fig. 1G; 0.61 ± 0.10 vs 0.75 ± 0.07 g), perineal fat content (Fig. 1H; 0.72 ± 0.06 vs 0.91 ± 0.03 g), as well as mesenteric fat (Fig. 1I;

0.47 ± 0.09 vs 0.67 ± 0.12 g) were significantly decreased in the HFD +EX group compared with the HFD group.

Voluntary exercise training reduces body weight via increased energy expenditure despite normal caloric intake in HFD fed mice

Change in body weight is controlled by energy intake and energy expenditure. To assess energy intake, food was weighed daily. Daily and cumulative caloric intake in all three groups was calculated at week 8 after the start date. At this point, although there was a significant increase in calorie intake in the HFD groups compared with the control group, we did not observe any caloric intake difference between the HFD and the HFD +EX groups (Fig. 2A and B). Energy expenditure, as measured by oxygen consumption over 24 h, was significantly increased in the HFD +EX group (5.32 ± 0.66 vs 4.72 ± 0.48 L/h/kg of lean mass) only during the night (Fig. 2C and D). Similarly, the total amount of carbon dioxide production over 24 h was also significantly increased in the HFD +EX group compared with the HFD group (3.92 ± 0.62 vs 3.52 ± 0.39 L/h/kg of lean mass) only during the night (Fig. 2E and F).

Long-term exercise training improves insulin sensitivity in HFD

To determine whether long-term voluntary exercise training can improve insulin sensitivity impaired by HFD, we measured both fasted and fed status glucose levels at 19 weeks of age. Although there was no significant difference in fasting plasma glucose levels between the HFD +EX and the HFD groups, fed plasma glucose levels in the HFD +EX group were significantly reduced compared with the HFD group (Fig. 3A and B). A glucose tolerance test also revealed that glucose tolerance was significantly improved in the HFD +EX group versus the HFD group ($P < .05$), especially after 30 and 60 min of glucose injection (Fig. 3C). The

insulin tolerance test also showed improvement associated with exercise training, with peak differences after 15 and 30 min of the insulin injection. This indicates that long-term exercise training improves systemic insulin sensitivity (Fig. 3D).

In support of the notion that long-term exercise training improves systemic insulin sensitivity in the HFD+EX group, insulin signaling in skeletal muscle was examined by immunoblotting for phosphorylation of AKT (protein kinase B) in gastrocnemius. Figure 3E and F shows that skeletal muscle phosphorylation of AKT was significantly impaired in the HFD group compared with the control group ($P < .05$), and voluntary exercise training remarkably reversed skeletal muscle phosphorylation of AKT in the HFD+EX group, indicating that there is significant improvement in skeletal muscle insulin signaling (Fig. 3E and F).

Voluntary exercise training reduces HFD-induced lipid accumulation in the liver and adipocytes size in white adipose tissue

Histological analysis shows that 12 weeks of HFD significantly increased lipid accumulation in the liver revealed by hematoxylin and eosin stain in liver sample sections, whereas voluntary exercise training remarkably reduced lipid accumulation in the liver (Fig. 4, left). In white adipose tissue, the cell size in HFD group mice was significantly increased compared with the control group; however, voluntary exercise training reduced the adipocytes size in the HFD+EX group (Fig. 4, right).

Effect of long-term voluntary exercise training on HFD-impaired central leptin signaling

To determine whether the long-term voluntary running wheel exercise training can improve the hypothalamic function that controls energy metabolism, we measured leptin-induced phosphorylation of STAT3 in the hypothalamus. Leptin-induced phosphorylation of STAT3 in

the ARC and VMH was almost completely blunted ($P < .05$) in the HFD group compared with the control group (54 ± 3 vs 5 ± 1 counts per slice). Voluntary exercise training partially restored ($P < .05$) leptin-induced STAT3 phosphorylation in the HFD-treated mice (19 ± 2 vs 5 ± 1 counts per slice), suggesting that voluntary exercise training improves central leptin signaling (Fig. 5).

Effect of long-term HFD and voluntary exercise training on POMC-expressing neurons

To determine the effect of HFD and exercise training on POMC-expressing neurons, immunolabeling with an anti-POMC antibody was assessed. It was found that 12 weeks of HFD significantly reduced ($P < .05$) the number of POMC neurons in the hypothalamus (26±3 counts per slice in the control group vs 16±2 counts per slice in the HFD group); however, long-term voluntary exercise training remarkably restored ($P < .05$) the number of POMC neurons in the HFD+EX group (23±2 counts per slice in the HFD+EX group vs 16±2 counts per slice in the HFD group; Fig. 6).

Long-term voluntary exercise training restores HFD-damaged neuronal proliferation in the hypothalamus

To elucidate the potential mechanism of HFD- and exercise-induced POMC-expressing neuron alteration, an endogenous proliferative marker Ki67 was used to determine. neuronal proliferation. Under high-fat conditions, the Ki67- positive cells showed significantly decreased ($P < .05$) proliferation compared with the control group (8±1 counts per slice in the control group vs 2±2 counts per slice in the HFD group). It was found that 12 weeks of voluntary exercise training significantly restored ($P < .05$) the loss of cell proliferation in the hypothalamus (4±1 counts per slice in the HFD+EX group vs 2±2 counts per slice in the HFD group) (Fig. 7). Long-term voluntary exercise training reduces HFD-induced apoptosis in POMC-expressing neurons

in the hypothalamus. To further investigate the potential mechanism of HFD- and exercise induced POMC-expressing neuron alteration, TUNEL assay was performed to determine the neuronal apoptosis among the three groups (Fig. 8). Although there was no apparent cell apoptosis occurring in the ARC of the control group, 12-week HFD significantly increased ($P < .05$) cell apoptosis, especially in the ARC, and voluntary exercise training strongly protected against the HFD-induced apoptosis in this area ($P < .05$). Furthermore, the apoptosis that specifically occurred in the POMC neurons was reduced by more than half in the exercise training group with HFD (10±3 counts per slice in the HFD group vs 4±2 counts per slice in the HFD+EX group).

Discussion:

In this study, we have demonstrated that exercise reduces apoptosis of POMC neurons in the ARC and reduces leptin insensitivity caused by diet-induced obesity. Decreased dysfunction in this population occurs concurrently with improvement across skeletal muscle, liver, and white adipose tissue. At the whole body level, these exercise induced adaptations emerge as reduced adiposity and improved glucose regulation.

Although over the past decade we have gained a better understanding of CNS function in regulating food intake and body weight homeostasis[33, 120], a gap of knowledge exists detailing how exercise training mechanistically induces weight loss via neurological control of energy balance and body weight in obese subjects. Given the facts, certain areas within the hypothalamus, such as ARC, VMH, DMH, and PVH, play important roles in regulating systemic metabolic homeostasis. However, to date, emerging evidence has shown that exercise training-induced improvements are associated with molecular changes that improve metabolic functions in most peripheral tissues, such as increased glucose uptake in skeletal muscle and adipose tissue as well as decreased lipids accumulation in adipose tissue and the liver, all of which contribute to enhanced insulin sensitivity. Although there is an increasing amount of studies demonstrating that exercise training enhances the brain function, including the effects of exercise on learning and memory in hippocampal neurons, the role of exercise training in improving metabolic function via CNS-mediated pathways has not yet been fully understood. Thus, it is worthwhile to investigate the CNS-associated mechanism(s) of exercise training to improve metabolic function, particularly under diet-induced obesity conditions. In this study, we demonstrated that, first, HFD-induced body weight gain and adiposity are reversed by voluntary exercise training mainly

through increased energy expenditure despite normal energy intake, and secondly, these effects may be associated with protection of POMC neurons and enhanced hypothalamic function response to leptin by voluntary exercise training under HFD conditions.

In the brain, POMC-expressing neurons are mainly located in the ARC of the hypothalamus and in the nucleus of solitary tract (NTS) in the brain stem. Genetically, when a null mutation of POMC gene is generated by targeting a gene in embryonic stem cell, hyperphagic and obesity phenotypes are displayed[191]. Human patients lacking POMC also confirm this obese phenotype[18]. Furthermore, a recent study has been published by Zhan and coworkers using the designer receptor exclusively activated by designer drugs system to selectively remove POMC-expressing neurons in these two areas. These researchers found that postnatal ablation of POMC neurons in the ARC nucleus (but not in the NTS) increased food intake, reduced energy expenditure, and ultimately resulted in obesity and metabolic and endocrine disorders[193]. Taken together, these findings indicate the importance and necessity of ARC POMC-expressing neurons in controlling whole-body homeostasis. An HFD rapidly induces neuron injury and eventually causes chronic inflammation in the hypothalamus, as confirmed by obese human subjects' MRI data[168]. In the ARC, POMC-expressing neurons are specifically affected by an HFD[96]. We hypothesize, thus, that damage to a critical neuronal type (POMC) for body weight control might play a role in obesity, and exercise training may play a role to prevent the damage induced by a HFD.

POMC-expressing neurons control both energy intake and energy expenditure. One interesting finding of this study is that there is no food intake difference between the HFD and HFD+EX groups observed, despite the fact that exercise training restored POMC-expressing

neurons significantly. This might be due to effects on counter regulators such as orexigenic agouti-related peptide (AgRP)-expressing neurons that also resides in the ARC. AgRP/NPY neurons control food intake, and ablation of AgRP neuron in adult mice has been shown to result in significantly reduced food intake[101]. It has also been reported that an HFD induces apoptosis of AgRP neurons[113]. These results suggest that long-term HFD and exercise training may have broad effects on both orexigenic and anorexigenic neurons in the ARC. Consistent with our findings, it has been reported that 6-week voluntary exercise training promotes leanness and prevents diet-induced obesity by increasing energy expenditure but not energy intake. These effects are associated with changes in the CNS centers that control energy homeostasis, particularly in the subset of neurons in the VMH, which is another primary satiety center in the hypothalamus, further proved the notion that exercise training may have broader effects than just particular neurons in the hypothalamus. In the same study, HFD-induced central leptin resistance, revealed by measuring food intake after central administration of leptin, was also significantly improved when followed by voluntary exercise training[26]. This suggests a potential mechanism of CNS-associated effects of exercise training on metabolic function. Our study further explored this notion by demonstrating central leptin signaling by immunolabeling phosphorylation of STAT3, a classic downstream pathway marker of leptin signaling transduction in the hypothalamus. We found that 12-week HFD treatment dramatically reduced pSTAT3 signals in the ARC and VMH nuclei, and that this impairment was significantly improved by voluntary exercise training. This was also true with even shorter periods of exercise training in diet-induced obese rats. Patterson and coworkers showed that 3 weeks of post-weaning exercise training reduced body weight gain and adiposity in selectively bred diet-

induced obese rats, and that these effects are associated with increased leptin-induced pSTAT3 expression in the ARC area [135]. Leptin directly activates hypothalamic POMC-expressing neurons[32], and deficiency of leptin signaling pathway activation in POMC-expressing neurons results in increased body weight[9].

We investigated the effects of exercise training in POMC expressing neurons directly. A 12-week HFD significantly decreased the number of POMC-expressing neurons, but this decrease was not observed in the HFD+EX group. To the best of our knowledge, this is the first study to show that voluntary exercise training has a beneficial role on POMC-expressing neuron turnover. Notably, turnover is the balance between neurogenesis and neuronal death. Most recently, neurogenesis has been described in the hypothalamus and has been shown to participate in the response of hypothalamic neuronal circuits to metabolic status[79]. Emerging evidence suggests that, in addition to the hippocampal area, active neurogenesis takes place in other regions of the adult rodent brain, including the hypothalamus, where a potential neurogenic niche has been identified. In adults, neurogenesis occurs at low rates in different areas of the brain. Moreover, the new neurons produced through adult life seem to contribute to physiological function of the entire body[91]. Neurogenesis in the ARC has shown to be essential for reducing and sustaining reduced body weight, and an HFD was shown to disrupt this neuronal proliferation process in mice with diet-induced obesity[73, 110]. To investigate the potential role of exercise in enhancing neuronal proliferation in the ARC that possibly promotes POMC-expressing neurons, we measured the endogenous proliferative marker Ki67's expression in the ARC area among the three groups. Consistent with previous findings, we found that neurogenesis occurs in adults at very low rates in different areas of the brain. We could detect

very few Ki67-positive cells in the ARC area of mice in the control group; interestingly, there was a significant reduction of Ki67-positive cells in the same area of the HFD group. Voluntary exercise training significantly restored the Ki67-positive cells in the hypothalamus. However, one limitation of this study is that Ki67 can only be detected in premature cells, thus making it impossible to co-label along with mature cell markers to determine their final destination. Although we observed that there is a significant increase of Ki67-positive cells in the ARC of the HFD+EX group compared with the HFD group, the total net contribution to the increase of POMC-expressing neurons associated with reduction of body weight and adiposity remains mostly unclear. Future studies should address the specificity of exercise training-induced neuronal proliferation and weigh the contribution of these proliferative cells that regulate whole-body metabolism.

Similar to our findings, Borg and coworkers have recently reported that 7 days of exercise training increased hypothalamic cell proliferation 3.5-fold above the sedentary mice. However, blocking cell proliferation via administration of the mitotic blocker cytosine-1-β-d-arabinofuranoside (AraC) did not affect food intake or body mass in obese mice, indicating that the proliferation of new neurons is not required for maintaining whole-body homeostasis by exercise training[17]. Therefore, to elucidate the potential mechanism of nutrition and exercise training in altering POMC-expressing neurons, we next investigated neuronal death by using a TUNEL assay. It has been reported that overnutrition, such as a long-term HFD, could induce hypothalamic cell inflammation via endoplasmic reticulum (ER) stress, and inflammatory signal transduction can lead to the activation of apoptotic signaling pathways[195]. In contrast to the neuronal proliferative study, the TUNEL assay revealed that although there is no obvious

apoptosis occurring in the control group, in the HFD group we found that more cell apoptosis accumulated in the ARC area of the hypothalamus, and most strikingly, we also observed that voluntary exercise significantly reduced neuronal apoptosis compared with the HFD group, leading to a potential protective mechanism in which exercise training rescues HFD-induced neuronal loss. Along with our findings, Yi and coworkers have reported that 26 weeks of moderate treadmill exercise training prevented Western-style dietinduced hypothalamic inflammation by decreasing microglia activation in the ARC, supporting the idea of exercise training in repairing neuronal damage in the hypothalamus[192]. The possible molecular mechanism of HFD-induced ER stress might be associated with IKK-β/NF-κB pathway in the hypothalamus, which an HFD could activate leading to a progression of ER stress in the hypothalamus and therefore impairing insulin and leptin signaling, thus resulting in energy imbalance[195]. Thus, IKK-β/NF-κB in the hypothalamus is a potential target pathway for exercise training-associated benefits in the hypothalamus. Interestingly, interleukin 6 (IL6) is a cytokine that has both proinflammatory and anti-inflammatory functions along with its metabolic effects on food intake suppression, energy expenditure induction, and body weight and adiposity reduction. The actions of IL6 might mediate to suppression of IKK-β/NF-κB activation in the hypothalamus and thus help to maintain normal function[194]. Notably, the IL6 is a highly exercise inducible cytokine in skeletal muscle (during contraction) as well as in neurons located in the hypothalamus. Indeed, the increased hypothalamic IL6 expression was observed in exercise trained rats[194], suggesting a possible molecular mechanism that exercise improves metabolic function at least partially via the IL6-IKK-β/NF-κB/ER stress-mediated pathway, thus preventing HFD-induced neuronal inflammation/apoptosis and eventual neuron loss. BDNF is another

element that influences neuronal survival and differentiation[15] and has a strong metabolic function in regulating body weight[187]. It has been reported that HFD reduces hippocampal levels of BDNF[111]. Interestingly, BDNF can be induced in the CNS by exercise and exercise training[112, 111], leading us to suspect that BDNF also plays an important role in protecting neurons from HFD-induced damages in the hypothalamus, which might be further enhanced by exercise training. Taking these facts together, it would be intriguing to investigate the relationship between exercise-induced hypothalamic IL6 and BDNF signaling in conjunction with hypothalamic function in the context of energy homeostasis.

Overall, this study has demonstrated the effects of voluntary exercise training on metabolic function that may be associated with CNS-mediated pathways by protecting POMC-expressing neurons and enhancing leptin signaling in the ARC nucleus of the hypothalamus. Although the cellular and molecular mechanisms behind this phenomenon need to be explored further, our findings regarding CNS-mediated pathways that potentially mimic the effect of exercise training to prevent hypothalamic neuron loss would make a highly logical and desirable strategy for the prevention and treatment of obesity in humans.

Chapter 2 Figures

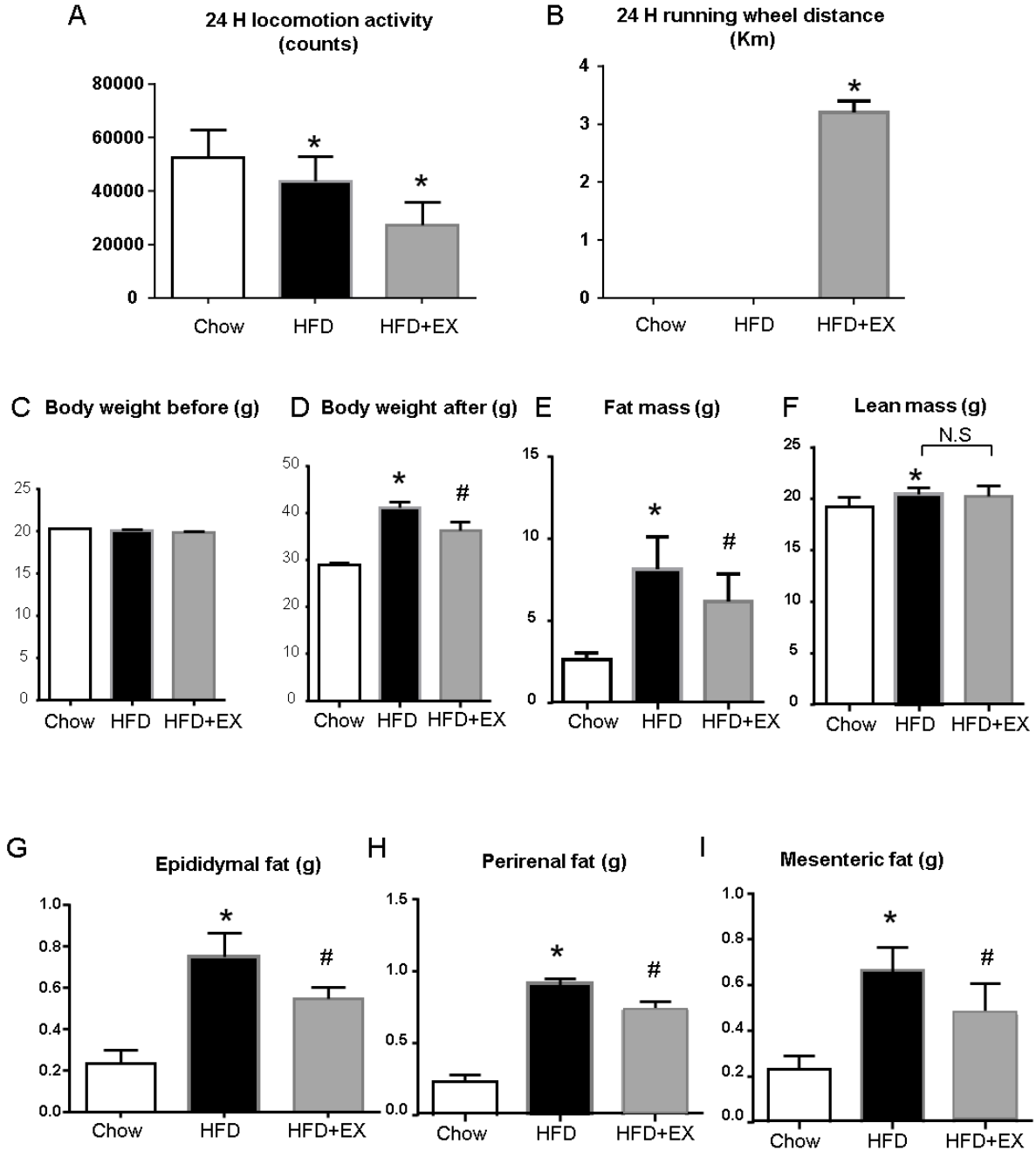


Figure 2.1 Chronic voluntary wheel running reduces high fat diet induced obesity and adiposity. (A) Locomotion behavior indicated by count data of beam breaks. **(B)** Running wheel distance traveled. **(C)** Body weight (grams) at the start of experiment. **(D)** Body weight (grams) at the end of the experiment. **(E)** Fat mass between groups. **(F)** Lean mass between groups at the end of experiment. **(G)** Epididymal fat mass (grams) at the end of the experiment. **(H)** Perineal fat mass (grams) at the end of experiment. **(I)** Mesenteric fat mass (grams) at the end of experiment. * indicates significance ($p<.05$) compared to chow; # indicates significance between HFD and HFD+Ex.

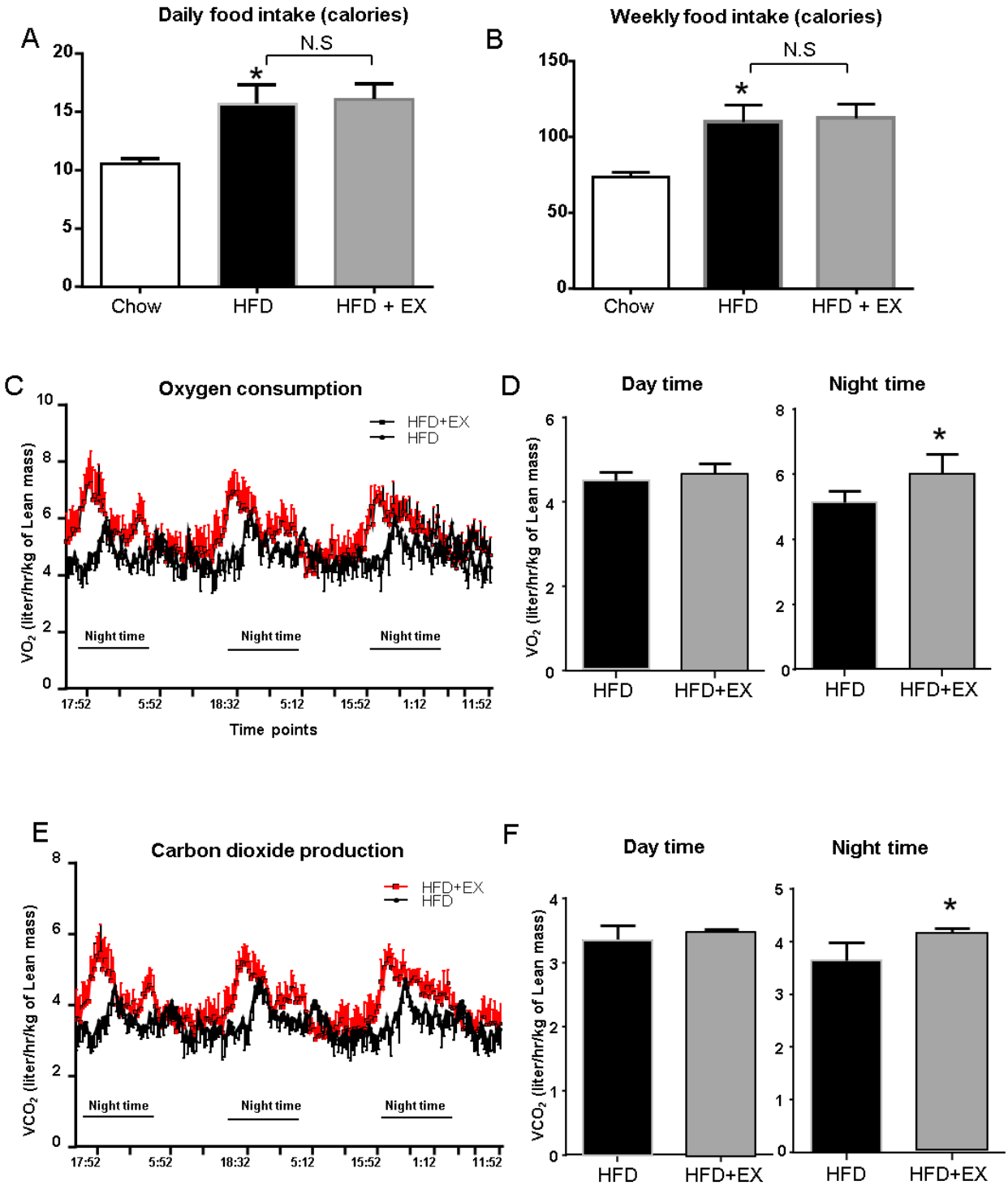


Figure 2.2 Energy intake and expenditure between chow, HFD, and HFD + Exercise groups. **(A)** Daily caloric intake between groups. **(B)** Weekly caloric intake between groups. **(C)** Energy expenditure as indicated by volume of oxygen consumption. **(D)** Oxygen consumption by light cycle. **(E)** Gas exchange as measured by carbon dioxide production. **(F)** Carbon dioxide production by light cycle. T-test significance ($p<.05$) indicated by *.

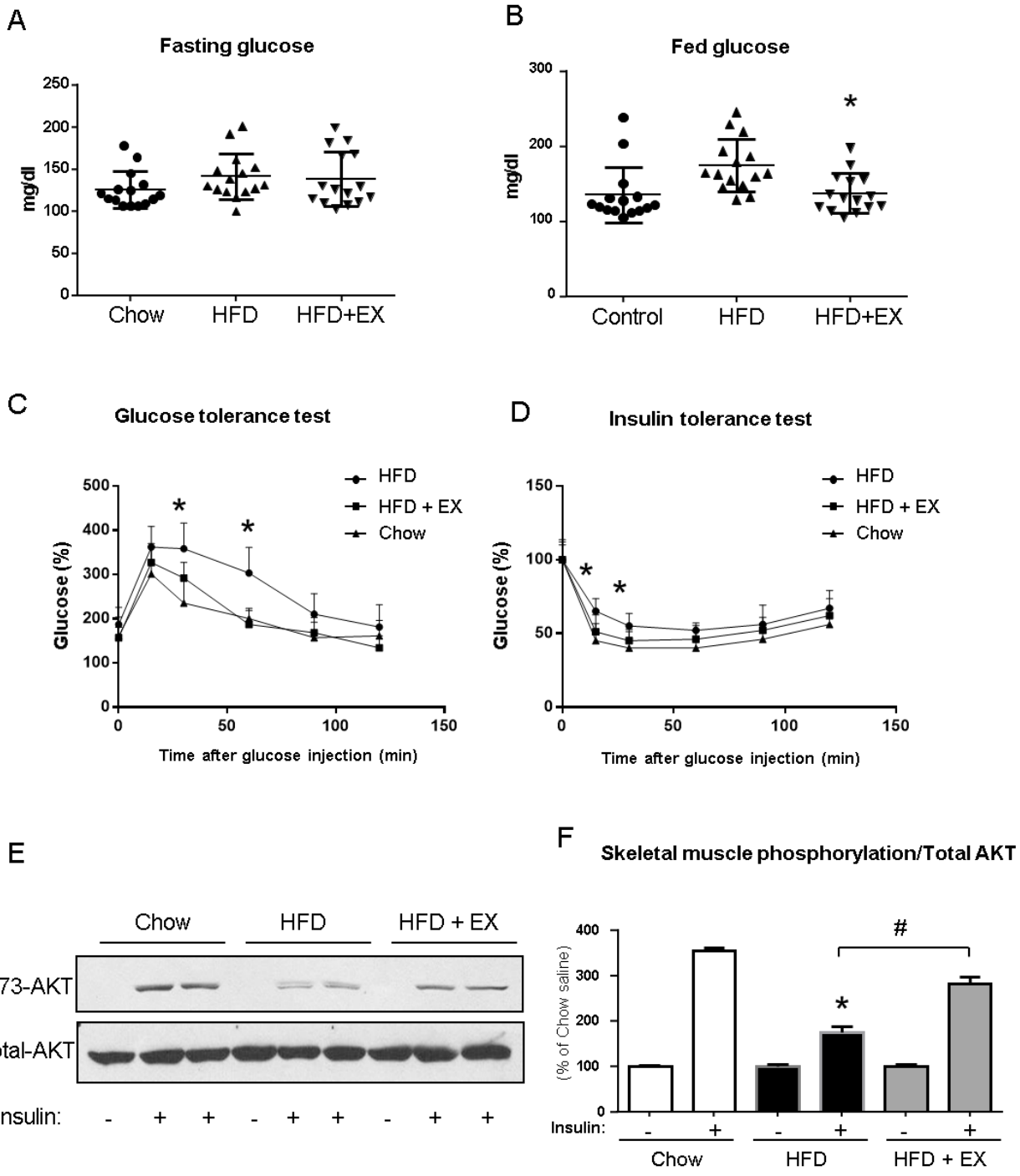


Figure 2.3 Exercise improves glucose tolerance and insulin sensitivity. (A) Fasting glucose levels. **(B)** Fed glucose levels. **(C)** Glucose tolerance test after intraperitoneal glucose injection. **(D)** Insulin tolerance test after intraperitoneal insulin injection. **(E)** Serine 473 phosphorylation of AKT (Protein Kinase B) after saline (-) or insulin (+) injection. **(F)** Relative change is phosphorylated AKT compared to chow group after saline administration. * indicates significance compared to chow, # indicates significance compared to HFD.

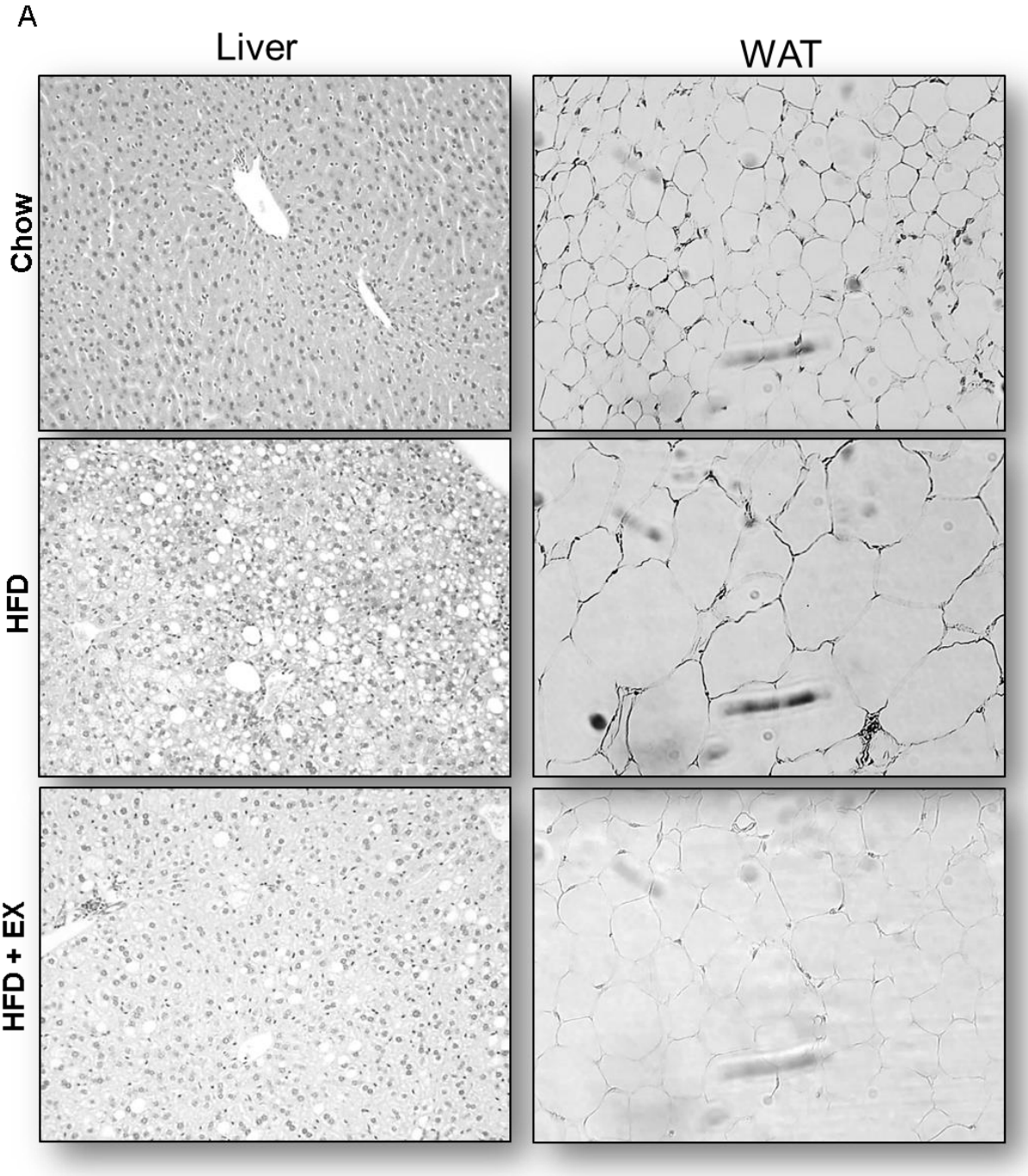


Figure 2.4 Exercise improves liver and adipose tissue morphology. (A) Liver lipid droplet accumulation (left) and white adipose cells between chow, HFD, and HFD + exercise.

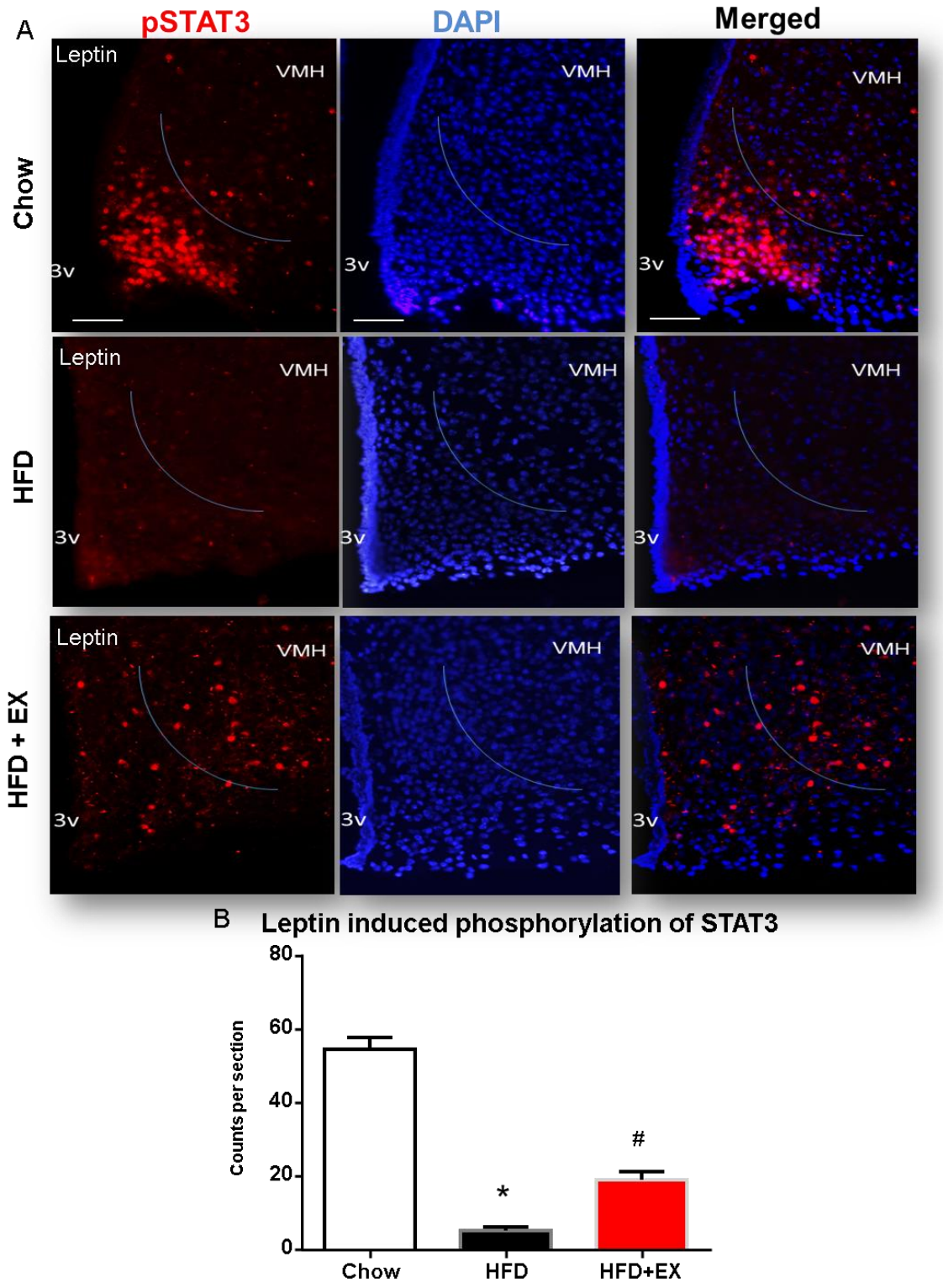


Figure 2.5 Exercise improves high fat diet induced hypothalamic leptin insensitivity. (A) Representative images of cells positive for phosphorylation STAT3 between chow, HFD, and HFD+Ex. Scale bars are 50µm. **(B)** Quantification of leptin induced phosphorylation of STAT3. * indicates significance compared to chow, # indicates significance compared to HFD.

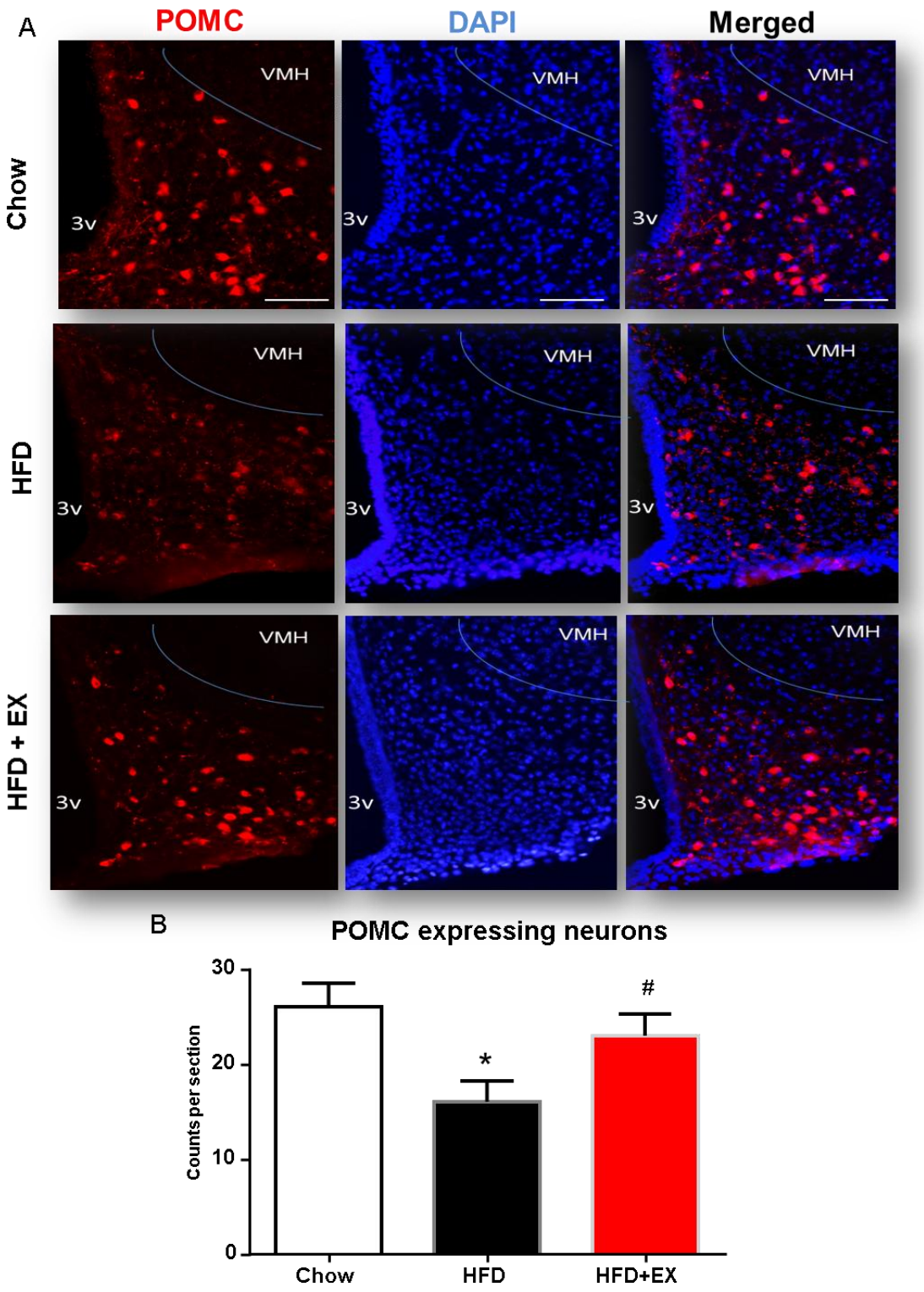


Figure 2.6 Exercise partially restores HFD induced loss of POMC neuron number. (A) Representative images of POMC neurons (red) and nuclei (blue) for chow, HFD, and HFD+Ex. Scale bars are 50µm. **(B)** Quantification of POMC neuron number. * indicates significance compared to chow, # indicates significance compared to HFD.

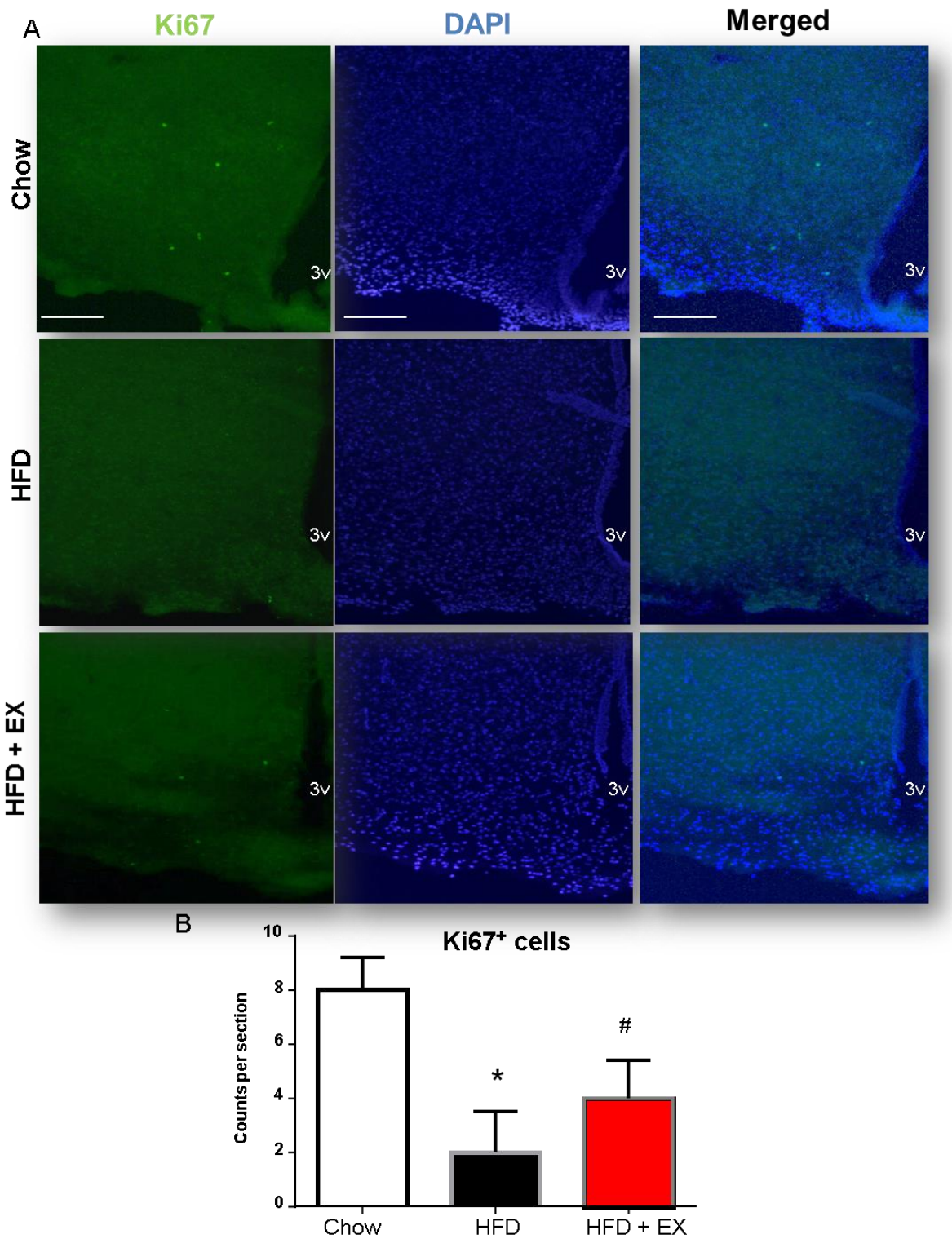


Figure 2.7 Exercise partially restores HFD induced suppression of proliferation. (A) Representative images of Ki67 positive cells (green) and nuclei (blue) for chow, HFD, and HFD+Ex. Scale bars are 100µm. **(B)** Quantification of Ki67 positive cells. * indicates significance compared to chow, # indicates significance compared to HFD.

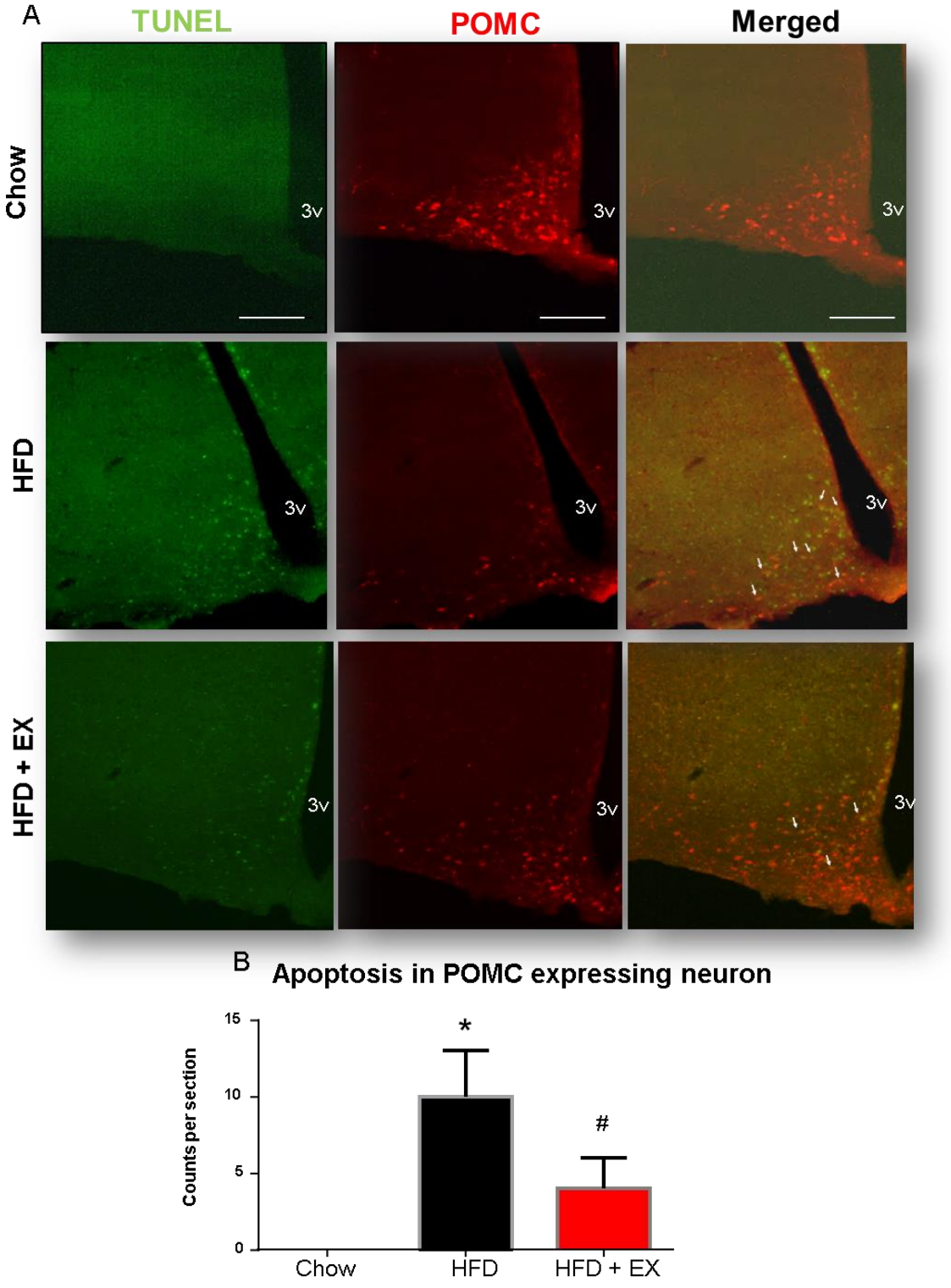


Figure 2.8 Exercise prevents POMC neuron apoptosis. (A) Representative images of POMC neurons (red) and apoptotic cells (green) for chow, HFD, and HFD+Ex. Scale bars are 100µm. **(B)** Quantification of Ki67 positive cells. * indicates significance compared to chow, # indicates significance compared to HFD.

Chapter Three:
AgRP/NPY Neuron Mediated Hunger is Modulated by Metabotropic Glutamate Receptor 1 During Fasting

Abstract: The potential to control feeding behavior via hypothalamic AgRP/NPY neurons has led to many approaches to modulate their excitability – particularly by glutamatergic input. In the present study using NPY-hrGFP reporter mice, we identify AgRP/NPY neuronal metabotropic glutamate receptor 1 (mGluR1) expression and test the effect of fasting on mGluR1 function. In the first experiment, we show *in vivo* that blocking metabotropic glutamate receptor 1 by antagonist 3-MATIDA lowers fasting induced refeeding. Using the same antagonist, we demonstrate *ex vivo* that enhanced mGluR1 function on AgRP/NPY neurons occurs as part of a normal physiological response to fasting. Conversely, using the pharmacological agonist dihydroxyphenylglycine (DHPG) we demonstrate the enhanced capacity of mGluR1 to drive firing of AgRP/NPY neurons after overnight fasting. Further, under synaptic blockade we demonstrate that DHPG acts directly on AgRP/NPY neurons to create a slow inward current. Using an *in vitro* approach, we show that emulation of intracellular signals associated with fasting by forskolin enhances mGluR1 induced phosphorylation of Extracellular Regulated-Signal Kinase (1/2) in GT1-7 cell culture.

Introduction

AgRP/NPY neurons release potent regulators of food seeking behavior[171] that respond to energy deficit [148,101] due to changes in circulating factors[34]. In addition, many pre-synaptic sources of glutamate contribute to the excitability of AgRP/NPY neurons[84, 99], particularly under the fasted condition. Fasting induces activation of Protein Kinase A (PKA) in arcuate AgRP/NPY neurons[122, 152]. PKA enhances function of mGluR1to increase IP3 accumulation[48] and promotes surface stability of mGluR1 by preventing internalization induced by arrestin2 and G-protein receptor kinase 2[118]. While previous reports found no detectable function of mGluR1 by agonist dihydroxyphenylglycine (DHPG) on AgRP/NPY neurons in fed mice[127], a gap in understanding exists regarding if energy deficit can switch on mGluR1 in AgRP/NPY neurons.

G-protein coupled receptor (GPCR) metabotropic glutamate receptor 1 (mGluR1) mRNA is expressed across the adult brain such as hypothalamus, hippocampus, globus pallidus, substantia nigra, thalamus, olfactory bulb, cortex, cerebellum, and brain, while mGluR1 protein is expressed mostly in cerebellum, olfactory bulb, and hypothalamus[176]. mGluR1's are primarily concentrated on post-synaptic structures[60] to depolarize the neuron by enhanced inositol-3-phosphate driven release of sequestered intracellular calcium from endoplasmic reticulum. Controversy exists over the presence and function of mGluR1 in the arcuate nucleus of the hypothalamus[176, 60, 75, 109].

Disorder of excitatory synaptic transmission via metabotropic glutamate receptors contributes to nervous system diseases ranging from chronic pain, cancer, autism, schizophrenia, Alzheimer's disease, Parkinson's disease, neuroinflammation, multiple sclerosis, epilepsy, stroke, obesity and diabetes – the diversity of these diseases owed to the broad regional distribution of metabotropic glutamate receptors. Indeed, clinical trials to test employment drugs

to manipulate mGluR1 have yielded limited results for treatment of epilepsy, pain, Alzheimer's, Parkinson's, anxiety/depression[60]. By understanding the metabolic mediators that influence mGluR1 function, new approaches targeted at enhancing effectiveness of mGluR1 modulation could hold the potential to be a new frontier for treatment of a broad range of disorders.

Here, we demonstrate a functional excitatory role of metabotropic glutamate receptor 1 mGluR1 on AgRP/NPY which drives refeeding after the fasted state. Antagonism of mGluR1 lowers fasting induced refeeding. We show that fasting increases AgRP/NPY neuron response to DHPG, while there is no effect on AgRP/NPY neurons of fed mice. Similarly, in GT1-7 hypothalamic cells, forskolin stimulation facilitates DHPG induced phosphorylation of ERK. Taken together, our data indicate that neuronal excitability by mGluR1 occurs secondarily to status of the ongoing cell signals.

Methods

Animal housing

B6.FVB-Tg(NPY-hrGFP)1Lowl/J mice were individually housed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23, revised 1996) an approved by the Institutional Animal Care and Use Committees of East Carolina University.

Fasting induced refeeding

Overnight fasting was conducted by placing animals in a fresh cage without food while fed animals were moved to a fresh cage and given food *ad libitum*. Alpha-dry bedding was used to prevent consumption of bedding during the fast. Food was removed between 5:00PM-7:00PM. On the subsequent day, animals were injected with 21.5μG of α-Amino-5-carboxy-3-methyl-2-thiopheneacetic acid (3-MATIDA) dissolved in DMSO by intracerebroventricular administration at 7:30AM (start of light phase) and food was presented at 8:00AM. This dose is the maximum solubility of 3-MATIDA that can be delivered within 2μl of administration, approximating to 1mg/kg[117]. Control animals received DMSO as vehicle control.

Intracerebroventricular cannulation

Mice were administered analgesic meloxicam and anesthetized with ketamine and xylazine. Mice were then cannulated for delivery of drug to lateral ventricle by intracerebroventricular cannulation. After a midline incision and orientation to the bregma, stereotaxic coordinates (-.5mM posterior, 1mM lateral, 2.5mM depth) were used for placement of a sterilized cannula and mice were included if placement was visually verified to the lateral ventricle by after sacrifice. Two screws were also implanted, approximately at the location of the ipsilateral lamboid

structure and at the contralateral ventricle. 3M carboxylate dental cement was used to secure the implants to the skull.

Immunofluorescence

We conducted immunofluorescent analysis as previously described[86]. Briefly, at least 3 closely matched sections were used for at least 3 mice in each group. Slices were washed in PBS and blocked in PBS with triton (.3%). Slices were incubated overnight in primary antibody. On the subsequent day, slices were washed and incubated in secondary antibody. After 90 minutes, slices were washed and mounted for fluorescent microscopy. Co-localization was manually determined by overlaying images and using cell counter plug-in on ImageJ. For imaging of mGluR1, a set of stains was also conducted without any primary antibody to verify that detection was above background levels. The mean value of no primary control images was subtracted from all images prior to calculation of Manders Overlap Co-efficient using 'Just Another Co-localization' plug-in.

Epifluorescence microscopy

cFOS and pERK co-localization with AgRP/NPY neurons was imaged using Leica DM6000FS epifluorescent microscope. Images for each marker were obtained with equal exposure, saturation, gain, gamma and shutter intensity. Representative images were treated with identical corrections.

Confocal Microscopy

Sections were imaged using an Olympus FV1000 laser scanning confocal microscope (LSCM). Acquisition software was Olympus FluoView FSW (V4.2). The objective used was 60X oil immersion (NA=1.35, Olympus Plan Apochromat UPLSAPO60X(F)). Images were 800x800

pixel with 2us/pixel dwell time. Detector noise was reduced by application of a 3X line scanning kalman filter. Images were acquired in sequential scan mode. Dapi was excited using the 405nm line of a multiline argon laser, emission was filtered using a 490nm dichroic mirror and 430-470nm barrier filter. GFP was excited using the 488nm line of a multiline argon laser, emission was filtered using a 560nm dichroic mirror and 505-540nm barrier filter. Alexafluor 594 was excited using a 559nm laser diode, emission was filtered using a 575-675nm barrier filter. Standardized laser power and detector gain settings were determined using test sections and zero detector offset was used for all images. Lateral optical resolution was .196um, and axial optical resolution was .902um. The pinhole aperture diameter was set to 105um (1 Airy disc). A 3X digital zoom was applied to all images used for colocalization analysis to ensure adequate sampling. Lateral pixel size was .088um/pixel, and axial pixel size was .390um/slice. Image processing was performed using ImageJ (V1.51f). Representative images were treated with identical corrections.

Patch clamp electrophysiology

We conducted cell attached voltage clamp recordings of AgRP/NPY neurons. Briefly, before 10:00AM mice were deeply anesthetized by isoflurane followed by intracardial perfusion with chilly n-methyl-D-glucamine solution (in mM: 92 NMDG, 2.5 2.5 KCl, 1.25 Nah2PO4, 30 NaHCO3, 20 HEPES, 25 Glucose, 2 Thiourea, 5 Na-Ascorbate, 3 Na-Pyruvate, .5 $CaCl_2$, 10 $MgSO_4$) and sliced into 200-300μM using VF200 Compresstome (Precisionary Instruments, Greenville NC). Slices recovered for an hour and were stored for recording in a BSK6 (Automate scientific, Berkley CA) in HEPES solution (in mM: 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2

$CaCl_2$, 2 $MgSO_4$). Recordings were conducted in normal aCSF (in mM: 119 NaCl, 2.5mM KCl, 1.25mM NaH2PO4, 24 NaHCO3, 12.5 Glucose, 2 CaCl2*H2O, 2 $MgSO_4$) on a bath. Neurons were visualized using Leica DM6000FS microscope with polarized differential interference contrast microscopy using infra-red illumination and fluorescent imaging (488nM). Using 3-7mOhm pipettes, cell-attached seals were obtained. For cell attached recordings to detect firing rate, gain was modified to enhance signal/noise ratio for clear detection of action potentials and holding potential was set to -50mV. Seals were stabilized and a 3-5 minute baseline period was recorded followed by perfusion of dihydroxyphenylglycine (DHPG) (50μM). Firing rates were counted only during recording periods where action potential amplitude exceeded any change in voltage using clampfit threshold counter. Equivalent length periods (.5-5minute) were set within each recording during perfusion of aCSF or DHPG. Firing rate (Hz) was calculated from dividing the number of events by the number of seconds. For whole cell recordings, gigaohm seals were obtained and the cells were broken into using negative pressure and signals were stabilized before any analysis periods. Current clamp recordings were stabilized for repeated firing under baseline condition. Voltage clamp whole cell recordings were conducted at a -60mV holding potential.

Cell Culture

Immortalized hypothalamic GT1-7 cell culture were raised in Dulbecco's Modified Eagle Medium. GT1-7 cells were pre-treated (1.75hr) with standard media alone, media with adenylyl cyclase stimulant forskolin (10μM), or media and forskolin (10μM). Next, we remade pre-treatment mix with or without mGluR1 agonist DHPG (50μM) and applied to cells (.25hrs). Cells were lysed and harvested. Cell Culture Protein concentration from cells within each well

was determined using Pierce bicinchoninic acid assay (BCA) (ThermoFisher Scientific). Samples were standardized by adding equal amount of protein and 4x loading buffer combined with variable volume of by lysis buffer for western blot. Due to limitations in protein, phosphorylated ERK was averaged across 4 experiments and total ERK, which was quite stable across conditions, was averaged across two independent experiments.

Western Blot: Equal protein content mixtures were loaded into 4-20% HCL gel then transferred to nitrocellulose membrane. Membrane strips were incubated overnight in 1:1000 diluted antibody in 5% milk for pERK (Cell Signaling Technology), total ERK. Samples were incubated for 2 hours in secondary antibody followed by ECL treatment and imaging with Chemidoc. Using ImageJ, images were inverted and mean intensity of equal-area selections was measured from each lane and a blank spot on the strip (background). Background was subtracted from mean intensity of each lane. Mean phosphorylated ERK was calculated across four different sets of experiments, while total ERK was conducted across two independent experiments.

Statistical analysis

2-way repeated measures ANOVA with Sidak correction for multiple comparisons was used to analyze food intake across refeeding period. Paired t-tests were used to determine differences between firing rate under aCSF and DHPG treatment, while unpaired tests were used for comparisons between fed and fasted neurons as well as for western blots. Statistical significance was determined by $p<.05$ (two-tailed). Data presented as Mean±SEM.

Results

Hypothalamic AgRP/NPY neuron mGluR1 expression

We searched for the presence of mGluR1 on AgRP/NPY neurons using overnight fasted NPY-hrGFP mice. Manders Overlap Coefficient reveals that AgRP/NPY neurons predominantly express mGluR1 in non-nuclear subcellular locations (.0685±.011) compared to nuclear localization marked by DAPI (.035±.002) (Figure 3.1). Both regions had detectable immunoreactivity relative to no primary control (.003±.000) Previous reports show hypothalamic mGluR1a/b[75, 60],and single cell transcriptome analysis demonstrate that AgRP/NPY neurons express GRM1[58]. To our knowledge this is the first visualization of mGluR1 on hypothalamic AgRP/NPY neurons.

Forskolin enhances mGluR1 function in GT1-7 hypothalamic cells

We selected adenylyl cyclase stimulant forskolin to mimic fasting induced AgRP/NPY neuron intracellular signals. Given the utility of ERK1/2 activity as a readout for Gq Protein-Coupled Receptor manipulation[130], we tested for the effect of mGluR1 agonism by DHPG (1.35±.026) with and without forskolin pre-treatment (.7976±.022) compared to control (.9031±.021) and forskolin alone (1.11 ± .013). We show *in vitro* that pre-treatment with forskolin enhances mGluR1 function by DHPG-induced phosphorylation of ERK1/2 ($p < .05$) (Fig 3.2). Of note, treatment with DHPG alone did not alter pERK1/2. This data clearly demonstrates conditional functionality of mGluR1.

Fasting enhances AgRP/NPY responsiveness to mGluR1 agonist dihydroxyphenylglycine

Using cell attached recordings of AgRP/NPY neurons, we confirmed that mGluR1 agonist DHPG (50μM) has no effect on firing rate (1.31±.2354) of AgRP/NPY neurons from

mice under the fed status (1.36±.237) (n = 12) (Figure 3.3A), but notably DHPG enhanced firing rate (4.58±.971) of AgRP/NPY neurons from mice under the fasted condition (2.62±.556) (n = 11) (Figure 3.3B). No observable effect of DHPG on AgRP/NPY neurons from fed mice is consistent with a previous report that there is no change in AgRP/NPY membrane potential by DHPG[95]. Notably, mGluR1 function after fasting can enhance neuronal firing of AgRP/NPY neurons even beyond the typically high fasting-induced firing rate. Because this pharmacological stimulation well exceeds normal glutamatergic inputs available to mGluR1, this strongly suggests that mGluR1 has the capacity to drive excitability under appropriate conditions.

mGluR1 antagonism reveals contribution to AgRP/NPY neuron activation during fasting

Bath application of mGluR1 antagonist 3-MATIDA (100μM) reduces AgRP/NPY firing rate ($p < .05$) (Figure 3.4) from brain slices (n = 11) of fasted mice. Because loss of mGluR1 function results in slowed firing (.2688±.078) compared to aCSF alone (.7245±.166), this data indicates mGluR1 contributes to increased fasting induced firing rate. This may co-occur with ionotropic glutamatergic function as reported elsewhere[99].

mGluR1 induces a slow inward current in AgRP/NPY neurons under synaptic blockade

Using whole cell voltage clamp recordings from AgRP/NPY neurons of fasted mice, we employed an ionotropic synaptic blockade with AP5, CNQX, and picrotoxin prior to perfusion of DHPG (Figure 3.5). Once isolated, DHPG perfusion results in slow excitatory currents (Figure 3.5B) in a subset of AgRP/NPY neurons (n = 4/16). Compared to blockade alone (.4393±1.614), DHPG induces a slow inward current (-16.22±5.34).The slow current observed occurs over the timescale of minutes as previously described under previous measurements of mGluR1 induced slow current[117, 57]. The average amplitude of observed slow currents is greater

than 10pA, which is a physiologically relevant influence on small neurons such as AgRP/NPY neurons[11].

mGluR1 antagonism reduces fasting induced refeeding

Next we investigated for a physiological function of mGluR1 on AgRP/NPY neurons. Based on the excitatory action of mGluR1 and the established role of AgRP/NPY neurons to control food seeking behavior during fasting, we tested the effect of mGluR1 antagonism during a fasting induced refeeding assay. Compared to DMSO control (.224±.038), central administration (n = 5) of mGluR1 antagonist 3-MATIDA reduces food intake (.066±.025) at the 60-90 minute mark during fasting induced refeeding ($p < .05$) (Figure 3.6). However, compared to DMSO control (.972±.058) this change was not substantial enough to alter cumulative food intake over two hours (.79±.098). Given that AgRP/NPY neurons are essential for ghrelin to drive feeding behavior[28], it is notable that this time coincides with reported decreases in ghrelin levels and our observed central effect by 3-MATIDA[52].

Fasting induced AgRP/NPY activation and phosphorylation of Extracellular Signal-Regulated Kinase

Next, we tested for immunoreactivity of pERK1/2 and cFOS in AgRP/NPY neurons of fed and fasted mice. We observe that in AgRP/NPY neurons of fasted mice, increased ($p < .05$) localization of neuronal activation marker cFOS (Fig 3.7A) and phosphorylation of ERK1/2 (Fig 3.7B). This suggests that factors contributing to phosphorylation of ERK1/2, including mGluR1, may be enhanced as part of the activation response to fasting. While previous reports demonstrate that fasting influences arcuate pERK1/2 and NPY immunoreactivity[114, 172], to our

knowledge this is the first report demonstrating that fasting alters pERK specifically in AgRP/NPY neurons.

Discussion

In the present study, we demonstrate that overnight fasting enhances function of metabotropic glutamate receptor 1 (mGluR1) on AgRP/NPY neurons in the hypothalamus, and that central antagonism of this receptor reduces refeeding behavior. Many previous reports have detailed the influence of hormonal[19, 83], intracellular signals[189, 123] and synaptic alterations[174, 182] that occur in AgRP/NPY neurons in response to fasting[150]. Here we relate fasting induced intracellular signals of AgRP/NPY neurons with enhanced post-synaptic mGluR1 function (Figure 8).

Despite multiple reports of mGluR1detection in medial basal hypothalamus [109, 58] to our knowledge, this report is the first to visualize mGluR1 localization specifically on AgRP/NPY neurons. Expression of mGluR1 is consistent with the presence of mRNA for GRM1[58]. Interestingly, while fasting does not alter GRM1 mRNA, we observe that mGluR1 immunoreactivity is enhanced. This discrepancy could be due to changes in translation rates, trafficking to subcellular locations more available to antibody binding, or a reduction in mGluR1 degradation. Given that this finding is limited to the quality of the mGluR1 antibody used in this study, control stains were conducted on slices that did not get treated with primary antibody.

mGluR1 antagonism with central administration of 3-MATIDA has a profound blunting effect on re-feeding behavior at a 60 to 90 minute time point. Within the first 60 minutes of re-feeding after an overnight fast, ghrelin is a dominant driver of hunger, but previous reports demonstrate that by minute 60 circulating ghrelin levels have waned[52]. Decreased availability of ghrelin results in a smaller pool of activated AgRP/NPY neurons. Once the pool of AgRP/NPY neurons falls below a critical point of around 800 into the 300-700 range or the 0-100 range[164], drive for food seeking behavior may is dependently reduced. Thus, mGluR1 serves as a

modulator of AgRP/NPY excitability to maintain the pool of activated AgRP/NPY neurons above threshold required to drive feeding. Loss of this excitatory drive by antagonism of mGluR1 results in reduced hunger that becomes apparent as reduced feeding behavior. While the antagonistic approach by 3-MATIDA yielded valuable information regarding reduction of food intake via reduced mGluR1 activity, one major limitation to studying mGluR1 is that central mGluR1 agonism leads to severe epileptic activity[169].

In AgRP/NPY neurons of fasted mice, *ex vivo* experiments with antagonist 3-MATIDA revealed a reduction in neuronal firing rate compared to aCSF. This effect establishes a physiological role of mGluR1 to contribute to fasting induced firing rate of AgRP/NPY neurons. A reduction of firing by AgRP/NPY neurons indicated that 3-MATIDA may have a role in reducing feeding.

Conversely, AgRP/NPY neurons exhibit responsiveness to metabotropic glutamate receptor 1 agonist dihydroxyphenylglycine (DHPG). Consistent with previous reports, we show that DHPG agonism has no detectable effect on AgRP/NPY neurons of fed mice[126]. This finding demonstrates that fasting enhances the capacity of mGluR1to mediate AgRP/NPY neuronal firing under physiological conditions in response to pharmacological stimulation. At this time it is unclear if this is due to a post-translational modification or directly related to our observation of enhanced immunoreactivity.

Consistent with visual evidence of mGluR1 on AgRP/NPY neurons and the observed change in firing rate, we report functional post-synaptic effects of DHPG directly on AgRP/NPY neurons. Voltage clamp electrophysiology revealed a DHPG-induced slow excitatory post-synaptic current in subset of AgRP/NPY neurons of fasted mice under ionotropic synaptic

blockade, similar to the slow-current observed in mitral cells[56, 117]. Given reports that a 10pA change to the rheobase of small neurons like AgRP/NPY neurons can result in substantial changes to their level of activation[11], this slow current is profound enough to influence excitability of AgRP/NPY neurons in a physiologically relevant way.

A number of groups have reported PKA activation in AgRP/NPY neurons of mouse hypothalamus during fasting[122, 174, 115] and that forskolin stimulates AgRP transcription in GT1-7 cells via PKA activation[122]. Further, PKA activation is critical for the calcium response to ghrelin[78]. As a synaptic modulator, PKA is known enhance AMPAR trafficking[64] and to blunt function of inhibitory metabotropic glutamate receptors[148]. Notably, in HEK293 cells transfected to express mGluR1, forskolin enhances mGluR1 response to glutamate[48], likely by preventing internalization of mGluR1 by reducing association with G-protein coupled receptor kinase 2 and arrestin 2[118]. Thus, we used forskolin to mimic the fasting induced intracellular signals and this resulted in enhanced mGluR1 induced p-ERK. This effect isn't explained by simple addition of forskolin or DHPG treatment. This *in vitro* experiment is consistent with enhanced function of mGluR1 in hypothalamic neurons in concordance with increased Protein Kinase A activity and phosphorylation of ERK during the fasted state. Of note, while forskolin is a classic adenylyl cyclase stimulant, it also enhances ERK phosphorylation. Given that ERK1/2 can also potentiate mGluR1 function[188], future studies should tease out if there is a dominant intracellular stimulus.

We observe enhanced positive cell counts for cFOS and phosphorylation of ERK in AgRP/NPY neurons of fasted mice. While it is unclear at this time what the physiological sources or role of this ERK phosphorylation is, mGluR1 appears to be one contributor. Co-appearance of cFOS and phosphorylation of ERK within the hypothalamic population of

AgRP/NPY neurons may reflect some relationship between the two and would be consistent with mGluR1 activation during fasting. However, this effect is in stark contrast to previous reports of FGF induced phosphorylation of ERK1/2 in inactivated AgRP/NPY neurons[106] and known roles of ERK to inhibit AgRP transcription mediated by KLF4[132]. The ability of mGluR1 to simultaneously drive firing and ERK phosphorylation marks a distinction from receptors with tyrosine kinase activity. Sorting out the discrepancy between activation and ERK phosphorylation warrants future investigation. We suggest a glutamatergic system capable of facilitating action potential induced release of AgRP filled vesicles and simultaneously slowing production of new AgRP – a neuronal phenomenon which would correspond to whole body satiation if the neurons are not also under the influence of ghrelin. This model would be consistent with the timing of the effect we observe by mGluR1 antagonism on feeding behavior.

Conclusion

In summary, our results demonstrate expression and function of mGluR1 on AgRP/NPY neurons that contributes to feeding behavior. Our results demonstrate an approach to hunger control via AgRP/NPY neuron excitability through intracellular signals that influence glutamatergic synapses by mGluR1 activation.

Chapter 3 Figures

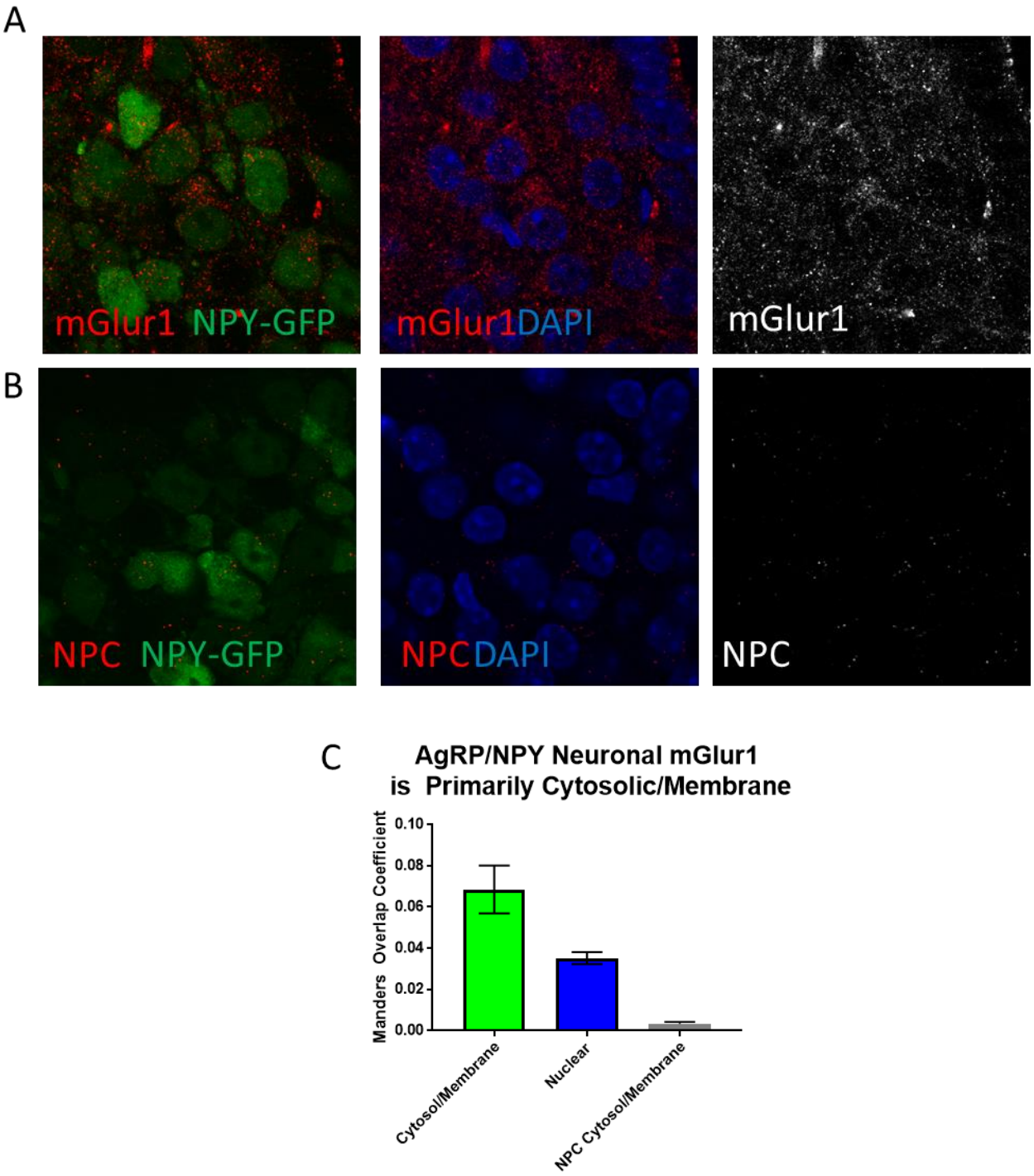
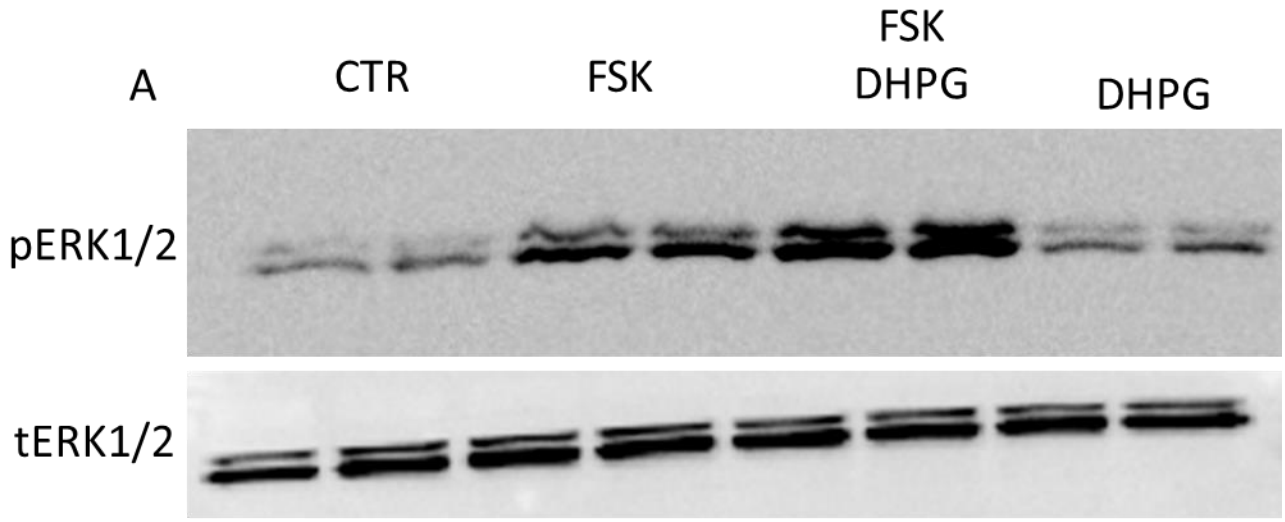


Figure 3.1 AgRP/NPY Neurons Express mGluR1a/b. (A) Representative images of mGluR1 a/b (red) expressing AgRP/NPY (green) neurons (*left*), close apposition of mGluR1 (red) to the nucleus of AgRP/NPY neurons (blue) (*middle*), and mGluR1 staining alone (grey) (*right*). **(B)** Representative images of no primary control stains (red) on AgRP/NPY neurons (green) (*left*), nuclei (blue) (*middle*), and no primary control grey scale (*right*). **(C)** Manders overlap coefficients for the proportion of cytosol/membrane covered by mGluR1a/b, nucleus covered by mGluR1a/b, and cytosol/membrane coverage in the no primary control condition.



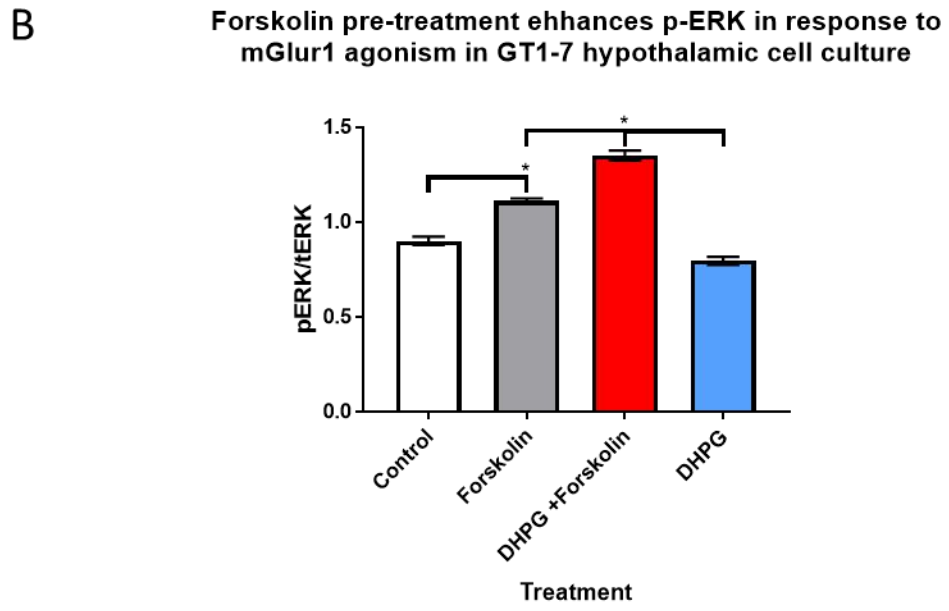


Figure 3.2 Forskolin enhances mGluR1 function. (A) Representative images of western blots for phosphorylation of ERK (1/2) and total ERK (1/2) **(B)** Bar graph of mean intensity for phosphorylated ERK (1/2) (n = 4) normalized to total ERK (n = 2). Student's t-test used for analysis of pre-determined comparisons between conditions. * indicates ($p<.05$).

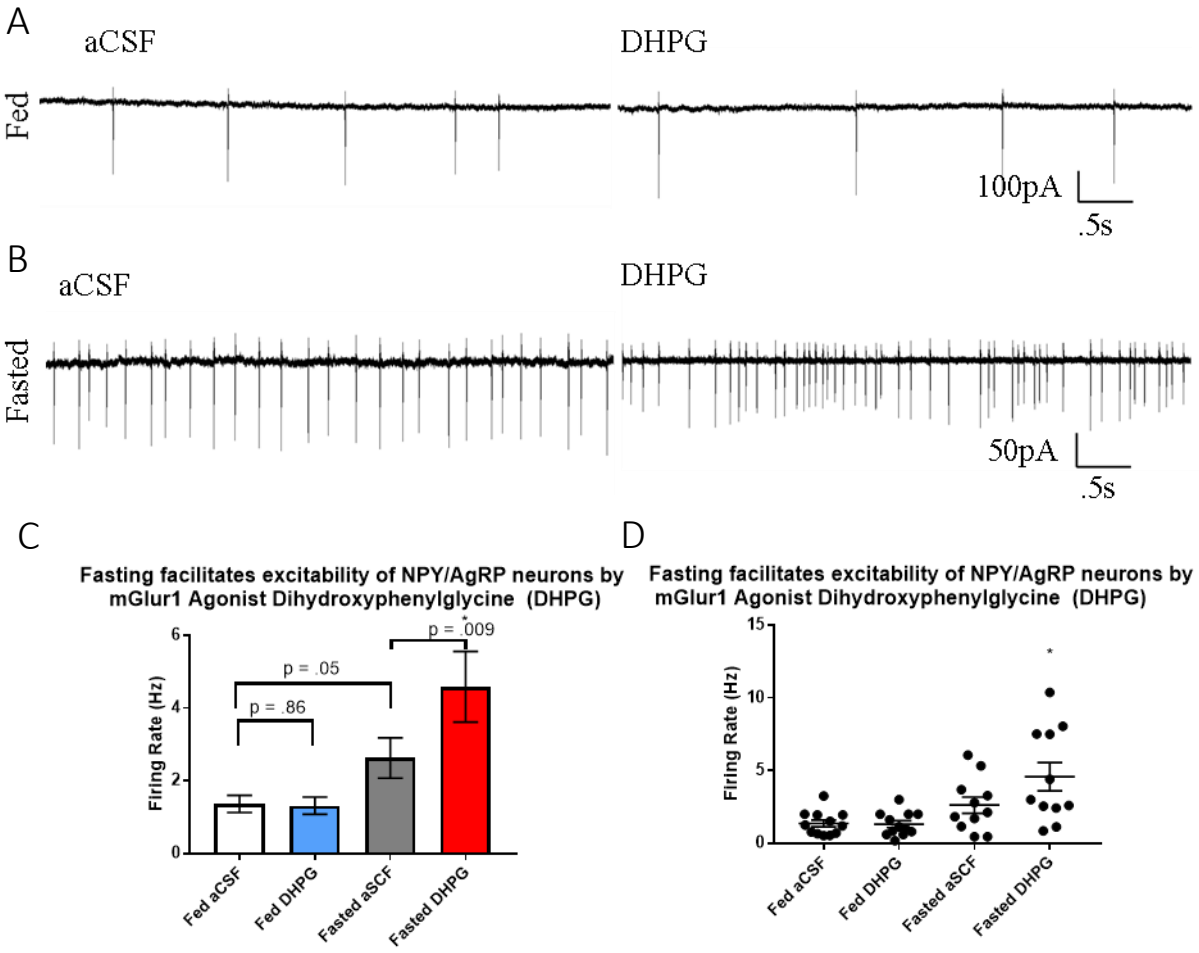


Figure 3.3: Group I metabotropic receptor agonist dihydroxyphenylglycine (DHPG) enhances firing rate of NPY/AgRP neurons specifically under the fasted condition. (A) Representative trace showing neuronal firing of AgRP/NPY neuron from a fed mouse under aCSF and DHPG. **(B)** Representative trace showing neuronal firing of AgRP/NPY neuron from fasted mouse under aCSF (middle left) and DHPG (middle right). **(C)** Bar graph (bottom left) of mean firing rate. **(D)** Dot plot of each individual neuron (bottom right). Standard t-test used to compare across fed and fasted conditions, matched pairs t-test used for detection of within condition differences. Means ±SEM (n = 11 fed; n = 12 fasted). Bar graph significance marked by * indicates p<.05.

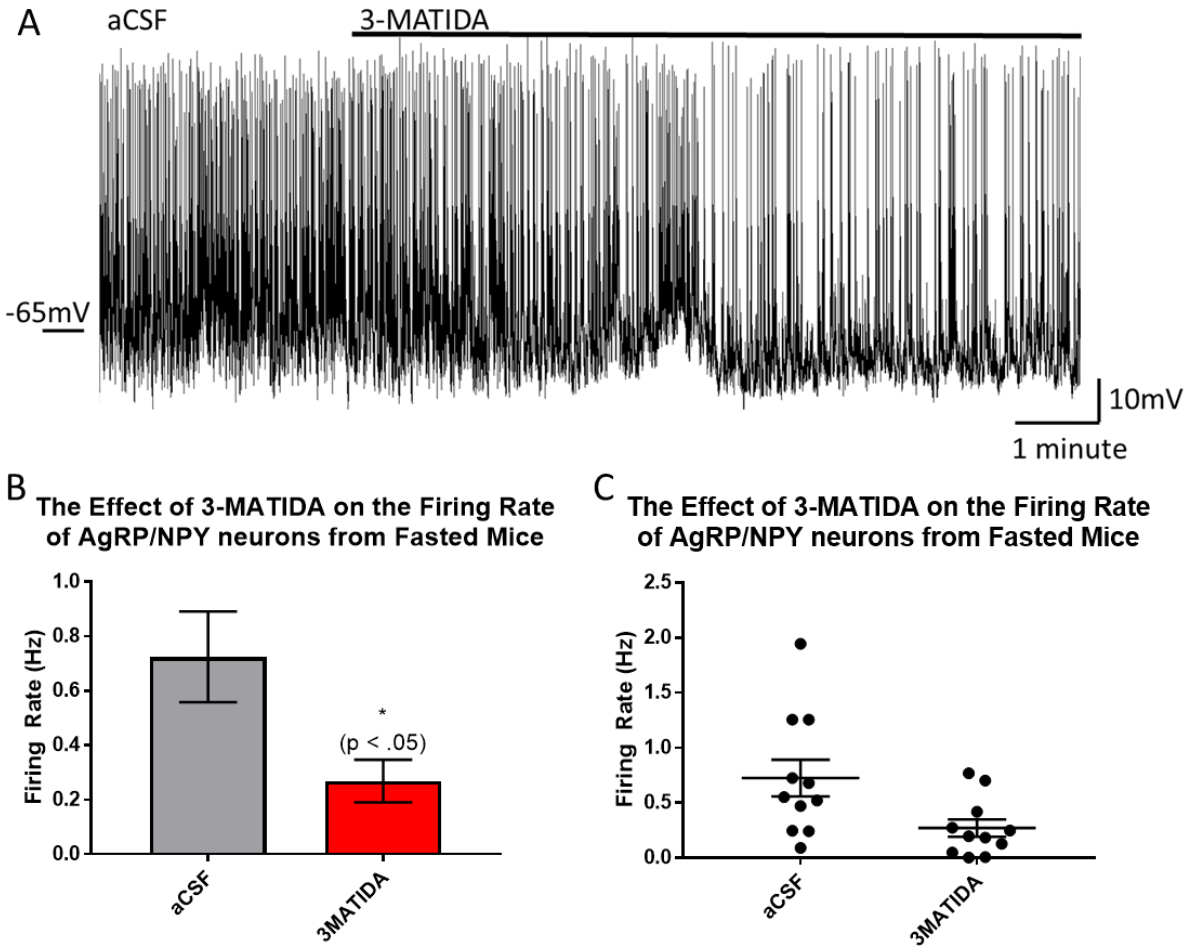


Figure 3.4: mGluR1 antagonist 3-MATIDA slows firing rate of NPY/AgRP neurons from fasted mice. (A) Representative whole cell recordings of neuronal firing of AgRP/NPY neuron from a fed mouse under aCSF and 3-MATIDA. **(B)** Bar graph of mean firing rate. **(C)** Dot plot of each individual neuron. Matched pair t-test used to compare across fed and fasted conditions. Means ±SEM (n = 11). Bar graph significance marked by * indicates p<.05.

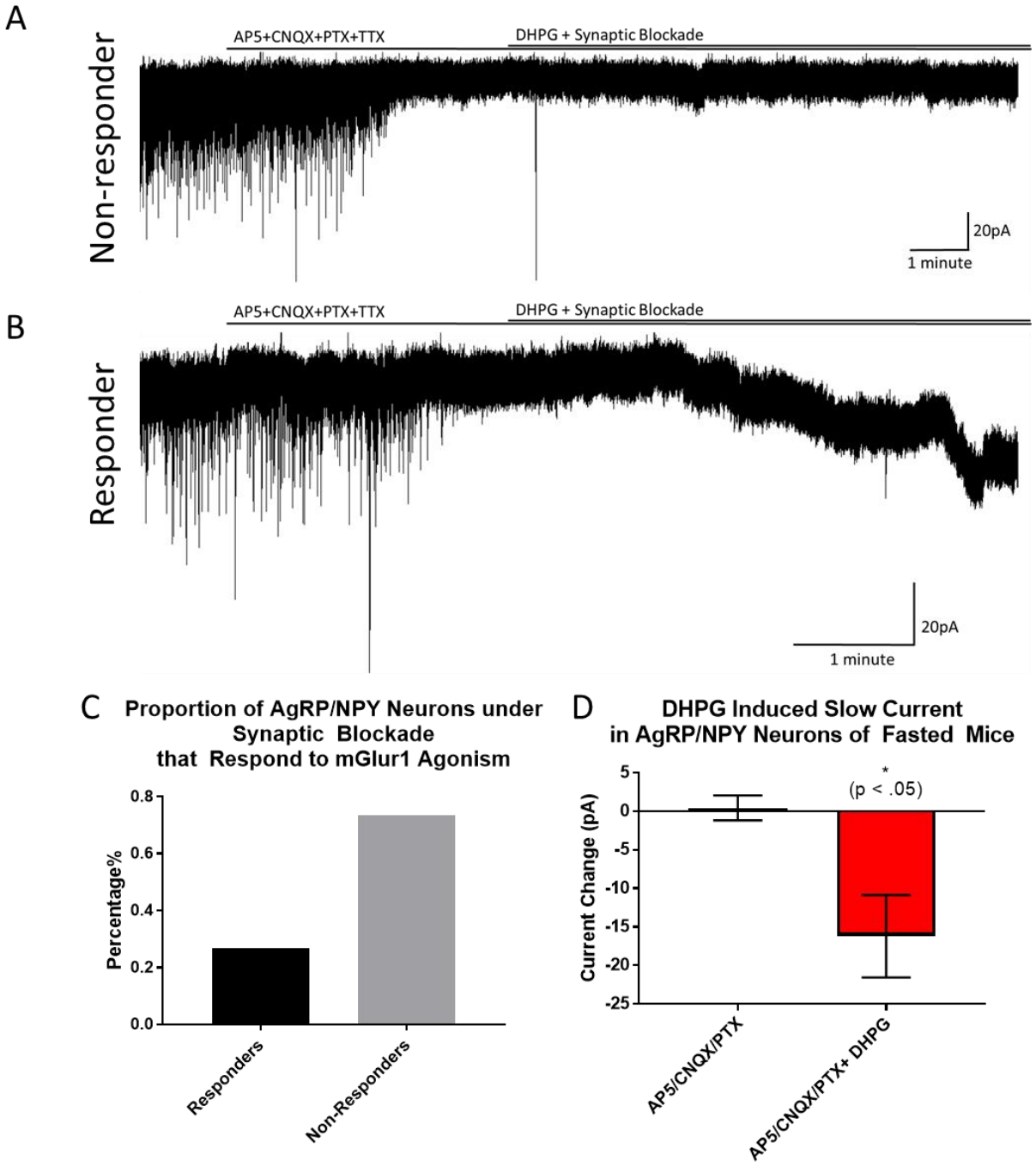


Figure 3.5: A subset of AgRP/NPY neurons under synaptic blockade exhibit a slow inward current in response to Group I Metabotropic Receptor Agonist Dihydroxyphenylglycine. (A) Representative whole cell voltage clamp recording of an AgRP/NPY neuron that does not respond to DHPG. **(B)** Representative whole cell voltage clamp recording of an AgRP/NPY neuron in response to DHPG. **(C)** Bar graph of proportion of AgRP/NPY neurons from fasted mice that are DHPG responders (4/16). **(D)** Bar graph of current change from previous condition. Matched pair t-test to compare blockade and blockade + DHPG conditions. Means ±SEM (n = 4).

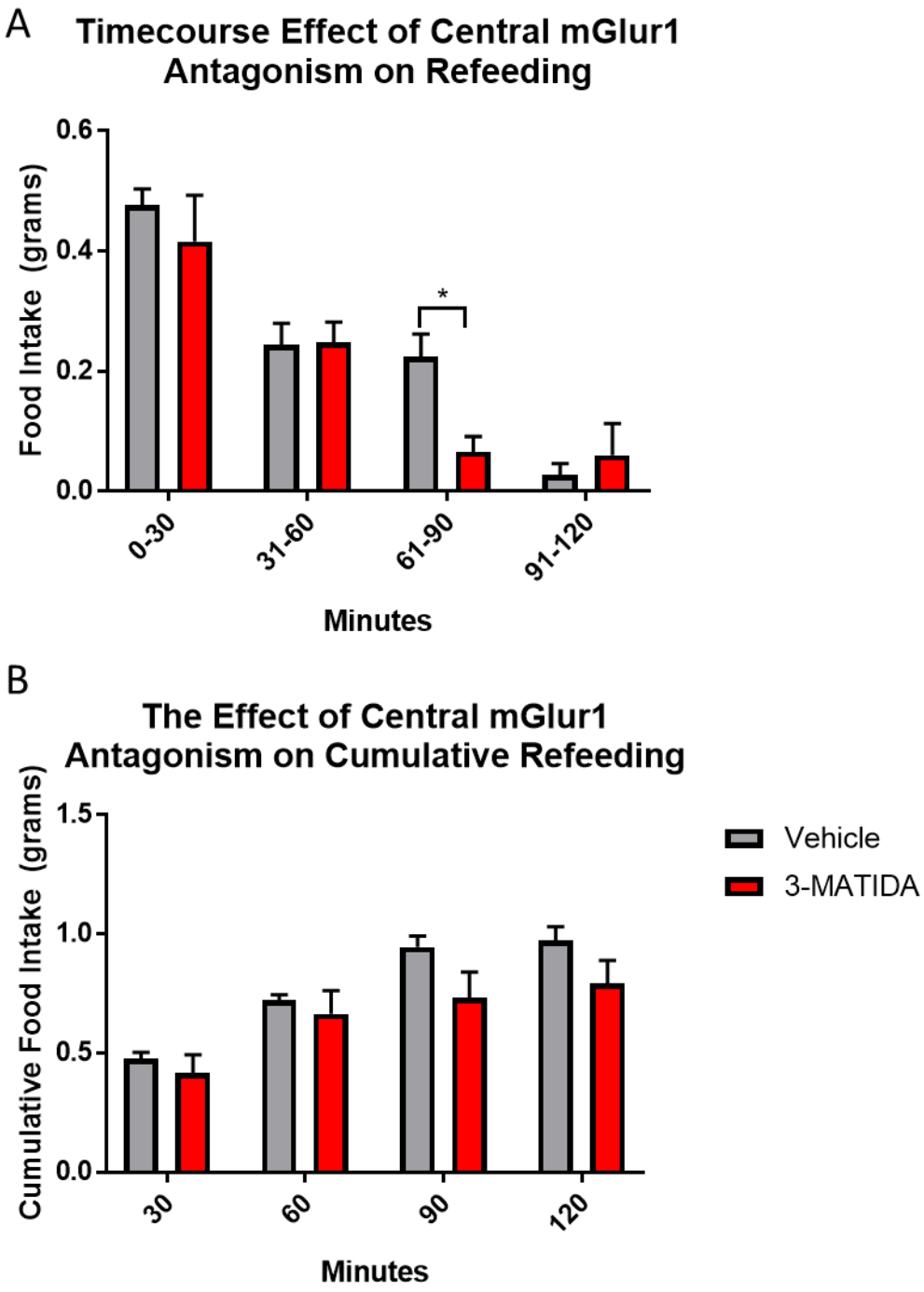


Figure 3.6: Central administration of mGluR1 antagonist 3-MATIDA reduces fasting induced refeeding. (A) Food intake values over two hours of refeeding broken into 30 minute epochs. **(B)** Cumulative food intake values over two hours. Two way ANOVA with Sidak multiple comparison test was used. * indicates ($p<.05$). n= 5 for each group.

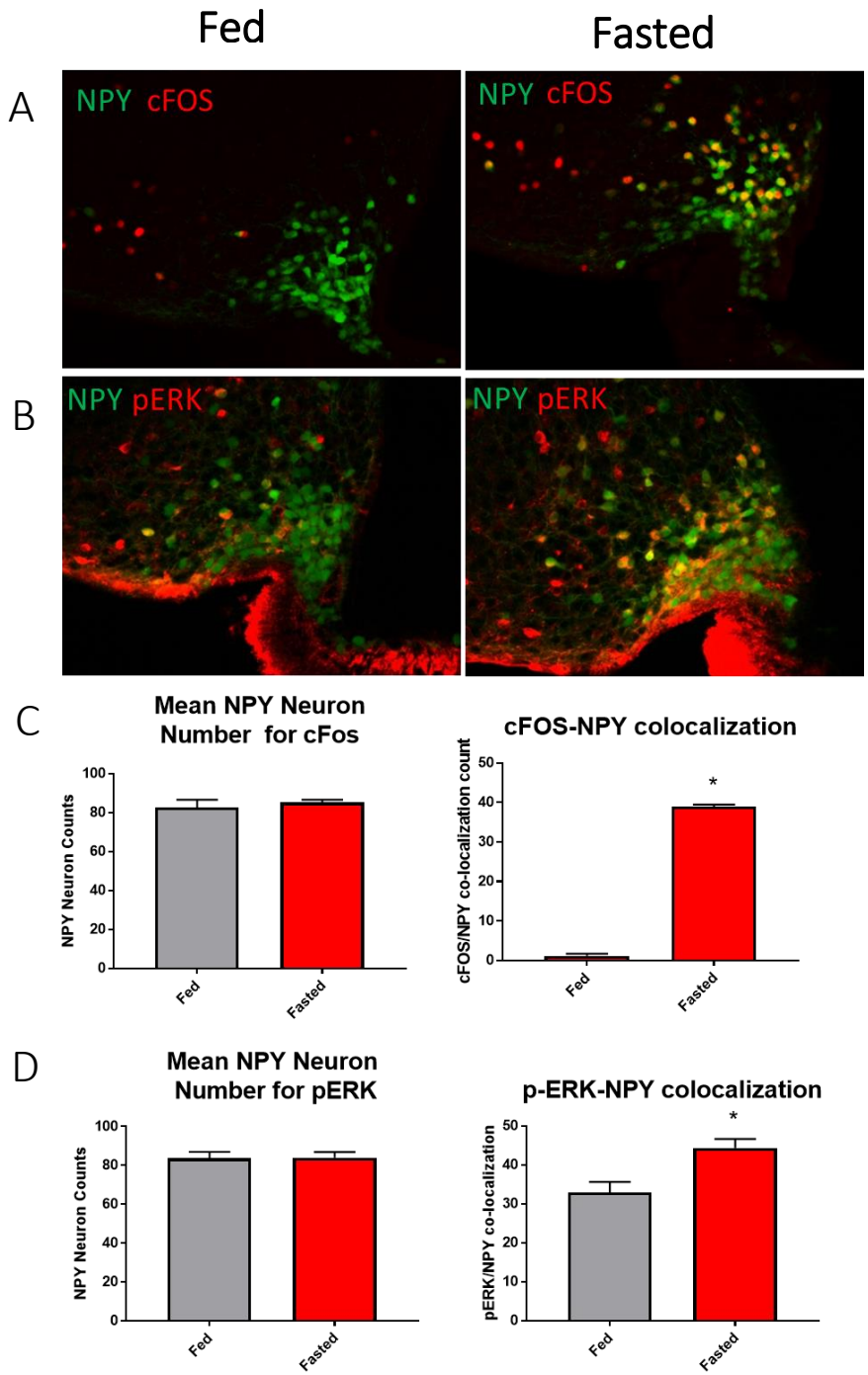


Figure 3.7: Fasting induces cFOS and phosphorylation of ERK1/2 in AgRP/NPY neurons. **(A)** Representative images of cFOS (red) and NPY/AgRP neurons (green) under fed and fasted conditions. **(B)** Representative images of pERK (red) and NPY/AgRP neurons (green) under fed and fasted conditions. **(C)** Count data for mean AgRP/NPY neurons per slice and mean number of AgRP/ neurons co-localized with cFOS. **(D)** Count data for mean AgRP/NPY neurons per slice and mean number of AgRP/NPY neurons co-localized with pERK. Bar graphs show Mean + SEM. * indicates ($p<.05$). n = 3 fed, 4 fasted mice for each set of stains.

Chapter 4: Summary and Conclusions

In modern western society with rampant energy surplus due to excess consumption of calorie dense foods and lack of activity, diseases related to energy surplus have reached epidemic proportion. As a critical node in the control of whole body energy balance, the ARC holds the potential to reverse or slow progression of many diseases. The purpose of this dissertation was to address adaptations of the ARC in response to two methods of energy surplus reduction; 1) increased energy expenditure and 2) reduced energy intake.

The ARC houses many critical populations of neurons that regulate energy balance. Two of these populations, known as POMC and AgRP/NPY neurons have been referred to as the yin/yang of energy balance[199]. POMC neurons reduce energy surplus by inducing satiety and increasing energy expenditure. Conversely, AgRP/NPY neurons increase feeding and lower energy expenditure. As effectors, these neuron populations co-regulate downstream targets such as the melanocortin receptor expressing neurons. As sensors, these neurons receive hormonal and neural inputs from sources throughout the body. In order to investigate the potential for increased expenditure and decreased intake to influence adaptations within the arcuate nucleus, two independent studies were conducted whereby we tested the hypotheses that 1) increase in energy expenditure by voluntary wheel running will result in improved hypothalamic hormone signaling and reduced POMC neuron turnover, and 2) decrease in energy intake by overnight fasting will result in enhanced mGluR1 mediated excitatory input onto AgRP/NPY neurons.

Taken together, this dissertation addresses a subset of adaptations that occur within the arcuate nucleus of the hypothalamus in response to common methods for reducing energy surplus (Figure 4.1). The data within this dissertation clearly demonstrate that 1) the damaging effects of diet-induced obesity on the hypothalamus and body are reversed by voluntary exercise,

and 2) hunger drives feeding behavior via enhancement of mGluR1 mediated AgRP/NPY neuronal excitability.

Exercise Reverses the Damage of Diet-Induced Obesity

We assessed if exercise reverses the damage of DIO to the ARC of the hypothalamus and peripheral organ systems. In DIO mice, voluntary exercise reduces bodyweight by decreasing fat mass (Figure 2.1). This decrease in fat mass is driven by increased energy expenditure without a change in food intake (Figure 2.2). Thus, exercise may serve as a viable treatment for the hallmark of obesity. In addition, exercise improves glucose tolerance and reduces insulin resistance – particularly at skeletal muscle (Figure 2.3). Along with these changes, exercise improves the morphology of liver and white adipose tissue evidenced by reduced lipid droplet accumulation and reduced adipocyte size (Figure 2.4). This suggests adaptation to substrate availability and lipid handling by reduced storage or increased oxidation. The reduction in adiposity likely contributes to reduced hyperleptinemia[12], which permits hypothalamic sensitivity to leptin and promotes ARC signaling via the leptin receptor indicated by phosphorylation of STAT3 (Figure 2.5). Blunted leptin signaling in the ARC is sufficient to induce obesity[31], thus reversal of this DIO phenomenon provides substantial justification towards the use of exercise as a therapy for obesity. Further, DIO leads to apoptosis of POMC neurons[113], while experimental loss of POMC neurons results in obesity[53]. Therefore, preservation of the number of POMC neurons in the population can have a profound effect on improvement of whole body energy balance. In our study, we demonstrate preservation of POMC neuron number by exercise (Figure 2.6) due to reduced apoptosis (Figure 2.7) which occurs under DIO in sedentary animals.

Hunger drives excitability of AgRP/NPY Neurons via enhanced mGluR1 function

Hunger promotes food-seeking behavior, primarily through activation of AgRP/NPY neuron[84, 150]. Many hormonal and neural factors contribute to activation[28, 86] or disinhibition [92, 11] of AgRP/NPY neurons. We demonstrated that AgRP/NPY neurons express mGluR1a/b in the cytosol and membrane (Figure 3.1) despite evidence that suggests that mGluR1 has little influence on depolarization of AgRP/NPY neurons[127] from fed mice. However, the intracellular milieu of AgRP/NPY neurons becomes markedly different during fasting, whereby an accumulation of cAMP drives activation of PKA[48, 118]. Given the known role of PKA to facilitate synaptic stability of excitatory ionotropic AMPA receptor while inhibiting Gi linked mGlur2/3[148], we used an *in vitro* model to test the impact of forskolin pre-treatment on the function of mGluR1 in immortalized hypothalamic cells. Consistent with our hypothesis, forskolin pre-treatment enhances the response to group I metabotropic receptor agonist DHPG (Figure 3.2) as indicated by phosphorylation of ERK (1/2).

In order to test if hunger is a physiological stimulus for AgRP/NPY neurons, we compared the effect of DHPG on AgRP/NPY neurons in fed and overnight fasted mice. We demonstrate that an overnight fast is sufficient to enhance mGluR1 mediated AgRP/NPY firing even beyond the high firing rate that occurs after fasting (Figure 3.3). This experiment shows that the ceiling for mGluR1 function is raised by overnight fasting.

Further, we demonstrated that mGluR1 signaling contributes to the increased firing of AgRP/NPY neurons that is typical of the fasted condition. Using current clamp electrophysiological recordings of AgRP/NPY neurons from fasted mice, we demonstrated that treatment with mGluR1 antagonist 3-MATIDA (Figure 3.4) reduces firing. This experiment shows that mGluR1 functions as part of the normal response to hunger.

Next, we determined that group I metabotropic receptors function directly on AgRP/NPY neurons. After application and action of ionotropic synaptic blockade, we demonstrated a slow inward current on AgRP/NPY neurons by application of DHPG (Figure 3.5). This experiment clearly demonstrates direct action of group I metabotropic receptors on AgRP/NPY neurons on a subset of AgRP/NPY neurons.

Based on structural and functional evidence of mGluR1s on AgRP/NPY neurons, we tested the effect of mGluR1 antagonism on feeding behavior in overnight fasted mice. Unexpectedly, feeding behavior was unchanged within the first hour of refeeding, but was blunted at the 60-90 minute time point. This time point corresponds with time course data showing that ghrelin levels are cut in half within 60 minutes of refeeding[52]. Therefore, we surmise that as the pool of ghrelin-recruited AgRP/NPY neurons drops, the role of glutamatergic inputs becomes increasingly important – particularly by mGluR1. Despite limited effectiveness in reduction of feeding behavior over the first hour, the early onset of satiation by mGluR1 antagonism indicates potential therapeutic value in modulating the tone of AgRP/NPY neurons via this receptors. Because ablating AgRP/NPY neurons in adult mice leads to starvation[101, 186], a less drastic approach via modulation of the integration of glutamatergic inputs may be desirable.

Interface of conducted studies on exercise and hunger

The studies comprising this dissertation paint the ARC as a dynamic nuclei that integrates metabolic inputs with the capacity for potent output to regulate energy balance. This dissertation clearly demonstrates that nutritional status modulates the ARC on POMC and AgRP/NPY neurons. Further, this dissertation identifies two approaches towards reduced energy surplus by 1) energy expenditure or 2) reduced intake. Adaptation to the ARC is stimulated under both of

these conditions. The adaptations identified in this dissertation may be leveraged to develop future therapeutic approaches towards maintenance of energy balance for treatment of obesity and diabetes. While the ARC is only comprised of a few thousand cells comprising a very small percentage of the organism's bodyweight, it has the capacity to control energy balance as coordinator of many organ systems.

Limitations of Presented Experiments

For the study regarding the effect of exercise to prevent the damage of diet-induced obesity on the arcuate nucleus, we were unable to identify the precise mechanism of neuroprotection and reduced apoptosis. In addition, despite the fact that proliferation of new cells was limited, we were unable to determine the identity of these cells as neurons, astrocytes, or glial cells. In terms of leptin signaling, our experiment did not determine whether enhanced phosphorylation of STAT3 was caused by improved transport across the BBB, improved access to leptin receptor, or some change to intracellular signaling cascade that permitted enhancement of phosphorylation. A final limitation of this study is that it investigated concurrent improvement in the ARC and peripheral organs, therefore it inherently lacks of determination of a causal role by the arcuate nucleus to promote improvement across peripheral organs.

Our study regarding the effect of hunger on AgRP/NPY neuronal mGluR1 was limited from a number of angles. First, quantification of AgRP/NPY expression of mGluR1a/b by western blot is not specific to this neuronal subtype using microdissection, thus western blot of medial basal homogenate may reflect changes in other neurons populations. Using immunofluorescent reactivity, it is not clear if mGluR1 is expressed specifically on a cell within a region of interest or if this is a dendrite overlaying that cell. A common approach for

determination of the proportion of neurons that express a gene is single cell quantitative polymerase chain reaction (qPCR), but we steered clear of this approach because it doesn't give any indication about the quantity of mRNA translated to mGluR1 or the amount present at a post-synaptic site. Further, if it is a post-translational modification that contributes to enhanced mGluR1 function by fasting the quantity of mGluR1 may be unchanged and largely irrelevant. Our *in vitro* investigation of mGluR1 was limited to measurement of ERK1/2 phosphorylation rather than direct measurement of activity IP3. In addition, the use of cutting edge mGluR1 manipulators such as positive/negative allosteric modulators may prove beneficial for *ex vivo* recordings. The *in vivo* investigation of mGluR1 function is limited to antagonistic approaches due to epileptic seizures induced by agonism. An approach that knocks out GRM1 and conditionally re-expresses it within AgRP/NPY neurons may be required to fully verify that reduced feeding behavior caused by 3-MATIDA administration is via AgRP/NPY neurons.

Towards the Future

Future studies should investigate the precise intracellular mechanisms involved in exercise induced neuroprotection of POMC neurons as this may yield specific targets for long term promotion of energy expenditure and satiety. On the other hand, studies should evaluate the effect of acute and chronic exercise on AgRP/NPY neurons to determine if energy expenditure promotes their activation in a manner consistent with hunger, and if these stimuli induce different adapts over time.

Chapter 4 Figures

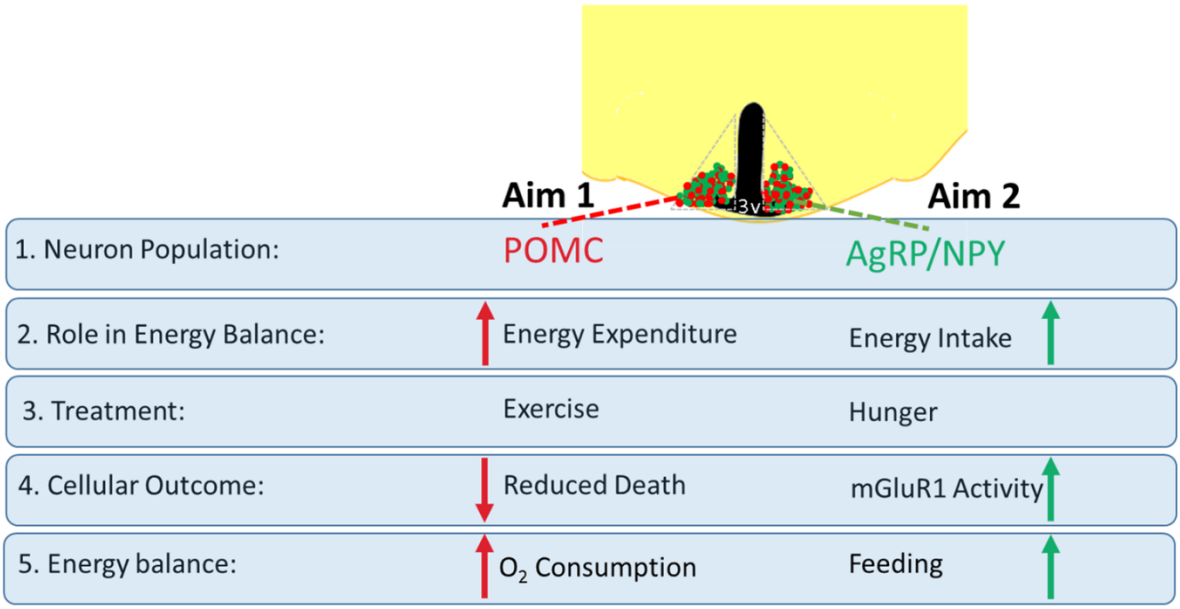


Figure 4.1 Adaptations of the arcuate nucleus of the hypothalamus in response to exercise and hunger. Aim 1 includes experimentation to determine the effect of exercise to prevent high-fat diet induced POMC neuron apoptosis to support of energy expenditure. Aim 2 includes experimentation to determine the effect of hunger on activation of AgRP/NPY neuronal activation via mGluR1 to drive energy intake.

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APPENDIX A: Institutional Approval

East Carolina University

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

October 23, 2013

Hu Huang, Ph.D.
Department of Kinesiology
Ward Sports Medicine Bldg.
ECU Brody School of Medicine

Dear Dr. Huang:

Your Animal Use Protocol entitled, "Central Nervous System Control of Metabolism Responses to Exercise and Diet - Experiments" (AUP #P085) was reviewed by this institution's Animal Care and Use Committee on 10/23/13. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

East Carolina University

Animal Care and Use Committee
252 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 8, 2016

Hu Huang, Ph.D.
Department of Kinesiology
ECDOI
ECU Brody School of Medicine

Dear Dr. Huang:

The Amendment to your Animal Use Protocol entitled, "Central Nervous System Control of Metabolism Responses to Exercise and Diet - Experiments" (AUP #P085a) was reviewed by this institution's Animal Care and Use Committee on December 8, 2016. The following action was taken by the Committee:

"Approved as amended"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure