AgRP neuron activity is required for acute exercise-induced feeding

By

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While much is known about the role of neuropeptide Y/agouti-regulated peptide (NPY/AgRP) and pro-opiomelanocortin (POMC) neurons to regulate energy homeostasis, little is known about how forced energy expenditure, such as exercise, modulates these neurons and how this relates to energy intake. Therefore, we investigated the effects of acute exercise on neuronal activity in the arcuate nucleus (ARC) of the hypothalamus. To accomplish this, we utilized immunohistochemistry and patch-clamp electrophysiology experiments on NPY-GFP transgenic mice immediately after an acute bout of treadmill exercise. Due to the ability of NPY/AgRP and POMC neurons to mediate energy homeostasis, food intake studies were also performed immediately after an acute bout of treadmill exercise. AgRP-Ires-cre transgenic mice were used to induce loss in AgRP neuronal activation by bilaterally injecting an inhibitory cre-recombinase–dependent Adeno Associated Virus (AAV-hM4Di-mCherry) to assess AgRP neurons in food intake post-exercise.

While we observed no difference in activation in POMC neurons, immediately after exercise, activation in ARC NPY/AgRP neurons is significantly increased compared to the sedentary control group; further confirmed by electrophysiology recording showing a significant increase in firing rate in NPY/AgRP neurons after acute exercise. Food intake was significantly increased immediately after an acute bout of exercise. This exercise-induced food intake was

abolished when AgRP neuron activation was inhibited. Neuronal inhibition of AgRP neurons had no effect of hypothalamic paraventricular nucleus (PVN) activation immediately after a bout of acute exercise.

Our results demonstrate NPY/AgRP activation is critical for acute exercise induced food intake in mice, thus providing insight into the subtle exercise induced response to facilitate energy replacement.

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List of Abbreviations

NPY/AGRP- Neuropeptide Y/agouti-regulated peptide

POMC- Pro-opiomelanocortin

ARC- Arcuate nucleus of the hypothalamus

AAV- Adeno Associated Virus

PVN- Paraventricular nucleus of the hypothalamus

CNS- Central Nervous System

α-MSH- α-melanocyte stimulating hormone

MC4Rs- melanocortin-4 receptors

Sim-1- Single-minded homolog 1

VMH- Ventromedial hypothalamus

DMH- Dorsomedial hypothalamus

CFR-Corticotrophin-releasing factor

PYY- Peptide YY

CSF-Cerebrospinal fluid

DREADD- Designer receptors Exclusively Activated by Designer Drugs

DIO- Diet Induced Obesity

PBS-Phosphate-buffered solution

PBST- Phosphate-buffered solution +.03% Triton

CNO- Clozapine-N-Oxide

VO_2- Volume of oxygen consumed

Operational Definitions

Immunohistochemistry (IHC) – A method of staining tissue with specific antibodies attached with fluorescence so that certain cell populations are distinguishable under a microscope.

cFOS- An early intermediate gene used as a marker for neuronal activity

Patch Clamp Electrophysiology- is a laboratory technique in electrophysiology used to study ionic currents in individual living cells, tissue sections, or patches of cell membrane. The technique is especially useful in the study of excitable cells such as neurons.

Cre-recombinase- An enzyme used to carry out site specific recombination events

DREADD system– DREADD receptors are introduced into neural tissue. Mutagenic G-Protein coupled receptors are introduced to this tissue. Gain or loss-of function of this tissue can be achieved using a typically pharmacologically inert compound such as Clozapine-N-Oxide.

Chapter 1: Introduction

General Information

Obesity has become an epidemic that continues to skyrocket every year, with a prevalence of almost 30% of humans worldwide being either overweight or obese [1]. A major cause of obesity is a homeostatic imbalance of energy, which is tightly controlled by the central nervous system (CNS) [2]. Regular exercise has been shown to be a beneficial intervention by improving many conditions associated with metabolic health, such as improving insulin sensitivity and reducing fat mass [3,4]. The CNS is also a major regulator of metabolic health. Specifically, the hypothalamus is a critical component for many of the homeostatic functions that occur in the body. Some of these functions include the regulation of heart rate [5], sleep cycles [6], and body temperature [7]. Another vital function of the hypothalamus is the control of energy homeostasis, with the arcuate nucleus and the sub-populations of neurons contained within it being especially important in fulfilling this role [8].

Roles of NPY/AgRP and POMC Neurons

The ARC contains two distinct neuronal populations, which when activated, have opposite effects on feeding behavior. These include the anorexigenic POMC neurons and the orexigenic NPY/AgRP neurons. The role of POMC neurons can be observed in POMC knockout mice [9]. After 3-months POMC knockout mice were over twice the weight of littermate controls. Conversely, when chemically activated, POMC neurons inhibit food intake [10]. POMC neurons suppress appetite by releasing α-melanocyte stimulating hormone (α-MSH), which binds to and activates melanocortin-4 receptors (MC4Rs) [11]. Mice deficient in MC4R's have a marked increase in weight, similar to mice deficient in POMC [12]. Evidence for ARC

POMC neuron function can also be found in the circulating factors that are able to stimulate them. This population of neurons can receive input from the hormones serotonin [13] and leptin [14], both of which inhibit food intake when elevated in the body [14–16] .

Whereas POMC neurons have an inhibitory effect on energy homeostasis, the opposite is true of their neighboring NPY/AgRP neurons. Activation of NPY/AgRP neurons in the ARC by both chemical and optogenetic stimulation results in an immediate and robust increase in food intake[17,18]. Ablation of these neurons results in a significant decrease in food intake and, if not reversed, can lead to starvation in adult mice [19,20]. NPY/AgRP neurons respond to energy deficit due to changes in circulating factors and synaptic inputs [21]. When activated, the NPY/AgRP neurons release the neuropeptides NPY, AgRP, and GABA (an inhibitory neurotransmitter). Central administration of NPY and AgRP has been shown to decrease energy expenditure and induce hyperphagia [22,23]. GABA released by NPY/AgRP neurons are another potent mediator of the orexigenic effects of this subpopulation. AgRP neuron-specific deletion of the vesicular GABA transporter results in mice resistant to diet induced obesity and a dulled response to ghrelin [24]. The GABA released by NPY/AgRP neurons also promotes food intake by the inhibition of many anorexigenic neurons located throughout the brain including the ARC POMC neurons [20,25].

Arcuate to Paraventricular Hypothalamus Circuit

The paraventricular nucleus of the hypothalamus is an important downstream site for the mediation of the ARC POMC and NPY/AgRP regulation of energy homeostasis, with both subpopulations sending dense projections to this area [26]. Lesions of the PVN and haploinsufficiency of Single-minded homolog 1 (SIM1) [27,28], which is expressed in the majority of PVN neurons, causes obesity. Activation of ARC NPY/AgRP neurons can inhibit

MC4Rs located in the PVN, and stimulation of the ARC NPY/AgRP terminals located in the PVN can cause a robust increase in food intake [12]. Recent studies have also shown that the activation of subsets of SIM1 expressing PVN neurons can markedly increase activation NPY/AgRP neurons in the ARC and increase feeding in sated mice, thus indicating a reciprocal circuit [29].

Acute Effects of Exercise on ARC and PVN activation

Hypothalamic signaling has been studied in depth in the context of its ability to affect metabolic changes. However, the role of exercise to activate neurons in ARC, especially at the subpopulation level, are still relatively undefined. In a recent publication using high intensity interval training in mice, it was reported that immediately after a single bout of acute exercise the resting membrane potential of ARC POMC neurons is increased, and an opposite effect was observed in ARC NPY/AgRP neurons [30]. In the same study, no difference in action potential frequency was observed immediately after a single bout of exercise in these neuron subpopulations. In a separate study, it was reported that rats who exercised above their lactate threshold had a significant increase in activation in the PVN and the ARC, but the specific subpopulations that were activated were not investigated [31].

In the He et al. study, which utilized high intensity interval training, volitional running could not be sustained, and electric stimulation was used to maintain running speed. The consequences of exercise induced by negative reinforcement, such as electric stimulation, may not accurately reflect the effects induced by exercise alone. In contrast**,** this thesis uses a more physiologically relevant exercise model to observe the acute effects of exercise on neuron activation in the ARC.

Hypothesis

We hypothesize that a bout of acute exercise will alter activation of arcuate hypothalamic neurons to induce food intake in sated state mice. We also hypothesize that inhibition of AgRP neurons attenuates exercise-induced food intake and increases neuronal activation in the PVN.

Purpose

This is an attempt to creating a better understanding of the changes that occur in the central nervous system immediately after exercise, and these might affect many of the metabolic changes that we observe peripherally. The purpose is to investigate the effects of acute exercise on hypothalamic neuronal activation that are integral in regulating energy homeostasis.

Significance

Extensive research has taken place on all the positive peripheral changes associated with increased amounts of exercise performed. Many of the predispositions to chronic illnesses such as diabetes and cardiovascular disease can be reduced dramatically just by increasing the amount of exercise that one performs. As of date there has been little research on the contributions of the CNS to theses exercise-induced health improvements. Most studies look at changes in body weight, dyslipidemia, and other risk factors. This study will seek to add to a new understanding of the CNS and the changes in activity that occur due to exercise. With this increased understanding of what occurs centrally, the road may be paved towards new interventions that target these populations and mimic some of the positive effects afforded by exercise

Chapter 2: Review of Literature

The Effects of Exercise on Food Intake

Exercise is a strategy often utilized to counteract obesity, since it lowers the energetic balance by increasing energy expenditure. However, it is possible that this might increase the drive to consume more energy to restore this balance. With this idea, many studies have been performed to measure the effect of exercise on appetite. For example, one study recruited lean healthy males and divided them into three groups, sedentary, low intensity exercise, and high intensity exercise [32]. The low and high intensity exercise were performed on bicycle ergometers at 30% and 70% of their VO_2 maxes respectively, for a duration of 30 min, while the sedentary subjects remained seated and could read quietly. Each subject acted as their own control and they were given a survey before and after their respective procedure to rate their level of hunger. Also, 15 minutes after the completion of their procedure, each subject was informed that an ad libitum meal was available and were instructed to collect the food whenever they felt hungry. There was a significant decrease in reported hunger immediately after the high intensity exercise, but no difference in food intake was observed among the three groups, suggesting high intensity of exercise might suppress appetite, but it doesn't affect the amount of food ingested after an acute bout of exercise. Another study measured the effects of exercise on both male and female rats over a 12-day period [33].The animals were divided into exercise and sedentary groups among their respective sexes. The exercise was performed on a treadmill at 21 m/min. for 60 minutes each day at the onset of the dark period. Food intake was measured twice daily, at the onset of the dark and light cycle. Male rats on average ate more than their female counterparts in both the exercise and sedentary groups for all time periods measured. There was a slight but significant decrease in food intake during the light cycle in male mice in the exercise

group compared to their sedentary male controls. There was no significant difference in cumulative food intake on average when light and dark cycle were combined in both the male and female exercise groups compared to their sedentary controls. A separate study investigated the effects that exercise had on 58 healthy young men after running for two hours [34]. For the sedentary portion of the study, subjects sat in a classroom for two hours and the following week they ran for a duration of 2 hours. They averaged a HR of 168 once the session was completed. After each session, they were given a buffet style meal and their food intake was measured. The exercise session resulted in a significant increase in food intake, with the subjects eating an average of 35 Kcal per person more compared to the sedentary condition. A separate study on the effects of acute exercise on caloric intake was performed with normal-weight young men and women. Subjects were divided into a sedentary group and an exercise group where treadmill exercise was administered for 5 consecutive days [35]. The men responded with an average increase of 208 kcal/d, whereas the women showed no significant change in food intake. Taken together, exercise induced changes to energy metabolism appear to have diverse outcomes on food intake. These changes could be due to the differences in the mode, intensity, and duration of exercise, as well as the metabolic profiles and age of the subjects.

The Effects of Exercise on the Hypothalamus

Exercise is a physiological challenge that disrupts the body's basal homeostatic function. This requires the body to respond rapidly to the stressors that exercise brings upon it. With the prominent role of the hypothalamus in the regulation of many of the body's homeostatic functions, this has led some researchers to look at the effects of exercise on this area of the brain. One area of interest being the ventromedial hypothalamus (VMH) due to its ability to help regulate glucose production, lipolysis, and its involvement in motor function [36–38]. To study

the regulation of hormonal and metabolic responses regulated by the VMH during exercise in rats, researchers prevented activation of the VMH in rats by injecting Marcaine, which inhibits neuronal activation, through a cannula aimed at the dorsal aspect of the VMH [39]. This was compared to another exercise group that received centrally-administered saline. The treatment and control group received the centrally administered injections 15 minutes before an acute bout of exercise. Rats were exercised on a treadmill for 20 minutes at a speed of 26m/min and blood sampling and liver biopsies were performed immediately once the exercise bout was completed. Mice who received the Marcaine treatment experienced a significant attenuation of exercise-induced increases in plasma concentrations of norepinephrine, epinephrine, glucose, as well as hepatic glucose production compared to the saline group. These results indicate that the VMH is involved in the regulation of glucose production during exercise possibly by enhancing epinephrine production, which is a potent stimulator of hepatic glucose production during exercise [40].

Another area of the hypothalamus that has been investigated in response to exercise is the paraventricular nucleus due to its ability to alter heart rate and blood pressure [41]. The PVN in 24-month-old rats was compared to 2-month-old rats to observe any differences in heart rate and blood pressure function, which often gets worst with age, and whether exercise has any effect on the GABAergic neurons, because GABAergic neurons account for 50% of the neurons in the PVN and can modulate heart rate and blood pressure [42]. The older mice were divided in 3 groups: sedentary, low exercise, and high exercise then compared to the 2-month-old mice that didn't receive any exercise treatment. Exercise occurred in the low exercise group 3 days a week compared to 5 days a week in the high group. Both groups ran at 12 m/min for a total of 60 minutes for 12 weeks. The number of PVN GABAergic neurons present in the sedentary group

was less than half of the young group, but the elderly exercise groups both had significantly more GABAergic neurons present compared to the sedentary group. Also, while not significant, the total GABAergic neurons expressed in the high exercise group was close to the 2-month-old group, indicating that exercise can induce neurogenesis in this area.

The Dorsomedial hypothalamus (DMH) has also been investigated during exercise. Numerous studies have shown that mice often lose weight if an exercise wheel is introduced to the cage [43,44]. Activation of DMH neurons decreases food intake and body weight [45]. This activation can occur via receptors that respond to corticotropin-releasing factor (CFR), a hormone that suppresses appetite [46]. Therefore, researchers investigated if DMH plays a role in the weight loss that often occurs when mice are given free access to an exercise wheel [47]. In this study mice were divided into groups that received either ICV injections of a CRF antagonist or saline and were given access to an exercise wheel for a week. Both groups received these injections every day for a week before the wheels were introduced. This central injection of a CRF antagonist significantly increased CRF mRNA levels in the DMH compared to mice who received saline injections in with free access to a running wheel, whereas CRF levels in the PVN and ARC remained unaffected. This central injection of a CRF antagonist also significantly attenuated the weight loss induced by voluntary exercise. Overall, these data suggest that elevated DMH CRF mRNA expression mediates aspects of the inhibitory effects of voluntary exercise on body weight.

There are currently few studies linking exercise to neuronal activity in ARC of the hypothalamus. A recent publication using a high intensity interval training protocol observed that while the resting membrane potential was altered in POMC and NPY/AgRP neurons immediately after a single bout of exercise, no changes in action potential were noted [30].

Another study demonstrated a significant increase in ARC neuronal activity when rats where given treadmill exercise above their lactate threshold, but this study didn't investigate the specific subpopulations that were activated in this area. A 40-day voluntary running wheel training study was previously conducted that showed an increase in the amount of neuropeptide Y present in the ARC and dorsomedial hypothalamus in adult male rats [48]. A separate study also found that 12 weeks of voluntary wheel training down-regulates leptin receptor mRNA in the ARC [49]. Both running wheel studies revealed an improvement in lean body mass compared to the non-exercise control groups. Additionally, our lab has also demonstrated a neuroprotective effect of exercise training on POMC neurons in obese mice [43]. This evidence supports an exercise-dependent mechanism to alter the neural circuitry within the ARC.

Possible Mechanisms That Could Affect Neuronal Activation in the ARC

The literature is far from complete regarding the effects of exercise on the ARC. Due to exercise's ability to have a substantial impact on energy homeostasis, there have been numerous studies investigating its effects on circulating factors that are involved in metabolic functions. These include ghrelin which promotes appetite, and leptin which counters the effects of ghrelin; both of which can alter POMC and NPY/AgRP neuronal activation [50,51]. Multiple studies have reported that there are no significant effects from chronic and acute exercise on endogenous leptin levels [52,53]. The effect of exercise on plasma ghrelin levels has been investigated in both human and rodent models, but results have been inconclusive [54–56]. However, a recent study performed by Mani et al. showed a significant increase in plasma acyl-ghrelin, an active form of ghrelin, in rodents after an acute bout of exercise [55]. Ghrelin receptors are highly expressed in the ARC of the hypothalamus, predominately on NPY/AgRP neurons [51]. Direct ghrelin microinjection into the ARC has been shown to stimulate food intake and increase

NPY/AgRP activity [57]; further, ghrelin receptor deficient mice showed a significant decrease in exercise endurance and decreased food intake after an acute bout of exercise [55]. Therefore, this indicates an exercise induced alteration in plasma ghrelin could alter ARC activation.

Peptide YY 3-36 (PYY) has also been implicated in energy homeostasis. PYY is another satiety hormone released by the gastrointestinal tract. Peripheral administration of PYY reduces food intake and reduces weight gain [58]. In the same study, administered PYY also decreases NPY mRNA in the hypothalamus and activates adjacent POMC neurons. This effect is absent in Y2 receptor deficient mice indicating that this hormone acts as a Y2 receptor agonist. Y2 receptors are expressed on NPY/AgRP neurons in the arcuate nucleus, indicating that PYY may act on these neurons through increased inhibition. PYY is significantly increased after a bout of acute exercise in humans [56], suggesting that exercise may cause a change in ARC activation through this hormone. Insulin, a hormone that is made and secreted by the pancreas, not only regulates glucose through peripheral action, but through the CNS as well [59].

Insulin receptors are expressed in many regions throughout the brain, including ARC POMC and NPY/AgRP neurons [60]. When centrally injected, insulin has an anorexigenic effect by stimulating POMC neurons and inhibiting NPY/AgRP neurons through increased hyperpolarization, indicating that this hormone not only plays a role in glucose homeostasis, but energy homeostasis as well [59,61,62]. Due to insulin's ability peripherally to influence the uptake of glucose in tissues such as skeletal muscle, the amount of glucose uptake can increase significantly due to the increased energy requirements induced by exercise [63]. This increase in glucose uptake results in an insulin independent mechanism during exercise. Without this decrease in secretion, an individual could quickly become hypoglycemic due to the increased glucose uptake. A decrease in plasma insulin could possibly cause an increase in activation of

NPY/AgRP neurons during exercise due to the decreased inhibition [64]. Also, exercise has been shown to alter levels of 5-HT in the hypothalamus [65]. 5-HT receptors are expressed on POMC neurons in the hypothalamus which can stimulate the melanocortin pathway [66], providing another possible circulating mechanism that could influence activity in the ARC by exercise.

Exercise training has also been shown to change the inherent excitability of PVN neurons through increased number of action potentials evoked in response to depolarizing stimulation when comparing mice with access to a running wheel compared to those without [67]. Increased cFOS expression in the PVN has also been observed in both mice and rats immediately after an acute bout of treadmill exercise [31,68]. Arcuate neurons have projections to/from the PVN. A retrograde analysis using a modified rabies virus estimates that both ARC POMC and NPY/AgRP neurons receive between 5-15% of their pre-synaptic input from this PVN area [26]. In the same analysis both ARC POMC and NPY/AgRP populations had a significant density of projections to the PVN. Activation of Subpopulations of SIM1 expressing neurons in the PVN have been shown to activate ARC NPY/AgRP neurons and increase food intake [29]. Taken together, these studies indicate that exercise induced PVN activation can possibly induce changes in activity in the ARC.

Chapter 3: Materials and Methods

Animal Care

All animal procedures were approved by the Institutional Animal Care and Use Committee for the University of East Carolina, Greenville (Greenville, NC). Two transgenic mouse lines were utilized; NPY-GFP mice that transcribed humanized *Renilla* Green Fluorescent Protein (hrGFP) under control of the mouse NPY promoter as well as AgRP-IRES-Cre mice in which Cre recombinase expression is observed in AgRP neurons. Mice were housed in a temperature-controlled environment (22–24 °C) with a 12-h light (07:30)/dark (07:30) cycle with standard mouse chow and water provided *ad libitum*.

Treadmill Protocol

Mice were randomly assigned to an exercise or sedentary control group using a random number generator in all experiments that the mice didn't serve as its own control. Male mice aged 12-13 weeks were used for all experiments unless otherwise specified. NPY-GFP mice were utilized for multiple experiments. To assess neuron activation immediately after an acute bout of exercise we randomly assigned cohort 1 (N=6) mice to a treadmill exercise or sedentary group. In cohort 2 male and female mice were used with a N value of 3 and 4 respectively. Patch-clamp physiological recordings were used to quantify changes in NPY-GFP neuron firing rated induced by exercise (N=15). In cohort 3 (N=10), food intake was assessed for the 8 hours following a bout of acute exercise. In cohort 4 (N=3), blood glucose and CSF glucose levels were measured in both the sedentary and acute exercise group. AgRP-IRES-Cre mice were also used in 2 separate experiments. In the first experiment (N=4) mice were divided into 4 groups, an exercise and sedentary group that received CNO injections prior to their bout of acute exercise, and mice

that received bilateral arcuate injections of AAV-HM4Di-mcherry prior to the experiment and received either CNO or saline injections before a bout of acute exercise; food intake was assessed for the 8 hours following a bout of acute exercise. The second experiment utilizing AgRP-IRES-Cre transgenic mouse line (N=3) also were divided into four groups, a sedentary and exercise group and two groups that received bilateral arcuate injections of AAV-HM4Di-mcherry that were treated with either saline or CNO prior to a bout of acute exercise to assess changes in neuronal activation in the PVN. All mice were familiarized to the treadmill prior to the acute exercise bout [Prior day 10 minutes rest on the treadmill followed by 5 min at the speed of 5 m/min and then for 5 min at the speed of 10 m/min]. On the day of the experiment mice were placed on the treadmill for a duration of 10 minutes to become acclimated before beginning the exercise protocol. After this 10-minute acclimation period the treadmill was increased to a speed of 13 m/min. for an hour, which in previous studies is estimated to be around 75% of the VO2 max in adult mice [69]. To control for any non-exercised effects of treadmill running (handling, novel environment, noise, and vibration), sedentary groups were placed on the top of the treadmill apparatus, in a cage with Alpha-dri bedding removed of food and water, for a period equivalent to exercise training. All conditions were maintained in DREADD experiments with the exception that either CNO or saline was injected 30 minutes prior to beginning the exercise protocol.

Blood/CSF collection and assessment of glucose concentration

Mice were anesthetized with 99.9% isoflurane immediately after the acute bout of exercise. Blood samples were immediately collected via tail incision and blood glucose levels

were measured. Directly after blood glucose collection an incision was made in the neck of the mouse to expose the cistern magna. A capillary tube was inserted into the cistern magna through the dura mater, and the CSF flowed into the capillary tube. The tube was then immediately connected to a 5 ml syringe through a polyethylene tubing to retrieve the CSF from the capillary tube. The glucose concentration of the CSF was then immediately measured.

Brain Tissue Preparation

Mice were anesthetized with 99.9% isoflurane and transcardially perfused with phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin. Brains were removed, stored in the same fixative for 24 hours, transferred into 30% sucrose at 4 ℃ overnight. Brains were then sliced coronally at 20-μm thickness on a freezing microtome (Leica VT1000) into five equal series. A single series of sections per animal was used in the histological studies.

Immunohistochemistry

For immunofluorescence, brain sections were washed in PBS and blocked in 3% normal donkey serum in PBS+.03% Triton (PBST) for 1 h at room temperature. Brain sections were then incubated overnight at room temperature in blocking solution containing primary antiserum (rabbit anti-POMC precursor, Phoenix Pharmaceuticals H-029-30, 1:3000; goat anti-Fos, Santa Cruz Biotechnology, sc-52-G, 1:500; rabbit anti dsRed, Clontech, 1:1000; rabbit ant SIM1, Millipore, 1:500). The next morning sections were extensively washed in PBS and then incubated in Alexa-fluorophore secondary antibody (A-21209, A-11039, 1:500) for 1 h at room temperature. After several washes in PBS, sections were mounted on glass slides.

For DAB staining, brain sections were washed and blocked in 3% normal donkey serum in PBST for 1 h at room temperature. Slices were then incubated in cFOS goat primary antiserum (goat anti-Fos, Santa Cruz Biotechnology, sc-52-G, 1:1000) overnight followed by biotinylated donkey anti-goat IgG (Vector; 1:1000) for 2 hr. Sections were then incubated in the avidin–biotin complex (ABC; Vector Elite Kit; 1:500) and incubated in 0.04% DAB and 0.02% cobalt chloride (Fisher Scientific), and 0.01% hydrogen peroxide.

Microscopy

The sections were imaged photographed digitally using a Leica DM6000F upright microscope. 20x objectives were used to image either the left or the right hemisphere in the arcuate area of the hypothalamus. POMC, cFOS, SIM1, DAPI and NPY-positive neurons throughout the image were counted. Quantification of immunofluorescent images was obtained using ImageJ Cell Counter plug-in function for marking and numbering of positive cells. Once positive cells were marked, ImageJ software was used to overlay images to quantify colocalization.

Stereotaxic AAV-HM4Di-mCherry injections

An inhibitory Cre-recombinase–dependent Adeno Associated Virus, AAV-hM4Di-mCherry was utilized to express hM4Di selectively inhibit ARC AgRP neurons in AgRP-IRES-Cre mice. This virus introduces a mutagenic inhibitory G-protein coupled receptor that is only stimulated by the pharmacologically inert clozapine-N-Oxide (CNO). 100-nL AAV injections were performed bilaterally into the ARC in 5-6-week *AgRP-Ires-Cre* mice (coordinates, bregma: anterior-posterior, -1.50mm; dorsal-ventral -5.95 and -5.80 mm; lateral, +/- 0.20 mm) with a

glass micropipette and air pressure injector system (Grass S48 Stimulator). Mice were individually housed and allowed 2 weeks to recover before the start of any in-vivo studies.

Electrophysiology

Animals were deeply anesthetized and decapitated. Brains were quickly removed into ice-cold N-methyl-D-glucamine solution consisting of (in mM) 92 NMDG, 20 HEPES, 25 Glucose, 30 $NaHCO_3$, 1.2 NaH_2PO_4, 2.5 KCl, 10 $MgSO_4$, .5 CaCl 5 sodium ascorbate, 3 sodium pyruvate, 2 Thiourea, oxygenated with 95% O_2/5% CO_2, measured osmolarity 310–320 mOsm/l. 300-μm-thick coronal sections were cut with a VF200 Compresstome (Precision Instruments, Greenville NC, USA) and incubated in oxygenated chilled for 10 min. Slices were transferred to oxygenated aCSF holding solution (92mM NaCl, 20mM HEPES, 25 mM Glucose, 30 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM KCl, 10 mM $MgSO_4$, .5 mM CaCl 5 mM sodium ascorbate, 3 mM sodium pyruvate, and 2 mM Thiourea) and stored in the same solution at room temperature in a BSK 6 (Automate Scientific, Berkley CA, USA) (20–24 °C) for at least 60 min before recording. A single slice was placed in the recording chamber where it was continuously superfused at a rate of 3–4 ml per min with oxygenated recording aCSF solution (125mM NaCl, 11mM Glucose, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 10mM $MgSO_4$, 2.4 mM CaCl, 1 mM MgCL). Neurons were visualized with an upright Leica DM6000F equipped with infrared differential interference contrast and fluorescence optics. Borosilicate glass microelectrodes (4–6 MΩ) were filled with internal solution.

To assess the effect of Exercise on ARC NPY/AgRP neurons. Cell-attached recordings (seal resistance, 1-2 GΩ) were made in voltage clamp mode with aCSF as internal solution and holding current maintained at V_h = -50 mV.

Food Intake

Food intake was be measured by limiting the amount of food in each cage to ~8g or 2 pellets. Food intake was measured 0.5, 1, 2, 4, and 8 hours post exercise treatment. All mice were individually housed at least 1 week prior to exercise treatment. Mice were placed in alpha dry bedding 48 hours prior to food intake measurements.

Calculating Estimated Energy Expenditure

To calculate the estimated energy expenditure of the mice undergoing the exercise treatment we used a previously validated equation for adult mice to predict their relative VO_2 (ml/kg/hr) (VO_2 = 5444 + [223 x Treadmill Velocity]) [69]. This was then divided by 60 to give us the relative VO_2 per minute (ml/kg/min). Then we multiplied this number by each mouse's body weight in kg to estimate the Absolute VO_2 of each mouse (ml/min). We then divided this number by 1000 to convert this number to L/min. One liter of O2 consumed expends 5 calories so the absolute VO_2 in L/min was then multiplied by 5 to estimate Kcal/min consumed. This number was then divided by 1000 to convert it to cal/min and multiplied by the number of minutes the mouse exercised on the treadmill (60 min.) to estimate the total energy expended during the exercise bout.

Calculating Excess Energy Consumption

To calculate the excess energy consumed in the exercise group compared to the sedentary group over the total time measured post-exercise (8-hours), we subtracted the total weight in grams of the food consumed from each mouse during the exercise condition and

subtracted this from the total food consumed during the sedentary condition. This difference was then multiplied by the metabolizable energy contained in their food source (3.20 Kcal/gram; Prolab Isopro RMH 3000).

Statistical Analysis

Results are reported as the mean ± SEM. Statistical analyses were performed using Prism 6.0 (GraphPad) software. Cumulative Food intake was analyzed by 2-way repeated measures ANOVA with Sidak correction for multiple comparisons set to *$p < 0.05$ for significance. Static data such as total neurons, neurons co-localized with cFOS, and firing rate was averaged and measured utilizing an unpaired t-test, and a one-way ANOVA when with an alpha value set at 0.05 for significance. All patch clamp recordings were reported offline using Clampfit 10.6 to measure electrophysiological recording

Chapter 4: Results

4.1 ARC NPY/AgRP neuron activation is increased and POMC neuron activation remains unchanged after a bout of acute exercise

Due to the ability of ARC NPY/AgRP and POMC neurons to mediate energy intake, the activities of POMC and NPY/AgRP neurons in mice was assessed immediately after an acute bout of exercise using immunohistochemistry. There was no difference in the number and activation (cFOS) of POMC expressing neurons in the exercise group compared to the sedentary group (Figure 1B, C). Conversely, the number of NPY/AgRP neurons that were colocalized with cFOS was significantly increased in the exercise group compared to the sedentary group (Figure 1E). An average of 23/48 (48% ± .7%) of NPY/AgRP neurons were active in each slice analyzed in the exercise group compared to the control average of 3/49 (6% ± .6%), indicating an increased number of NPY/AgRP neurons activated by an acute bout of exercise.

4.2 ARC NPY/AgRP neuron firing rate is increased after an acute bout of exercise *ex vivo*

Next, we aimed to confirm our immunofluorescent findings through cell-attached patch-clamp recordings. Recordings were made in NPY-GFP neurons of the ARC in NPY-GFP mice immediately after performing a bout of acute exercise and compared to sedentary mice, to record differences the number of action potentials observed. We observed a significant increase in firing rate of arcuate NPY/AgRP neurons immediately after exercise compared to sedentary controls, with the exercise group averaging a firing rate of 2.068 ± 0.3267 Hz compared to 0.0668 ± 0.195 Hz in the control group. (Figure 4C).

4.3 Food Intake is Increased Immediately Post-Exercise

An increase in energy expenditure induced by exercise can cause an energy imbalance in the body. After a bout of acute exercise, we observed a significant increase in the number of active NPY/AgRP neurons. Therefore, we assessed whether an hour of acute exercise resulted in any changes in subsequent food intake. Cumulative food intake was significantly increased in response to exercise compared to the sedentary group at the 1,2,4, and 8-hour time points measured (Figure 2A). The total food intake consumed over 8 hours after a bout of acute exercise was also significantly higher compared to mice under the sedentary control condition with the exercise group averaging 1.4 ± 0.0765 grams consumed compared to 1 ± 0.076 gram of chow consumed by the control group (Figure 2B). Using a previously validated metabolic equation [69] , we were able to estimate the total excess energy expended by the exercise group. We then compared this estimated energy expenditure to the excess calories consumed by the exercise group versus the sedentary group. There was no significant difference between the two measures with the estimated energy expenditure averaging 1172 ± 34.67 more calories expended compared to the sedentary control, and an average of 1215 ± 288.8 excess calories consumed by the exercise group, indicating that the excess energy intake may possibly be used to balance the excess energy expended during the acute bout of exercise (Figure 2C).

4.4 Blood and CSF glucose levels were elevated immediately post-exercise

NPY/AgRP neuron activity has been previously showed to increase during periods of low glucose concentrations. To elucidate if this might be a potential cause of activation in this neuron population, both blood and cerebrospinal fluid (CSF) glucose levels were measured immediately after a bout of acute exercise. In line with previous studies [55,70], both parameters were

significantly increased with the CSF glucose increasing 148% ± 0.29% and the blood glucose increasing 165% ± 2.3% in the exercise group compared to the control group immediately post-exercise (Figure 3A-B).

4.5 AgRP neuron inhibition abolishes exercise-induced food intake

To determine whether AgRP neurons play a role in the observed acute exercise-induced refeeding, an inhibitory Cre-recombinase–dependent AAV, AAV-hM4Di-mCherry was injected bilaterally into the ARC of AgRP-Ires-cre mice. There was no difference in food intake between DREADD treated mice that received saline injections (1.413 ± 0.077 gram average 8 hours post-exercise) and untreated mice that received a CNO injection before a bout of acute exercise (1.326 ± 0.076 gram average 8 hours post-exercise). DREADD treated mice that received CNO injections to induce inhibition of AGRP neurons before an acute bout of exercise had a significant decrease in food intake (0.6375 ± 0.121 gram average 8 hours post-exercise) after a bout of acute exercise compared to the other two exercise groups. (Figure 5B)

4.6 PVN Neuron activation is increased immediately post-exercise and this activation is not mediated by AgRP activation

To determine if AgRP neuronal activation was necessary for the increase in PVN neuronal activity from an acute bout of exercise, activation in the PVN was measured immediately after an acute bout of exercise where AgRP neurons were inhibited. SIM1 staining was used to quantify PVN neurons. There was no significant difference in the number of SIM1 expressing neurons between the sedentary, exercise, and DREADD groups that received either CNO or saline before the bout of exercise (Figure 6B). All three exercise groups showed an increase in cFOS

colocalization in SIM1 expressing neurons in the PVN ,with the exercise group and the DREADD groups that were injected with CNO and saline averaging 55% ± 6.2%, 49% ± 7.3%, and 47% ± 6.5% colocalization with cFOS respectively immediately after an acute bout of exercise compared to the 26% ± 8.5% colocalization in the sedentary group. There was no significant difference in the amount of cFOS expression in SIM1 neurons between the three exercise groups (Figure 6).

Chapter 5: Discussion

In this study we investigated the effect of an acute bout of exercise on the activity of NPY/AgRP and their adjacent POMC neurons in the arcuate nucleus of the hypothalamus, as well as the role NPY/AgRP neuron activation plays in the in the feeding response post-exercise. In NPY-GFP reporter mice, exercise was able to increase the number of activated arcuate NPY-GFP neurons as well as the firing rate in this subpopulation compared to the sedentary control mice. POMC and NPY/AgRP neurons regulate energy homeostasis by mediating energy intake and energy expenditure. Activation of ARC NPY/AgRP neurons has been shown to decrease energy expenditure and promote food intake [17,18]. Whereas, postnatal ablation of ARC POMC neurons increased food intake and reduced energy expenditure [71]. When the estimated increase in calories expended during an acute bout of exercise was calculated and compared to the excess calories consumed by the exercise group compared to the sedentary group there was no significant difference. This combined with the increase in NPY/AgRP activation post-exercise may suggest a potential compensatory mechanism to conserve energy due to the energy deficit caused by an acute bout of exercise.

Consistent with the increase in NPY/AgRP neuron activation, an acute bout of exercise was also found to significantly increase food intake immediately post-exercise. Furthermore, this exercise-induced increase in food intake was completely abolished while AgRP neuron inhibition was induced through the DREADD system. Previous studies from our lab have shown that a chronic increase in energy expenditure via voluntary exercise can have a beneficial role on POMC neuron degeneration in a diet induced obesity (DIO) mouse model [43]. We have also observed that chronic exercise has the ability to attenuate DIO-induced hypothalamic apoptosis and augmented POMC and NPY-expressing in the ARC hypothalamus of an Alzheimer's disease

mouse model [72]. However, these studies only focused on the chronic effects of exercise. It is possible that this acute activation of the NPY/AgRP neurons observed in this study may contribute to this therapeutic effect that we have previously observed.

ARC NPY/AgRP neurons are typically known to increase food intake partially due to their ability to inhibit MC4Rs in the PVN. Inhibition of these neurons typically suppresses the release of their inhibitory neurotransmitters allowing α-MSH to maintain its excitatory effect to promote satiety. Consistent with a previous study [31] , increased activation of the PVN of the hypothalamus immediately after exercise was also observed in our study. Inhibition of the ARC NPY/AgRP neurons during exercise decreased the amount of food consumed but had no effect on the activation of SIM1 expressing neurons in the PVN, suggesting that AgRP neuron activation is required for exercise induced food intake (Figure 6B). Krashes et al. previously reported subpopulations of SIM1 expressing neurons that, when activated, can increase energy intake and increase ARC NPY/AgRP activation [29]. Therefore, it is possible that this PVN activation mediates acute exercise-induced AgRP neuronal activation.

Contrary to a previous study by He et al. [30], we observed increased depolarization of NPY/AgRP neurons and the unchanged activity of POMC neurons immediately after an acute bout of exercise. The same study also reported no significant difference in the frequency of action potentials in arcuate NPY-GFP expressing neurons immediately after a bout of acute exercise [73]. Differences in these findings could be attributed to the protocol of exercise used, with a bout of high intensity interval exercise being utilized by this group. In addition, an electric stimulus to coax the mice to stay on the treadmill was utilized, which possibly generated a stress rather than an exercise response. In contrast, due to the lower speed used in the current study during the bouts acute exercise, running without stimulation was easily maintained. Therefore,

unnecessary stress that might cause different responses not due to the acute bout of exercise was minimized. Our exercise protocol was designed based on previous literature to mimic a VO2 of around 75% of the VO2max in un-trained mice [69]. The protocol in He et al.'s group was chosen due to it being previously shown to reduce food intake in mice [55], which may have made it a non-objective experimental condition. The appropriated intensity and the lack of the undue stress provided by electrical stimulation in the current study represented a more physiologically relevant exercise response. ARC NPY/AgRP neuron activation is normally seen when food is restricted, but this activity can also be altered by other stressful conditions such as a painful stimulus that can alter plasma hormone levels [25,62]. Single bouts of exercise have been shown to alter circulating hormone levels [55,56,65,74]. A number of the circulating hormones have been shown to modulate activity levels of the NPY/AgRP neuron population in the ARC [13,55,63]. Some of these include acylated ghrelin that activates AgRP neurons, which was recently reported to be increased immediately after a bout of acute exercise [55], and a decrease in the ARC NPY/AgRP inhibiting hormone insulin [55,63]. NPY/AgRP neurons also can serve as a regulator of glucose homeostasis, due to this subpopulations ability to sense glucose concentrations. Low glucose concentration has been shown to activate AgRP neurons [75]. However, consistent with findings from other studies [55,76], blood glucose and CSF glucose were increased immediately after a bout of acute exercise (Figure 3 A-B), indicating that this is likely not the reason for the increase in activation observed.

The effect of exercise on food consumption has previously been studied in both humans and rodents across several modes of exercise. While many of these studies demonstrated a decrease in food-intake immediately after exercise [32,77], increased food consumption has also been noted [34], as well as no significant difference in the amount of food consumed [4,73].

These results are controversial due differences in the distinct exercise intensity, duration, mode, as well as the age and gender of the subjects who performed these bouts of exercise. In our studies, we observed an increase in food intake after an acute bout of treadmill exercise. Importantly, this observed significant increase was revealed at either the 1 or 2-hour point of all the food intake experiments performed in this study, which is consistent with the acute, robust increase in food intake that is typically observed when ARC AgRP neurons are activated. This may suggest an energy compensatory effect in mice that undergo increased forced energy expenditure. This is supported by the calculated estimated increase in calories expended during the bout of acute exercise almost matching the increase in calories consumed in the exercise group compared to the sedentary group. Importantly, when AgRP neuron activation was inhibited through the DREADD system, this acute exercise-induced energy intake was completely abolished, indicating that AgRP neuronal activation is required for acute exercise-induced food intake.

Conclusion and Further Study

In this study. we observed the effects of a single bout of exercise increases arcuate NPY neuron activation immediately after exercise, while POMC neurons activation remained unaffected. Notably, this exercise induced energy deficit also causes a significant increase in food intake immediately post-exercise. Inducing loss of function of this neuron sub-population significantly negates this increase in food intake. Therefore, these data demonstrate exercise-induced NPY/AgRP activation is critical in inducing food intake post exercise in sated mice.

Future studies should identify possible mechanisms underlying exercise-induced NPY/AgRP neuron activation, with particularly focus on PVN activation. Inhibition of these

PVN neurons through a SIM1-Cre transgenic could elucidate the causes of the neuronal changes we observed. Also, the long-term effects of exercise training at this modality should be investigated. While usually modest in the absence of caloric restriction, a chronic increase in the time spent exercising, typically leads to a decrease in body weight [4,33,78]. With the ARC's ability to reorganize cellular synaptic plasticity depending on its metabolic state, it's possible that the activation observed can be altered with a chronic exercise regimen. This would serve to help us gain a better understanding of the adaptations that occur centrally in response to exercise both acute and chronic

Chapter 6: Figures

Figure 1

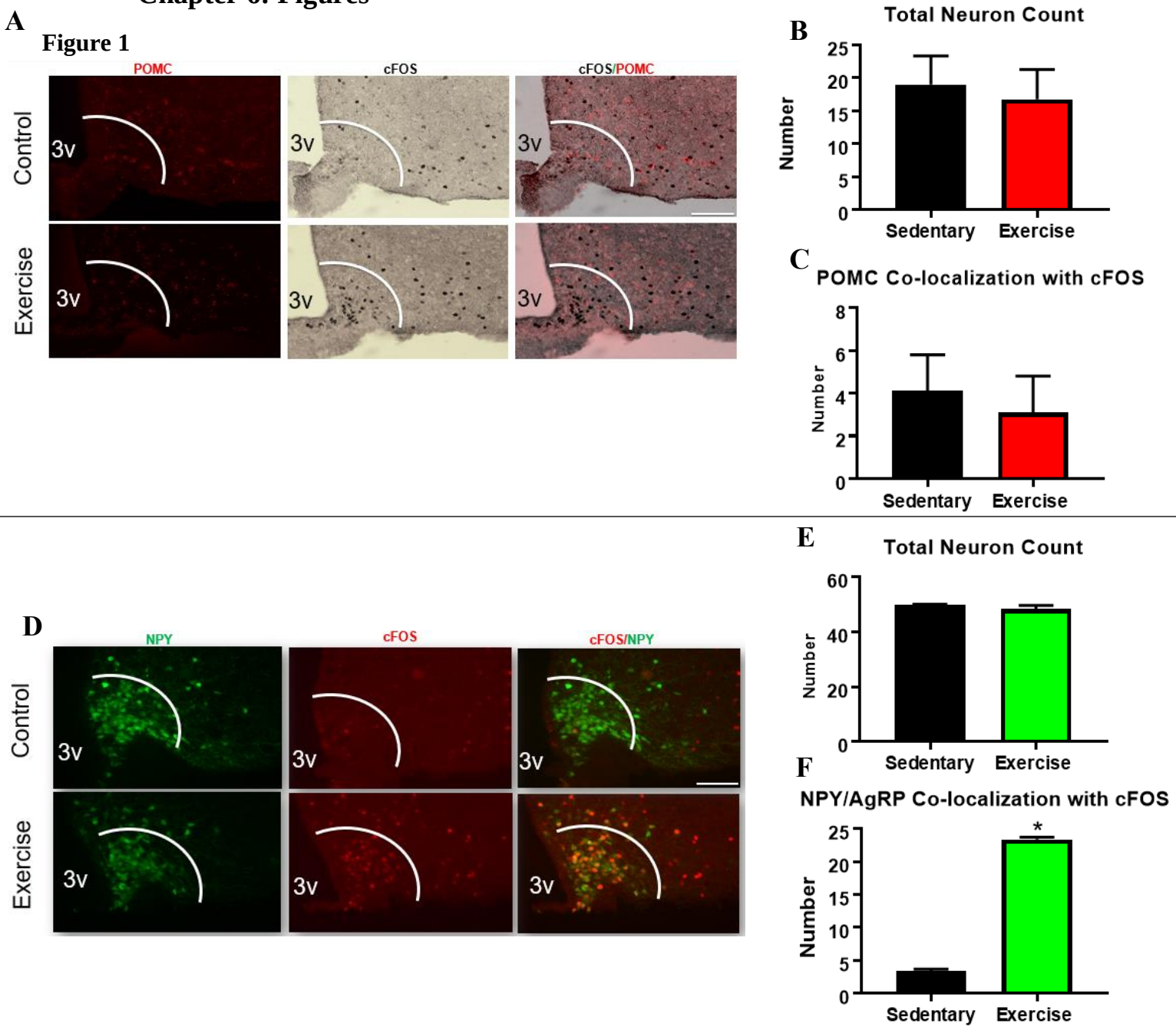

Fig 1. **(A)** The expression of cFOS in POMC expressing neurons of arcuate sections from the sedentary control and exercise groups in male mice immediately after an acute bout of exercise. **(B)** Average POMC neuron count per slice. **(C)** Average number of neurons that are co-localized with cFOS. **(D)** The expression of cFOS in NPY/AgRP neurons of arcuate sections from the sedentary control and exercise groups immediately after an acute bout of exercise. **(E)** Average NPY/AgRP neuron count per slice **(F)** NPY/AgRP neuron's that are co-localized with cFOS. 3V, third ventricle; scale bars represent 50 µM. Bar graphs show Mean + SEM. (N = 6 per group), * indicates $p < 0.05$ vs sedentary group.

Figure 2

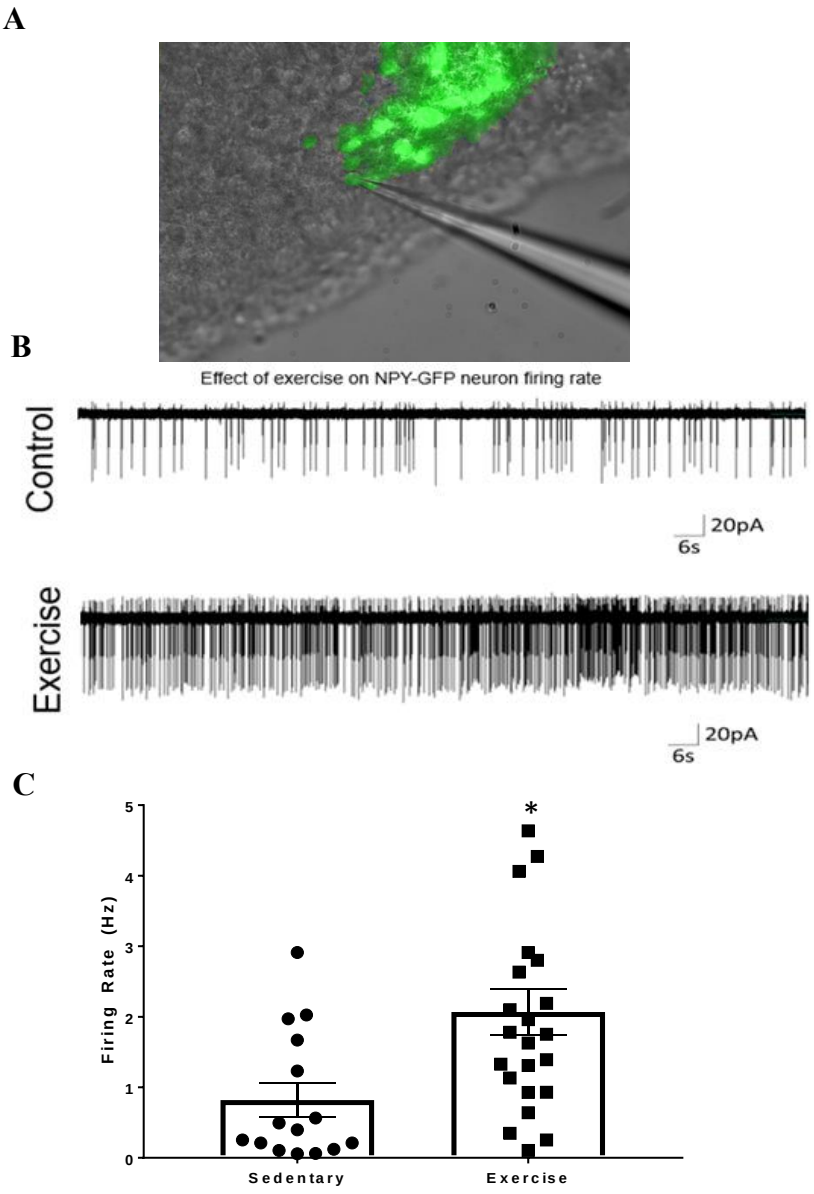

Fig 2. **(A)** Representative Image of patch-clamp pipette sealed to AgRP/NPY neuron **(B)** Representative trace of AgRP/NPY neuron after sedentary and exercise conditions **(C)** Graph comparing calculated firing rate of NPY/AGRP neurons in male and female mice. Data expressed as mean ± SEM. (N=15) * indicates $p < 0.05$ vs sedentary group

Figure 3

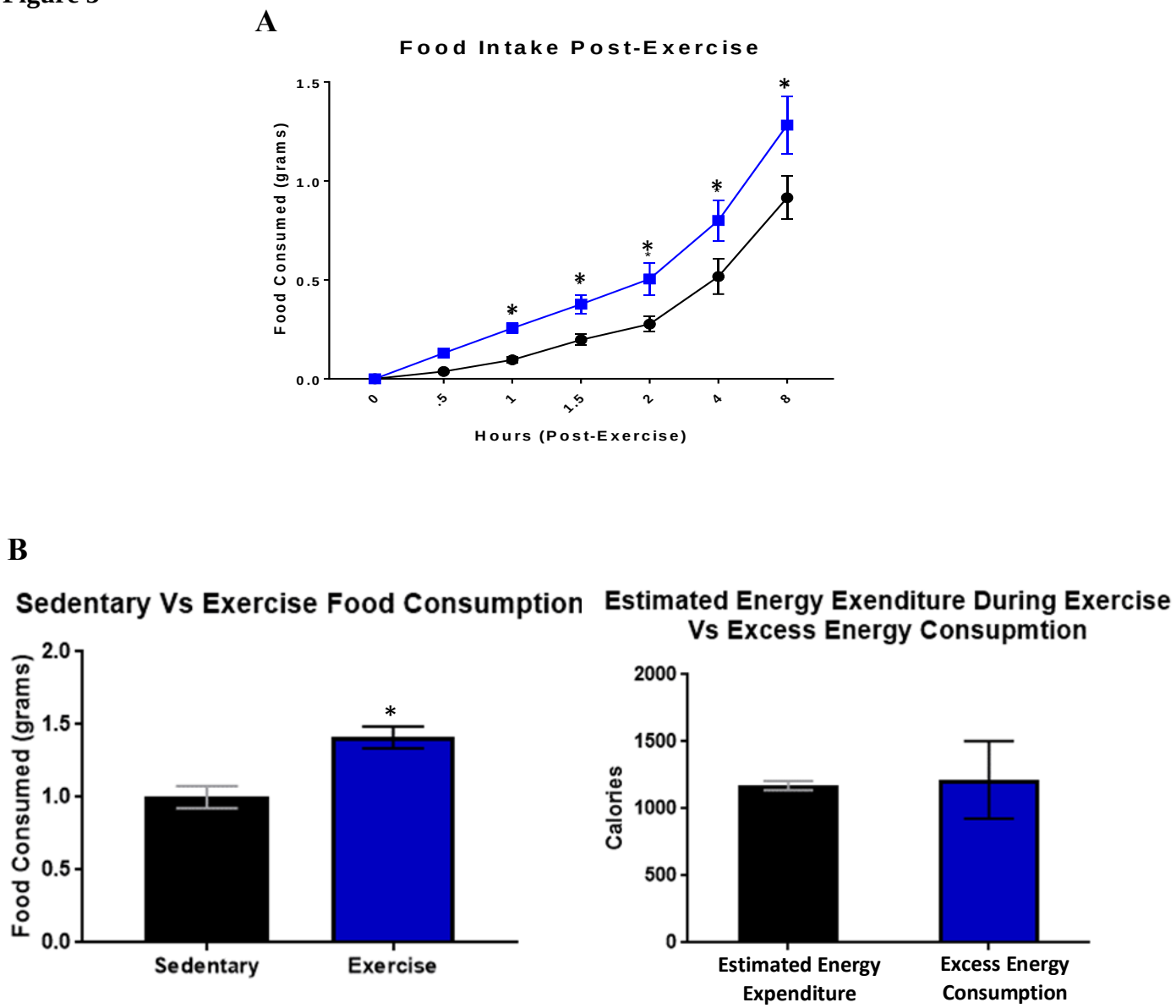

Fig 3. **(A)** Average cumulative food intake in male mice over an 8-hour period. **(B)** Total food consumed after 8-hour time period. **(C)** Estimated energy expenditure of the exercise groups during the 1 hour of acute exercise compared to the difference in calorie consumption from the exercise group compared to the sedentary group. Bar graphs show Mean + SEM. (N = 10 per group), * indicates $p < 0.05$ vs sedentary group.

Figure 4

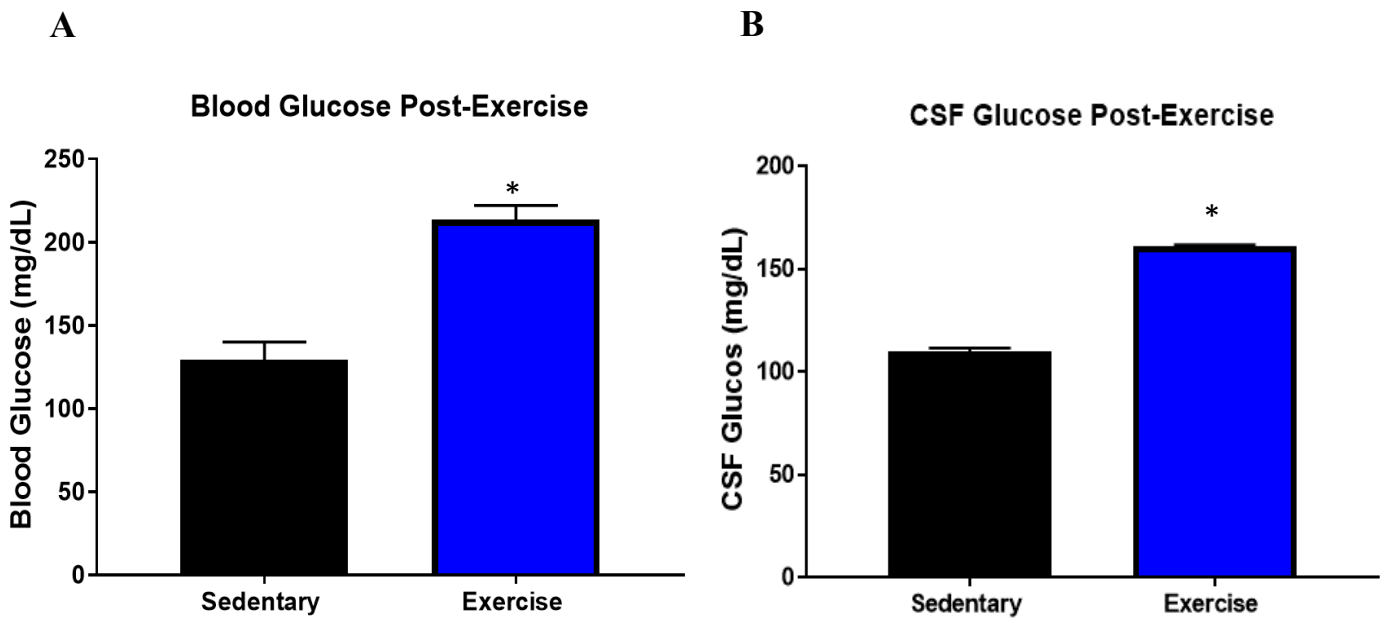

Fig 4. **(A**) Average blood glucose levels of male mice immediately after a bout of acute exercise **(B)** Average CSF glucose levels of mice immediately after a bout of acute exercise. Bar graphs show Mean + SEM. (N = 3 per group), * indicates $p < 0.05$ vs sedentary group.

Figure 5

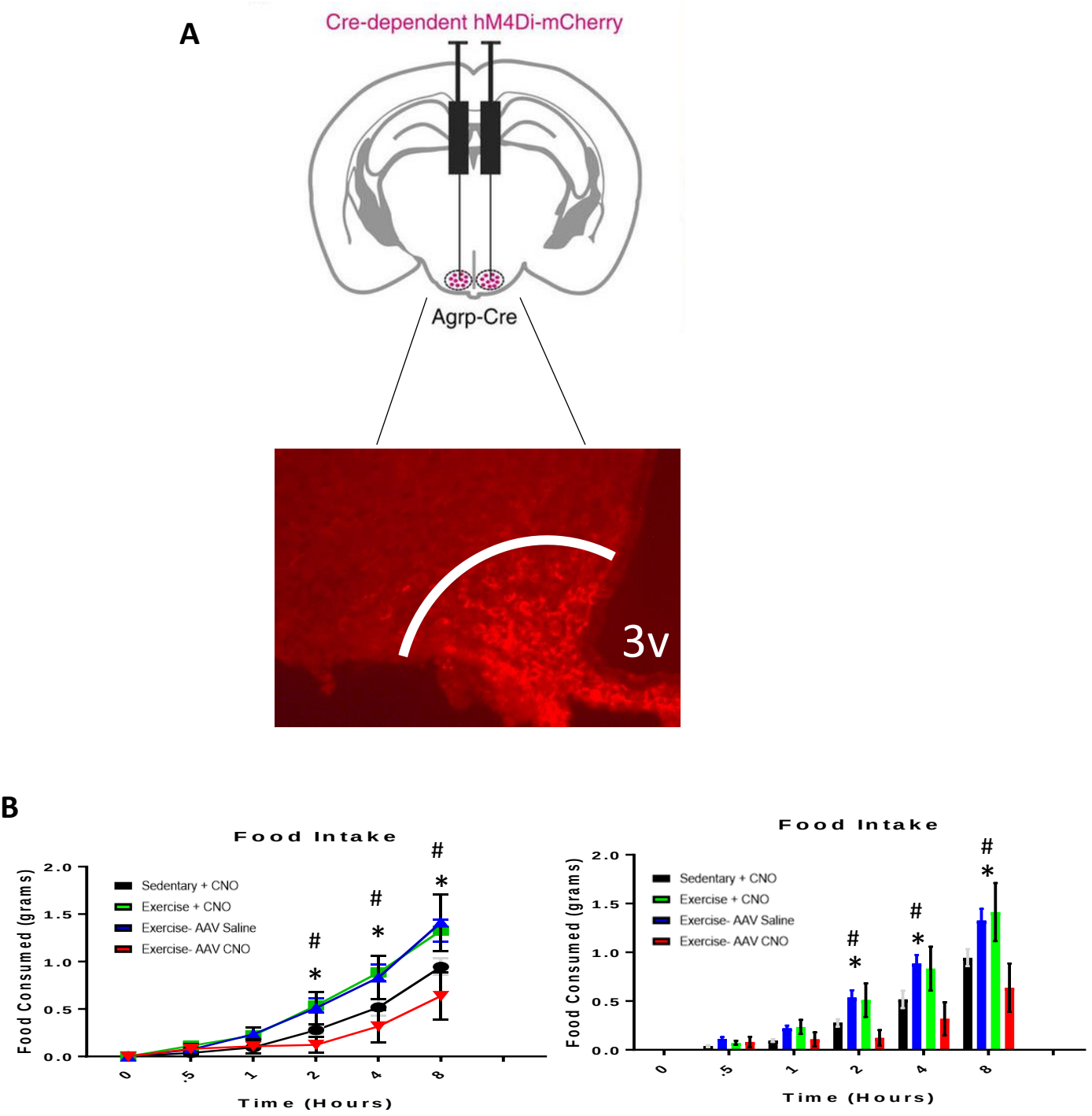

Fig 5. **(A)** Top, targeting scheme for hM4Di-mCherry. Bottom, localization of hM4Di-mCherry. 3V, third ventricle. **(B-C)** Graph comparing cumulative food intake over an 8-hour period in male mice. # indicates significance between Exercise + CNO and Sedentary + CNO groups ($p < 0.05$); * indicates a significant difference between Exercise- AAV Saline and Exercise – AAV CNO groups ($p < 0.05$) (N=4). (CNO and saline injections of .03 mg/kg of body weight, i.p. were applied 30 minutes prior to exercise)

Figure 6

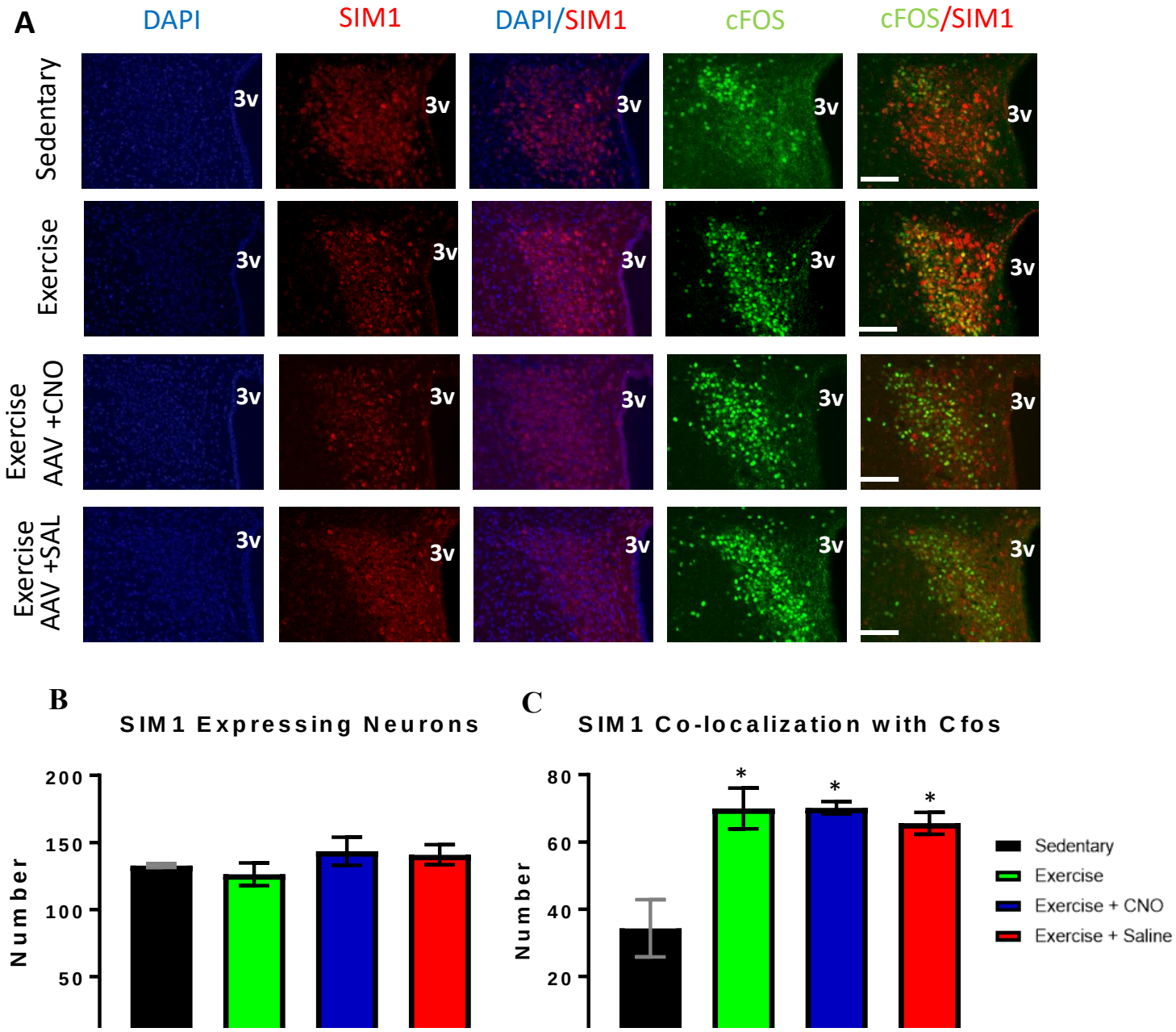

Fig 6. **(A)** Immunofluorescence of SIM1 and cFOS-positive cells in the PVN from the sedentary, exercise, exercise AAV + Saline, and AAV + CNO groups of male mice immediately after a bout of acute exercise **(B)** Quantification of SIM1-positive cells in the PVN among the 4 groups. **(C)** Quantification of SIM1-positive cells in the PVN among the 4 groups. 3V, third ventricle; scale bars represent 50 µM. Bar graphs show Mean + SEM. (N = 3 per group), * indicates $p < 0.05$ vs sedentary group.

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Appendix A

East Carolina University

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

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

October 18, 2016

Hu Huang, Ph.D.
Department of Kinesiology
ECDOI
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 #P085a) was reviewed by this institution's Animal Care and Use Committee on October 18, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle 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. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

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

SM/jd

Enclosure