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Neuromedin U Acts via the Paraventricular Nucleus of the Hypothalamus and the Dorsal Raphe Nucleus to Regulate Motivation for High-Fat Food

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Neuromedin U Acts via the Paraventricular Nucleus of the Hypothalamus and the Dorsal Raphe Nucleus to Regulate Motivation for High-Fat Food

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Motivation for high-fat food is thought to contribute to excess caloric intake in obese individuals. A novel regulator of motivation for food may be neuromedin U (NMU), a highly-conserved neuropeptide that influences food intake. Although these effects of NMU have primarily been attributed to signaling in the paraventricular nucleus of the hypothalamus (PVN), NMU has also been found in other brain regions involved in both feeding behavior and motivation. We investigate the effects of NMU on motivation for food and food intake, and identify the brain regions mediating these effects. The motivational state for a particular reinforcer (e.g., high-fat food) can be assessed using a progressive-ratio schedule of reinforcement under which an increasing number of lever presses are required to obtain subsequent reinforcers. Here, we used a progressive-ratio operant responding paradigm in combination with assessments of food intake, craving-like behavior, and locomotor activity to evaluate the effects of NMU in rats, and identify the brain regions mediating these effects. We found that peripheral administration of NMU decreases operant responding for high-fat food in rats. Evaluation of Fos-like immunoreactivity in response to peripheral NMU indicated the PVN and dorsal raphe nucleus (DRN) as sites of action for NMU. NMU infusion into either region mimics the effects of peripheral NMU on food intake and operant responding for food. Moreover, NMUR2 knockdown in the PVN inhibited the incubation of craving-like behavior, suggesting a complex role for NMU in regulating changes in motivation for food. NMU-containing projections from the lateral hypothalamus (LH) to the PVN and DRN were identified as an endogenous source of NMU; a pathway linking the PVN to the nucleus accumbens shell (NAcSh) was identified as a possible downstream mechanism regulating

food motivation, and characterized as potentially enkephalinergic. Together, these data suggest a LH-PVN-NAcSh and a LH-DRN pathway as mechanisms for regulating high-fat food craving and motivation through NMUR2.

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List of Abbreviations

CRH	Corticotropin Releasing Hormone
NMU	Neuromedin U
CNS	Central Nervous System
NMUR2	Neuromedin U Receptor 2
GPCR	G-Protein Coupled Receptor
PVN	Paraventricular Nucleus of the Hypothalamus
DRN	Dorsal Raphe Nucleus
NAcSh	Nucleus Accumbens Shell
VTA	Ventral Tegmental Area
PENK	Preproenkephalin
DREADD	Designer Receptor Exclusively Activated by Designer Drugs
FR	Fixed Ratio
PR	Progressive Ratio
PBS	Phosphate-Buffered Saline
BSA	Bovine Serum Albumin
DMSO	Dimethylsulfoxide
DAB	Diaminobenzidine
FACS	Fluorescence-Activated Cell Sorting

M1/M2	Motor Cortex
ARC	Arcuate Nucleus of the Hypothalamus
VMH	Ventromedial Hypothalamus
LH	Lateral Hypothalamus
IP	Intraperitoneal
A/P	Anterior/Posterior
M/L	Medial/Lateral
D/V	Dorsal/Ventral
aCSF	Artificial Cerebrospinal Fluid
ICV	Intracerebroventricular
GFP	Green Fluorescent Protein
ENK	Enkephalin
HC	Hippocampus
ANOVA	Analysis of Variance
PFC	Prefrontal Cortex
NMS	Neuromedin S
shCTRL	(animals) treated with AAV-2 expressing a scrambled control hairpin
shNMUR2	(animals) treated with AAV-2 expressing a short hairpin RNA against NMUR2
NAc	Nucleus Accumbens

DAMGO	D-Ala2, N-Me-Phe4, Glyol5-enkephalin
EBSS	Earle's Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PCR	Polymerase Chain Reaction
HM3D	AAV-2-CaMKII-HM3D-GFP
CNO	Clozapine-N-Oxide
HPA	Hypothalamic-Pituitary-Adrenal

Chapter 1: Introduction

1.1 Obesity Resulting from Maladaptive Consumption Behavior

Obesity is a serious health concern in the United States; affecting more than a third of adults[1]. It is comorbid with some of the leading causes of preventable death, including heart disease, stroke, and certain types of cancer [2].

Food intake is driven not only by caloric need but also by emotional need, particularly the desire for rewarding, or reinforcing food [3-5]. The reinforcement obtained by eating these foods increases the likelihood that they will be consumed again. Whereas historically this reinforcement system was effectively balanced by limited exposure, calorically dense foods are now readily available and hence overconsumed[6].

As foods high in fat are among the most potent at driving obesity and consumption of them is reinforcing [3, 4, 7, 8], examination of the neural circuitry mediating this response becomes vital. However, the neural circuitry regulating food reinforcement is little-studied, and poorly understood.

Current treatments suffer from low efficacy, significant negative side effects, or both, and generally fail to address the notion that motivation for food underlies excess caloric intake [5, 9-14]. As such, new targets are needed to treat this disorder. The consumption of foods, especially high-fat foods, evokes both homeostatic and hedonic responses; homeostatic regulation of food intake is dependent on intact hypothalamic circuitry while hedonic regulation of food intake is dependent on mesolimbic dopamine and serotonin pathways [15-18]. There are considerable challenges to developing effective therapeutics to target both homeostatic and hedonic aspects of food intake.

1.2 Different food types and their effects on consumption/reinforcement

The difficulties in separating out the homeostatic and hedonic aspects of feeding behavior are compounded by the differential effects of specific food types, or diets. Sucrose-rich diets, for example, have both satiating and reinforcing effects, and depend on a combination of hypothalamic and limbic circuitry in order to maintain the associated behaviors [19-24]. However, they do not lead to significant rates of obesity, and sucrose

itself is not considered obesogenic [7, 25]. High levels of dietary protein, similarly, do not lend themselves to the induction of obesity in either humans or rodent models of consumption; however, they display a distinct pattern of neuronal activation in areas of the brain associated with feeding (such as the hypothalamus) [3, 26]. For our purposes, we focus on the consumption of a high-fat diet, with 45% of kcal coming from fat. Both human and rodent studies demonstrate that such a diet is, in fact, obesogenic, with extended consumption leading to increased bodily obesity[27]. Importantly, high-fat diets are also highly reinforcing, activating regions of the brain associated with both feeding and behavioral reinforcement [28-30]. As such, diets rich in fat are preferentially consumed over those lower in fat, with individuals displaying significantly increased consumption as compared to eqicaloric amounts of sucrose-rich or protein-rich food [21, 31-33].

Exposure to, and long-term consumption of high-fat food introduces a positive feedback loop, whereby consumption of reinforcing high-fat food increases future consumption – up to a point [28, 29]. This effect is accentuated by the cumulative effects of obesity, which, in and of themselves, are an anxiogenic, and a mood suppressant, resulting in higher rates of depressive behaviors in animal models, and greater diagnosed and self-reported negative aspect in human patients[34]. This suppressant effect, in turn, leads to greater consumption of the anxiolytic and reinforcing high-fat foods, as it offers both a positive and a negative reinforcement. The high-fat food provokes a hedonic response, and must be consumed in ever-greater quantities to compensate for the negative effects of obesity; meanwhile, greater quantities of food are necessary to address the homeostatic requirements of maintaining the increased body mass [35-38].

1.3 Maladaptive Consumption is Driven by Central Mechanisms

As the reinforcing aspects of high-fat food result in combined homeostatic and hedonic dysregulation, it is important to recognize that this maladaptive consumption behavior it triggers is driven by CNS mechanisms. That is, the reinforcement feedback loop driving overconsumption of obesogenic food is mediated in great part by signaling within the brain [17, 39, 40]. Unfortunately, the neural circuitry regulating food

reinforcement is little-studied, and poorly understood. As such, new targets are needed to treat this disorder. The consumption of foods, especially high-fat foods, evokes both homeostatic and hedonic responses [15-18]. Homeostatic regulation of food intake is dependent on intact hypothalamic circuitry while hedonic regulation of food intake is dependent on mesolimbic dopamine and serotonin pathways [17, 29, 41-43]. Hypothalamic activity plays a key role in energy balance, as demonstrated by lesion studies, and there is considerable evidence linking dysregulation of hypothalamic circuitry to obesity [44-47]. In particular, the PVN is a key structure regulating obesity, through leptin, oxytocin, neuropeptide Y, melanocortin, and corticotropin releasing hormone (CRH) signaling[46, 48-56]. Enkephalin, in particular, acts via the PVN to stimulate consumption of a high-fat diet [21, 57].

Continued consumption of food containing high levels of fat can lead to obesity; these foods are considered to be obesogenic. Rats placed on diets of energy-dense, high-fat food display as much as a 44% increase in body fat over those on a standard diet, and therefore are used to model diet-induced obesity [13, 58]. However, the reinforcing aspects of these diets are relatively understudied, with the connections between feeding circuitry and reinforcement pathways receiving minor attention. Exposure to a high-fat diet has been shown to cause dysregulation of NAc dopamine signaling [4, 59-61]. Similar effects can be produced by dysregulation of hypothalamic enkephalin; furthermore, food reinforcement has been linked to the functionality of the NAc, and NAc-driven feeding on high-fat foods has been successfully blocked via hypothalamic inactivation [57, 62-69]. As such, the NAc also represents a vital structure for decreasing the reinforcing effects of obesogenic food.

1.4 Neuromedin U as a Regulator of Consumption and Reinforcement

A promising target for the regulation of motivation for high-fat food is neuromedin U (NMU), a neuropeptide that is widely distributed within the CNS. NMU binds NMU receptor 2 (NMUR2), a G-protein-coupled receptor (GPCR), resulting in decreases in food intake [70-74]. Knocking out NMU induces a hyperphagic and obese phenotype in mice, while overexpression of the peptide results in a hypophagic, lean

phenotype [75-77]. This is reinforced by a small number of genome-wide association studies, implicating both NMU and NMUR2 as potential regulators of food intake in humans [78, 79]. Interestingly, NMUR2 has been found within the paraventricular nucleus of the hypothalamus (PVN), a key feeding center[80], and its effects on feeding point toward a potential for regulating the hedonic aspects of consumption[32, 43, 81, 82]. Depletion of the receptor within the PVN not only produces the opposing effect – increased food intake and weight gain – but does so only for animals consuming a high-fat diet [32]. Moreover, this knockdown induces a greater preference for foods rich in dietary fat, in rodents [32], suggesting that PVN NMUR2 may play a role in human dietary preference and obesogenic food consumption.

Importantly, recent research has indicated that NMUR2 signaling may directly incorporate both traditional feeding and reinforcement circuitry. NMUR2 expression has been identified in pathways projecting from the dorsal raphe nucleus (DRN) to the nucleus accumbens shell (NAcSh), a well-established reinforcement circuit, where it regulates cocaine-evoked locomotion [82]. Similarly, a classical reinforcement pathway linking the ventral tegmental area (VTA) to the NAcSh was recently identified as NMU-driven, and capable of regulating the reinforcing effects of amphetamines[81], and the NAc has been shown to regulate alcohol reinforcement via NMU[43]. Here, we demonstrate the first evidence of NMUR2 signaling within a novel PVN-NAcSh pathway, that regulates the reinforcement value of a high-fat diet.

1.5 Experimental Summary of Specific Aims

This project targets three fundamental weaknesses in obesity research. First, current understanding – and treatment – of obesity is heavily symptom-focused, looking to reducing gross consumption or fat absorption without addressing the underlying mechanisms. As a result, it fails to alter the behaviors leading to overconsumption. Second, there is little investigation, or mechanistic understanding, of the reinforcing and craving components of obesogenic feeding behavior. As NMUR2 signaling may alter the incubation of craving-like behaviors, and reinforcement signaling, it represents a potential mechanism for understanding and potentially treating obesity. And finally,

there is a lack of understanding of precisely how NMUR2 is capable of regulating food reinforcement, and motivated behavior. Our study represents an effort to address these three issues in parallel, unified through a single novel pharmacotherapeutic target, NMUR2. We will link homeostatic food consumption studies with the motivation pathways driving the intake of obesogenic food.

Specific Aim 1. Demonstrate the role of NMUR2 signaling in models of motivation for food, and identify central sites mediating the motivational effects of NMUR2 signaling.

Recent findings in rats indicate that regulation of NMUR2 signaling in the PVN can alter preference for, and bingeing on, high-fat diets [32]. Our preliminary data suggest that systemic administration of NMU is capable of decreasing operant responding for a high-fat diet on a progressive ratio schedule of reinforcement. We will reinforce this data, identify central targets of NMU through immunohistochemical labeling and Fos-like immunoreactivity, and infuse NMU directly into these locations to demonstrate that the identified targets mediate the behavioral effects of NMU. We will also employ our AAV-RNAi methodology to knock down levels of NMUR2 specifically in the PVN, and examine the effects on motivation and craving for high-fat food using progressive ratio responding and cue reactivity. We hypothesize that NMU-NMUR2 signaling regulates motivation and craving for high-fat food, and that these effects are mediated by the PVN.

Specific Aim 2. Identify neuronal pathways connecting NMUR2-positive neurons in the PVN to the NAc.

Our preliminary data provide support for the existence of neuronal connections linking the PVN and the NAcSh. We hypothesize that NMUR2-positive neurons in the PVN project directly to the NAcSh. Within the PVN, preliminary data indicate that preproenkephalin (PENK) mRNA increases in response to NMU administration. Using neuronal tracers and immunohistochemistry, we will visualize the PVN-NAcSh pathway, and determine the expression of PENK and NMUR2 within PVN-NAcSh neurons. Further, we will use AAV vectors to express designer receptors exclusively activated by

designer drugs (DREADDs) in the PVN-NAcSh pathway, and ascertain the pathway's potential to mediate motivation for, and consumption of high-fat food. We hypothesize that NMUR2 neurons connecting the PVN and the NAcSh utilize enkephalin, and that the PVN-NAcSh pathway regulates the reinforcement value of high-fat food.

Chapter 2: General Experimental Methods

2.1 Animal Models

Male Sprague-Dawley rats (Harlan, Inc., Houston, TX) weighing 225-250 grams (at the start of the experiment) were used for all experiments. Rats were housed two to a cage, except when necessary to separate animals for feeding assays. Colony environment was maintained at 71^{o}F and 30-50% relative humidity, with lights-on between 06:00 and 18:00. All animals were allowed to habituate a minimum of seven days before testing, surgery, or treatment.

2.2 Surgical Procedures

2.2.1 Injection of a Viral Vector

Rats were anesthetized with an isoflurane vaporizer system (VetEquip, Pleasanton, CA) and secured on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A small midline incision was made in the shaved scalp in order to expose the skull and bilateral holes were drilled through the skull above the target sites.

5 µL syringes with 26 ga blunt-tipped needles (Hamilton Co., Reno, NV) were inserted bilaterally to the target coordinates, based on the methods of Benzon et al. 2014[32]. PVN coordinates were adjusted for a 10^{o} outside angle and were set at (A/P -0.18, M/L +0.15, D/V -0.82) from bregma. Injections were delivered at a rate of 0.2µL/30 seconds over a period of 5 minutes. Following injection, the needle remained in place for 3 minutes before removal. All incisions were stapled closed and post-operative care was administered following Benzon et al. 2014[32]. Animals were given 7 days to recover after surgery before training resumption, and a minimum of 14 days post-surgery before testing, to allow for full viral expression. Surgeries were performed between 09:00 and 17:00.

All viruses used are monosynaptic, infecting only single neurons rather than replicating and spreading.

2.2.2 Insertion of Guide Cannula(e)

Rats were anesthetized with an isoflurane vaporizer system (VetEquip, Pleasanton, CA) and secured on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A small midline incision was made in the shaved scalp in order to expose the skull and bilateral holes were drilled through the skull above the target sites.

Bilateral guide cannulae were implanted based on the methods of Kasper et al. 2016[82]. For animals receiving peptide infusions, PVN coordinates were adjusted for a 10^{o} outside angle and were set at (A/P -0.18, M/L +0.15, D/V -0.82) from bregma. DRN coordinates were adjusted for a 30^{o} outside angle, with internal cannula inserted at (A/P -0.71, M/L +0.32, D/V +0.83) from bregma.

For animals receiving colchicine treatment, a single guide cannula was implanted pointed to the lateral ventricle (A/P +0.14, M/L +0.23, D/V -0.54) from bregma. All incisions were stapled closed and post-operative care was administered following Benzon et al. 2014[32]. Surgeries were performed between 09:00 and 17:00.

2.3 Behavioral Assays

2.3.1 Food Intake

Rats were separated into individual home cages for assessment of food intake. Feeding was assessed separately for standard diet (Teklad Mouse/Rat Diet 7912, Harlan, Inc., Houston, TX), containing 17% energy from fat, and high-fat diet (Open Source Diets formula D12451, Research Diets Inc., New Brunswick, NJ, USA), containing 45% energy from fat. Shortly prior to dark cycle start (17:45), all food was removed from animal cages, though they were supplied with water *ad libitum*. At dark cycle start, (18:00), pre-weighed metal hoppers containing standard or high-fat diet were introduced into the animal cages.

Food weight was measured immediately before dark cycle start, and monitored for three consecutive 24-hour periods (18:00-18:00) following experimental start, with timepoints taken at 2, 4, and 24 hours following each dark cycle start. Food intake was calculated as change in food weight between two successive measurements in a given day and averaged across test days.

2.3.2 Operant Conditioning

Between 13:00 and 17:00, rats were placed in standard rat operant chambers (Med Associates, Georgia, VT) housed in ventilated sound attenuating cubicles with fans (Med-Associates, Inc.). Each chamber was equipped with two response levers, a food tray between the levers, and a house light opposite the levers. Responding on the lever associated with food delivery resulted in delivery of a high-fat food pellet (45% energy from fat, 45mg, Bioserv F06162, Frenchtown, NJ), and the blinking of a white cue light at 1 Hz for 5 seconds. A second, inactive lever was placed in the chamber to distinguish between activity-induced changes in response, and reinforcer-seeking behavior. Responses on the inactive lever were recorded, but no pellets were delivered for inactive lever presses.

2.3.2.1 Fixed Ratio Responding

Animals were taught to self-administer pellets of high-fat food in the aforementioned operant chambers. Self-administration training consisted of daily 30-minute sessions during which rats were trained to press the active lever on a fixed ratio (FR) schedule of reinforcement to obtain pellets of high-fat food. Rats were not food restricted prior to, or during operant conditioning, to decrease the probability of confounding effects. Sufficient responses on the active lever in order to receive a reinforcer depended on the test program, with one, three, or five active lever presses necessary per reinforcer delivery. Successful reinforcer delivery resulted in the deposition of a high-fat food pellet in the food tray, and the blinking of the cue light. Rats were initially trained on a FR1 schedule of reinforcement, where only a single active lever press was necessary for reinforcer delivery. This training was repeated daily until rats maintained at least 85% of total responses on the active lever, and at least 10 reinforcers were earned per session, over a three day period. Once the rats met this acquisition criterion, and demonstrated stable FR1 responding, they were moved to an FR3 schedule, where 3 active lever presses were required in order to receive a reinforcer. Following stability on the FR3, rats were moved to an FR5 schedule. Animals unable to maintain FR5 responding were excluded from further testing.

2.3.2.2 Progressive Ratio Responding

After animals reached criterion on the FR5, they were moved to a progressive ratio (PR) schedule, where earning each successive high-fat food pellet and cue delivery within the session requires a greater number of responses (1,2,3,6,9,12,15,20,25,32,40,50,62,77,95) on the active lever. Responses on the active, reinforced lever within a 60-minute test session were quantified, and compared to inactive, unreinforced lever responses, and total reinforcers delivered.

2.3.2.3 Cue Reactivity

Rats were placed in operant chambers and allowed to press on an FR1 schedule. Cue deliveries were replicated, including the light cue, the sound of the pellet dispenser, and the sound of the pellet dropping into the food tray. However, no pellets were delivered, regardless of responses. Cue reactivity tests were administered immediately before animals entered a 30-day forced abstinence period, during which rats were confined to their home cages, and were not exposed to the cues, the high-fat food pellets, or the operant chambers. Following the forced abstinence, a second cue reactivity test was administered.

2.3.3 Locomotor Activity

Locomotor activity was monitored and quantified using a modified open field system (San Diego Instruments, CA, USA). Clear Plexiglas chambers (40x40x40cm) were surrounded by a 4x4 photobeam matrix positioned 4cm from the floor of the chamber. Consecutive beam breaks within the central 16x16cm of the box were recorded as central ambulation. Peripheral beam breaks in the surrounding perimeter were recorded as peripheral ambulations. Central and Peripheral beam breaks were summed up to give a measure of total ambulation. Vertical activity was also recorded using a row of 16 photobeams positioned approximately 16cm above the chamber. Beam breaks in these beams were recorded as vertical activity. One-quarter inch of corncob bedding was placed on the floor of each chamber. Rats were habituated for one hour daily in the locomotor chambers, before locomotor activity assessments began. Locomotor activity

was assessed following treatment, and continued for 2 hours. . Locomotor testing was undertaken between 0900 and 1700.

2.4 Euthanasia

Rats were anesthetized with 5% inhaled isofluorane, before being injected with a solution composed of 0.2 mL ketamine (DOSE) and 0.3 mL xylazine (DOSE). Following induction of anesthesia, rats were transcardially perfused with ice cold 1X phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS.

2.5 Immunohistochemistry

Following euthanasia, brains were extracted, and allowed to post-fix for 24 hours in 4% paraformaldehyde in 1X PBS. Brains were then cryoprotected in 1X PBS, 20% glycerol, and 0.01% sodium azide, and sliced into 40μm sections.

2.5.1 Fos-like Immunoreactivity

To identify the central targets enabling NMU-mediated alterations in food intake and reinforcement, rats were given IP NMU and Fos-like immunoreactivity was examined in CNS sites associated with feeding, reinforcement, and NMUR2 mRNA expression. Rats were injected with either 0.3 mg NMU/kg, in a saline and 10% DMSO solution, or an equal volume of vehicle (total volume 3 mL), and euthanized two hours later. Euthanasia was performed between 13:00 and 15:30. Brains were extracted, cryoprotected and sliced into sections as previously described. Sections were analyzed for Fos expression using a protocol modified from Radulovic et al. 1998[83]. Briefly, sections were then washed twice, at 10 minutes per wash, in 0.1M PBS and incubated with fresh 0.3% H_2O_2 in 0.1M PBS for 30 minutes with shaking. Immediately afterwards, sections were washed a further 3 times in 0.1M PBS and placed in blocking solution (0.1M PBS, 0.1% BSA, 0.2% Triton X-100, 2% goat serum) for 60 minutes at room temperature with shaking.

Following blocking, sections were incubated with rabbit anti-Fos antibody (sc-52, Santa Cruz Biotechnology, Inc., Dallas, TX; 1:20 000) for two days at 4^{o}C. Once primary incubation was completed, sections underwent four successive ten-minute washes in

0.1M PBS, with shaking and were then incubated with biotin-SP-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA; 1:500) in blocking solution for two hours at room temperature. Another series of 4 ten-minute washes in 0.1M PBS was applied, during which time ABC solution (Vector Labs, Burlingame, CA) was prepared. Sections were incubated in ABC solution for 1 hour at room temperature and the series of washes was repeated.

Sections were incubated in diaminobenzidine (DAB, D5905-50TAB, Sigma-Aldrich, St. Louis, MO) solution for 8 minutes on ice, with shaking, and then 3 drops of 0.3% H_2O_2 were added to reveal staining. After 45 seconds of exposure, 0.1M PBS was added to stop the reaction. Sections were washed 4 times, for 10 minutes each, in 0.1M PBS, mounted on Superfrost microscope slides and allowed to dry for 2 hours. Slides were rehydrated in 0.1M PBS for 20 minutes, followed by ethanol dehydration and two 3-minute Citrisolv (Fisher Scientific) washes and were then coverslipped with DPX mounting medium (Fisher Scientific). Prepared slides were imaged on a DMI4000 Inverted TCS SPE confocal microscope (Leica Biosciences, Buffalo Grove, IL) Images were exported to ImageJ software and the number of Fos-like immunoreactive nuclei in the regions of interest was quantified by three experimenters blinded to the treatment conditions and averaged across experimenters to eliminate bias.

2.5.2 Immunofluorescence

Sections of brain were washed 3X in 1X PBS, 5 minutes/wash, to remove residual sodium azide, and incubated in 1% SDS for 5 minutes for antigen unmasking. Sections were washed 3X more in 1X PBS, 5 minutes/wash, and incubated for 1 hour in a blocking solution containing 0.3% Triton X-100 in 1X PBS and 3% of each serum appropriate to the secondary antibodies. Primary antibodies were diluted in blocking solution and incubated on brain slices overnight (20 hours) at room temperature.

Sections were washed 3X in 1X PBS, 5 minutes/wash, and then secondary antibodies were applied, and allowed to incubate at room temperature for 2 hours with shaking. Sections were washed 3 times, for 5 minutes each, in 1X PBS, mounted on Superfrost microscope slides and allowed to dry for a minimum of 2 hours. Slides were

rehydrated in 1X PBS for 20 minutes, followed by ethanol dehydration and two 3-minute Citrisolv (Fisher Scientific) washes and were then coverslipped with DPX mounting medium (Fisher Scientific). Prepared slides were imaged on a DMI4000 Inverted TCS SPE confocal microscope (Leica Biosciences, Buffalo Grove, IL) Images were exported to ImageJ software. Colocalization was performed with the JACoP plugin, through ImageJ, and fluorescence thresholds were applied for improved quantification and visualization of immunolabeling.

2.6 Flow Cytometry

Rats were anesthetized with 5% inhaled isofluorane, and decapitated. Structures of interest were isolated via microdissection. Briefly, isolated tissue was placed in Hibernate A to improve cell survival, then prepared using the Worthington Papain Dissociation System, with the incorporation of trehalose to improve cell stability and viability[84]. Flow cytometry was performed with a FACS Aria machine, through the UTMB Flow Cytometry and Cell Sorting Core Facility, and the assistance of Mark Griffin.

2.7 Real-Time Polymerase Chain Reaction

Following flow cytometry, mRNA was collected from each of the resulting cell populations. An Arcturus PicoPure kit, together with a Qiagen RNeasy Mini Kit, was used to extract and isolate mRNA, under RNase-free conditions, and mRNA quality was analyzed using a NanoDrop 2000 spectrophotometer. The mRNA was then converted into cDNA using the iScript cDNA synthesis kit. Equal concentrations of cDNA were used together with SYBR Green Master Mix, and gene-specific forward and reverse primers to create triplicate reactions in a 96-well PCR plate, and reaction volume was brought up to 20 uL with RNase-free water. Samples were run on an Applied Biosystems 7500 Fast Real-Time PCR system, and resulting experimental data were normalized to housekeeping genes, and fold change from baseline was determined.

2.8 Viral Vectors

Hairpin RNA were previously designed and validated as targeting NMUR2 rat mRNA. [32] Briefly, a 24-nucleotide sequence, was identified within the mRNA

sequence (Ensembl transcript ID ENSRNOT00000018967) using predefined search algorithms, and synthesized (Sigma–Aldrich, Saint Louis, MO, USA) as described previously. A pAAV-shRNA and a plasmid already containing the control hairpin, shCTRL were donated by Dr. Ralph DiLeone, PhD. The hairpin vector co-expressed eGFP under the control of an RNA Polymerase II promoter and terminator. The hairpin oligonucleotides were cloned into the pAAV shRNA plasmid, and RNAi viruses were produced through a triple-transfection, helper-free method and purified as previously described.

2.9 Data Analysis

Data were analyzed using Graphpad Prism statistical software (Graphpad Prism 7, San Diego, CA, USA).

Chapter 3: Regulation of Motivation for Food by Neuromedin U in the Paraventricular Nucleus and the Dorsal Raphe Nucleus

3.1 Introduction

It has been established that NMU signaling plays a key role in feeding behavior, and may play a role in motivational disorders[43, 70, 73, 80-82]. However, the central targets of NMU have not been fully characterized, and its direct effects on motivation for food remain unstudied. To that end, behavioral, viral, and immunohistochemical techniques were used in order to establish the effects of peripheral NMU administration on food consumption and motivation, to identify the CNS structures on which NMU acts, and confirm their significance as direct mediators of NMU-driven behaviors. Activation of NMUR2, through ICV or intra-PVN administration of NMU, downregulates food intake, leading to decreased body weight in animal models[70, 72, 73, 80]. In turn, knockdown of the receptor within the PVN has been shown to increase food intake, weight gain, and preference for high-fat foods[32]. The ability to alter this preference for highly reinforcing, or motivating foods, in particular, is suggestive of a potential role of NMU signaling in the regulation of motivation for food.

NMU has been primarily studied within the PVN, where NMU mRNA is highly expressed[72, 85]. NMU-NMUR2 signaling in the PVN, in particular, has been implicated in the activation of the hypothalamic-pituitary-adrenal axis[86, 87]. Both acute and chronic regulation of the associated pathways have been shown to alter food intake, and motivation for food[88]. Moreover, not only does NMU decrease food intake, but decreased food intake has been shown to increase CNS expression of NMU[89]. As such, NMU represents a demonstrable homeostatic control for feeding, with potential hedonic regulatory effects, though the hedonic modulation has yet to be confirmed. NMU mRNA has been found in a wide range of regions, including the motor cortex (M1/M2), and the arcuate nucleus (ARC)[90]. This suggests that, while previous studies may have identified an element of NMU signaling with regards to feeding, further understanding of the regions and pathways involved is essential to interpreting the behavioral effects of NMU.

To that end, we set out to identify and characterize the behavioral phenotype produced by NMU administration, and the central targets of this treatment. Peripheral NMU, administered with dimethylsulfoxide (DMSO) to promote brain access, decreases lever pressing for high-fat food pellets on a progressive ratio schedule of reinforcement, a model of motivation. Furthermore, we show that peripheral NMU induces changes in Fos-like immunoreactivity in both feeding and reinforcement-associated brain regions. We present neuroanatomical data linking NMU modulation of standard and high-fat food intake with specific brain regions, and show that food reinforcement is regulated by administration of NMU into the PVN and dorsal raphe nucleus (DRN). Finally, immunohistochemical studies demonstrate that these regions are endogenously innervated by NMU-positive projections from the lateral hypothalamus (LH), a region known to be involved in both high-fat food consumption and reinforcement.

3.2 Specific Methods

3.2.1 Animal Subjects

Male Sprague-Dawley rats (N=96; Harlan, Inc., Houston, TX) weighing 225-250 grams (at the start of the experiment) were used for all experiments. Rats were housed two to a cage, except when necessary to separate animals for feeding assays. Colony environment was maintained at 71^{o}F and 30-50% relative humidity, with lights-on between 06:00 and 18:00. All animals were allowed to habituate a minimum of seven days before testing, surgery, or treatment.

At the conclusion of the studies, animals were euthanized with 5% isoflurane and transcardially perfused for 5 minutes with 1X PBS (75mL), followed by 15 minutes of 4% paraformaldehyde (225mL). Brains were immediately extracted, post-fixed overnight in 4% paraformaldehyde in 1X PBS, and cryoprotected in a solution of 20% glycerol and 0.01% sodium azide in 1X PBS at 4^{o}C for a minimum of 24 hours. After cryoprotection, brains were frozen on dry ice and sliced into 40μm coronal sections using a microtome (SM 2010R, Leica Biosystems, Buffalo Grove, IL). Slices were stored in 0.01% sodium azide in 1X PBS at 4^{o}C until use. Targeting for injections and cannula placement were assessed by examining native fluorescence under a confocal microscope (DMI4000 Inverted TCS SPE confocal microscope, Leica Biosystems). Brains in which one or more

cannulae (n=6) or injections (n=10) were off-target were excluded from further analysis, and the corresponding animals' data was not considered.

Experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals[91]* and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

3.2.2 Feeding and Peripheral NMU Administration

Rats were separated into individual home cages for assessment of food intake. Feeding was assessed separately for standard diet (Teklad Mouse/Rat Diet 7912, Harlan, Inc., Houston, TX), containing 17% energy from fat, and high-fat diet (Open Source Diets formula D12451, Research Diets Inc., New Brunswick, NJ, USA), containing 45% energy from fat. All rats were weighed prior to the onset of the dark cycle for calculation of NMU dosage. Animals received a single intraperitoneal (IP) injection of NMU (0.3 mg/kg; 046-39, Phoenix Pharmaceuticals, Inc., Burlingame, CA) in saline with 10% dimethylsulfoxide (DMSO) or vehicle alone, fifteen minutes prior to dark cycle start (17:45) on each test day (total volume 3 mL).

Food weight was measured immediately before dark cycle start, and monitored for three consecutive 24-hour periods (18:00-18:00). Food intake was calculated as change in food weight between two successive measurements and averaged across test days.

3.2.3 Operant Conditioning and Peripheral NMU Administration

Between 13:00 and 17:00, rats were placed in standard rat operant chambers (Med Associates, Georgia, VT). Each chamber contained two retractable levers, with a cue light placed above each lever and a food tray situated between the levers. A house light was illuminated throughout the testing session. Responding on the lever associated with food delivery resulted in delivery of a high-fat food pellet (45% energy from fat, 45 mg, Bioserv F06162, Frenchtown, NJ). Rats were first trained in half-hour sessions on a fixed ratio (FR) 1 schedule, where a single response on the correct lever is needed to receive a pellet. A response on the incorrect lever triggers a 10 second timeout (during which responses on both levers are recorded, but do not count towards food pellet acquisition).

Once the percentage of responses on the correct lever exceeded 85% for three consecutive days, animals were advanced to an FR 3 and then an FR5 schedule, which require 3 and 5 correct responses for pellet delivery, respectively. Once animals have reached this criterion on the FR 5, they move to a progressive ratio (PR) schedule, where earning each successive high-fat food pellet within the session requires a greater number of responses (1,2,3,6,12,15,20,25,32,40,50,62,77,95). Correct responses – that is, responses on the reinforced lever – were quantified, along with the total number of pellets earned within a 60-minute test session. On test day, animals received IP injection of 0.3 mg/kg NMU in DMSO, or vehicle alone fifteen minutes prior to testing (total volume 3 mL). Following the operant test, with NMU treatment on a PR schedule, animals were returned to their home cages for a 30-day abstinence period, during which they were not given access to the operant chambers, or high-fat food. At the end of the abstinence period, rats were again dosed with NMU (0.3 mg/kg in DMSO, IP) 15 minutes prior to being returned to the operant chambers. In the chambers, rats were given 60 minutes to lever-press; however, active lever presses only delivered the cues previously associated with high-fat food delivery, in order to examine craving behavior.

3.2.4 Analysis of Fos-like immunoreactivity

To identify the central targets enabling NMU-mediated alterations in food intake and reinforcement, rats were given IP NMU and Fos-like immunoreactivity was examined in CNS sites associated with feeding, reinforcement, and NMUR2 mRNA expression. Rats were injected with either 0.3 mg NMU/kg, in a saline and 10% DMSO solution, or an equal volume of vehicle (total volume 3 mL), and euthanized two hours later. Euthanasia was performed between 13:00 and 15:30. Brains were extracted, cryoprotected and sliced into sections as previously described. Sections were analyzed for Fos expression using a protocol modified from Radulovic et al. 1998[83]. Briefly, sections were then washed twice, at 10 minutes per wash, in 0.1M PBS and incubated with fresh 0.3% H_2O_2 in 0.1M PBS for 30 minutes with shaking. Immediately afterwards, sections were washed a further 3 times in 0.1M PBS and placed in blocking solution (0.1M PBS, 0.1% BSA, 0.2% Triton X-100, 2% goat serum) for 60 minutes at room temperature with shaking.

Following blocking, sections were incubated with rabbit anti-Fos antibody (sc-52, Santa Cruz Biotechnology, Inc., Dallas, TX; 1:20 000) for two days at 4^{o}C. Once primary incubation was completed, sections underwent four successive ten-minute washes in 0.1M PBS, with shaking and were then incubated with biotin-SP-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA; 1:500) in blocking solution for two hours at room temperature. Another series of 4 ten-minute washes in 0.1M PBS was applied, during which time ABC solution (Vector Labs, Burlingame, CA) was prepared. Sections were incubated in ABC solution for 1 hour at room temperature and the series of washes was repeated.

Sections were incubated in diaminobenzidine (DAB, D5905-50TAB, Sigma-Aldrich, St. Louis, MO) solution for 8 minutes on ice, with shaking, and then 3 drops of 0.3% H_2O_2 were added to reveal staining. After 45 seconds of exposure, 0.1M PBS was added to stop the reaction. Sections were washed 4 times, for 10 minutes each, in 0.1M PBS, mounted on Superfrost microscope slides and allowed to dry for 2 hours. Slides were rehydrated in 0.1M PBS for 20 minutes, followed by ethanol dehydration and two 3-minute Citrisolv (Fisher Scientific) washes and were then coverslipped with DPX mounting medium (Fisher Scientific). Prepared slides were imaged on a DMI4000 Inverted TCS SPE confocal microscope (Leica Biosciences, Buffalo Grove, IL) Images were exported to ImageJ software and the number of Fos-like immunoreactive nuclei in the regions of interest was quantified by three experimenters blinded to the treatment conditions and averaged across experimenters to eliminate bias.

3.2.5 Viral tracing and localization of NMUR2 and NMU

Regions demonstrating changes in Fos-like immunoreactivity were investigated to determine their potential to have reacted directly to the NMU treatment, and characterize endogenous sources of NMU for these regions. For viral tracing of signaling pathways, rats were anesthetized with an isoflurane vaporizer system (VetEquip, Pleasanton, CA) and secured on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A small midline incision was made in the shaved scalp in order to expose the skull and bilateral holes were drilled through the skull above the target sites.

Guide cannulae were implanted based on the methods of Kasper et al. 2016[82]. PVN coordinates were adjusted for a 10^{o} outside angle and were set at (A/P -0.18, M/L +0.15, D/V -0.82) from bregma; DRN coordinates were adjusted for a 30^{o} outside angle, with internal cannula inserted at (A/P -0.71, M/L +0.32, D/V +0.83) from bregma.

Animals received a guide cannula pointed to the lateral ventricle (A/P +0.14, M/L +0.23, D/V -0.54) from bregma. Additionally, each animal was given an interstitial injection of 2 μL of a replication-incompetent retrograde tracer, Rb-ΔG-B19-GFP into the PVN or the DRN, at above coordinates, at a rate of 0.2 μL/30 seconds over a period of 5 minutes. Following injection, the needle remained in place for 3 minutes before removal.

All incisions were stapled closed and post-operative care was administered following Benzon et al. 2014[32]. Animals were given 10 days to recover after surgery to allow for maximal viral expression. At this point, rats were given 75 μg colchicine in 1 μl artificial cerebrospinal fluid (aCSF), intracerebroventricularly (ICV), via the implanted guide cannula, in order to block axonal transport. Two days after colchicine administration, animals were euthanized and tissues were taken for immunohistochemistry as described above. Surgeries and euthanasia procedures were performed between 09:00 and 17:00.

Immunohistochemistry was performed as previously published[32]. Briefly, sections of brain were washed 3X in 1X PBS, 5 minutes/wash, to remove residual sodium azide, and incubated in 1% SDS for 5 minutes for antigen unmasking. Sections were washed 3X more in 1X PBS, 5 minutes/wash, and incubated for 1 hour in a blocking solution containing 3% normal donkey serum, 3% normal goat serum, and 0.3% Triton X-100 in 1X PBS. Primary antibodies against NMU (rabbit anti-NMU, 1672285, Thermo Scientific, Houston, TX; 1:100) and GFP (chicken anti-GFP, GFP-1020, Aves Labs, Tigard, OR; 1:1 000) were diluted in blocking solution and incubated on brain slices overnight (20 hours) at room temperature.

Sections were washed 3X in 1X PBS, 5 minutes/wash, and then secondary antibodies were applied. Fluorescent secondary antibodies, Alexa Fluor 568 goat anti-rabbit (A-11011, Invitrogen, Carlsbad, CA) and Alexa Fluor 488 donkey anti-chicken

(703-545-155, Jackson Immunoresearch, West Grove, PA) were used in 1X PBS at 1:200. For NMUR2 immunohistochemistry, no goat serum was used in the blocking, and the primary and secondary antibodies used were rabbit anti-NMUR2 (NBP1-02351, Novus Biologicals, Littleton, CO; 1:150) and Alexa Fluor 488 donkey anti-rabbit (NC0241229, Jackson Immunoresearch; 1:100) respectively. Slices were washed again, mounted, coverslipped and imaged as described above under Fos analysis. Colocalization was performed with JACoP software, through ImageJ, and fluorescence thresholds were applied for improved quantification and visualization of immunolabeling.

3.2.6 Feeding and Central NMU Administration

Having identified NMUR2-positive brain regions that showed altered Fos-like immunoreactivity following peripheral administration, we moved to central administration of the peptide to confirm that NMU-NMUR2 signaling in these regions mediates food intake and reinforcement. Following acclimation, rats received cannulae implantation surgery as described above in the "Viral Tracing and NMUR2 localization" section. In this instance, guide cannulae were implanted bilaterally targeting the PVN and unilaterally targeting the DRN at the previously stated coordinates. Animals were given a minimum of 7 days to recover after surgery before beginning behavioral experiments.

Feeding assays were performed as above, with the exception of NMU treatment. Rather than receiving peripheral NMU, animals received site-specific infusions of either aCSF or 0.3 nmol NMU per cannula in aCSF (total volume 2 µl per side, over five minutes), delivered via implanted guide cannula immediately prior to dark cycle start (18:00).

3.2.7 Operant conditioning and Central NMU Administration

Animals were trained to respond for high-fat food pellets in operant chambers, as described above in the "Operant Conditioning and Peripheral NMU Administration" section. After criterion was reached on PR responding (85% of responses on active lever), animals received cannulation surgeries targeting the PVN and DRN, as described in the previous section. Following surgical recovery, an additional week of operant training was administered to confirm that all animals returned to criterion following

surgery. As demonstrated with ICV-administered NMU prior to feeding by Wren et al. 2002[80], a dose of 0.3 nmol NMU reduces food intake. PR testing was performed as described above, with interstitial infusions replacing intraperitoneal injections, and infusions immediately preceding testing. Cannulated animals received 0.3 nmol NMU in aCSF, or vehicle (total volume 2µl per side, over five minutes) immediately prior to testing, followed by a 48-hour washout period prior to retesting. During this period, PR testing was performed to ensure the responsiveness of the animals had not been altered. Following the testing period, animals were euthanized, as described above and brains were examined to confirm cannula targeting.

3.3 Results

3.3.1 Dose selection of NMU

Peripheral doses were chosen based on Peier et al. 2011[92], which indicated 0.3mg/kg NMU as the lowest dose producing the changes in core body temperature which accompany NMU's anorectic effects. Interstitial doses of NMU were selected based on the work of Wren et al. 2002[80], which demonstrated a dose-effect of ICV NMU on feeding. As they describe, doses below 0.1 nmol do not produce a significant behavioral effect, and significant reductions in feeding are seen at 0.3 nmol and 1 nmol. In order to minimize animal usage, a single behaviorally relevant dose of 0.3 nmol was used, as previously identified.

3.3.2 Peripheral NMU training and acquisition

Previous work demonstrates that NMU is capable of regulating food intake. Here, we expand on this work, training rats to lever-press in operant chambers on PR schedules in order to understand the role of NMU on food reinforcement. After training and stabilization on each successive response schedule, performance was assessed to ensure that rats were learning the tasks. Acquisition rates for the operant tasks were monitored and compared across treatment groups. Average responding for NMU-treated and control animals was measured daily, with a minimum of three days (<15% day-to-day variability) required for behavioral patterns to be considered stable. No significant differences ($n=13\text{-}14$, $p>0.05$ by repeated-measures ANOVA) in acquisition or stable responding were identified on FR1, FR3, FR5 or PR schedules, either before or after

surgery (Figure 3.1, green data). Similarly, no effects were found in total reinforcements earned (Figure 3.1, blue data) or inactive lever presses during the training sessions (Figure 3.1, red data).

3.3.3 Behavioral effects of peripheral NMU administration

The effects of NMU signaling on total food consumption have been previously examined. These data demonstrate that intra-PVN and ICV NMU can regulate intake of a standard diet in rodents and that NMUR2 mediates preference for high-fat food. Here, we demonstrate that IP administration of NMU significantly decreases consumption of both a standard diet and a high-fat diet (Figure 3.2). NMU (0.3mg/kg) significantly reduced standard diet intake compared to vehicle treatment at the 2-hour timepoint ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=6 per group; Figure 3.2A). A similar significant effect of peripheral NMU (0.3mg/kg) was also observed upon high-fat diet intake vs. vehicle at the 2-hour timepoint ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=6 per group; Figure 3.2B). There are no significant differences of NMU treatment on either diet after 2 hours, presumably due to the short half-life of NMU[93]. No taste aversion was noted across test sessions, consistent with previous testing demonstrating that NMUR2 signaling does not affect sucrose preference.

Based on our previous work indicating that NMUR2 regulates preference for a high-fat diet[32], we investigated the effects of NMU on motivated behavior. To study NMU as a mediator of motivation for food, we used operant conditioning on a PR schedule. The PR schedule specifically quantifies reinforcement efficacy[94, 95]. Therefore, increased levels of responding on this schedule are associated with increased motivation for the high-fat food reinforcer. We found that the number of lever presses for pellets of high-fat food was significantly decreased by peripheral NMU (0.3mg/kg) treatment ($p<0.05$ by unpaired t-test, n=11 per group), as compared to vehicle (Figure 3.3A). However, the number of reinforcers earned in animals treated with peripheral NMU only trended towards significance ($p>0.05$ by unpaired t-test, n=11 per group), as compared to vehicle (Figure 3.3B). No significant effect was found on the number of inactive lever presses as a result of NMU treatment ($p>0.05$ by unpaired t-test, n=11 per

group), as compared to vehicle (Figure 3.3C). This suggests that NMU may suppress the motivation for high-fat food reinforcers.

Additionally, we sought to determine if NMU signaling is capable of regulating responsiveness to cues associated with high-fat food, in the absence of the food itself. This cue reactivity is associated with craving behavior, as seen in drugs of abuse[96, 97]. Animals underwent a 30-day forced abstinence period, during which they remained in their home cages, and were not exposed to high-fat food, before being returned to the operant chambers and allowed to press solely for the food-associated cues. We found that the number of lever presses for cues associated with high-fat food, in the absence of the food itself, was significantly decreased by peripheral NMU (0.3mg/kg) treatment ($p<0.05$ by unpaired t-test, n=11 per group), as compared to vehicle (Figure 3.4A). However, significance was not reached in the number of cue deliveries behaviors ($p>0.05$ by unpaired t-test, n=11 per group), making this data suggestive, but not conclusive regarding the ability of NMU to alter craving (Figure 3.4B). As expected, no significant effect was found on the number of inactive lever presses as a result of NMU treatment ($p>0.05$ by unpaired t-test, n=11 per group), as compared to vehicle (Figure 3.4C).

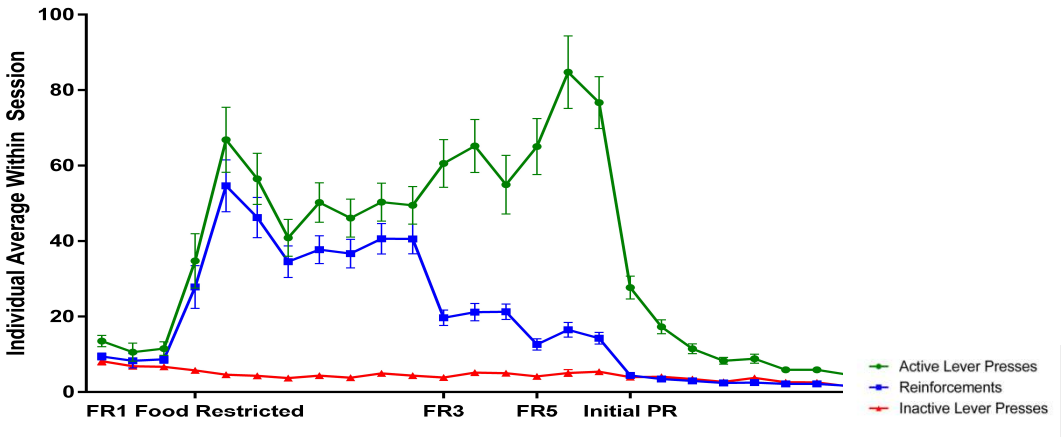

Figure 3.1. Acquisition of the operant task. Rats were introduced into the operant chambers, and allowed to lever-press for 45mg pellets of high-fat food, accompanied by the flashing of a cue light. Animals were not food restricted, to avoid hunger effects. All animals included in the study successfully learned the operant tasks.

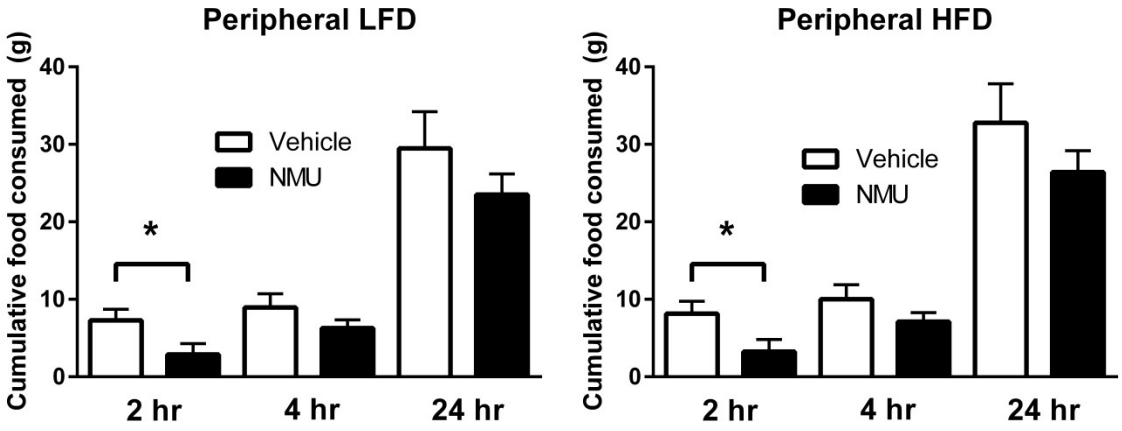

Figure 3.2. Effects of peripheral NMU administration on consumption of low- and high-fat diets. Rats were given 3 mg/kg NMU in 10% DMSO, IP, 15 minutes before the beginning of the dark cycle, and food consumption was monitored at 2, 4, and 24 hours post administration. NMU-treated animals show significantly decreased intake of both low- and high-fat food at the 2 hour timepoint, but this effect loses significance over the course of the dark cycle. $p<0.05$ by unpaired t-test, n=11 per group)

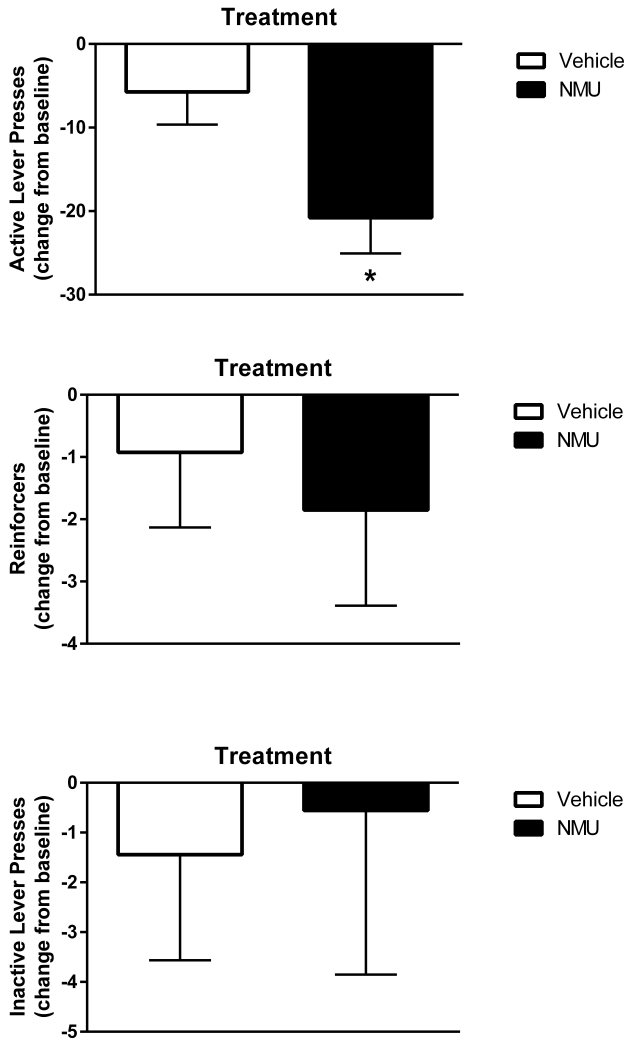

Figure 3.3. Peripheral NMU administration prior to progressive-ratio responding. Rats trained to respond on a progressive ratio were treated with 3 mg/kg NMU in 10% DMSO, IP, 15 minutes prior to introduction to the operant boxes. NMU treatment significantly decreases active lever-pressing for high-fat food($p<0.05$ by unpaired t-test, $n=11$ per group), but does not reach significance in altering reinforcers earned or inactive lever presses. Results were normalized to individual animal baselines.

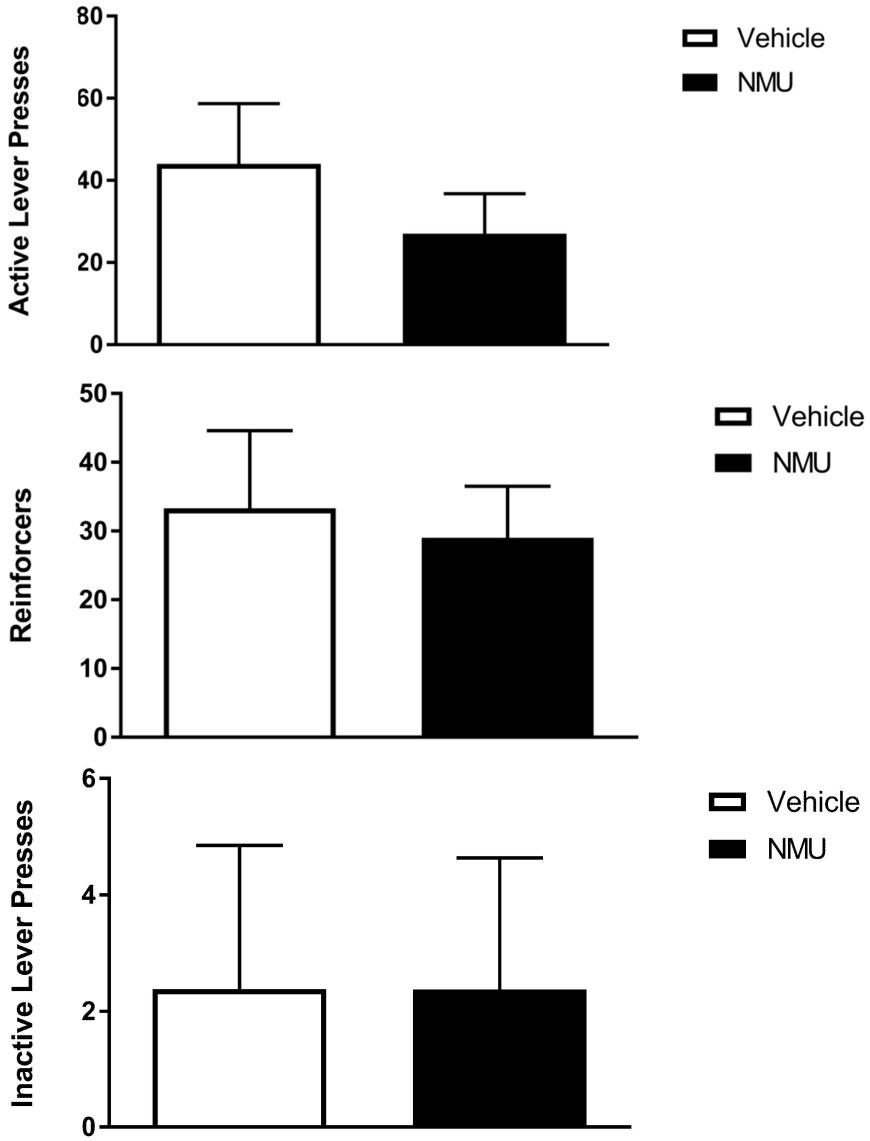

Figure 3.4. NMU treatment immediately following forced abstinence inhibits lever-pressing for cues associated with high-fat food. Following a 30-day forced abstinence period, rats were treated with 3 mg/kg NMU in 10% DMSO, IP, 15 minutes prior to introduction to the operant boxes. NMU treatment significantly decreases active lever-pressing for food cues ($p<0.05$ by unpaired t-test, n=11 per group), but does not reach significance in altering cue deliveries earned or inactive lever presses

3.3.4 Effects of NMU on Fos-like immunoreactivity

To identify candidate brain regions mediating the NMU-induced changes in behavior, we investigated changes in expression of Fos, an immediate early gene and indicator of neuronal activation[98, 99], following peripheral NMU treatment (Figure 3.5). While the majority of studies of NMU-NMUR2 in the CNS have focused solely on the PVN[72, 80, 87], we identified the DRN as another structure responding to peripheral NMU administration. Both the PVN (Figure 3.5A, top) and DRN (Figure 3.5A, bottom) displayed significantly lower Fos-like immunoreactivity following peripheral NMU (0.3mg/kg) treatment, compared to vehicle ($p<0.05$ by unpaired t-test, n=6 animals per group). Since the PVN and DRN displayed decreased Fos-like immunoreactivity over vehicle in response to peripheral NMU administration (Figure 3.5B), we further investigated these two brain regions as potential mediators of the effects of NMU on feeding and reinforcement. No significant treatment-dependent differences were noted in other feeding areas, such as the ventromedial hypothalamus (VMH) (Figure 3.6A, B, top row) or regions associated with memory, such as the hippocampus (HC) (Figure 3.6A, B, middle row). While significant increases in motor cortex (M1/M2) activity were found (Figure 3.6A, B, bottom row, $p<0.05$ by unpaired t-test), this is consistent with previous findings from ICV administration of NMU and has been characterized as an indirect behavioral output following activation of stress pathways[90]. As such, it was not further investigated as a direct site of action for NMU's effects on motivated consumption of food.

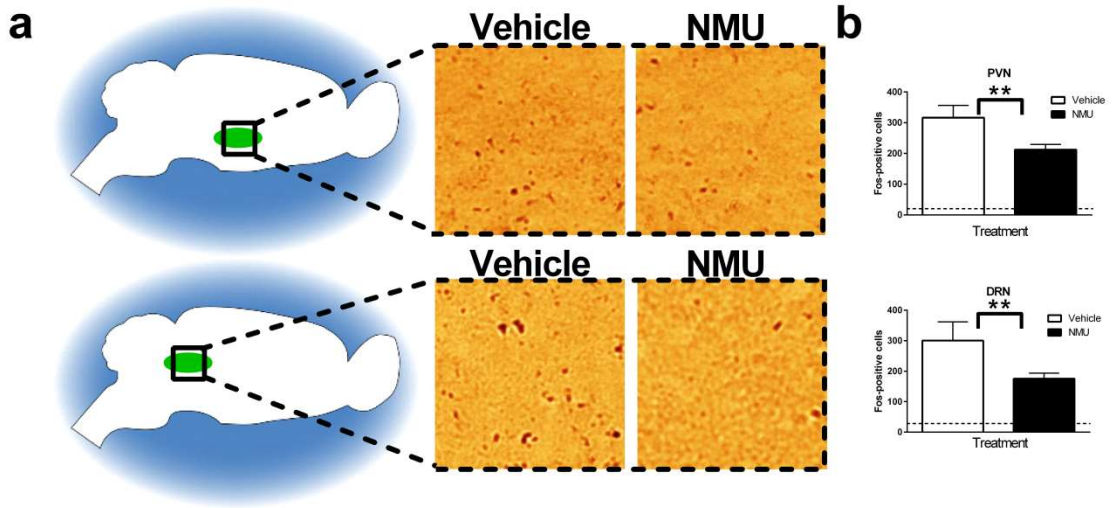

Figure 3.5. Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) and dorsal raphe nucleus (DRN) is significantly altered by peripheral Neuromedin U (NMU). (A) Representative images of vehicle-treated and NMU-treated PVN (top row) and DRN (bottom row). 20x magnification. **(B)** Significantly fewer Fos-positive cells are found in PVN and DRN of NMU-treated animals, as compared to vehicle-treated animals. (n=3 per group, **$p<0.01$ by unpaired t-test). Dashed line indicates Fos-like immunoreactivity in naïve tissue.

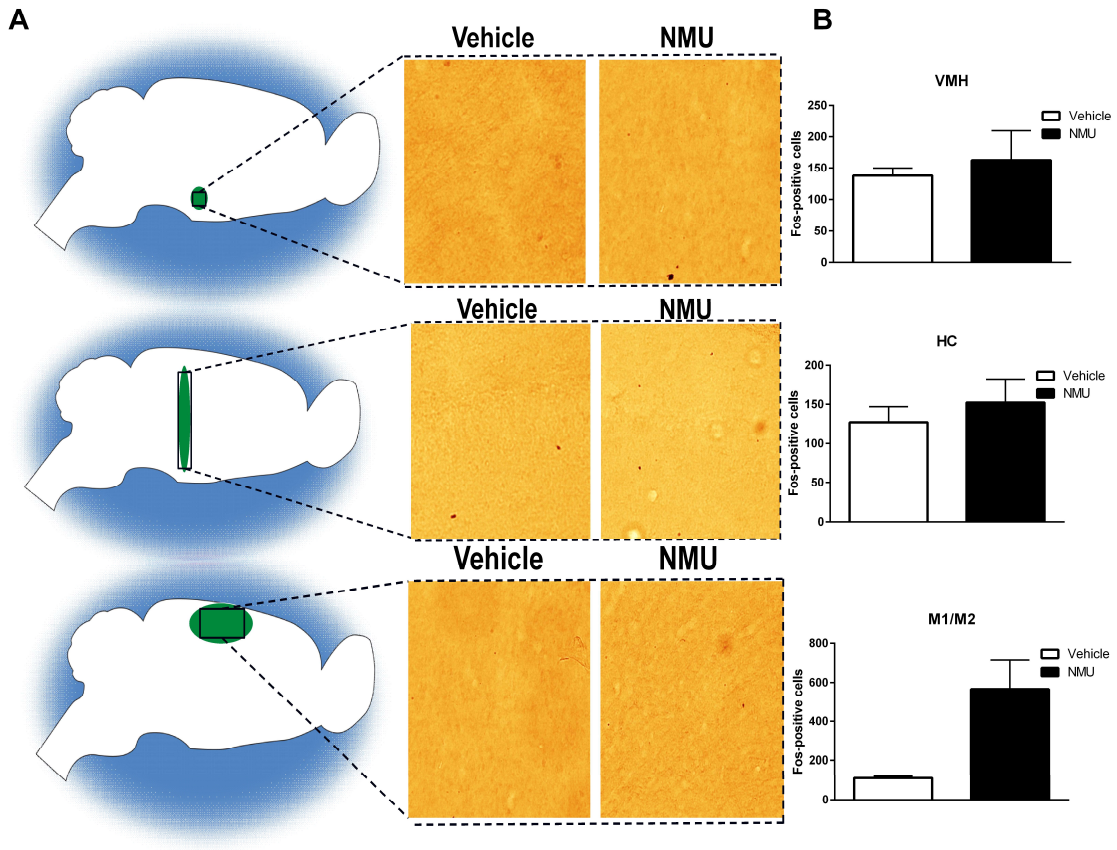

Figure 3.6. Fos-like immunoreactivity in the ventromedial hypothalamus (VMH), hippocampus (HC), and motor cortex (M1/M2). (A) Representative images of vehicle-treated and NMU-treated VMH (top row), HC (middle row) and M1/M2 (bottom row). 20x magnification. **(B)** No significant effects of peripheral NMU treatment are seen on Fos-like immunoreactivity in the VMH or HC. However, significantly more Fos-positive cells are found in the M1/M2 of NMU-treated animals, as compared to vehicle-treated animals. (n=3 per group, **p<0.01 by unpaired t-test).

3.3.5 Central NMU training and acquisition

We have demonstrated that peripheral NMU regulates food reinforcement, and identified several central targets of NMU. Here we confirm that animals treated centrally with NMU had no initial differences in learning or acquisition. After training and stabilization on each successive response schedule, performance was assessed to ensure that rats were learning the tasks. Acquisition rates for the operant tasks were monitored and compared across treatment groups. Average responding for NMU-treated and control animals was measured daily, with a minimum of three days (<15% day-to-day variability) required for behavioral patterns to be considered stable. No abnormalities in acquisition or stable responding were identified on FR1, FR3, FR5 or PR schedules, either before or after surgery, for animals receiving PVN cannulation (Figure 3.7, top, green line). Additionally, no disruptions were seen in total reinforcements earned (Figure 3.7, top, blue line) or inactive lever presses during the training sessions (Figure 3.7, top, red line). Similarly, animals receiving cannulation of the DRN display no abnormalities in acquisition of the operant task, or re-acquisition following surgery. Active lever-pressing was responsive to the assigned task (FR1, FR3, FR5, PR), and stabilized on each (Figure 3.7, bottom, green line). Corresponding patterns were found in reinforcements earned (Figure 3.7, bottom, blue line), displaying an understanding of the relationship between the task's required responses and the reinforcer delivery. Inactive lever presses during the training sessions (Figure 3.7, bottom, red line) remain minimal throughout the study, though slight, expected increases are seen upon the introduction of a novel, more difficult operant task.

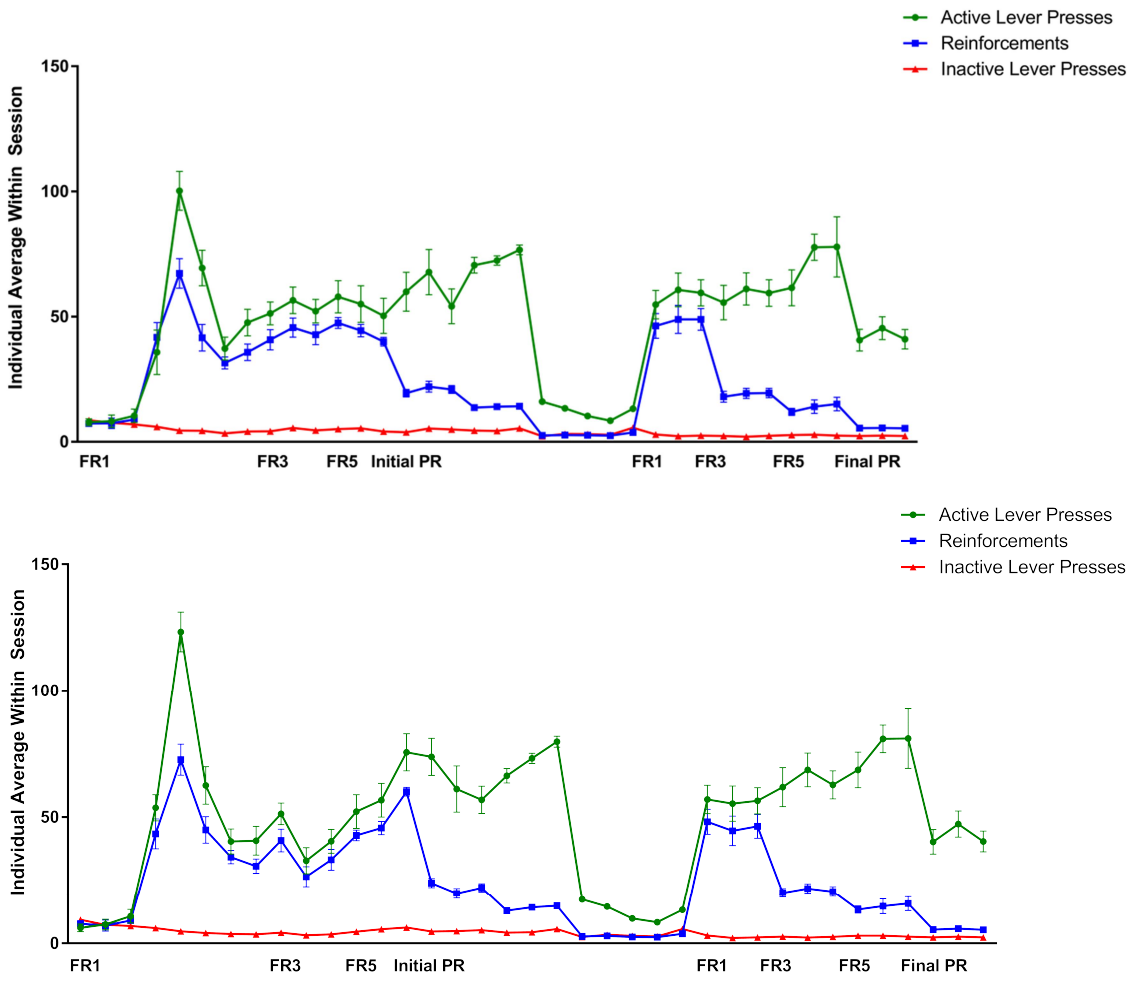

Figure 3.7. Acquisition of the operant task. Rats were introduced into the operant chambers, and allowed to lever-press for 45mg pellets of high-fat food, accompanied by the flashing of a cue light. Animals were not food restricted, to avoid hunger effects. All animals included in the study, including those receiving cannulation of the PVN (top) and cannulation of the DRN (bottom) successfully learned the operant tasks. Animals that did not survive the surgery (n=1), or did not recover fully (n=2) where stricken from the data.

3.3.6 Behavioral effects of centrally-administered NMU

Since peripherally administered NMU produces significant effects on total food consumption and operant behavior and reduces Fos expression in brain regions associated with NMU-NMUR2 signaling, our goal was to ascertain whether these brain regions specifically mediated the NMU-driven behavior. In agreement with the literature describing NMUR2 mRNA localization[90], NMUR2 expression was identified in both the PVN (Figure 3.8A) and DRN (Figure 3.8B). Additional NMUR2 immunofluorescence was detected in several other regions known to express NMUR2 mRNA, including the prefrontal cortex, ventral tegmental area, and nucleus accumbens.

Following training, surgery, and recovery (Figure 3.9A), animals receiving either intra-PVN (Figure 3.9B) or intra-DRN (Figure 3.9C) infusions of NMU (0.3mg/kg) were given access to food as described above and average consumption of standard and high-fat diet was measured at intervals over a 24-hour period. Intra-PVN NMU decreased standard diet intake at 2 and 4 hours post-treatment ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=5 per group) and high-fat diet intake at 2 and 4 hours post-treatment ($p<0.05$ and $p<0.01$, respectively, by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=5 per group), versus aCSF (Figure 3.10A, C). Intra-DRN NMU decreased standard diet intake at 2, 4, and 24 hours post-treatment ($p<0.01$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=8 per group) and high-fat diet intake at 2, 4, and 24 hours post-treatment ($p<0.01$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=7 per group) as compared to vehicle baseline (Figure 3.10B, D). In correspondence with the activity patterns identified in the Fos expression experiment (Figure 3.5), NMU administration into either the PVN or DRN was sufficient to induce a significant decrease in intake of a standard or high-fat diet.

Mirroring our peripheral administration studies, animals trained to lever-press for high-fat food pellets were treated with intra-PVN or intra-DRN infusions of NMU (0.3nmol/kg) or aCSF immediately prior to testing sessions. Intra-PVN NMU decreased PR responding for high-fat pellets, as compared to vehicle (Figure 3.11, left; $p<0.05$ by unpaired t-test, n=8 per group). Intra-DRN NMU administration caused a decrease in PR

responding, significantly greater than that produced by vehicle treatment ($p<0.05$ by unpaired t-test, n=8 per group) (Figure 3.11, right). This suggests that NMU acts directly via the PVN and DRN to regulate not only consumption of, but also motivation for high-fat food.

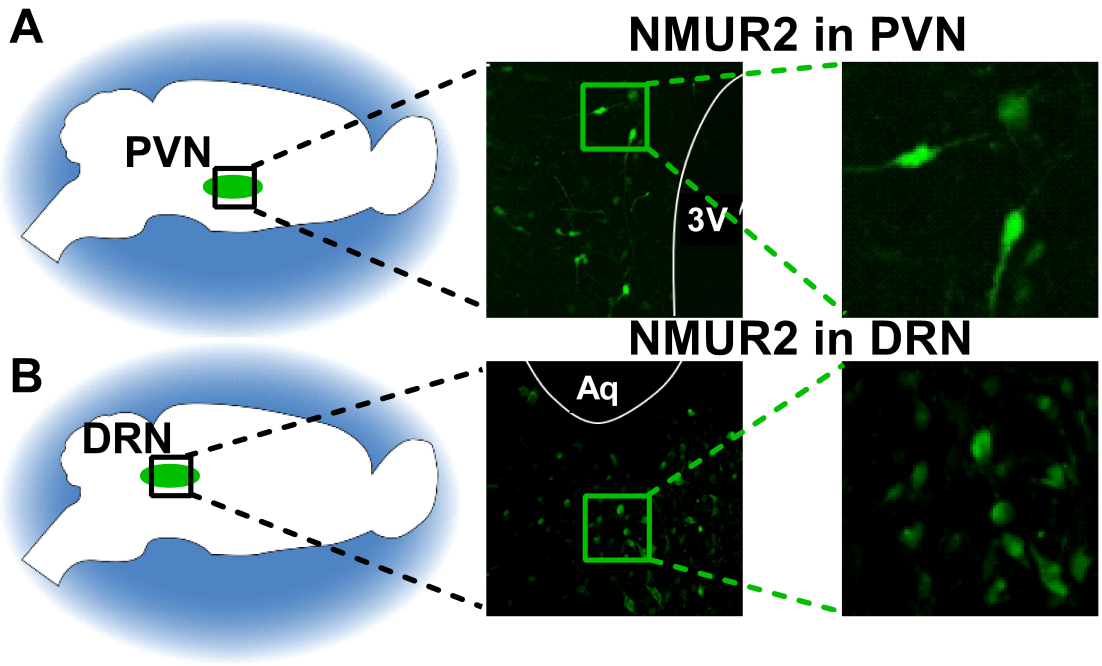

Figure 3.8. Localization of Neuromedin U Receptor 2 (NMUR2) in the paraventricular nucleus of the hypothalamus (PVN) and dorsal raphe nucleus (DRN). (A) NMUR2 is expressed in the PVN. (B) NMUR2 is expressed in the DRN. 3V indicates third ventricle; Aq indicates cerebral aqueduct. 20x magnification.

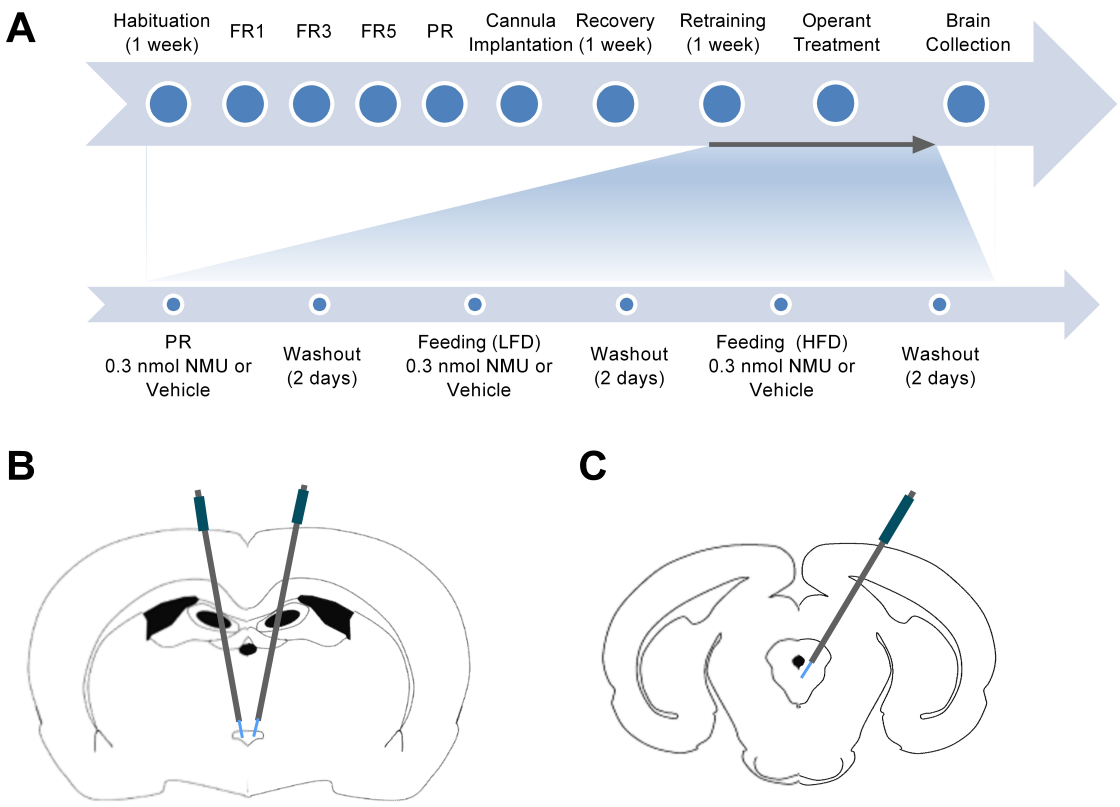

Figure 3.9. Timeline and targeting. (A) Timeline of experiments involving central Neuromedin U (NMU) administration. FR refers to fixed-ratio responding. PR refers to progressive-ratio responding. LFD and HFD refer to low-fat diet and high-fat diet, respectively. (B) Cannula targeting for the paraventricular nucleus of the hypothalamus (A/P -1.80 mm). (C) Cannula targeting for the dorsal raphe nucleus (A/P -7.10 mm).

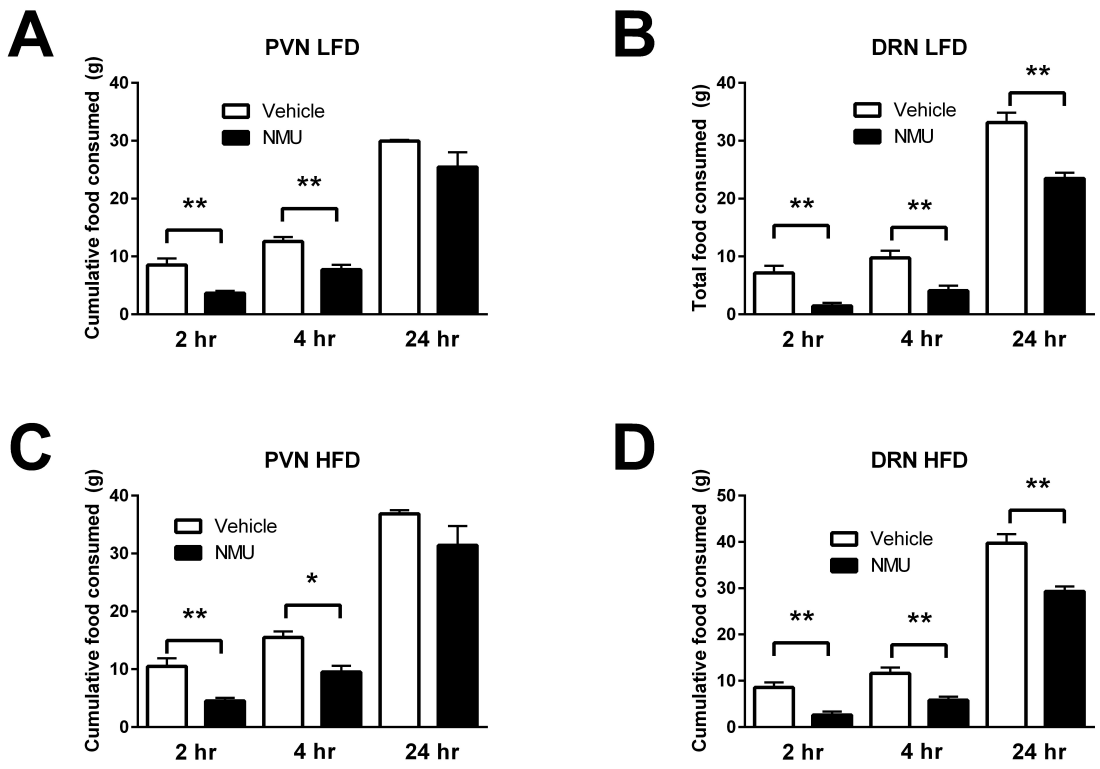

Figure 3.10. NMU administration into the PVN and DRN decreases intake of low- and high-fat food. (A) NMU infused into the PVN reduces LFD intake at 2 and 4 hours post-treatment (n=5), **p<0.01 by Sidak's multiple comparisons test). **(B)** NMU infused into the DRN reduces intake of a LFD at 2, 4, and 24 hours post-treatment (n=6, *p<0.01 by Sidak's multiple comparisons test). **(C)** NMU infused into the PVN reduces HFD intake at 2 and 4 hours post-treatment (n=5, *p<0.05, **p<0.01 by Sidak's multiple comparisons test). **(D)** Intra-DRN NMU reduces intake of a HFD at 2 hours, 4 hours, and 24 hours post-treatment (n=6, **p<0.01 by Sidak's multiple comparisons test).

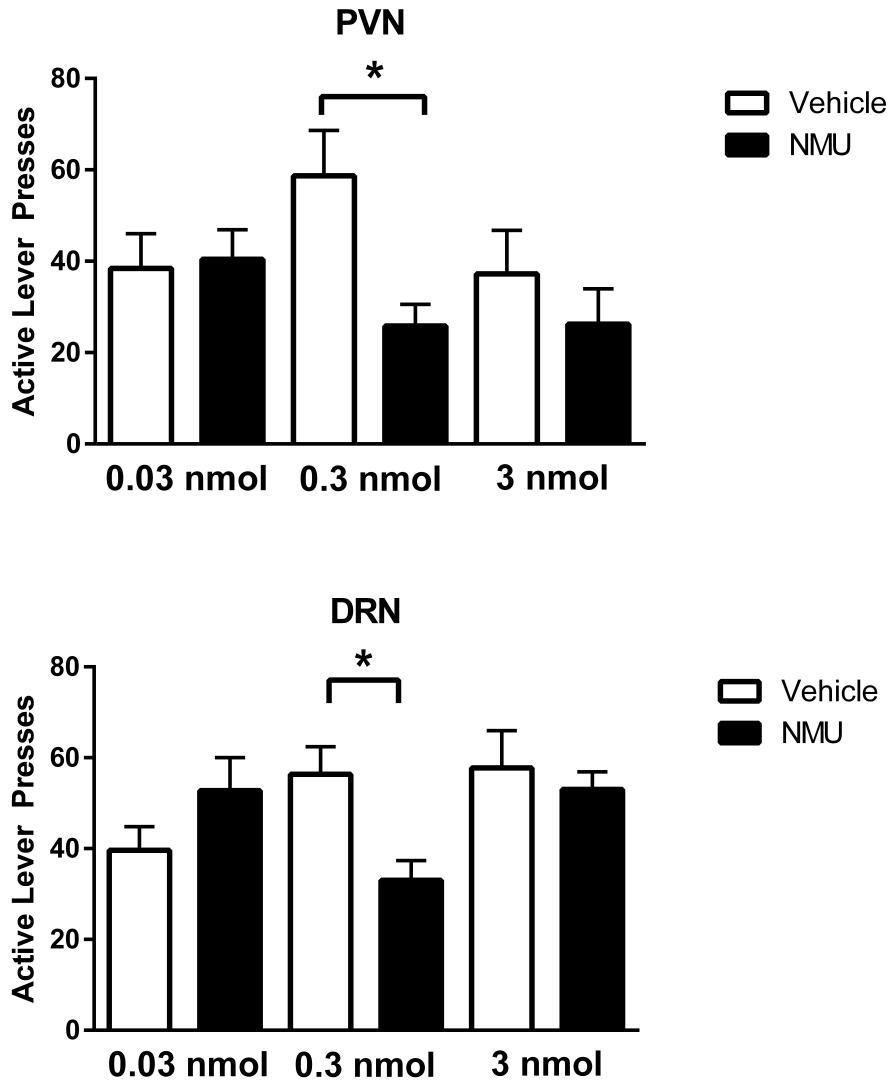

Figure 3.11. Infusions of NMU into the PVN or DRN dose-dependently decrease PR responding for high-fat food. (Top) 0.3 nmol NMU infused into the PVN decreases motivated responding for high-fat food (n=6, *p<0.05 by multiple-comparisons ANOVA). No significant effects are found at a dose of 0.03 nmol or 3 nmol. **(Bottom)** 0.3 nmol NMU infused into the DRN decreases motivated responding for high-fat food (n=8, **p<0.01 by multiple-comparisons ANOVA). 0.03 nmol treatment trends towards a significant increase in responding, and no significant effects are found at a dose of 3 nmol NMU.

3.3.7 NMU-containing neurons in the LH project to the PVN and DRN

While NMU in the PVN and DRN is behaviorally relevant, the source of NMU-containing neurons that innervate these brain regions has not yet been established. To explore this neurocircuitry, animals were treated with a combination of ICV colchicine and an attenuated, replication-incompetent rabies virus, Rb-ΔG-B19-GFP, targeted at the PVN (Figure 3.12A) or DRN (Figure 3.12B). Rb-ΔG-B19-GFP serves as a retrograde tracer by infecting via the presynaptic terminal and being transported in a retrograde direction thus labeling the cell body of the projection neurons. Colchicine blocks axonal transport resulting in an accumulation of NMU in the cell body. This combination allows for visualization of afferent pathways producing NMU. Analysis focused on regions known to project to the PVN and DRN. Fluorescent immunohistochemistry was used to enhance the native GFP signal expressed from the attenuated rabies virus (Figure 3.12A and 3.12, left column) and visualize NMU (Figure 3.12A and 3.12B, middle column) in the cell bodies of projection neurons. NMU immunoreactive cell bodies were observed in the LH, but not in the arcuate nucleus (ARC) or the prefrontal cortex (PFC) (Figure 3.12A and 3.12B). GFP-labeled cells were found in the LH and to a lesser extent, the ARC, following injection of the viral tracer into the PVN (Figure 3.12A, left column), and in the LH and PFC following injection of the viral tracer into the DRN (Figure 3.12B, left column). Colocalization of total NMU immuno-labeling (red) and GFP corresponding with the attenuated rabies virus (green) (Figure 3.12A and 3.12B, right column) was quantified using Image J (LH-PVN 25.32±5.10%, ARC-PVN 5.86±0.64%, PFC-DRN 15.21±1.60%, LH-DRN 4.70±1.75% colocalization). The data indicate that NMU-producing neurons project primarily from the LH to the PVN (Figure 3.12A) and the DRN (Figure 3.12B, n=3 animals/group).

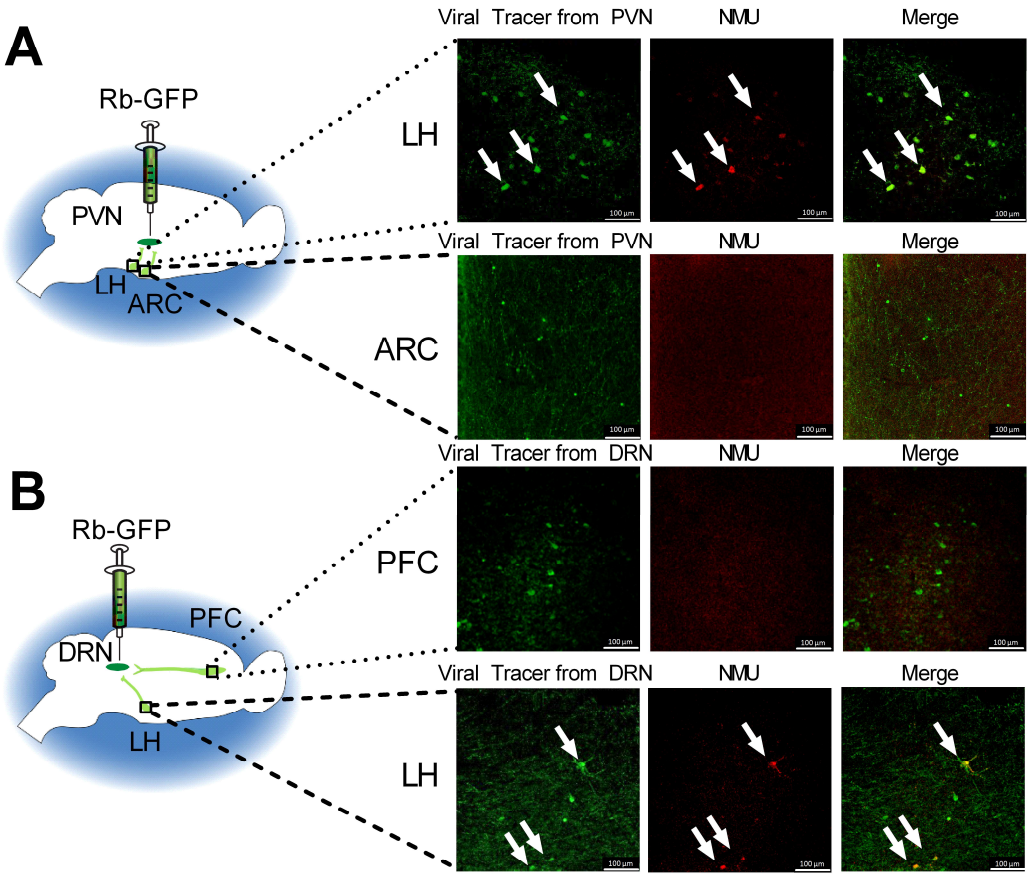

Figure 3.12. Neuromedin U (NMU)-containing neurons in the lateral hypothalamus (LH) project to the paraventricular nucleus of the hypothalamus (PVN) and dorsal raphe nucleus (DRN). (A) Attenuated, replication-incompetent Rb-ΔG-B19-GFP is injected into the PVN, and colchicine is introduced ICV following full viral expression. Minimal colocalization is seen in the ARC (A, bottom), while several NMU-positive neurons colocalize with the tracer in the LH (A, top). (B) Rb-ΔG-B19-GFP is injected into the DRN, and colchicine is introduced ICV following full viral expression. No noteworthy colocalization of NMU and tracer is identified in the PFC (B, top), while a number of neurons in the LH express both the tracer from the DRN, and NMU (B, bottom). 20x magnification.

3.4 Discussion

One of the understudied aspects of obesity is the motivation for high-fat foods. Such foods are powerful drivers of obesity that contribute to, maintain, and promote overeating[3-5, 7, 8]. This is due, in part, to the highly reinforcing properties of high-fat foods. Our work builds on previous data showing that NMUR2 signaling in the brain is capable of regulating preference for, and consumption of high-fat foods[32]. Consistent with previously published data [73, 80], we show that systemic NMU administration decreases food intake. Systemic NMU was co-administered with DMSO to ensure that NMU would cross the blood brain barrier. This allowed us to evaluate the expression patterns of Fos, an immediate early gene marker of neuronal activity[98, 99], and to identify potential anatomical targets in the brain for NMU binding. Two such regions, the PVN and DRN, demonstrate decreases in Fos-like immunoreactivity following NMU administration. Our immunohistochemistry demonstrates that the PVN and the DRN not only display decreased Fos in response to peripheral NMU, but also display high levels of NMUR2. This is consistent with previous studies of NMUR2 mRNA[100]; intriguingly, it also suggests that NMU may act through other mechanisms than classical stress signaling through both the PVN and DRN, such as inhibiting the signaling of orexigenic ghrelin neurons. Importantly, the presence of the receptor in Fos-responsive regions suggests that even peripherally administered NMU may act on the PVN and DRN to modulate food intake and reinforcement.

Direct infusion of NMU into the PVN supports previous research indicating that hypothalamic NMU suppresses food intake[80, 101], and extends these findings by demonstrating that PVN NMU signaling regulates motivation for high-fat food. Importantly, a connection has been implied between PVN–mediated feeding effects and the nucleus accumbens, a key structure for the regulation of reinforcement behavior[102]. Recent research has since confirmed the existence of a pathway linking the PVN to the nucleus accumbens, and demonstrates that the pathway is capable of regulating social aspects of reinforcement, suggesting a potential downstream mechanism by which the PVN may mediate food reinforcement.

In addition, we have expanded our understanding of the neuroanatomical substrates for NMU by identifying the DRN as a novel site of action for NMU-NMUR2 based regulation of food intake and motivation for food. Indeed, we find behavioral effects lasting more than 24 hours after NMU administration into the DRN. While the half-life of NMU in the CNS is unknown, studies of the peptide in the periphery support a half-life on the order of minutes, rather than hours [182]; this, in turn, suggests the induction of lasting alterations in signaling, as the neuropeptide itself is unlikely to maintain a significantly high concentration for such a period. While the role of the DRN on feeding is not fully elucidated, its regulation of reinforcement behavior via serotonin signaling has been reported [103-105], and recent research demonstrates that food reinforcement activates serotonin neurons within the DRN[106]. As ICV NMU has been demonstrated to alter serotonin expression in the brain [85, 107], and the behavioral effects of NMU are at least partially regulated by serotonin receptor function[108], NMU may regulate food reinforcement via serotonin signaling downstream of the DRN.

Pathways linking the LH and PVN, as well as the LH and DRN, have been unexplored with regards to NMU regulation of reinforcement. The LH has, however, been investigated in both non-food, and food reinforcement, and projections to the PVN and DRN have been characterized [109-111]. The production of NMU by these projections, and the observed downstream regulation of feeding and food reinforcement by NMU release, is consistent with the alterations of NMU mRNA in response to energy balance. This suggests that endogenous regulation of the feeding and food reinforcement behaviors identified here may be driven by NMU-producing LH-PVN and LH-DRN neurons. There is also potential for alternative ligands driving endogenous NMUR2 signaling in the PVN; neuromedin S (NMS) has also been shown to bind NMUR2 in the PVN, producing anorectic effects [86, 112]. However, NMS has not been identified in the DRN, or implicated in raphe-dependent regulation of feeding behavior.

Taken together, these data indicate that NMU-NMUR2 signaling in the PVN and DRN assists in regulating consumption of, and motivation for high-fat food, a key element in the development of, or resistance to, obesity. These studies highlight the emerging role of NMU signaling in reinforcement, elucidate the neurocircuitry mediating

its behavioral effects, and identify an endogenous source of the peptide. The combined result is the identification of specific NMU-NMUR2 signaling pathways as mediators of motivation for high-fat food. Motivated consumption of high-fat foods is a potent driver of obesity in both humans and animals, thus dysregulated NMU signaling pathways may underlie overconsumption of obesogenic food.

Chapter 4: Incubation of Craving-like Behaviors for High-Fat Food is Regulated by Neuromedin U Receptor 2 in the Paraventricular Hypothalamus

4.1 Introduction

Obesity is a serious health concern in the United States; affecting more than a third of adults[1]. It is comorbid with some of the leading causes of preventable death, including heart disease, stroke, and certain types of cancer [2]. As such, it is essential to understand the mechanisms underlying obesity. However, mechanistic understanding based on traditional notions of simply regulating total food intake has resulted in consistently low-efficacy treatments, and often significant negative side effects [3-9].

Two key factors have traditionally been disregarded, and are now rising into prominence: dietary composition, and motivation. First, that our conception of the development of obesity must be rooted in obesogenic food, not generalized consumption. Recent studies have demonstrated that consistent consumption of a low-fat diet, even to the point of satiety, is insufficient to induce an obese phenotype [10]. Sucrose-rich diets, for example, do not lead to significant rates of obesity, and sucrose itself is not considered obesogenic [11, 12]. High levels of dietary protein, similarly, do not lend themselves to the induction of obesity in either humans or rodent models of consumption; however, they display a distinct pattern of neuronal activation in areas of the brain associated with feeding (such as the hypothalamus) [13, 14]. For our purposes, we focus on the consumption of a high-fat diet, with 45% of kcal coming from fat. Studies demonstrate that such a diet is, in fact, obesogenic, with extended consumption leading to increased bodily obesity[15]. Importantly, high-fat diets are highly reinforcing, activating regions of the brain associated with both feeding and behavioral reinforcement [16-18]. As such, diets rich in fat are preferentially consumed over those lower in fat, with individuals displaying significantly increased consumption as compared to eqicaloric amounts of sucrose-rich or protein-rich food [10, 19-21].

Second, we must explore the motivation for intake, the fact that high-fat food consumption is self-reinforcing, leading to a "craving-like" phenotype where individuals have an increased propensity to seek out and consume high-fat food. Human studies have demonstrated that motivation for high-fat (or other high-fat) food underlies excess caloric

intake, and is a key factor in the maintenance of obesity[11, 14, 22, 23], Animal models of high-fat food intake demonstrate an increased preference for the food over a lower-fat diet, and show patterns of neuronal activation associated with high-fat food "craving," or the motivation to respond in an attempt to acquire the food [10, 14, 23]. Importantly, this craving-like behavior, similar to that seen in dieting humans, has been shown to incubate during periods of abstinence, resulting in an increase in craving behaviors following the abstinence period, and an increased probability of relapse [24]. Individuals remain abstinent from high-fat foods for extended periods in an effort to exert behavioral self-control, inhibit disordered eating patterns, and lose body weight [25-29]. However, both human and animal models of craving show that reinforcement seeking is elevated after extended abstinence periods, with longer abstinence increasing the strength of the seeking behavior [30]. This aspect of craving behavior is particularly well-studied in the context of drugs of abuse. Brain structures and signaling mechanisms involved in reinforcement and drug-craving overlap heavily with those known to play a role in feeding behavior [31-33]. However, food reinforcement and the incubation of craving-like behaviors remain understudied as mechanisms of overeating and dietary noncompliance.

As such, it is essential to examine the signaling mechanisms mediating motivated consumption of high-fat food, in order to understand the craving behaviors underlying its overconsumption. The paraventricular nucleus of the hypothalamus (PVN) has been heavily studied for its role in food intake and body weight [19, 32, 34-39]). However, the role of the PVN in regulating craving for food is poorly understood. Previous research has identified a PVN-localized G protein-coupled receptor, NMUR2, as a candidate for the regulation of responses to food-associated cues and the incubation of craving-like behaviors [40-42]. PVN NMUR2 has been established as a regulator of food intake via binding of the anorectic peptide neuromedin U (NMU). Dysregulation of this receptor has also been shown to produce increases in food intake [43-47]. Perhaps most importantly, NMUR2 has also been implicated in regulating the preference for high-fat food, strengthening its role as a driver of high-fat food intake [10]. Moreover, central NMU administration has been shown to regulate the reinforcement value of both alcohol and high-fat foods, with NMU decreasing intake of both [40, 41]. Though NMU has been linked to reinforcement, and NMUR2 has been tied to changes in body weight and food

preference, the ability of NMUR2 to modulate food-associated cues and abstinence-induced changes in food craving are not known [10, 40, 41].

In this study we make use of a progressive-ratio operant response paradigm for high-fat food, combined with forced abstinence to induce craving behavior, to investigate the effects of PVN-specific depletion of NMUR2 on abstinence-induced increases in craving for high-fat food in rats.

4.2 Specific Methods

4.2.1 Animal Subjects

Male Sprague-Dawley rats (n=28; Harlan, Inc., Houston, TX) weighing 225-250 grams (at the start of the experiment) were used for all experiments. Colony environment was maintained at 71^{o}F and 30-50% relative humidity, with lights-on between 06:00 and 18:00. Experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals[91]* and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

4.2.2 Operant Conditioning

Operant conditioning was performed as described previously[141]. Briefly, between 13:00 and 17:00, rats were placed in standard rat operant chambers (Med Associates, Georgia, VT). Responding on the lever associated with food delivery resulted in delivery of a high-fat food pellet (45% energy from fat, 45mg, Bioserv F06162, Frenchtown, NJ), and the blinking of a white cue light at 1 Hz for 5 seconds. A second, inactive lever was placed in the chamber to distinguish between activity-induced changes in response, and reinforcer-seeking behavior. Responses on the inactive lever were recorded, but no pellets were delivered for inactive lever presses. Rats were first trained in half-hour sessions (1 session per day) on a fixed ratio (FR) 1 schedule, where a single response on the active lever is needed to receive a pellet and cue delivery.

Once the percentage of responses on the active lever exceeded 85% for three consecutive days, animals were advanced to an FR3 and then an FR5 schedule, which require 3 and 5 correct responses for pellet delivery, respectively. After animals reached this criterion on the FR5, they were moved to a progressive ratio (PR) schedule, where

earning each successive high-fat food pellet and cue delivery within the session requires a greater number of responses (1,2,3,6,9,12,15,20,25,32,40,50,62,77,95) on the active lever. Responses on the active, reinforced lever within a 60-minute test session were quantified. Test values were taken as three-day averages, following stability on a given schedule.

All rats received a 30-day forced abstinence period, during which rats were confined to their home cages, and were not exposed to the cues, the high-fat food pellets, or the operant chambers. Following the abstinence period, a cue-reactivity test was administered. Rats were placed in operant chambers and allowed to press on an FR1 schedule. Cue deliveries were replicated, including the light cue, the sound of the pellet dispenser, and the sound of the pellet dropping into the food tray. However, no pellets were delivered, regardless of responses. Following the cue reactivity test, rats were allowed to re-establish responding on an FR1 schedule, and were re-stabilized on FR1, FR3, FR5, and PR schedules, in order to examine the effects of abstinence on consumption of, and motivation for high-fat food (Figure 4.1).

4.2.3 Virus Design and Production

Hairpin RNA were previously designed and validated as targeting NMUR2 rat mRNA [32]. Briefly, a 24-nucleotide sequence, was identified within the mRNA sequence (Ensembl transcript ID ENSRNOT00000018967) using predefined search algorithms, and synthesized (Sigma–Aldrich, Saint Louis, MO, USA) as described previously. A pAAV-shRNA and a plasmid already containing the control hairpin, shCTRL were donated by Dr. Ralph DiLeone, PhD. The hairpin vector co-expressed eGFP under the control of an RNA Polymerase II promoter and terminator. The hairpin oligonucleotides were cloned into the pAAV shRNA plasmid, and RNAi viruses were produced through a triple-transfection, helper-free method and purified as previously described.

4.2.4 Virus Delivery

Viral administration was performed as described previously[32]. In order to knock down expression of NMUR2 in the PVN, we stereotaxically injected 2µl/side of

AAV-2-shNMUR2-GFP or AAV-2-shCTRL-GFP into each rat, following initial training, based on the methods of Benzon et al. 2014[32]. PVN coordinates were adjusted for a 10^{o} outside angle and were set at (A/P -0.18, M/L +0.15, D/V -0.82) from bregma. Injections were delivered at a rate of 0.2µL/30 seconds over a period of 5 minutes. Following injection, the needle remained in place for 3 minutes before removal. All incisions were stapled closed and post-operative care was administered following Benzon et al. 2014[32]. Animals were given 7 days to recover after surgery before being returned to operant chambers, and a further 7 days to allow for full viral expression before stabilization and testing. Surgeries were performed between 09:00 and 17:00.

4.2.5 Euthanasia and Targeting

Following completion of the series of operant tasks, rats were euthanized, and viral targeting was analyzed as previously described[32]. Briefly, rats were transcardially perfused with ice cold 1X phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were extracted, cryoprotected in 1X PBS, 20% glycerol, and 0.01% sodium azide, and sliced into 40µm sections. Sections were examined for green fluorescence, indicative of viral expression. Injection sites were noted to allow for elimination of unsuccessful treatments. Data from rats with off-target injections or viral expression in structures other than the PVN (n=3) were excluded from the study.

4.2.6 Data Analysis

Data were analyzed using Graphpad Prism statistical software (Graphpad Prism 7, San Diego, CA, USA). Body weight, acquisition, and abstinence effects were analyzed using a repeated measures ANOVA with a Sidak post-hoc analysis to account for the multiple treatment groups and time periods under consideration.

4.3 Results

4.3.1 Training and Acquisition

The effects of targeted NMUR2 knockdown on gross food consumption behavior have been previously examined[32]. These data demonstrate that inhibition of NMUR2 within the PVN increases *ad libitum* intake of high-fat foods, and increases preference for

foods rich in fats. Here, we expand upon this work, training rats to lever-press in operant chambers on both FR and PR schedules in order to understand the role of NMUR2 signaling in the PVN on food reinforcement and food-associated cues. After training and stabilization on each successive response schedule, rats received an shRNA-mediated knockdown of NMUR2 (shNMUR2), or infusions of a scrambled shRNA (shCTRL) into the PVN (Figure 4.1A). Viral targeting data was confirmed following the operant response experiments. Bilateral infusion sites for each animal were confirmed as present in the PVN, and the extent of viral fluorescence was used to ensure spread was limited to the structure (Figure 4.1B,C).

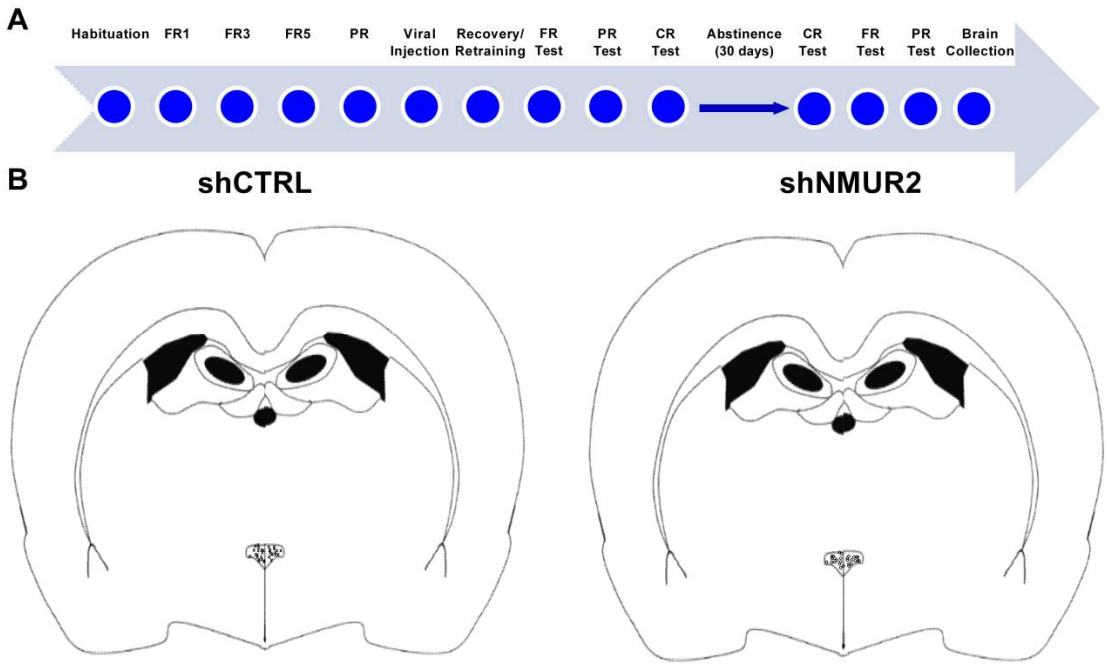

Figure 4.1 Behavioral timeline and targeting. A) Timeline showing the order of testing and treatment for animals with Neuromedin U Receptor 2 (NMUR2) knocked down in the paraventricular nucleus of the hypothalamus (PVN). B) Coronal representations of the viral target area for animals receiving the control virus (shCTRL), left, or the knockdown virus (shNMUR2), right, into the PVN. X and O represent injection sites for shCTRL and shNMUR2 animals, respectively.

As NMUR2 has been shown to influence body weight[32], we compared body weight in shCTRL and shNMUR2 animals over the course of the study (Figure 4.2). Treatment groups were balanced by body weight, and no significant differences in weight developed following viral injection and expression ($n=13\text{-}14$, $p>0.05$ by repeated-measures ANOVA). Moreover, no significant differences in body weight developed following forced abstinence, allowing for consideration of behavioral patterns independently of bodily obesity ($n=13\text{-}14$, $p>0.05$ by repeated-measures ANOVA). These effects are consistent with our previous data[32]; while PVN knockdown of NMUR2 leads to increased body weight on a high-fat diet, no increase in weight gain was observed on a standard diet.

In order to confirm that any behavioral effects of shNMUR2 were not mediated by gross changes in learning and memory or physical movement, acquisition rates for the operant tasks were monitored and compared across treatment groups. Average responding for shCTRL and shNMUR2 was measured daily, with a minimum of three days (<15% day-to-day variability) required for behavioral patterns to be considered stable. No significant differences ($n=13\text{-}14$, $p>0.05$ by repeated-measures ANOVA) in acquisition or stable responding were identified on FR1, FR3, FR5 or PR schedules, either before or after surgery (Figure 4.3).

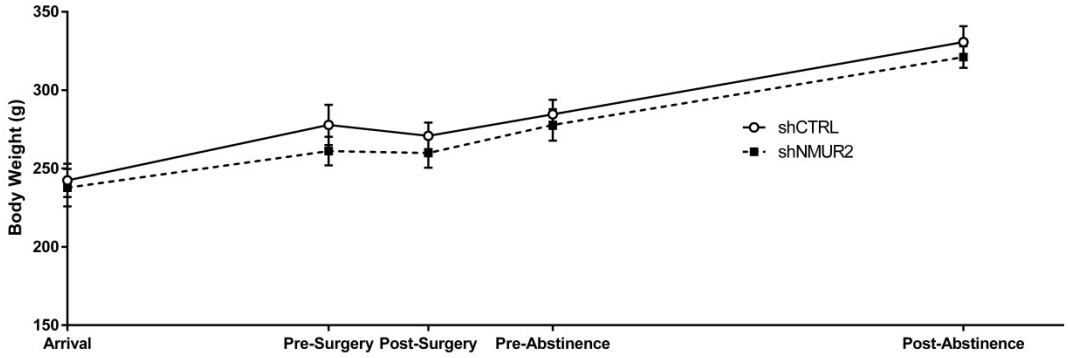

Figure 4.2. Animal body weights throughout the study. Animals were fed a standard rodent diet, and received an shRNA knockdown of Neuromedin U Receptor 2 (shNMUR2) or a control hairpin (shCTRL) in the paraventricular nucleus of the hypothalamus. Body weights were assessed throughout the study upon arrival at the facility, prior to viral surgery, following surgical recovery, prior to forced abstinence, and following forced abstinence. No significant differences in body weight were found between the treatment groups at any time point.

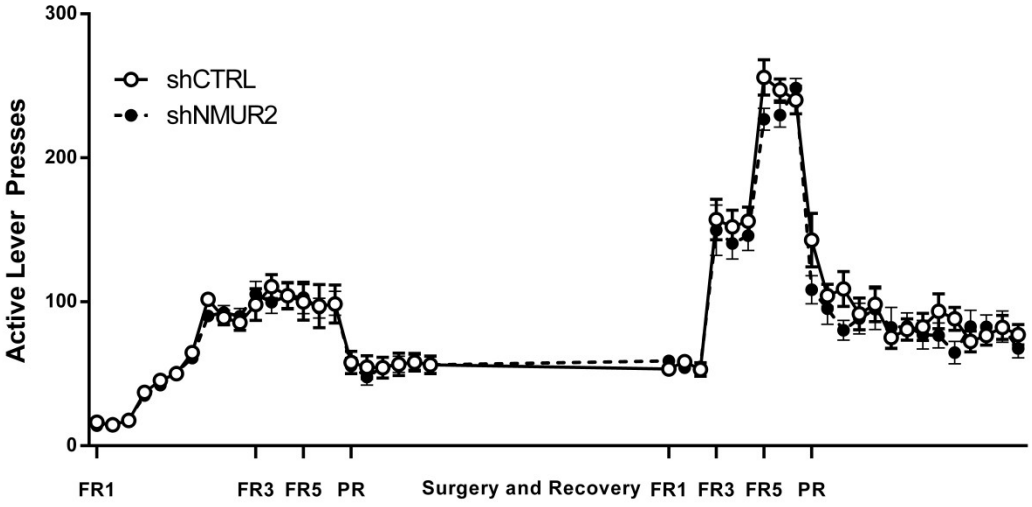

Figure 4.3. Acquisition and maintenance of operant responding. Operant responding for pellets of high-fat food was quantified on fixed-ratio 1, 3, and 5 schedules (FR1, FR3, FR5, respectively), as well as on a progressive ratio (PR) schedule. Treatment groups were balanced by responding prior to viral surgery, and responding was monitored following surgery to control for failures to re-acquire. No significant differences in FR or PR responding were found between animals receiving the control virus (shCTRL) or the knockdown virus (shNMUR2) on acquisition or maintenance of operant responding.

4.3.2 Effects of PVN NMUR2 Knockdown on Food Taking After Abstinence

Addiction-like behavior is mediated in part by an individual's propensity to taking of the abused substance[131, 142]. Here we demonstrate that following abstinence, fixed-ratio operant conditioning, a classical measure of taking behavior, is dysregulated in rats receiving a knockdown of NMUR2 in the PVN (Figure 4.4). Prior to abstinence, NMUR2 knockdown had no significant effect on high-fat food taking following full expression of the virus (Figure 4.4A). After thirty days of forced abstinence from high-fat food, rats expressing the control virus significantly increased taking behavior over their pre-abstinence baseline (n=13-14, *$p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=14 per group) (Figure 4.4A). This increase is consistent with previous work analyzing incubation of taking behavior following forced abstinence[97]. In contrast, shNMUR2 rats did not display a significant incubation in taking behavior over pre-abstinence responding, and lever-pressed significantly less than control animals following the abstinence period (n=13-14, *$p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=14 per group) (Figure 4.4A). This pattern is consistent with reinforcements earned by shNMUR2 and shCTRL rats in these sessions (Figure 4.4B). No significant difference was found between shCTRL and shNMUR2 reinforcements earned prior to abstinence, but following abstinence shNMUR2 rats received significantly fewer high-fat food reinforcers (n=13-14, *$p<0.05$ by repeated-measures ANOVA). Moreover, no significant difference (n=13-14, $p>0.05$ by repeated-measures ANOVA) in overall activity was noted in shCTRL or shNMUR2 animals before or after abstinence, as measured by responses on the inactive lever (Figure 4.4C).

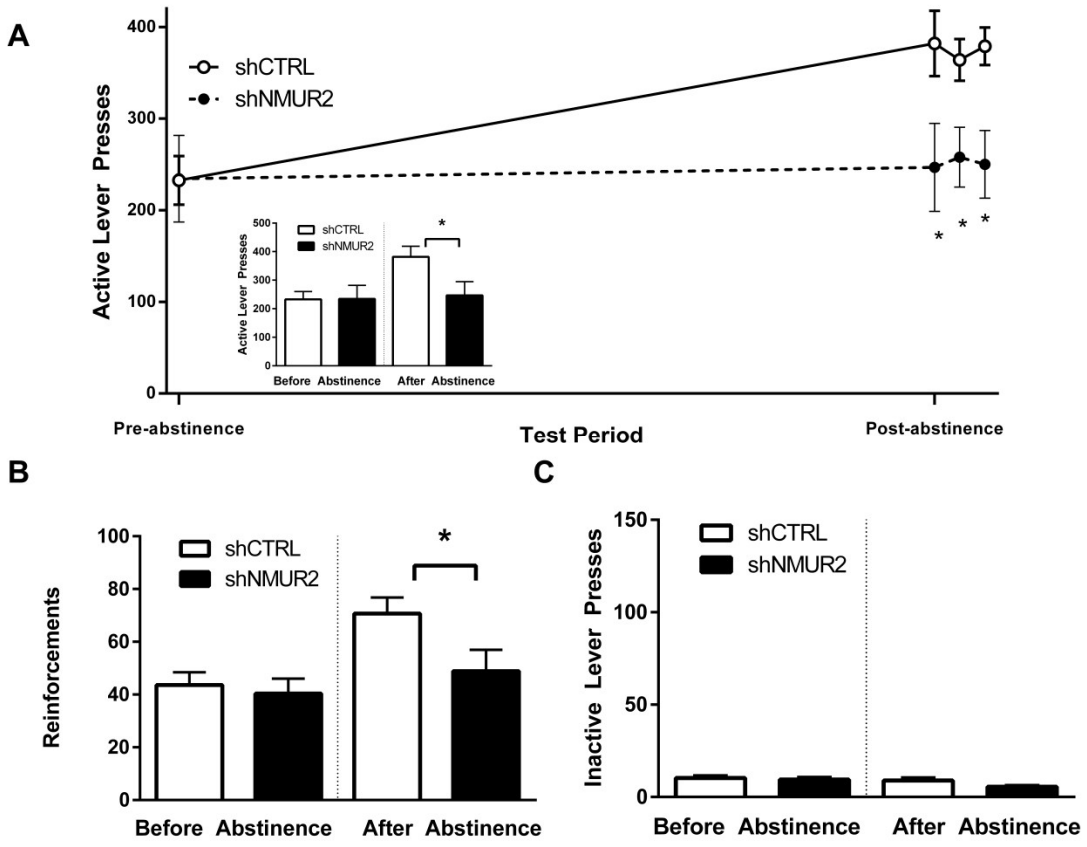

Figure 4.4. Knockdown of neuromedin U receptor 2 (shNMUR2) in the paraventricular nucleus of the hypothalamus blunts incubation of food taking. A) shNMUR2 animals do not display a difference in fixed-ratio (FR) responding from control (shCTRL) animals following full viral expression. However, after a 30-day forced abstinence period, shCTRL animals significantly increase responding over their pre-abstinence baseline, and respond significantly more than shNMUR2 animals. B) shNMUR2 does not alter earned high-fat reinforcers on a FR schedule prior to abstinence. Following forced abstinence, shCTRL animals significantly increase reinforcers earned over both their pre-abstinence baseline, and over shCTRL animals. C) No significant difference in inactive lever responding is found across treatment groups before or after abstinence.

4.3.3 Effects of PVN NMUR2 Knockdown on Food Reinforcement After Abstinence

A clear effect of PVN NMUR2 knockdown is also visible on the incubation of reinforcement for high-fat food. While no initial effect was found, following full viral expression, shNMUR2 rats show significantly decreased incubation of responding on a progressive-ratio schedule, as compared to control animals (Figure 4.5A, $p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=13-14 per group). Both treatment groups display the expected incubation in motivated responding for high-fat food, following the thirty-day abstinence period (Figure 4.5A, $p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=13-14 per group), with a robust increase in responding across treatments. This pattern is consistent with reinforcements earned by shNMUR2 and shCTRL rats in these sessions (Figure 4.5B). Importantly, no significant difference was found between shCTRL and shNMUR2 reinforcements earned prior to abstinence, but following abstinence shNMUR2 rats received significantly fewer high-fat food reinforcers (n=13-14, $*p<0.05$ by repeated-measures ANOVA) as compared to shCTRL rats. Thus, the incubation of reinforcement for high-fat food was blunted in shNMUR2 animals. No changes in overall activity were identified, as measured by responses on the inactive lever (Figure 4.5C), reinforcing the notion that the identified abstinence-induced motivational changes were, in fact, separate from the previously-described effects of NMU-NMUR2 signaling on gross activity.

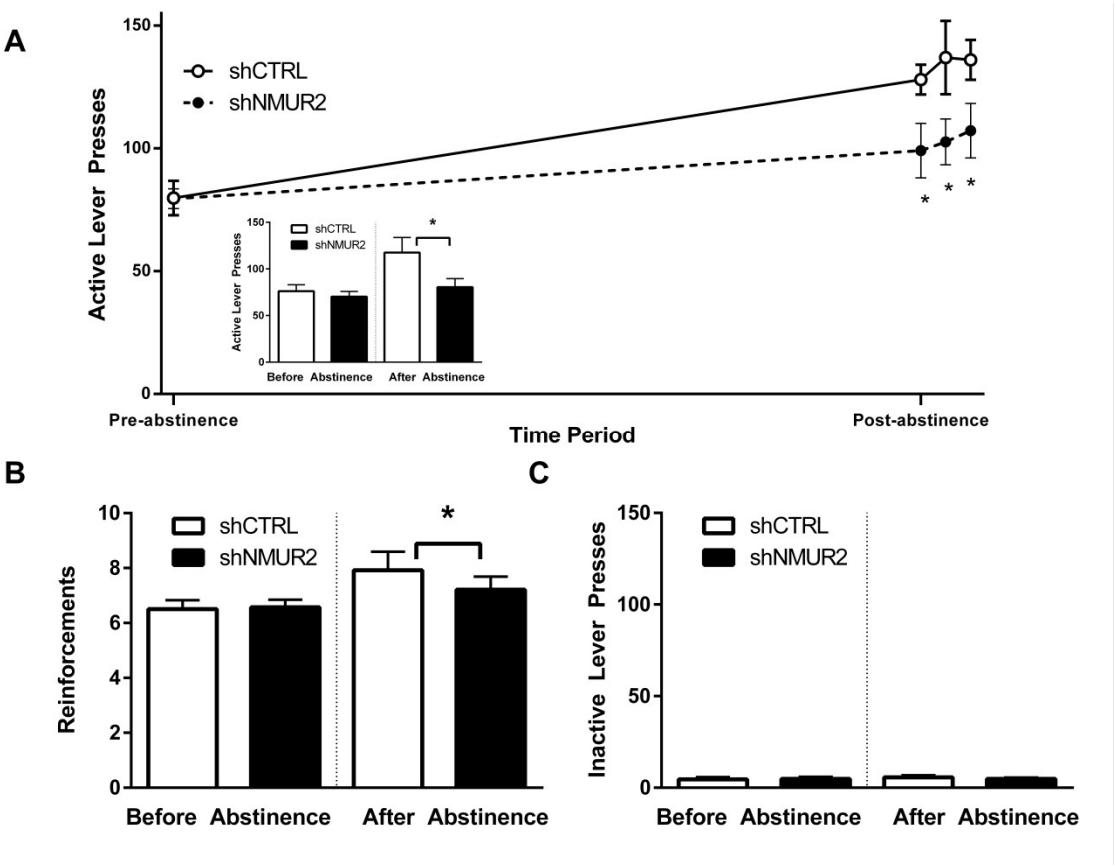

Figure 4.5. Knockdown of neuromedin U receptor 2 in the PVN blunts incubation of food motivation. A) shNMUR2 rats do not display a significant difference in progressive-ratio (PR) responding from shCTRL rats. However, after a 30-day forced abstinence period, shCTRL rats respond significantly more than prior to abstinence, and significantly more than shNMUR2 rats. B) shNMUR2 does not alter the number of high-fat reinforcers earned on a PR schedule. Following forced abstinence, shCTRL rats significantly increase reinforcers earned over their pre-abstinence baseline, and earn significantly more reinforcers than shNMUR2 rats. C) No significant difference in inactive lever responding is found across treatment groups before or after abstinence.

4.3.4 Effects of PVN NMUR2 Knockdown on Cue Reactivity

Craving for abused substances is widely studied using cue reactivity, an operant measure of the animal's behavioral response to cues associated with the substance in question[97, 143, 144]. shNMUR2 rats trained to lever-press, as above, for pellets of high-fat food show a significant decrease in cue reactivity, as compared to shCTRL animals (Figure 4.6A, $p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=14 per group). This phenotype remains following a thirty-day abstinence period. Intriguingly, while shCTRL animals show the expected significant increase in reactivity to food-associated cues following the abstinence period (Figure 4.6A, $p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, n=14 per group), no such pattern is found in shNMUR2 animals, who fail to demonstrate a significant incubation effect. These differences in active lever presses are consistent with those seen in reinforcements earned by shNMUR2 and shCTRL rats in these sessions (Figure 4.6B). A significant difference was found between shCTRL and shNMUR2 reinforcements earned prior to abstinence, with shNMUR2 rats receiving significantly fewer high-fat reinforcers (n=13-14, $*p<0.05$ by repeated-measures ANOVA). This effect was maintained following abstinence. These differences are not reconciled by changes in overall activity, as no significant effect was found on responses on the inactive lever across treatment groups, neither before nor after the abstinence period (Figure 4.6C). As such, it appears that NMUR2 knockdown in the PVN induces a lasting decrease in cue reactivity, which is resistant to abstinence-induced incubation of craving-like behavior, and separate from motility- or activity-based limitations.

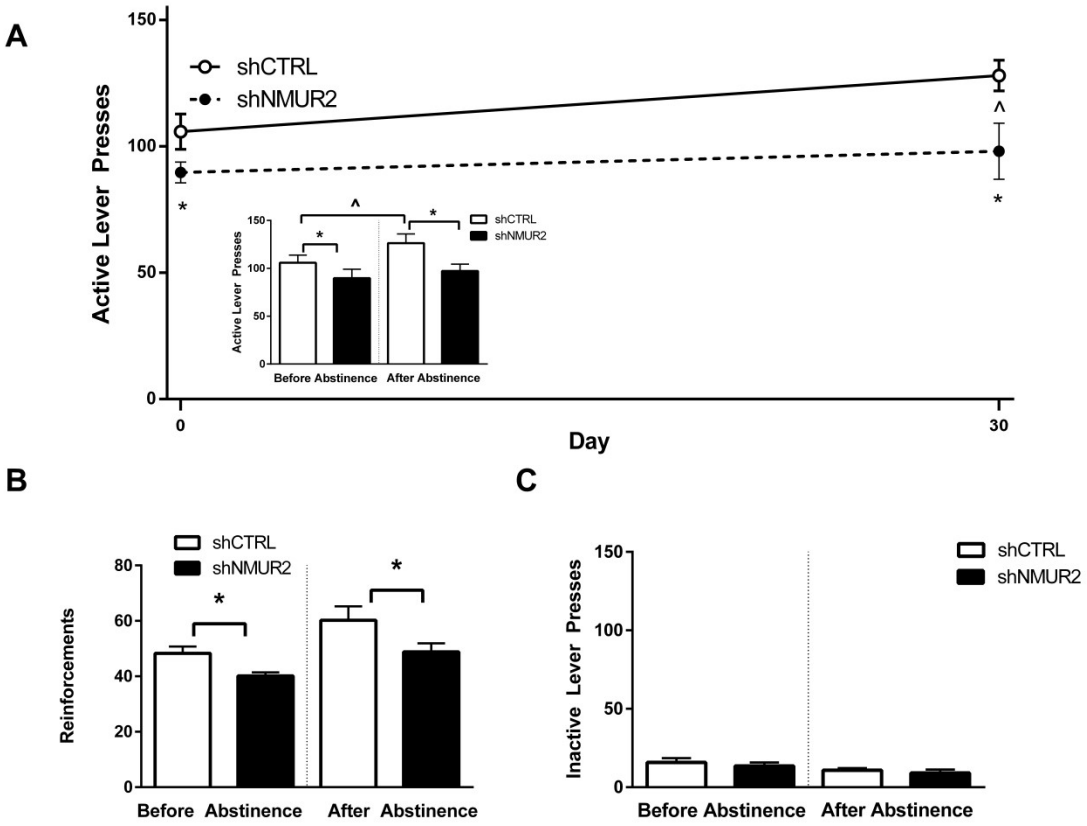

Figure 4.6. Knockdown of neuromedin U receptor 2 in the PVN blunts food seeking, and incubation of food seeking. A) shNMUR2 animals respond significantly less for cues associated with high-fat food than control (shCTRL) animals do following full viral expression, and maintain significantly lower responding following forced abstinence. Additionally, shCTRL animals display significantly increased active lever-pressing in response to food cues following forced abstinence, while no such increase was found in shNMUR2 animals. B) While both treatment groups earn significantly more cue deliveries following forced abstinence, shNMUR2 animals earn significantly fewer cue deliveries than shCTRL animals both before and after forced abstinence. C) No significant difference in inactive lever presses was found across treatment groups either before or after forced abstinence.

4.4 Discussion

This study builds on previous work showing that that food craving incubates during abstinence [24] by identifying NMUR2 as a receptor that mediates the incubation of craving-like behavior for high-fat foods. We tested this hypothesis by knocking down expression of NMUR2 in the PVN of adult rats, followed by measuring fixed-ratio and progressive-ratio operant responding, and cue reactivity, both before and after a forced abstinence period. We observed a clear decrease in the incubation of fixed-ratio responsiveness in PVN shNMUR2 animals, which is not attributable to changes in activity. Importantly, no initial effect was found on FR responding after full viral expression. However, after thirty days of forced abstinence, the shCTRL group significantly increased its responding for pellets of high-fat food, while PVN shNMUR2 animals did not display a significant increase over pre-abstinence levels. After abstinence, PVN shNMUR2 animals responded significantly less for high-fat food than did shCTRL animals. This is consistent with previous work demonstrating that that abstinence-induced incubation behavior is distinct from both *ad libitum* and binge-type intake patterns [33, 48-51].

PVN NMUR2 depletion was sufficient to blunt the incubation of reinforcement for high-fat food, after forced abstinence. Similar to fixed-ratio responding, no initial effect was seen on motivation for high-fat food. However, after forced abstinence PVN shNMUR2 animals displayed significantly decreased incubation of motivated responding for high-fat food. By contrast, we demonstrated that knockdown of NMUR2 in the PVN results in decreased reactivity to high-fat food cues before abstinence, as well as impaired incubation of seeking behavior for food cues. As food-associated cues have previously been shown to induce time-dependent incubation of craving-like behavior for high-fat foods [24, 52-55], the inhibition of cue reactivity in PVN shNMUR2 animals may be responsible for the blunted abstinence-induced incubation of craving-like behavior. This association is strengthened in that no effect was seen on overall activity, or lever-switching behavior as measured by inactive lever presses. This suggests that activity-dependent operant testing is a valid measure of motivation, and that the incubation effects are unlikely to be a result of impaired activity, associative capabilities, or recall. NMUR2 expression within the PVN may, therefore, offer an indicator of susceptibility to craving-

like behaviors, as abstinence-induced craving is a key indicator of propensity to craving and relapse behavior [31, 50, 51, 56].

It is important to note that NMUR2 depletion did not significantly affect body weight at any point in the study. This agrees with previous findings showing that NMUR2 in the PVN does not influence body weight of rats fed a standard rodent diet [10]. Moreover, the intermittent access to high-fat food provided by operant conditioning produced no significant effects on body weight, regardless of treatment. These data are consistent with previous studies on *ad libitum* access to standard or high-fat diets in PVN-specific NMUR2 knockdown animals [10], which indicate that significant weight gain is seen only in those animals maintained on a high-fat diet for extended periods.

Acute administration of the NMU peptide has recently been shown to inhibit motivation for high-fat reinforcers [40], and depleting the receptor within the PVN produces minimal acute effects on animals fed a standard diet [10]. However, a key characteristic of craving-like behavior, abstinence-induced increases in responsiveness, is attenuated by the depletion. These data provide an intriguing addition to previous findings that abstinence increases responding for, and consumption of, sucrose reinforcers [24, 57-59]. Moreover, those authors show similar increases in cue reactivity for sucrose-paired cues, both in operant and binge-like studies [33, 48, 57, 60]. Our data expand on these findings, demonstrating the strength of abstinence-induced incubation of craving-like behavior in terms of a more powerful natural reinforcer [18], and that this incubation effect is regulated by PVN NMUR2. These findings shed light on the mechanisms behind abstinence-induced craving. As Avena and Hoebel 2003 [58, 59] demonstrate, even 8 days of abstinence from sucrose can induce hyperreactivity to subthreshold doses of other reinforcers. Additionally, the ability of PVN NMUR2 to regulate the incubation of craving-like behavior has significant implications for co-abuse. Grimm et al 2006 [61] demonstrates that incubation of craving-like behavior for drugs of abuse, such as cocaine, can be stimulated by previous associations with sucrose reinforcers. As NMUR2 regulates both food- and drug-induced reinforcement [40, 41], it is possible that PVN NMUR2 represents a window into the mechanisms driving future abuse potential not only for high-fat foods, but for multiple substances of abuse.

Intriguingly, these findings support a bottom-up model of craving-like behavior. In contrast to top-down models, in which the prefrontal cortex (PFC) projects to regions such as the nucleus accumbens (NAc) to regulate craving-like behaviors, the bottom-up model views the projections to the PFC as major elements in the regulatory process [180-181]. Indeed, the PVN projects not only to regions known to mediate this bottom-up regulation, such as the NAc [102], but to the PFC directly [179]. Moreover, viral tracing studies, including our own (Figure 5.1), retain the possibility of collaterals, wherein projections from the PVN to other structures such as the NAc may continue on to innervate the PFC. As depletion of NMUR2 in PVN projections produces significant effects on craving-like behavior, it appears that there is a regulation of PFC circuitry by PVN NMUR2 in vivo; moreover, that PVN NMUR2 plays a role in the induction of incubation of craving-like behavior, as its depletion severely inhibits the process. However, the direct mechanism by which the depletion alters PFC-driven craving-like behaviors remains a question for future study, as accumbal, amygdalar, and direct hypothalamic signals, received from the PVN NMUR2 neurons and influencing the PFC, may be responsible.

Taken together, our data demonstrate a novel role for NMUR2 in the PVN: regulation of the incubation of craving-like behavior for high-fat food. While we have previously shown that depletion of PVN NMUR2 increases food intake and weight gain of ad libitum high-fat diet [10], these data demonstrate a differential effect on *reinforcement*-based models of abstinence-induced food craving. Depletion of NMUR2 in the PVN dampens the incubation of food craving-like behaviors during abstinence, inhibiting motivation for high-fat food and its associated cues following a diet, or abstinence period. Thus, PVN NMUR2 represents a potential mechanism for regulating relapse potential and dietary noncompliance by severely blunting the incubation of craving-like behavior.

Chapter 5: NMUR2 Drives an Enkephalin-Mediated PVN-NAc Pathway to Regulate Motivation for High-Fat Food

5.1 Introduction

Although homeostatic food consumption is believed to be regulated primarily by the hypothalamus[46, 149, 150], reinforcement is generally associated with nuclei outside the hypothalamus, the Nucleus Accumbens (NAc) in particular[22, 151]. Anorexigenic and orexigenic signals may be mediated in part by general hypothalamic regulation of feeding and satiety[150, 152, 153]. However, their behavioral effects may be superseded by drivers of reinforcement, such as the enkephalins, acting on downstream, mesocorticolimbic regions[66, 151, 154-156]. This downstream effect may offer some explanation as to why highly reinforcing foods, such as those high in fat, are among the most overconsumed.

The enkephalins, endogenous opioid peptides, have been of particular interest in the study of feeding and reinforcement[28, 63, 110, 114, 157]. A wide range of studies have investigated multiple aspects of feeding regulation, including the effects on macronutrient selection, energy balance, gross consumption, and motivation for food[20, 21, 63, 155]. Indeed, both animal and human studies indicate that opioid signaling in general, and enkephalin signaling in particular are capable of regulating food intake and reinforcement[28, 110, 119, 156]. Several brain regions have demonstrated responsiveness to enkephalin treatment, regulating feeding behavior, but enkephalin in the NAc produces perhaps the greatest effects on reinforcement and palatability when it comes to food[28, 57, 69, 156]. Moreover, the demonstrated role of the NAc as a downstream regulator of feeding, and a key site for many forms of reinforcement behavior, suggest that it is an ideal target for the reinforcing aspects of high-fat food [4, 24, 59, 155].

The NAc expresses high levels of enkephalins in areas receiving projections from key feeding structures such as the nucleus of the solitary tract and the basolateral amygdala[24, 157], and to activate both mu and delta opioid receptor signaling pathways in response to their activation[154, 156]. Though the role of enkephalins in accumbal-

driven feeding has been evaluated, their role as a downstream integrator of hypothalamic feeding signals remains understudied.

While we have demonstrated the importance of high-fat food in the development of obesity, and the role of PVN NMU-NMUR2 signaling in regulating motivation for high-fat food, we have yet to examine downstream pathways or mechanisms. However, it has been previously shown that intra-accumbal infusions of the mu opioid receptor agonist D-Ala^2, NMe-Phe^4, Glyol^5-enkephalin (DAMGO) regulate high-fat food intake, and motivation for highly palatable foods[22, 63]. Moreover, a recent publication by Dolen et al. 2013 [102] has demonstrated the presence of a novel pathway linking the PVN to the nucleus accumbens shell (NAcSh), which regulates social aspects of reinforcement. As such, we investigate the role of the PVN-NAc pathway in mediating the downstream effects of PVN NMUR2 signaling, and the potential for enkephalin as a mediator of these effects.

5.2 Specific Methods

5.2.1 Animal Subjects

Male Sprague-Dawley rats (N=36; Harlan, Inc., Houston, TX) weighing 225-250 grams (at the start of the experiment) were used for all experiments. Rats were housed two to a cage, except when necessary to separate animals for feeding assays. Colony environment was maintained at 71^{o}F and 30-50% relative humidity, with lights-on between 06:00 and 18:00. All animals were allowed to habituate a minimum of seven days before testing, surgery, or treatment.

At the conclusion of the studies, animals were euthanized with 5% isoflurane and transcardially perfused for 5 minutes with 1X PBS (75mL), followed by 15 minutes of 4% paraformaldehyde (225mL). Brains were immediately extracted, post-fixed overnight in 4% paraformaldehyde in 1X PBS, and cryoprotected in a solution of 20% glycerol and 0.01% sodium azide in 1X PBS at 4^{o}C for a minimum of 24 hours. After cryoprotection, brains were frozen on dry ice and sliced into 40µm coronal sections using a microtome (SM 2010R, Leica Biosystems, Buffalo Grove, IL). Slices were stored in 0.01% sodium azide in 1X PBS at 4^{o}C until use. Targeting for injections and cannula placement were

assessed by examining native fluorescence under a confocal microscope (DMI4000 Inverted TCS SPE confocal microscope, Leica Biosystems). Brains in which one or more cannulae or injections were off-target were excluded from further analysis, and the corresponding animals' data was not considered.

Experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals[91]* and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

5.2.2 Operant Conditioning

Operant conditioning was performed as described previously[141]. Briefly, between 13:00 and 17:00, rats were placed in standard rat operant chambers (Med Associates, Georgia, VT). Responding on the lever associated with food delivery resulted in delivery of a high-fat food pellet (45% energy from fat, 45mg, Bioserv F06162, Frenchtown, NJ), and the blinking of a white cue light at 1 Hz for 5 seconds. A second, inactive lever was placed in the chamber to distinguish between activity-induced changes in response, and reinforcer-seeking behavior. Responses on the inactive lever were recorded, but no pellets were delivered for inactive lever presses. Rats were first trained in half-hour sessions (1 session per day) on a fixed ratio (FR) 1 schedule, where a single response on the active lever is needed to receive a pellet and cue delivery.

Once the percentage of responses on the active lever exceeded 85% for three consecutive days, animals were advanced to an FR3 and then an FR5 schedule, which require 3 and 5 correct responses for pellet delivery, respectively. After animals reached this criterion on the FR5, they were moved to a progressive ratio (PR) schedule, where earning each successive high-fat food pellet and cue delivery within the session requires a greater number of responses (1,2,3,6,9,12,15,20,25,32,40,50,62,77,95) on the active lever. Responses on the active, reinforced lever within a 60-minute test session were quantified. Test values were taken as three-day averages, following stability on a given schedule.

5.2.3 Surgical Procedures

Viral administration was performed as described previously[32]. In order to express the DREADD in PVN neurons, we stereotaxically injected 2µl/side of AAV-2-CaMKII-HM3D-GFP or AAV-2-CaMKII-GFP into each rat, following initial training, based on the methods of Benzon et al. 2014. PVN coordinates were adjusted for a 10^{o} outside angle and were set at (A/P -0.18, M/L +0.15, D/V -0.82) from bregma. Each animal also received bilateral guide cannulae, implanted into the NAcSh based on the methods of Kasper et al. 2016[82]. NAcSh coordinates were adjusted for a 10^{o} outside angle and were set at (A/P +0.14, M/L +0.20, D/V -0.57) from bregma.

Injections were delivered at a rate of 0.2µL/30 seconds over a period of 5 minutes. Following injection, the needle remained in place for 3 minutes before removal. All incisions were stapled closed and post-operative care was administered following Benzon et al. 2014[32]. Animals were given 7 days to recover after surgery before being returned to operant chambers, and a further 7 days to allow for full viral expression before stabilization and testing. Surgeries were performed between 09:00 and 17:00.

5.2.4 Euthanasia and Targeting

Following completion of the series of operant tasks, rats were euthanized, and viral targeting was analyzed as previously described[32]. Briefly, rats were transcardially perfused with ice cold 1X phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were extracted, cryoprotected in 1X PBS, 20% glycerol, and 0.01% sodium azide, and sliced into 40µm sections. Sections were examined for green fluorescence, indicative of viral expression. Injection sites were noted to allow for elimination of unsuccessful treatments. Data from rats with off-target injections or viral expression in structures other than the PVN (n=3) were excluded from the study.

5.2.5 Immunohistochemistry

Sections of brain were washed 3X in 1X PBS, 5 minutes/wash, to remove residual sodium azide, and incubated in 1% SDS for 5 minutes for antigen unmasking. Sections were washed 3X more in 1X PBS, 5 minutes/wash, and incubated for 1 hour in a blocking solution containing 0.3% Triton X-100 in 1X PBS and 3% of each serum

(donkey, goat). Primary antibodies were diluted in blocking solution and incubated on brain slices overnight (20 hours) at room temperature.

Sections were washed 3X in 1X PBS, 5 minutes/wash, and then secondary antibodies (AlexaFluor 488, 555, or Cascade Blue) were applied, and allowed to incubate at room temperature for 2 hours with shaking. Sections were washed 3 times, for 5 minutes each, in 1X PBS, mounted on Superfrost microscope slides and allowed to dry for a minimum of 2 hours. Slides were rehydrated in 1X PBS for 20 minutes, followed by ethanol dehydration and two 3-minute Citrisolv (Fisher Scientific) washes and were then coverslipped with DPX mounting medium (Fisher Scientific). Prepared slides were imaged on a DMI4000 Inverted TCS SPE confocal microscope (Leica Biosciences, Buffalo Grove, IL) Images were exported to ImageJ software. Colocalization was performed with the JACoP plugin, through ImageJ, and fluorescence thresholds were applied for improved quantification and visualization of immunolabeling.

5.2.6 Flow Cytometry

Rats were anesthetized with 5% inhaled isofluorane, and decapitated. Structures of interest were isolated via microdissection. Isolated tissue was placed in Hibernate A to improve cell survival, then prepared for analysis as follows.

32 mls of Earle's Balanced Salt Solution (EBSS) were added to the albumin ovomucoid inhibitor mixture and contents were allowed the contents to dissolve while preparing the other components. Solution was mixed before using and equilibrated with O_2:CO_2. 10% trehalose (w/v) was added[84], and solution was re-equilibrated. 5 mL of EBSS were added to a papain vial. Vial 2 was placed in a 37°C water bath for ten minutes or until the papain was completely dissolved and the solution appeared clear. If solution appeared alkaline, it was equilibrated with 95% O_2:5%CO_2. 500 µLs of EBSS were added to a DNase vial and mixed gently to avoid shear denaturation. 250 µls of this solution were added to the vial containing the papain. This preparation contained a final concentration of approximately 20 units/ml papain and 0.005% DNase. Tissue was placed in the papain solution, and gently minced to increase surface access to the papain solution. Air in the vial was displaced with sterile O_2:CO_2, and the vial was immediately

capped. The vial containing the tissue was then incubated at 37°C with constant agitation on a rocker platform for 90 minutes.

Following incubation, the mixture was triturated with a 10 mL pipette. The cell suspension was removed, placed in a sterile screwcapped tube and centrifuged at 300g for 5 minutes at room temperature. 2.7 mL EBSS were mixed with 300 µL reconstituted albumin-ovomucoid inhibitor solution in a separate sterile tube, and 150 µLs of DNase solution was added to this separate tube. Supernatant was discarded from the centrifuge tube, and the cell pellet was immediately resuspended in DNase dilute albumin-inhibitor solution. A discontinuous density gradient was then prepared by adding 5.0 mL of albumin-inhibitor solution to a centrifuge tube, carefully layering cell suspension on top, then centrifuging at 70g for 6 minutes at room temperature. The supernatant was then discarded, and pelleted cells were resuspended in Hibernate A for flow cytometric analysis. Flow cytometry was performed with a FACS Aria machine, through the UTMB Flow Cytometry and Cell Sorting Core Facility, and the assistance of Mark Griffin.

5.2.7 Real-Time Polymerase Chain Reaction

Following flow cytometry, mRNA was collected from each of the resulting cell populations. An Arcturus PicoPure kit was used together with a Qiagen RNeasy Mini kit to extract and isolate mRNA, under RNase-free conditions, as follows:

Cells were pelleted in a microcentrifuge tube by centrifuging at 3,000 x g for 10 minutes. Supernatant was disposed of. Cell pellet was resuspended in one mL of cell suspension media (0.9mL of 1xPBS/10%BSA;0.1mL of 0.5M EDTA). Cell suspension was centrifuged at 3,000 x g for 5 minutes. Supernatant was disposed of. Cells were extracted with 100uL of Extraction Buffer, and the cell pellet was resuspended gently by pipetting. Solution was incubated at 43^{o}C for 30 minutes. The sample was centrifuged at 3,000 x g for 2 minutes. The supernatant containing the extracted RNA was pipetted into a new microcentrifuge tube, avoiding pickup of pelleted material. The RNA Purification Column was preconditioned by pipetting 250 uL Conditioning Buffer onto the purification column filter membrane, and incubating at room temperature for 5 minutes. The purification column was centrifuged in a collection tube at 16,000 x g for 1 minute.

100 uL of 70% Ethanol were added to the cell extract, and mixed by pipetting up and down. Then the cell extract and Ethanol mixture were pipetted into the preconditioned purification column. In order to bind RNA to the column, the column was centrifuged for 2 minutes at 100 x g, immediately followed by a centrifugation at 16,000 x g for 30 seconds to remove flowthrough. 100 uL of Wash Buffer were then pipetted into the purification column and the column was centrifuged for 1 minute at 8,000xg.

Following centrifugation, 10 uL DNase I Stock Solution were added to 30 uL Buffer RDD in an independent centrifuge tube, and mixed by gently inverting. The 40 uL DNase incubation mix was pipetted directly into the purification column membrane, and incubated at room temperature for 15 minutes. Following incubation 40 uL of PicoPure RNA Kit Wash Buffer 1 were pipetted into the purification column membrane, and the column was centrifuged at 8,000 x g for 15 seconds. Flowthrough was discarded.

100 uL Wash Buffer 2 was then added to into the purification column and the column was centrifuged for 1 min at 8,000xg. Another 100 uL Wash Buffer 2 were pipetted into the purification column and the column was centrifuged for 2 minutes at 16,000 x g. Following centrifugation, the column was examined for residual wash buffer; if buffer remained in the column, the column was re-centrifuged at 16,000 x g for 1 minute. The purification column was then transferred to a new 0.5 mL microcentrifuge tube, and 11 uL Elution Buffer were pipetted directly onto the membrane of the purification column. The mRNA quality and concentration in the resulting solution was analyzed using a NanoDrop 2000 spectrophotometer.

The mRNA was then converted into cDNA using the iScript cDNA synthesis kit. Briefly, reactions were set up for each mRNA sample, containing equal concentrations of RNA template. 4uL of 5x iScript Reaction Mix were added to each reaction, together with 1uL of iScript Reverse Transcriptase, and the reaction volume was brought up to 20uL using nuclease-free water. Reactions were incubated for 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, 5 minutes at 85^{o}C, and held at 4oC for the next step. A NanoDrop 2000 spectrophotometer was used to analyze cDNA concentration, to confirm equal amplification, and ensure that equal concentrations of cDNA template were introduced into each PCR well.

Nuclease-free 96-well PCR plates were prepared, at 4^{o}C, with samples run in triplicate for the gene of interest, corresponding housekeeping genes (18S and GAPDH) for each animal, and no-template controls. Each well contained 10 uL of SYBR Green Master Mix, 1uL of forward and reverse primer for the gene in question, 1uL of iTaq DNA Polymerase, and equal concentrations of cDNA template. Total volume for each well was brought up to 20uL using RNase-free water. Equal concentrations of cDNA were used together with SYBR Green Master Mix, and gene-specific forward and reverse primers to create triplicate reactions in a 96-well PCR plate, and reaction volume was brought up to 20 uL with RNase-free water. The plate was sealed with an optical adhesive cover, and briefly centrifuged to spin down contents and eliminate air bubbles. Samples were run on an Applied Biosystems 7500 Fast Real-Time PCR system, and resulting experimental data were normalized to housekeeping genes, and fold change from baseline was determined.

5.2.8 Data Analysis

Data were analyzed using Graphpad Prism statistical software (Graphpad Prism 7, San Diego, CA, USA). Food consumption was analyzed using a repeated-measures ANOVA with a Sidak post-hoc analysis for multiple comparisons. Mean PR responding was analyzed by two-way ANOVA, and multiple comparisons were applied to within-session binning. Locomotor activity was normalized to vehicle baseline and analyzed by two-way t-test.

5.3 Results

5.3.1 Characterization of PVN-NAcSh Pathway Neurons

While NMUR2 signaling in the PVN and ENK in the NAcSh are behaviorally relevant, no relationship between them is established. To explore this neurocircuitry, rats were treated with 1 of 2 adeno-associated viral vectors: either AAV-6, a retrograde tracer injected into the NAcSh, or AAV-2, an anterograde tracer injected into the PVN. Viral expression of the fluorophores was immunoenhanced. PVN neurons projected to the NAcSh (Figure 5.1), confirming the findings of Dolen et al. 2013 [102] with viral tracing in both anterograde and retrograde directions, with a NAcSh-PVN AAV-6 tracer (Figure 5.1A, B), and a PVN-NAcSh tracer (Figure 5.1C, D). Immunolabeling of PVN-NAcSh

pathway neurons for Enkephalin and NMUR2 demonstrates colocalization within the pathway, showing that both NMUR2 and ENK are present in at least a subset of PVN-NAcSh neurons (Figure 5.2 A). To reinforce these findings, and display a clearer relationship between NMUR2 and ENK, we treated animals with NMU, and isolated PVN neurons expressing NMUR2 using flow cytometry. Analysis of the collected cells indicates a significant upregulation of PENK mRNA in response to NMU treatment, specific to NMUR2-positive neurons in the PVN (Figure 5.2 B, $p<0.05$ by two-way t-test, n=3 animals/group). These data reinforce the understanding that NMUR2 and ENK are co-expressed in the PVN, and that NMUR2 activation regulates ENK signaling.

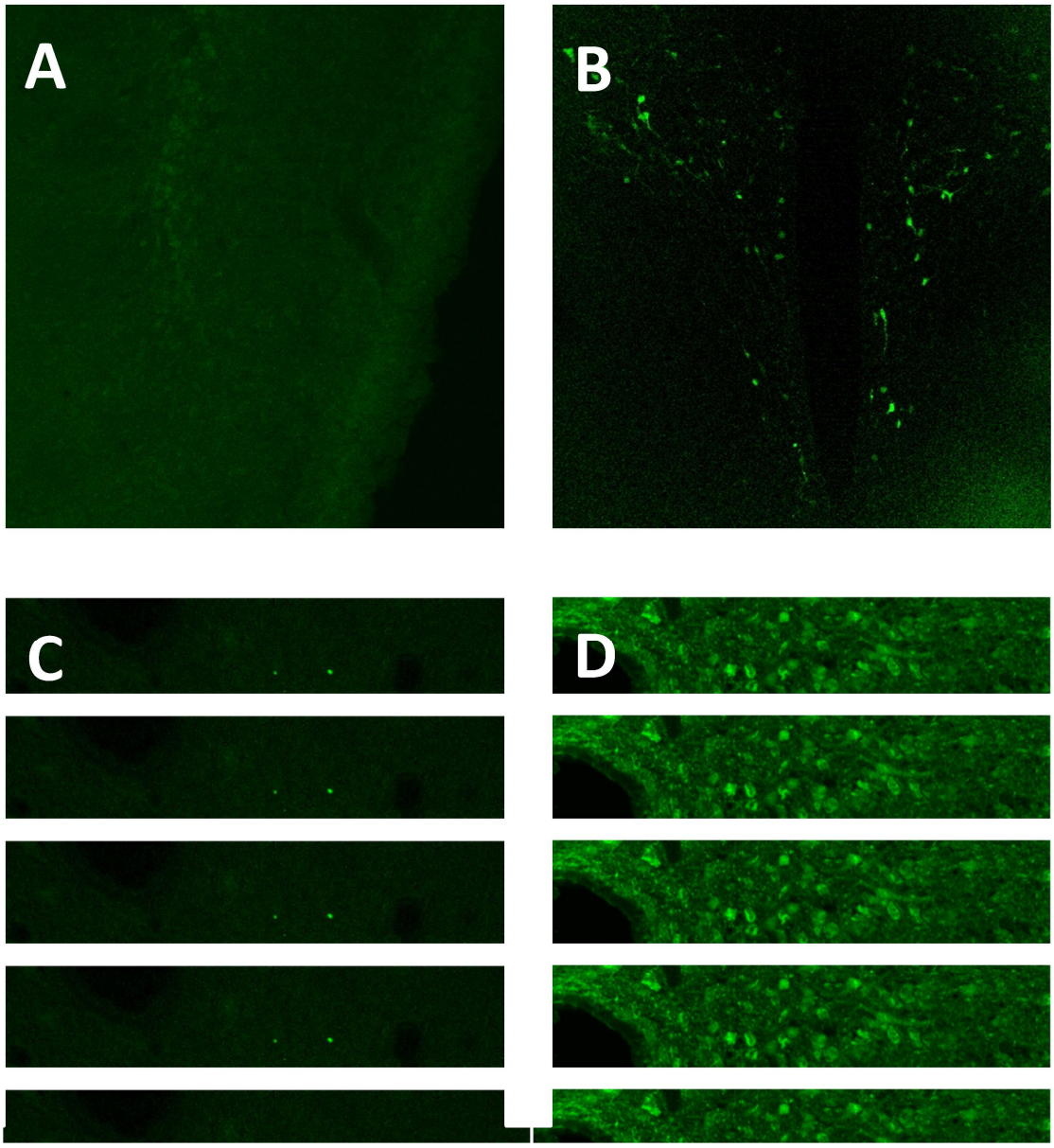

Figure 5.1. Tracing of the PVN-NAcSh pathway from the retrograde (A,B) and anterograde directions using AAV-6 and AAV-2 expressing GFP.

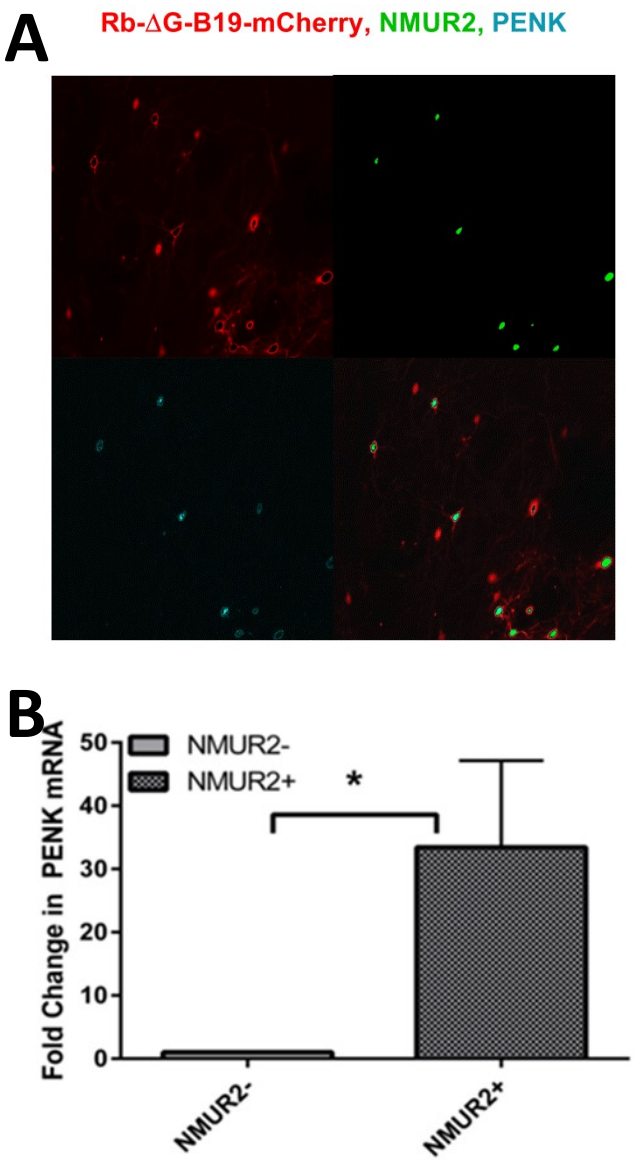

Figure 5.2. Characterization of PVN NMUR2 neurons. A) A retrograde tracer labels PVN neurons projecting to the NAcSh (red). We find coexpression of the tracer with NMUR2 (green), and PENK (cyan). B) FACS isolation of PVN NMUR2 neurons following NMU treatment shows that PVN NMUR2 neurons express more PENK mRNA in response to NMU. ($p<0.05$ by two-way t-test, n=3 animals/group).

5.3.2 Training and Acquisition

While we have previously examined the effects of PVN stimulation by NMU on food intake and motivation[141], the link between the PVN and the NAcSh have not yet been investigated. Our previous work demonstrates that NMU is capable of regulating motivation-associated behaviors for high-fat food through the PVN[32, 141], while ENK produces similar effects through the NAcSh[20, 24, 114]. Here, we expand on this work, training rats to lever-press in operant chambers on PR schedules in order to understand the role of the PVN-NAcSh pathway on food reinforcement. After training and stabilization on each successive response schedule, rats received an infusion of the DREADD-expressing AAV-2-CaMKII-HM3D-GFP (HM3D) or a control virus (CTRL) expressing only a fluorescent tag. (Figure 5.3A). Bilateral infusion sites for each animal were localized to the PVN (Figure 5.3B, left), and bilateral cannulation sites were placed at the location in the NAcSh receiving projections from the PVN (Figure 5.3B, right).

In order to confirm that any behavioral effects of pathway activation were not mediated by gross changes in learning and memory or physical movement, acquisition rates for the operant tasks were monitored and compared across treatment groups. Average responding for CTRL and HM3D was measured daily, with a minimum of three days (<15% day-to-day variability) required for behavioral patterns to be considered stable. No significant differences ($n=13$-14, $p>0.05$ by repeated-measures ANOVA) in acquisition or stable responding were identified on FR1, FR3, FR5 or PR schedules, either before or after surgery (Figure 5.4, green line). Importantly, rats acquired the tasks properly, demonstrating understanding of the tasks through the proportional increases or decreases in reinforcers earned with each new task (Figure 5.4, blue line). As expected, no significant differences were found on inactive lever pressing either, with minimal activity on the lever throughout training and testing (Figure 5.4, red line), save for slight increases following the introduction of novel tasks.

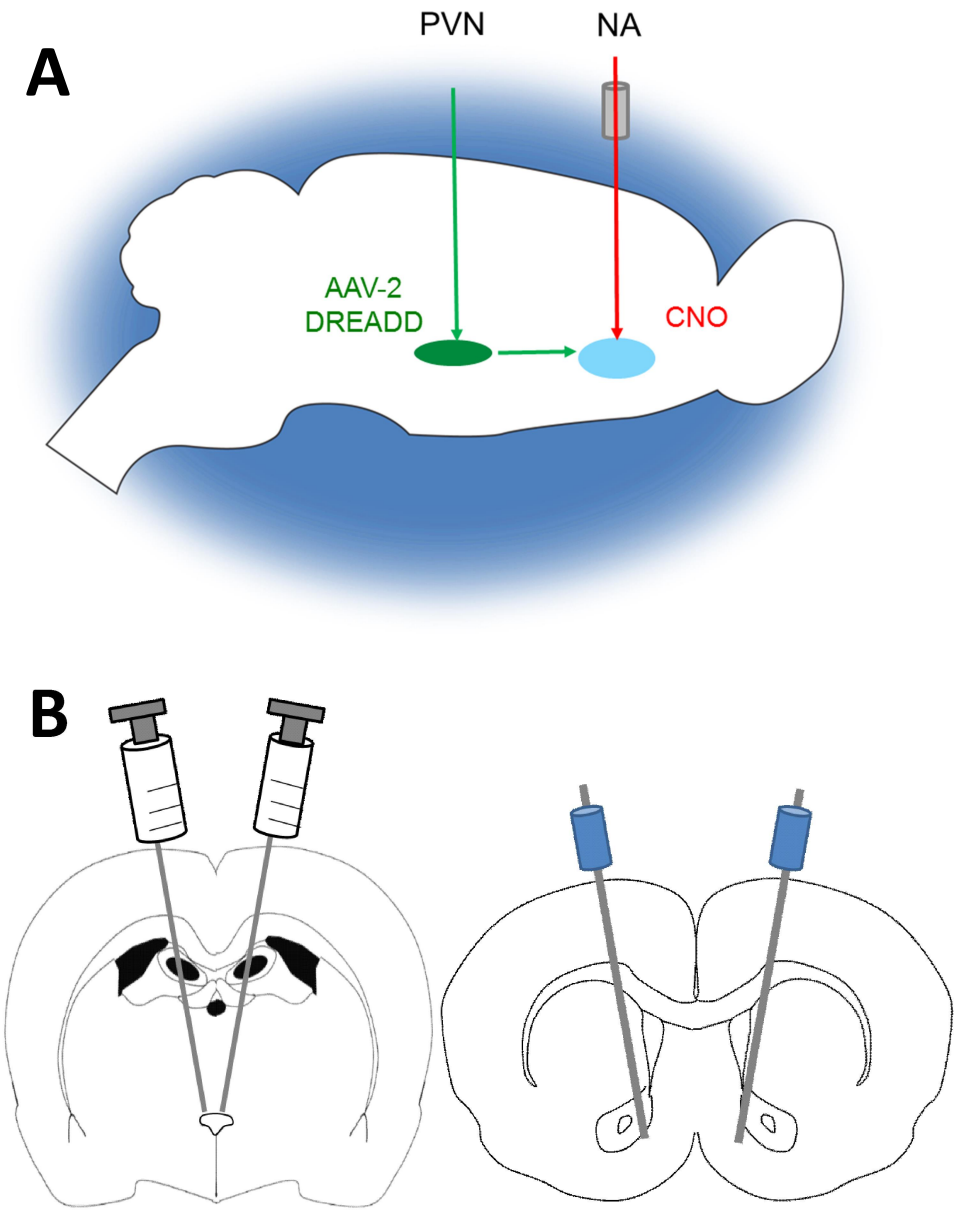

Figure 5.3. Experimental strategy and targeting. A) An AAV-2 viral vector expressing either a control virus, or an active DREADD, is injected into the PVN, and the NAcSh is cannulated. Once the virus has reached full expression, CNO is injected via the cannulae into the NAcSh to activate the PVN-NAcSh pathway specifically. B) Targeting sites for the viral injection (PVN, left), and the cannulation (NAcSh, right).

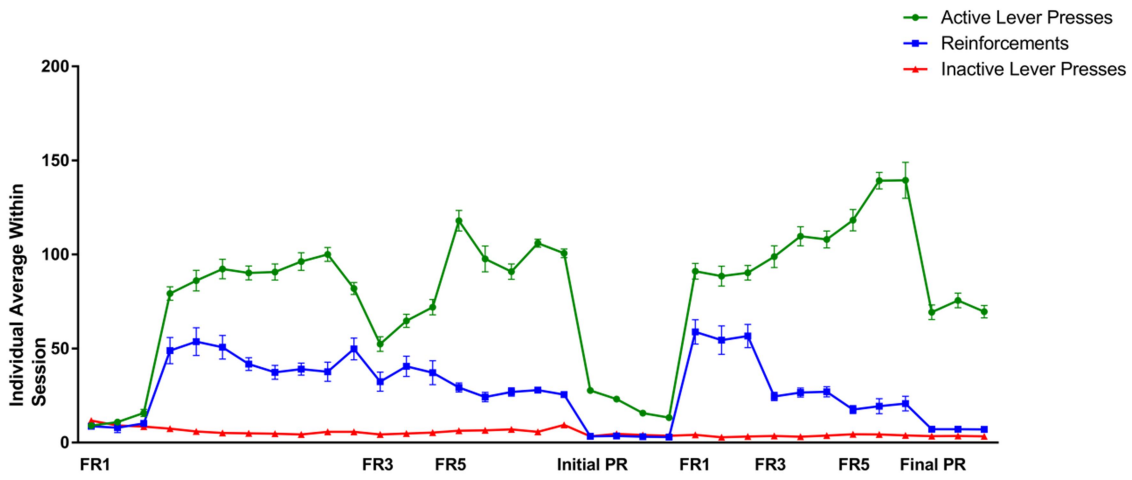

Figure 5.4. Training and acquisition. Rats were introduced into the operant chambers, and allowed to lever-press for 45mg pellets of high-fat food, accompanied by the flashing of a cue light. Animals were not food restricted, to avoid hunger effects. All animals included in the study successfully learned the operant tasks. Training and acquisition data were used to ensure that groups receiving the different viruses were balanced in baseline lever-pressing, and reinforcements earned.

5.3.3 Effects of DREADD Activation and Inhibition of PVN-NAcSh Pathway on Feeding

Since NMU in the PVN, and ENK in the NAcSh produce significant effects on total food consumption and operant behavior[24, 80, 114, 141], our goal was to ascertain whether activation of the PVN-NAcSh pathway specifically mediated these behaviors. Initially, we sought to confirm that these effects were mediated by NMUR2 signaling in the pathway, giving animals either CNO or aCSF, and NMU or the DMSO vehicle (Figure 5.5). However, the combination of treatments and the high variability in response resulted in high variance which prevented detection of significant differences. Established effects of NMU on motivated behavior for high-fat food were disrupted, even in animals given only aCSF, and the CTRL and HM3D animals responded differently to the vehicle-vehicle (CNO-, NMU-) condition (Figure 5.5A). Moreover, effects varied greatly from animal to animal, and rates of lever-pressing did not correlate well with reinforcers earned (Figure 5.5B). Animals, even those in the control groups, displayed abnormally high inactive lever-pressing (Figure 5.5C). Due to the lack of clarity in the data, additional experimentation with all these conditions was considered impractical, and we focused our future studies solely on the activation of the PVN-NAcSh pathway.

Following training, surgery, and recovery (Figure 5.3A), animals receiving intra-NAcSh infusions of CNO (2 μL of 1 μM) were given access to food as described above and average consumption of standard and high-fat diet was measured at intervals over a 24-hour period. Intra-NAcSh CNO decreased standard diet intake at 2 and 4 hours post-treatment ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=13-14 per group, Figure 5.6A,B). . High-fat diet consumption, however, was not significantly reduced in HM3D animals, as compared to control animals (Figure 5.6C,D), due to high variability.

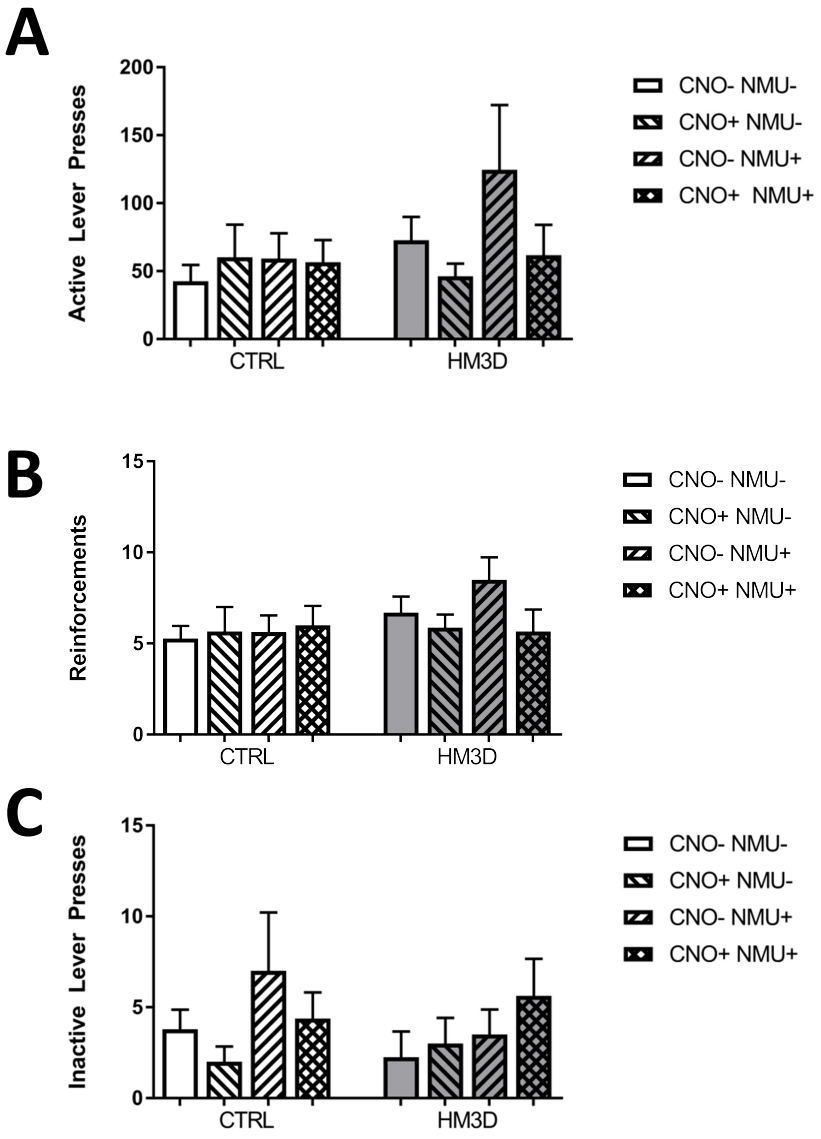

Figure 5.5. PR responding with cotreatment of NMU and CNO. A) No significant effects of NMU or CNO are found on active lever-pressing in CTRL rats. CNO does not affect active-lever pressing in HM3D rats. NMU has a highly variable effect on lever-pressing. B) No significant effects of NMU or CNO are found on reinforcers earned in CTRL rats; HM3D rats show minor, statistically insignificant decreases in reinforcers earned after CNO treatment, but increase earning with NMU. C) CNO and NMU effects on inactive-lever pressing are highly variable in the control rats, and enhancing in HM3D rats.

5.3.4 Effects of DREADD Activation and Inhibition of PVN-NAcSh Pathway on Operant Responding

Mirroring our NMU administration studies, animals trained to lever-press for high-fat food pellets were then treated with intra-NAcSh infusions of CNO (2 μL of 1 μM) or aCSF immediately prior to testing sessions. CNO delivery into the NAcSh increased PR responding for high-fat pellets in CTRL animals, while decreasing it in HM3D animals (Figure 5.7A,B; $p<0.05$ by two-tailed t-test, n=13-14 per group). Similarly, intra-NAcSh CNO administration caused a trend towards an increase in reinforcers earned on a PR schedule in CTRL animals, and a significant decrease in reinforcers earned in HM3D animals (Figure 5.7B,C, $p<0.05$ by two-tailed t-test, n=13-14 per group). No significant effect of virus alone (vehicle CTRL vs vehicle HM3D) or CNO alone (vehicle CTRL vs CNO CTRL) was found ($p>0.05$ by multiple-comparisons ANOVA, n=13-14 per group) (Figure 5.7). No significant effects of CNO treatment were found on inactive lever presses in CTRL or HM3D animals (Figure 5.7E,F). Importantly, the motivational effect was observable by evaluating patterns of burst responding during the operant session, with the number of bursts significantly lower in HM3D CNO-treated animals, but trending towards a slight increase in CTRL CNO-treated animals (Figure 5.8; $p<0.05$ by two-way T-test, n=13-14 per group). This suggests that activation of the PVN-NAcSh pathway acts directly to regulate not only consumption of, but also motivation for high-fat food.

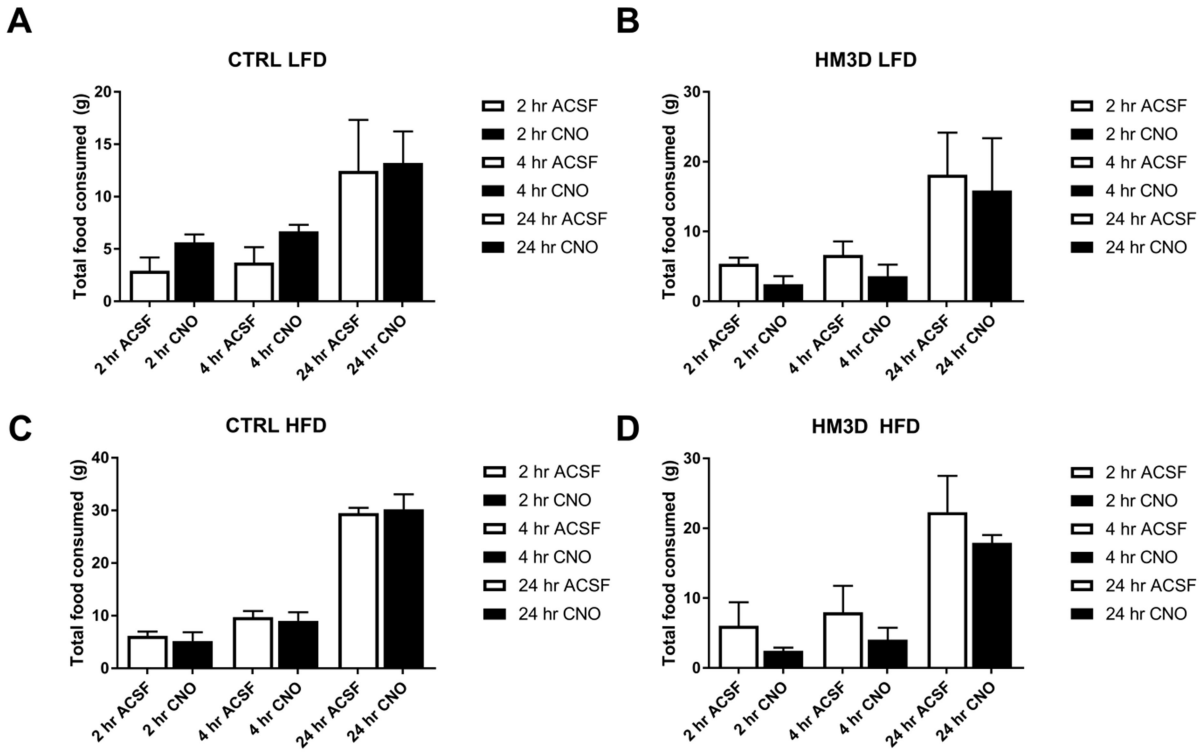

Figure 5.6. Regulation of low-and high-fat diet consumption by PVN-NAcSh pathway activation. A) CTRL animals do not significantly alter low-fat food consumption after CNO. B) HM3D animals consume significantly less high-fat food after CNO treatment. C) CTRL animals do not significantly alter high-fat food consumption after CNO treatment. D) HM3D animals consume significantly less high-fat food following CNO infusion into the NAcSh.

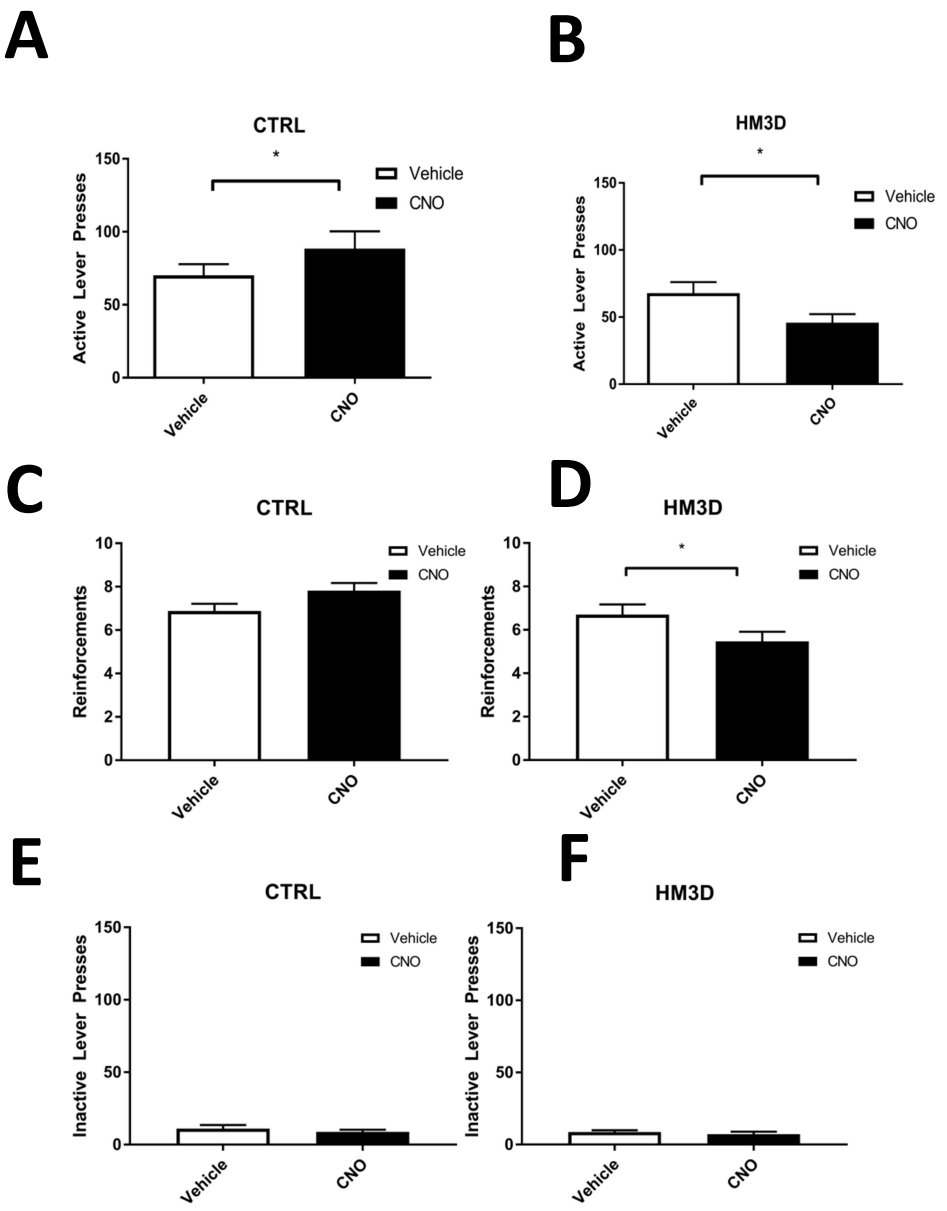

Figure 5.7. DREADD activation of the PVN-NAcSh pathway inhibits motivation for high-fat food. A) CTRL rats work significantly more for high-fat food after pathway activation. B) HM3D rats lever-press significantly less for high-fat food after PVN-NAcSh pathway activation. C) CTRL rats do not earn significantly more reinforcers following pathway activation. D) HM3D animals earn significantly fewer reinforcers after pathway activation. E) CTRL animals do not have significantly fewer inactive lever presses following CNO treatment. F) HM3D rats do not have significantly fewer incorrect lever presses than after CNO treatment.

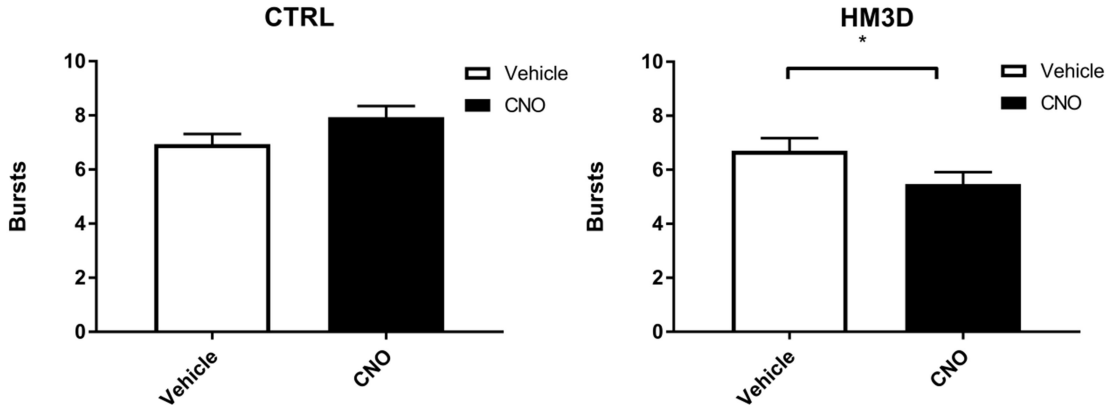

Figure 5.8. DREADD activation of the PVN-NAcSh pathway inhibits burst-responding for high-fat food. A) CTRL rats show no change when treated with CNO. B) HM3D rats produce significantly fewer bursts of responding after pathway activation

5.3.5 Effects of DREADD Activation and Inhibition of PVN-NAcSh Pathway on Locomotor Activity

In order to ascertain any potential side effects on gross activity that might be affecting our findings, we assessed locomotor activity immediately following intra-NAcSh infusions of CNO (2 μL of 1 μM) or an equal volume of aCSF. Animal CNO data was normalized to vehicle baselines, and CTRL and HM3D groups were compared in different measures of activity (Figure 5.7). No effect of CNO treatment was found on peripheral activity ($p>0.05$ by two-way t-test, n=13-15 per group, Figure 5.9A). Similarly, no effect was found on central, or exploratory activity ($p>0.05$ by two-way t-test, n=13-15 per group, Figure 5.9B). Rearing behavior, in which the animal stands on its hind legs to investigate the surroundings, also displayed no significant difference across treatment groups ($p>0.05$ by two-way t-test, n=13-15 per group, Figure 5.9C). A complete assessment of total locomotor activity, unsurprisingly, also indicates that viral activation of the PVN-NAcSh pathway has no significant effect on gross activity ($p>0.05$ by two-way t-test, n=13-15 per group, Figure 5.9D). This suggests that activation of the PVN-NAcSh pathway has no significant effect on overall mobility and activity, targeting feeding and motivated behaviors more specifically. .

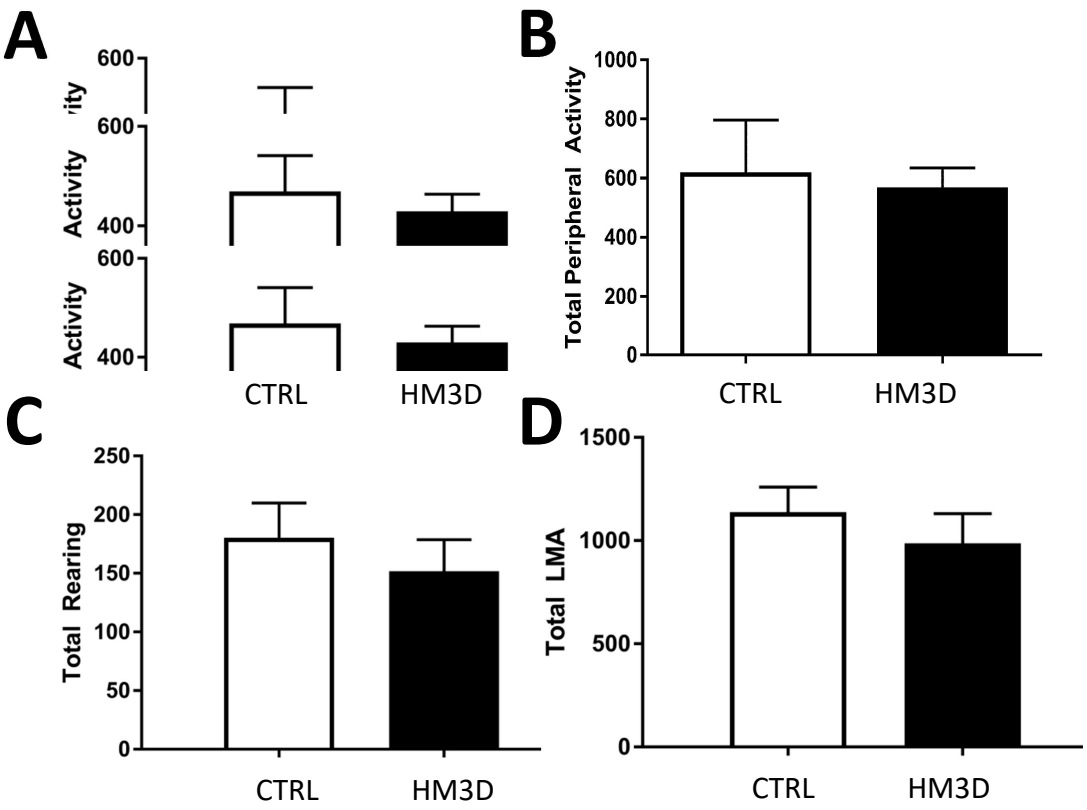

Figure 5.9. Locomotor effects of PVN-NAcSh pathway activation. A) Activating the pathway does not significantly alter total central locomotor activity. B) Activating the pathway does not significantly alter total peripheral locomotor activity. C) Activating the PVN-NAcSh pathway does not significantly alter total rearing, though there is a slight trend towards a decrease. D) Activating the PVN-NAcSh pathway does not significantly alter total locomotor activity, though there is a trend towards decreased activity following CNO treatment in HM3D animals.

5.3.6 Effects of DAMGO in the NAc on Feeding

Having identified the effects of PVN-NAc pathway activation on standard and high-fat food consumption, we sought to ascertain whether the effects were mediated by ENK. Following training, surgery, and recovery, animals receiving intra-NAcSh infusions of DAMGO (2.5 µg/2 µl) were given access to food as described above and average consumption of standard and high-fat diet was measured at intervals over a 24-hour period. Intra-NAcSh DAMGO increased standard diet intake at 2 and 4 hours post-treatment ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=13-14 per group, Figure 5.10A). . A similar effect was seen with animals on a high-fat diet, with consumption increased at 2 and 4 hours post-treatment ($p<0.05$ by multiple-comparisons ANOVA with Sidak post-hoc analysis, n=15 per group, Figure 5.10B).

5.3.7 Effects of DAMGO in the NAc on Operant Responding

Following our CNO administration studies, animals trained to lever-press for high-fat food pellets were then treated with intra-NAcSh infusions of DAMGO (2.5 µg/2µL) or aCSF immediately prior to testing sessions. DAMGO delivery into the NAcSh decreased PR responding for high-fat pellets, as compared to aCSF (Figure 5.11A; $p<0.05$ by multiple-comparisons ANOVA, n=21 per group). Similarly, intra-NAcSh DAMGO administration caused a decrease in reinforcers earned on a PR schedule (Figure 5.11B, $p<0.05$ by multiple-comparisons ANOVA, n=13-14 per group). No significant effect of DAMGO was found ($p>0.05$ by multiple-comparisons ANOVA, n=13-14 per group) (Figure 5.11C) was found on inactive lever presses. This suggests that activation of the NAc by DAMGO, a mu opioid agonist, inhibits motivation for high-fat food without altering lever-pressing capabilities.

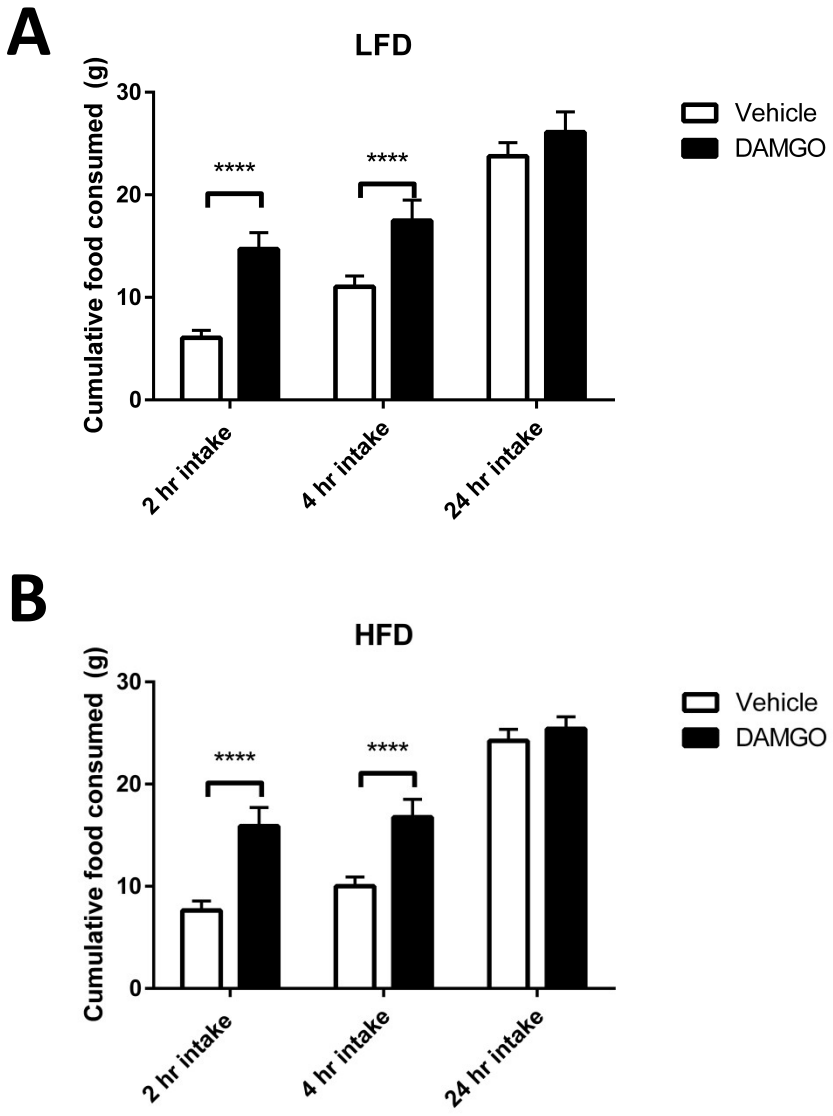

Figure 5.10. DAMGO infusion into the NAcSh increases consumption of low- and high-fat food. A) Animals infused with DAMGO into the NAcSh show increased low-fat diet intake at 2, 4, and 24 hours post-treatment. B) Animals infused with DAMGO into the NAcSh show increased high-fat diet intake at 2, 4, and 24 hours post-treatment.

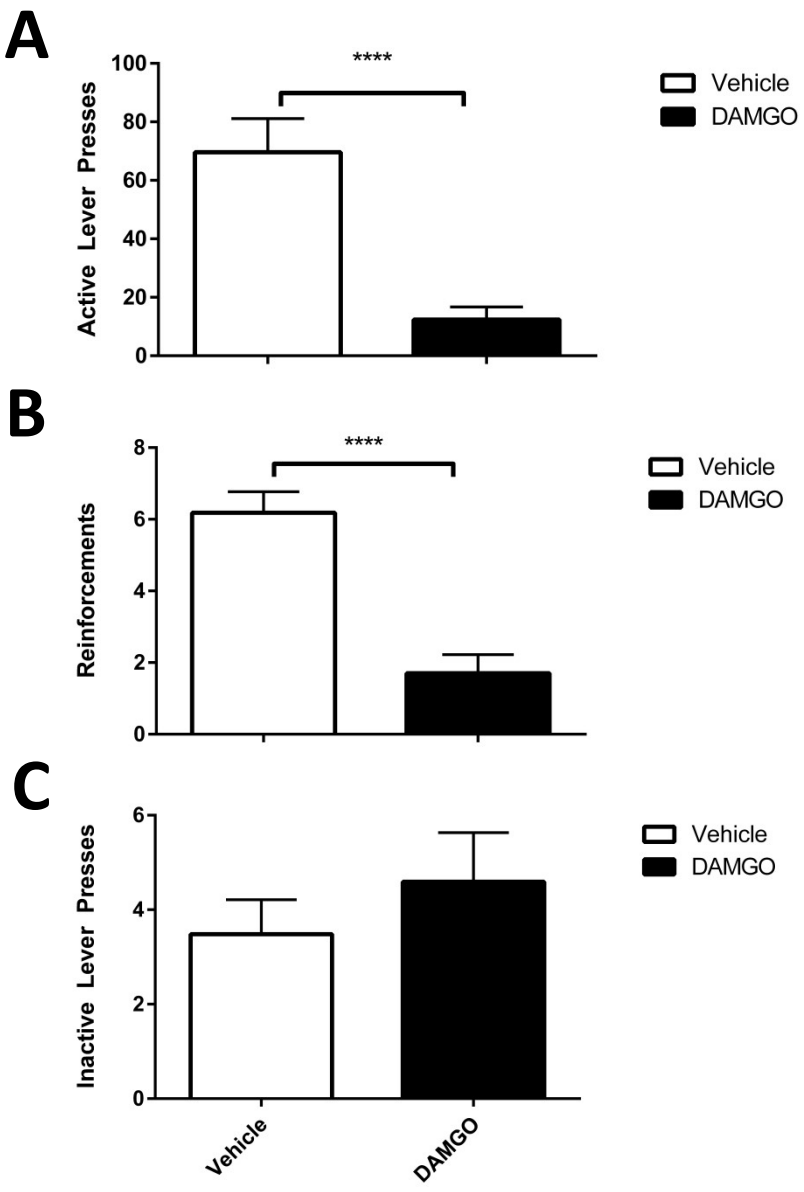

Figure 5.11. PR responding is inhibited immediately after DAMGO infusion. A) Active lever presses on a PR schedule are severely inhibited immediately following DAMGO treatment. B) Reinforcers earned on a PR schedule are severely inhibited immediately following DAMGO treatment. C) There is no significant change in inactive lever presses on a PR schedule immediately following DAMGO treatment, though there is a trend towards increased inactive lever presses.

5.3.8 Effects of Enkephalin Release in the NAc on Locomotor Activity

In order to ascertain any potential side effects on gross activity that might be affecting our findings, we assessed locomotor activity immediately following intra-NAcSh infusions of DAMGO (2.5 μg/2 μL) or an equal volume of aCSF. No effect of DAMGO treatment was found on peripheral activity ($p>0.05$ by two-way t-test, n=15 per group, Figure 5.12A). Similarly, no effect was found on central, or exploratory activity ($p>0.05$ by two-way t-test, n=15 per group, Figure 5.12B). Rearing behavior, in which the animal stands on its hind legs to investigate the surroundings, also displayed no significant difference across treatment groups ($p>0.05$ by two-way t-test, n=15 per group, Figure 5.12C). A complete assessment of total locomotor activity, also indicates that DAMGO infusion into the NAc has no significant effect on gross activity ($p>0.05$ by two-way t-test, n=15 per group, Figure 5.12D). This suggests that acute activation of the mu opioid receptor in the NAc by DAMGO has no significant effect on overall mobility and activity, though a trend towards decreased activity is visible. .

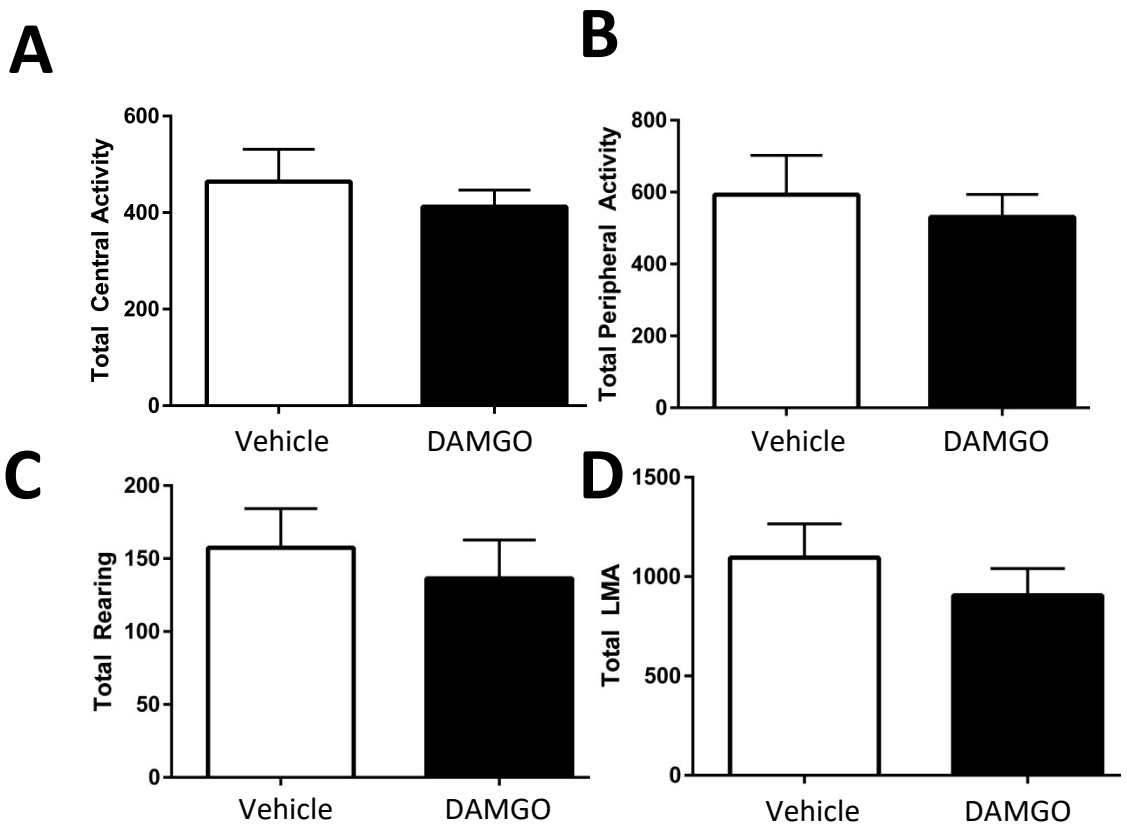

Figure 5.12. Immediate locomotor effects of intra-NAcSh DAMGO. A) DAMGO treatment does not significantly alter total central locomotor activity, though there is a trend toward decreased activity. B) DAMGO treatment does not significantly alter total peripheral locomotor activity immediately following treatment. C) DAMGO infusion into the NAcSh does not significantly alter total rearing activity immediately following treatment. D) DAMGO infusions into the NAcSh do not significantly reduce total locomotor activity, though there is a strong trend towards decreased activity as compared to vehicle control.

5.3.9 Time-Dependency of DAMGO Effects

As the effects of intra-NAc DAMGO[63] have previously been shown to invert 2 hours post-treatment, with a strong compensatory upregulation of activity, we investigated locomotor and operant response to DAMGO 2 hours post-treatment. Animals trained to lever-press for high-fat food pellets were treated with intra-NAcSh infusions of DAMGO (2.5 μg/2μL) or aCSF, and then returned to their home cages for 2 hours prior to testing sessions. DAMGO delivery into the NAcSh increased PR responding for high-fat pellets, as compared to aCSF (Figure 5.13A; $p<0.05$ by multiple-comparisons ANOVA, n=21 per group). Similarly, intra-NAcSh DAMGO administration caused an increase in reinforcers earned on a PR schedule (Figure 5.13B, $p<0.05$ by multiple-comparisons ANOVA, n=13-14 per group). No significant effect of DAMGO was found ($p>0.05$ by multiple-comparisons ANOVA, n=13-14 per group) (Figure 5.13C) on inactive lever presses. The reversal in responding can be seen across the course of the session, with DAMGO-treated animals responding far less in the immediate treatment condition (Figure 5.134A), and consistently more across the first third of the later session (Figure 5.14 B). Oddly enough, we see a decrease in responding in the vehicle animals 2 hours after treatment, as compared to their immediate treatment condition. It is possible that this may be a stress, or circadian rhythm effect, however, given the multiple disruptions required for the later timepoint, and the difference in testing hour.

A similar pattern is found in locomotor activity. Whereas immediately following DAMGO administration, animals showed a minor trend toward decreasing locomotor activity, 2 hours post-administration we see a significant upregulation of locomotor behavior. Rats previously habituated to the locomotor chambers were given intra-NAcSh infusions of DAMGO (2.5 μg/2 μL) or an equal volume of aCSF, and given 2 hours before the beginning of the locomotor assay. DAMGO treatment significantly increased peripheral activity ($p<0.05$ by two-way t-test, n=8 per group, Figure 5.15A). Similarly, an increase was found on central, or exploratory activity ($p<0.05$ by two-way t-test, n=8 per group, Figure 5.15B). Rearing behavior, in which the animal stands on its hind legs

to investigate the surroundings, did not display significant difference across treatment groups ($p>0.05$ by two-way t-test, n=8 per group, Figure 5.15C). However, a complete assessment of total locomotor activity, confirms that indicates that DAMGO infusion into the NAc, as expected, significantly increases gross activity ($p<0.05$ by two-way t-test, n=8 per group, Figure 5.15D) 2 hours after administration. Consistent with the literature[63], these data indicate that the effects of DAMGO in the NAc are time-dependent.

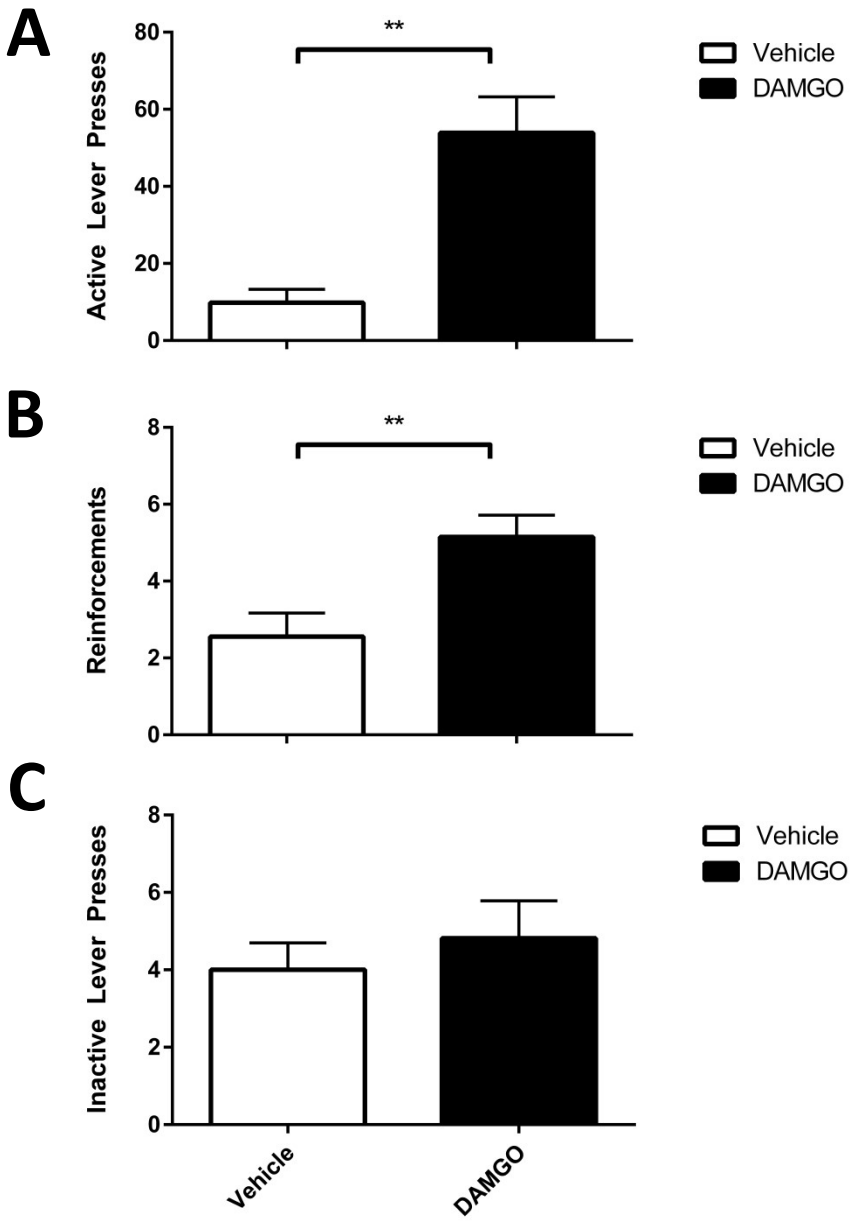

Figure 5.13. PR responding is increased 2 hours after DAMGO infusion. A) Active lever presses on a PR schedule are significantly increased 2 hours after DAMGO treatment. B) Reinforcers earned on a PR schedule are significantly increased 2 hours after DAMGO infusion into the NAcSh. C) There is no significant change in inactive lever presses 2 hours after DAMGO treatment, though there is a trend towards increased inactive lever presses.

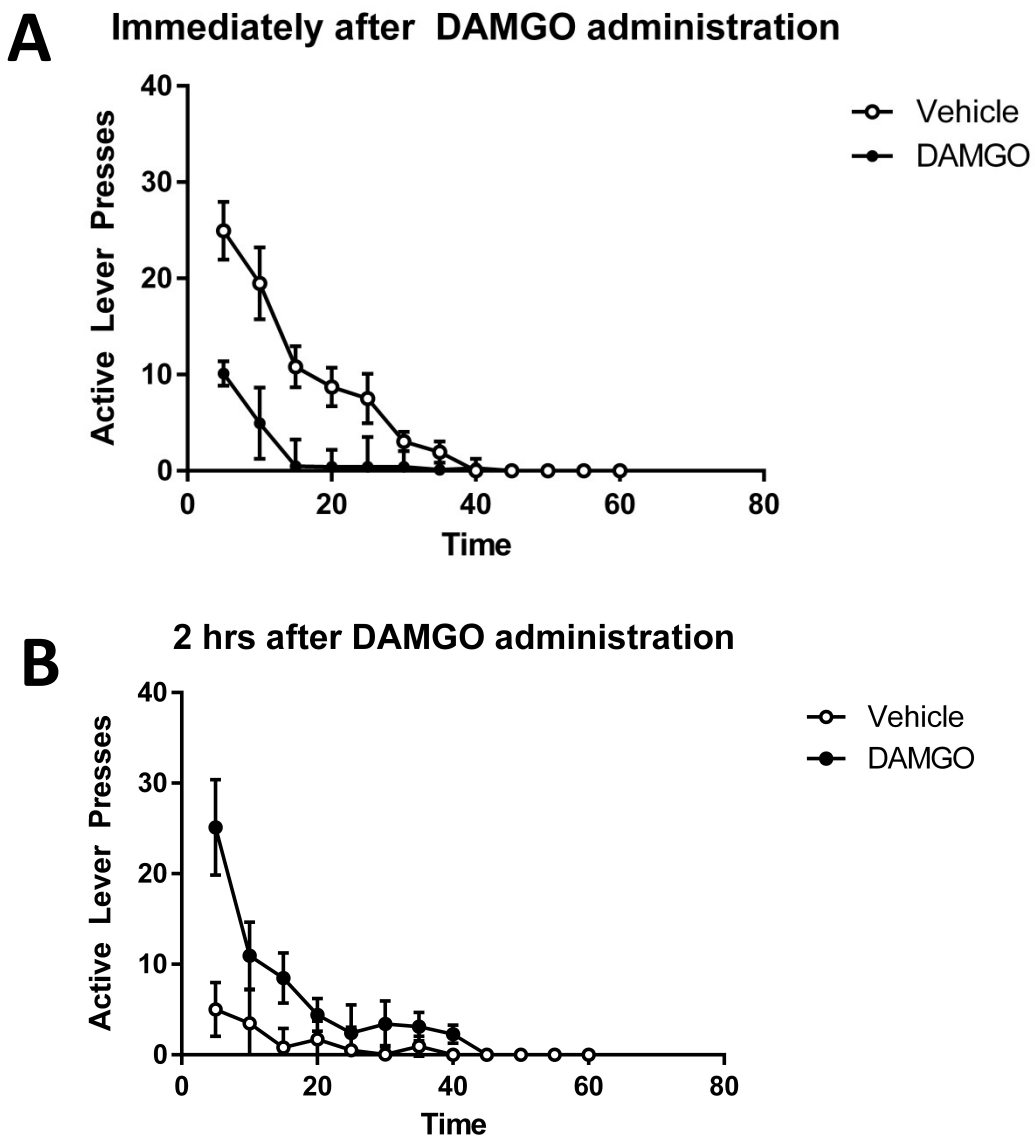

Figure 5.14. Time course of responding in DAMGO treated animals. A) DAMGO-treated animals respond significantly less for high-fat food pellets over the course of the testing session immediately following DAMGO infusion. B) DAMGO-treated animals lever-press significantly more for high-fat food pellets over the course of the testing session 2 hours after DAMGO treatment, while vehicle-treated animals greatly decrease their responding 2 hours after treatment.

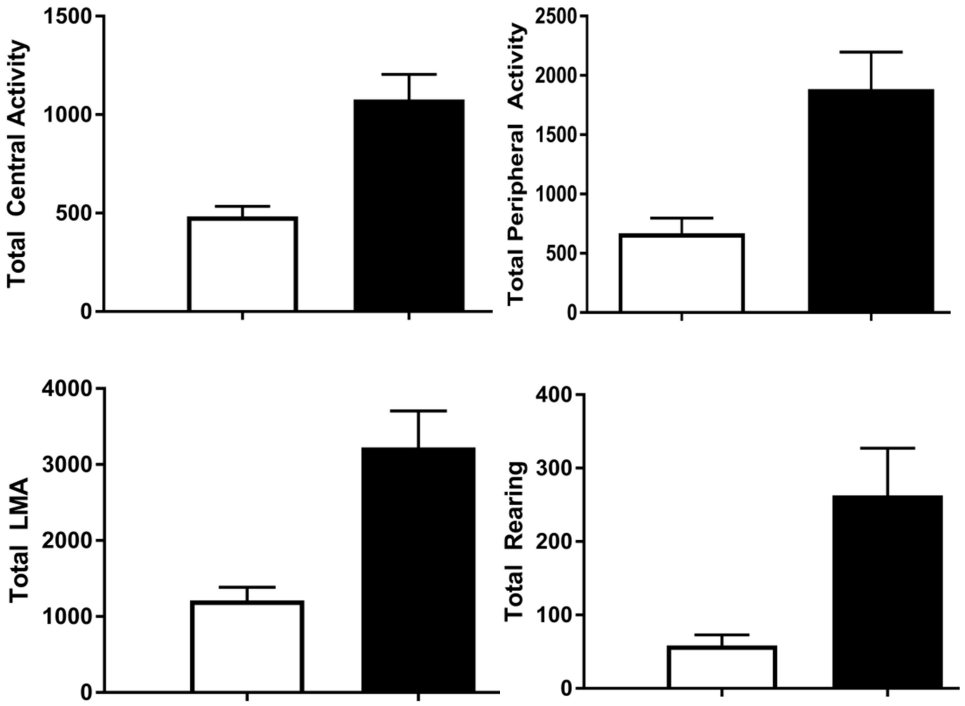

Figure 5.15. Locomotor effects of intra-NAcSh DAMGO 2 hours after administration. A) DAMGO treatment significantly increases total central locomotor activity 2 hours after treatment, consistent with the literature. B) DAMGO treatment significantly increases total peripheral locomotor activity 2 hours after treatment. C) DAMGO infusion into the NAcSh significantly increases total rearing activity 2 hours after treatment. D) DAMGO infusion into the NAcSh significantly increases total locomotor activity 2 hours after treatment.

5.4 Discussion

Our findings support the previously described work of Dolen et al. 2013 [102] in showing that a PVN-NAc pathway exists, and regulates reinforcement; moreover, we build on the enkephalins' established accumbal effects [24] to suggest NMUR2 signaling as a possible mechanism driving accumbal enkephalin and its regulation of high-fat food intake and motivation. We can also draw several other conclusions from this work. First, the PVN-NAc pathway could be driven by NMU and ENK, with NMU treatment leading to enkephalin upregulation in the PVN-NAc pathway. Second, the pathway regulates motivation for high-fat foods, not solely social reinforcement. Third, the effects appear to be time-dependent; the initial effects of DAMGO mimic those of NMU, but previously described compensatory mechanisms alter the long-term effects of DAMGO treatment. As such, the pathway may mediate the reinforcement effects of PVN NMUR2 signaling, through ENK, but total food consumption is differentially regulated. We can examine these findings on both behavioral and biochemical levels.

The initial viral tracing and colocalization studies revealed a potential downstream mechanism for the effects of PVN NMUR2 on motivated behavior for high-fat food. We confirmed the existence of the PVN-NAc pathway, as reported in Dolen et al 2013 [102], and reinforced the data through the use of both anterograde and retrograde tracers to identify the locations of both synapses, and cell bodies. We also demonstrate for the first time that the pathway expresses NMUR2, postsynaptically, in the PVN, making it a target for PVN NMU. Importantly, this implicates the PVN-NAc pathway as a mediator for the great body of PVN NMU literature on feeding, as well as the growing evidence of reinforcement effects. Additionally, we demonstrate, both through immunohistochemistry and RT-PCR, that these pathway neurons co-express NMUR2 and pre-proenkephalin (PENK), an enkephalin precursor. Treatment of animals with NMU, and isolation of PVN-NAc pathway neurons shows an augmentation of PENK expression, suggestive of accumbal enkephalin release in response to NMU. Therefore, a PVN-NAc pathway linking key feeding and reinforcement structures has been identified, shown to co-express NMUR2 and a known accumbal mediator of motivation, and to produce that mediator in response to NMU treatment.

Importantly, we further demonstrate that specific activation of the PVN-NAc pathway through excitatory DREADDs replicate many of the effects of PVN NMU treatment on food consumption and motivated behavior. Animals expressing the excitatory DREADD in the pathway decrease standard diet intake in response to accumbal CNO. Similar decreases are seen in consumption of high-fat food, but high variability in rat food consumption eliminated statistical significance. We find effects in excitatory DREADD animals on progressive-ratio responding and motivation for high-fat food that are consistent with findings from PVN NMU administration. Responding for high-fat food is decreased, as is total reinforcements earned, with no significant effect on inactive lever responding. In contrast, no significance was found in CNO-treated animals expressing the inhibitory DREADD in the PVN-NAc pathway, suggesting that there is insufficient native activity in the pathway for inhibition to produce a novel phenotype. These effects may be consistent with endogenous opioid signaling; they show a similar acute phenotype to opioid administration in the NAc. Kelley et al. [24, 155] have repeatedly shown that enkephalin agonism, particularly mu-opioid agonism, in the NA regulates food intake – including high-fat food intake – as well as offering a controlling role in motivation for food. While this is not conclusive evidence of a causative role, it does suggest that at least acutely, activation of the PVN-NAc pathway produces behavioral phenotypes similar to NAc enkephalin treatment.

Additionally, we find that these behavioral effects are consistent with a timeline of enkephalin effects. Animals treated with intra-NAc enkephalin produce initial deficits in a range of behaviors, including both operant responding and locomotor activity; previous research, however, has demonstrated that approximately two hours post-treatment, the animals will increase locomotor responding to significantly above baseline levels[63]. Here we demonstrate a similar pattern in HM3D rats treated with CNO, activating the PVN-NAc pathway. Initial deficits are found in locomotor activity, as well as operant responding for high-fat food, and are consistent with – though smaller in magnitude than – those produced by DAMGO infusions into the NAc. Interestingly, however, we find opposing long-term effects, when comparing PVN NMU treatment, PVN-NAc pathway activation, and intra-NAc DAMGO. Activation of the pathway, whether by NMU or CNO, appears to produce lasting effects on consumption of both

standard and high-fat food, well beyond the 2-hour inhibition seen in DAMGO animals. In contrast, rats undergoing mu-opioid agonism in the NA show increased consumption through the 24-hour timepoint. While there may indeed be an initial inhibition of food consumption by DAMGO, consistent with locomotor and operant data, the 2-hour resolution seems insufficient to demonstrate this.

As such, enkephalin does appear to be implicated in the activation of the PVN-NAc pathway, and its regulation of food intake, locomotor activity, and motivation. However, the contrasting data indicate that, at a minimum, pathway activation, whether by NMU or CNO, produces a biochemical response more complex than simply flooding the NAc with a mu-opioid agonist. Two components in particular offer some explanation: location and effect. First, the enkephalin – and DAMGO – literature originating from sources such as the Kelley lab[24, 155], the premier source for NAc and enkephalin studies, is primarily focused on a different region of the NAc. In order to achieve consistent and complete NAc coverage, the Kelley lab prefers rather more lateral targeting coordinates, aiming more towards the NAc core, a distinct subregion of the NAc. Our tracing data, however, indicate PVN-NAc projections tend to be distinctly more dorsomedial, and primarily in the NAc shell, consistent with other hypothalamic feeding projections to the NAc. As the core is more widely associated with motor expression of reinforcement behaviors[158, 159], as opposed to reinforcement itself, this may explain some of the differences as targeting effects. Moreover, our immunohistochemical and RT-PCR studies demonstrate a role for enkephalin within the PVN-NAc pathway. In particular, we show co-expression of NMUR2 and PENK in the pathway, and an upregulation of PENK in response to NMU treatment. This may be due to enkephalin release, into the NAc, consistent with the initial inhibitory effects of DAMGO treatment; alternatively, it may indicate a blockade of processing and release, inducing the more lasting changes in food intake that are seen in NMU-treated animals. To clarify the role of the pathway, we look to undertake a microdialysis analysis of activation of PVN-NAc neurons, both by CNO and NMU. This should confirm the role of enkephalin release, and offer some insight into the lasting regulatory effects that differ from blanket mu-opioid agonism.

Our data suggest a downstream mechanism by which PVN NMU-NMUR2 signaling regulates motivation for, and consumption of high-fat food. We link key feeding and reinforcement centers, and demonstrate that this PVN-NAc pathway is NMUR2 positive, and capable of regulating the behaviors produced by NMU administration into the PVN. Moreover, we indicate the potential role of the pathway in enkephalin signaling, altering NAc enkephalin in such a way as to influencing food intake and reinforcement. Taken together, these data suggest that NMUR2 within the PVN may regulate motivation for, and consumption of, high-fat food through enkephalin signaling in a PVN-NAc pathway.

Chapter 6: Summary and Conclusions

6.1 General Conclusions

Obesity remains a serious health concern, with over a third of American adults currently considered obese[1]. Moreover, obesity is comorbid with a wide range of disorders, ranging from heart disease to stroke, cancer, and diabetes[2]. As such, it is essential to identify the mechanisms leading to, and maintaining obesity. One such factor is food intake; the overconsumption of food, particularly foods high in dietary fat, is obesogenic[7, 160]. This overconsumption phenotype is maintained, despite the known negative side effects of obesity, due in part to the reinforcing aspect of high-fat foods. Both human and animal studies have demonstrated that high-fat foods in particular are a significant factor not only in the induction of obesity, but that consumption of highly reinforcing foods underlies excess caloric intake in obese individuals[3-5].

The high reinforcement efficacy of high-fat food leads to significant difficulties in successfully treating obesity. Both pharmacological and lifestyle interventions are able to induce certain amounts of weight loss, but both suffer from severe drawbacks. No more than ~10% of body weight is generally lost, even in ideal scenarios[127]. In addition, compliance with the treatment regimen remains poor, due to difficulty of major lifestyle alterations, and severe side effects from even the FDA-approved pharmacological treatments[161, 162]. As a result, more than 90% of individuals attempting to lose weight fail to do so long-term, relapsing, resuming maladaptive consumption behavior, and regaining weight within 5 years of initial treatment[10-14]. It is postulated that the reinforcing aspects of obesogenic food may play a key role in this behavioral paradigm, with the strength of the reinforcer, and craving for the high-fat food driving the high rates of relapse to overconsumption[3-5].

Of particular interest are neuronal pathways that have been identified as overlapping between feeding behaviors and reinforcement behaviors, with reinforcers such as exercise, drugs of abuse, and high-fat food having been shown to act through some of the same reinforcement circuits[39, 117, 163]. The nucleus accumbens shell, in particular, has been implicated in reinforcement signaling, mediating reinforcement effects for substances such as cocaine, as well as high-fat foods[22, 110, 164]. In

addition, recent research identifies a novel pathway to the NAcSh from the PVN [102], a key region for the regulation of energy balance and homeostasis. The PVN, too, has been reported to mediate reinforcing effects of drugs of abuse, and natural rewards such as sucrose[20, 119, 165-167], suggesting that this PVN-NAcSh pathway may play a key role in regulating the reinforcing properties of high-fat food.

The mechanisms underlying food reinforcement, however, remain relatively understudied. As previously addressed, there are many compounds and mediators capable of regulating food intake, or weight gain, but little investigation into the CNS reinforcement signaling that instigates and maintains high-fat food consumption. Virtually every mediator of CNS signaling has been implicated in regulation of food intake, either directly or indirectly, in one region of the brain or another, and many compounds targeting these signaling pathways have been investigated. However, most identified regulators either inhibit food intake in maladaptive fashions (e.g. methamphetamine[168, 169]), or do not produce a sufficiently large, sustained decrease in consumption in humans[170]. Moreover, of these regulators and their targets, relatively few have been associated specifically with altering the reinforcement value of food – Lorcaserin, a serotonin 2C receptor agonist recently approved for obesity treatment, being the noteworthy exception[171-173].

These concerns led us to the consideration of NMU-NMUR2 signaling, due to its demonstrated ability to decrease food intake via the PVN[80]. Importantly, NMUR2 is also known to be associated with obesity in humans, supported by multiple genome-wide associated studies, indicating a certain degree of translational potential[78, 79]. Our previous work builds upon this base, demonstrating that knockdown of NMUR2 in the PVN is capable of increasing animals' preference for high-fat food, and their increased food intake, and weight gain on a high-fat diet. Recent work from Vallöf et al. 2016 provides auxiliary support, indicating a role for NMU signaling in driving accumbal mechanisms of reinforcement for alcohol and amphetamines[43, 81]; Kasper et al. 2016[82] does much the same, confirming the ability of NMU to regulate behaviors associated with motivation for cocaine. These data strongly support the potential of

NMU-NMUR2 signaling to regulate food reinforcement, linking PVN NMUR2 to classical NAc reinforcement circuitry.

Our overarching purpose in this study is to examine the hypotheses that CNS NMUR2 signaling regulates intake of, and motivation for high-fat food in rats. This work demonstrates that PVN, and incidentally, DRN, NMUR2 regulates intake of, and motivation for high-fat food, and that these effects are mediated by downstream signaling through a PVN-NAcSh pathway. Specifically, we demonstrate that peripheral treatment with NMU is sufficient to inhibit consumption of standard and high-fat food in rats, and to significantly decrease motivated responding for pellets of high-fat food. Moreover, this treatment causes changes in Fos-like immunoreactivity in the PVN and DRN, and that both these regions receive NMU from LH projections, suggesting that they are responsive to endogenous NMU signaling capable of regulating food intake and reinforcement. Direct infusion of NMU into either region sharply inhibits intake of both diets, for a longer period than the peripheral treatment, as well as decreasing progressive-ratio responding for high-fat food. In addition, we demonstrate that knocking down NMUR2 within the PVN inhibits the incubation of craving-like behavior during abstinence from high-fat food. This confirms that PVN NMUR2 not only regulates food reinforcement acutely, but may drive long-term alterations in reinforcement behavior. We further demonstrate that the effects of PVN NMU on intake of, and motivation for high-fat food can be replicated through DREADD activation of the PVN-NAc pathway, and that the behavioral effects seen are consistent with regulation of the mu opioid receptor by accumbal ENK. We demonstrate colocalization of NMUR2 and PENK in the pathway, and show alteration of PENK in PVN-NAc pathway neurons in response to peripheral NMU. These data suggest that LH NMU is trafficked to the PVN, where it binds NMUR2 on neurons projecting to the NAcSh. Moreover, this may lead to the alteration of accumbal mu opioid signaling through the regulation of ENK release from the PVN-NAc pathway, and, in turn, influence motivation for, and consumption of obesogenic, high-fat food.

6.2 Specific Aim 1

In Specific Aim 1, we demonstrate the ability of NMU to regulate the reinforcement value of high-fat food, and identify the CNS targets mediating these effects. Acute peripheral NMU treatment (3 mg/kg i.p. in 10% DMSO) 15 minutes before the start of the dark cycle decreased intake of both standard and high-fat food. We next examined the effect in operant conditioning, administering the same dose 15 minutes before introduction of the trained rats into the operant chambers, and allowing them to press on a PR schedule. Acute peripheral NMU significantly decreased motivation for high-fat food, with NMU-treated animals lever-pressing less often for high-fat food, and receiving fewer pellets of food. As it had previously been established that NMU can act centrally (and it has since been established that NMU crosses the blood-brain barrier[174]), we investigated central activity triggered by peripheral NMU treatment, measuring Fos-like immunoreactivity 2 hours after treatment. We found that Fos-like immunoreactivity was decreased in the PVN and DRN, both regions established to have NMU-like immunoreactivity in previous studies. This suggested that NMU could inhibit consumption of, and motivation for highly reinforcing food through binding to its CNS receptor, NMUR2, in one or both of these locations.

In order to support this relationship, we applied immunohistochemistry and AAV tracers to both the PVN and DRN. This allowed us to confirm the presence of NMUR2 in both the PVN and DRN, and to demonstrate, for the first time, an endogenous CNS source of NMU: the LH sends NMU-producing projections to the PVN and DRN. In order to confirm that PVN and DRN NMUR2 played a key role in the feeding and reinforcement effects of NMU treatment, we treated rats with intra-PVN or intra-DRN NMU, immediately before the start of the dark cycle (or, alternatively, introduction into an operant chamber). Rats receiving NMU into either region displayed behavioral phenotypes identical to, if more pronounced than, those produced by the peripheral treatment. As such, consistent with previously identified effects of NMU on standard diet intake and drug-associated behaviors, we were able to confirm that NMU in the PVN and DRN inhibits consumption of, and motivation for high-fat food.

A separate cohort of rats received intra-PVN shRNA against NMUR2, expressed by an AAV-2 vector. These animals allowed us to examine the effects of PVN NMUR2 depletion on consumption of, and motivation for, high-fat food. We found that, consistent with our previous work[32], body weight is not affected by the depletion, as rats are maintained on a standard diet. Intriguingly, we also do not see an initial difference in operant responding for pellets of high-fat food, on a FR or PR schedule, despite an initial decrease in reactivity to cues associated with the food. This suggests two important points: first, that the intake and motivation effects of the PVN NMUR2 depletion require the incubation of craving-like behavior to develop. While receptor agonism may produce a significant effect acutely, even full viral RNAi expression appears insufficient to significantly alter the motivation to consume high-fat food. Second, these data indicate a novel role for PVN NMU-NMUR2 signaling, as a regulator of craving and cue reactivity. PVN depletion of NMUR2 severely blunts CR for high-fat food, and this effect persists following even extended periods of abstinence. Given that there are some established links between the PVN and cue behavior[109, 175, 176], this suggests an unsurprising conclusion: PVN NMUR2, despite not being well-known, is not an isolated 'silver bullet' mechanism for overconsumption of high-fat food. Rather, it likely acts through multiple downstream pathways, altering reinforcement and cue behaviors differently over the course of the experiment.

These data complement existing findings on the role of NMU signaling in feeding and reinforcement behaviors. Investigation of NMU as a mediator of taking and reinforcing behaviors has previously been undertaken from multiple directions, including alcohol, amphetamine, and cocaine[43, 81, 82]. The Jerlhag lab, for example, has tested central (ICV) administration of NMU with ethanol intake[43]. Moreover, they have demonstrated that ICV NMU disrupts conditioned place preference, a key indicator of the reinforcing properties of a substance, for both ethanol and amphetamines[43, 81]. Their work, and that of Kasper et al. 2016[82], demonstrate that NMU treatment similarly inhibits amphetamine- and cocaine-evoked locomotion. Coupled with the identification of PVN NMU-NMUR2 signaling as a mediator of NMU's anorexigenic effects, these data reinforce the notion of NMU-NMUR2 signaling as a mechanism controlling the

regulation, or dysregulation of the overlapping signaling pathways involved in reinforcement behavior. .

The reinforcement-related effects of NMUR2 signaling in VTA-NAc and DRN-NAc pathways, as well as the unexpected response from NMUR2 depletion in the PVN, suggest that the story may be more complex than previously imagined, however. Multiple NMUR2 signaling pathways appear to converge on the NAc to regulate reinforcement[39, 117]; this actually offers some potential for specific targeting, potentially altering solely food- or drug reinforcement, for example. Unfortunately, insufficient data exists to fully tease apart any potential homeostatic and hedonic separation between the NMUR2 signals, particularly given that individual labs and investigators have focused their investigation of NMU reinforcement effects on particular – and different – brain regions. That said, our previous work indicates a potential for selectivity, with a PVN-specific depletion of NMUR2 increasing high-fat food intake, and preference, significantly over standard rodent chow[32]. Our operant studies, with a separate cohort of rats with PVN NMUR2 depletion, however, indicate minimal phenotypic or behavioral differences in treated animals, as compared to those expressing a scrambled control hairpin. Specifically, decreased reactivity is seen to cues associated with high-fat foods, but intake of, and motivation for such foods remain unaltered. Only following an abstinence period do the intake and reinforcement effects of the depletion become apparent, with depletion significantly inhibiting the incubation of craving-like behavior following a 30-day abstinence period. This discrepancy may suggest different pathways mediating the cue- and food-reinforcement effects, represents an different perspective from the incubation effects found with sucrose exposure and abstinence, by researchers such as Grimm and Avena[97, 113]. In particular we identify a potential mechanism mediating not only food intake and motivation for reinforcing foods, but the incubation of the craving-like effect, and thus, relapse to overconsumption of high-fat food.

6.3 Specific Aim 2

In Specific Aim 2, we sought to identify and characterize downstream projections mediating the effects of PVN NMUR2 signaling on intake of, and motivation for high-fat food. Through the use of fluorescently labeled AAV vectors as anterograde and retrograde tracers, we confirmed the existence of the neuronal pathway linking the PVN to the NAcSh. As we suspected that this pathway was mediated by NMUR2 signaling, we made use of immunohistochemistry to characterize it as NMUR2-positive. This represents the first evidence of NMUR2 signaling linking hypothalamic feeding circuitry arising in the PVN, reinforcement and motivation pathways in the NAc. To investigate whether signaling through this PVN-NAcSh pathway mediated the effects of PVN NMUR2 on consumption of, and motivation for high-fat food, we made use of DREADDs. We infused AAV-2 expressing either an excitatory or inhibitory DREADD into the PVN; rather than simply activate or inhibit all PVN neurons, we cannulated the NAcSh, at the location established by our tracing work, to allow us to confirm that the PVN-NAcSh pathway in particular mediates the previously identified behavioral effects. Rats receiving infusions of CNO into the NAcSh before the beginning of the dark cycle showed moderate decreases in food intake on a standard diet. This effect, interestingly enough, was not found on a high-fat diet, due in part to great variability in individual consumption.

However, we do find that CNO treatment immediately before the operant task induces a significant decrease in motivation for high-fat food in HM3D animals, consistent with the effects of intra-PVN NMU. Animals expressing the HM4D virus, which leads to pathway inhibition, did not display a significant behavioral change; power analysis following the first cohort indicated that, if the trends were maintained, more than 60 additional animals would be required to demonstrate any significant effect. Due to the impracticality of running 3-4 more cohorts (with applicable controls), we pursued the role of PVN-NAcSh pathway excitation. Importantly, no significant effect is found on locomotor activity, regardless of central or peripheral location, and rearing remains unaffected. This suggests that pathway activation produces an effect more specific for feeding and reinforcement than general locomotion. Further, the lack of effect on central

or peripheral motion offers a contrast to some of the other NMUR2-mediated signals known to originate in the PVN, such as the activation of the hypothalamic-pituitary-adrenal (HPA) axis. While Jethwa et al. 2006[177] demonstrates a role for NMU through leptin, corticosterone, and stress signaling, for example, here we find that activation of the NMUR2-expressing PVN-NAc pathway does not appear to increase or decrease characteristic stress behavior. That said, it is known that PVN NMUR2 can induce some of its behavioral effects, including decreases in food intake, through the regulation of the HPA axis, indicating that NMU does not act solely on cue- or food reinforcement to inhibit motivation for, and consumption of high-fat food. These data offer an interesting counterpoint to our findings with the Fos-like immunoreactivity, however, as peripheral administration of NMU was shown to decrease Fos-like immunoreactivity in the PVN, while activation of the PVN-NAc pathway with the HM3D DREADD results in similar behavioral effects. We must consider, however, the response of other, particularly paraventricular sites, in response to the peripheral treatment, as well as the demonstrated abilities of NMU to regulate the hypothalamic-pituitary-adrenal axis [183]. As NMUR2 has been shown to be both Gi- and Gq-coupled [71], this too may help to explain the apparent differences in signaling that result in similar behavioral effects. To be sure, however, it would be valuable to investigate the effects of intra-PVN NMU on PVN Fos-like immunoreactivity, and to identify the resulting changes in Fos-like immunoreactivity in neurons projecting to the NAc.

Previous work, particularly by the Kelley lab, has demonstrated that food intake, the reinforcement properties of high-fat food, may be mediated by enkephalin signaling in the NAc[24, 154, 155]. In particular, they demonstrate that infusions of ENK, and more specifically, the mu opioid agonist DAMGO, into the NAc result in increased food intake, and increased motivation for highly palatable food. As we have shown intake of, and motivation for high-fat food to be regulated by a NMUR2 pathway from the PVN to the NAc, we suspected that the pathway might be mediating its effect by means of ENK. Through immunohistochemical labeling of the pathway, we confirm that the PVN-NAcSh pathway does, in fact, express ENK, and furthermore, it coexpresses ENK and NMUR2. As no direct link between the two had been previously established, however, we labeled NMUR2 neurons in the PVN, treated the rats with NMU, and then

microdissected out the PVN. By dissociating the tissue, and applying flow cytometry, we were able to isolate the NMUR2 neurons, and use PCR to investigate changes in PENK mRNA in NMUR2 neurons in response to NMU. Oddly enough, we find an increase in PENK mRNA following NMU treatment. While this stands as something of an unexpected result, given the Kelley lab's demonstration of intra-NAc ENK increasing food consumption, we have yet to determine whether this is due to ENK release in the NAcSh, or an alternate mechanism, such as decreased conversion of PENK to ENK, for example. . That said PVN NMUR2 is colocalizes with ENK, and increased PENK in response to NMU treatment supported the notion that the PVN-NAcSh pathway might mediate food intake and motivation through enkephalin signaling.

To that end, we infused DAMGO into the NAcSh, at the same coordinates at which we had previously administered CNO. Infusions of DAMGO or vehicle were performed before the beginning of the dark cycle, immediately prior to the operant task, or following acclimation to locomotor chambers. We found that DAMGO infusions into the NAcSh increased food intake over a 2, 4, and 24 hour period post-treatment; while these data are consistent with the Kelley lab's administration of DAMGO to the accumbens core, we were somewhat surprised to find the effect counter to the patterns of NMU administration. After all, NMU infusions into the PVN decreased food intake, and NMU appears to increase PENK mRNA in the PVN-NAcSh pathway. The effects were further confounded by our operant studies, which indicate that DAMGO infusions at our target coordinates severely inhibit motivation for high-fat food, with DAMGO-treated rats lever-pressing significantly less and earning significantly fewer reinforcers. Additionally, we found a minor decrease in locomotor activity in DAMGO-treated animals, contrasting sharply with conventional literature on DAMGO's effects in the NAc.

Further examination, however, lends a clue: previous studies show that DAMGO effects differ greatly depending on the time period considered, with DAMGO initially suppressing activity, then increasing it several hours post-treatment[63]. This provided a reasonable explanation for the opposing results of our feeding, locomotor, and progressive-ratio studies: they take into account different time periods following the

treatment. To that end, we repeated DAMGO infusions into the NAcSh, and returned rats to their home cages for 2 hours before placing the rats in the test chambers. Two hours after administration, rats responded significantly more for pellets of high-fat food, earned significantly more reinforcers, and showed increased locomotor activity. This compensatory increase indicates that the enkephalinergic effects on NAcSh-driven motivation are indeed time-sensitive, and that the motivational effects of PVN-NAcSh pathway activation are, at a minimum, consistent with mu opioid agonism by DAMGO in the NAcSh. That said, neither administration of NMU into the PVN, nor activation of the PVN-NAcSh pathway produces ad libitum feeding effects consistent with DAMGO administration into the NAcSh.

There are, however, a number of confounding factors, including dose and specificity. The DAMGO infusion, sufficient to induce a prolonged activation of accumbal mu opioid receptors, likely produces far greater activation and coverage than potential ENK release from the PVN-NAc pathway. And while we have shown that PVN-NAcSh neurons express ENK in the synaptic terminals, we have not yet confirmed that ENK is released by these neurons, or what range of ENK concentrations is normally found before, and after ENK release. Thus, there is the potential that our dose of DAMGO is outside the normal range released by the pathway, preventing a direct comparison of the effects. Moreover, we know that the NAcSh contains neurons expressing the mu opioid receptor that are not directly targeted by the PVN-NAcSh pathway, so DAMGO treatment induces a wider range of neuronal activation than pathway activation alone. Similarly, we know that NMU infusion into the PVN, while capable of activating the PVN-NAcSh pathway, also regulates other NMUR2 signals, including leptin-mediated regulation of food intake, and stress signaling via the HPA axis[86, 177]. These limitations may help explain some of the discrepancy between the increased *ad libitum* feeding effects of intra-NAcSh DAMGO, and the decreases seen with intra-PVN NMU and PVN-NAcSh pathway activation.

6.4 Summary and Future Directions

In these studies, we sought to identify a role for NMU in the motivated consumption of high-fat food, and characterize underlying neurocircuitry that mediate its behavioral effects. Investigation of NMU, an endogenous anorexigenic peptide that has been demonstrated to regulate reinforcement-like behaviors for drugs of abuse, offers an insight into potential mechanisms driving obesity. In particular, we find that NMU signaling, through NMUR2, may address some of the key weaknesses in our existing mechanistic understanding of obesity: the reinforcing nature of obesogenic foods, and the propensity to relapse and return to overconsumption of these foods. Both clinical and preclinical studies have established the importance of inhibiting these maladaptive consumption behaviors, if obesity is to be reduced, and if weight loss strategies are to be successful long-term[3-5]. Genome-wide association studies in humans go one step further, supporting the potential for NMUR2 as an obesogenic regulator that may show translational relevance beyond the preclinical, affecting consumption behavior and obesity in both humans and rodents[78, 79].

Thus, the value of NMUR2 as a potential target is enhanced; as an endogenous peptide, it appears to lack the significant side effects of current weight loss treatments[75, 90, 107, 112], and recent research indicates that the peptide itself is capable of crossing the blood-brain barrier[174]. Moreover, we have established that NMUR2 is capable of regulating preference for high-fat foods, and that it may, in fact, differentially affect weight gain on high-fat, but not standard diets[32]. Our latest work builds upon this, demonstrating that NMU signaling directly regulates not only food consumption, but obesogenic food consumption, not only consumption, but motivation to consume. Importantly, we also find that NMUR2 is a major player in relapse behavior, with animals expressing lower levels of NMUR2 in the PVN showing minimal or reduced incubation of craving-like behavior.

These findings offer great potential for NMUR2 signaling as a key mechanism underlying obesity. In particular, we hope to expand our work to both human and animal samples, and investigate the effects of individual variation in NMU and NMUR2 levels.

As we have found high variation in expression levels, and have established significant behavioral effects of NMU and NMUR2, we believe that differences in endogenous expression may offer some insight into propensity for overconsumption of high-fat foods, and thus, obesity. To test this, we have begun investigation into protein and mRNA expression levels for NMU and NMUR2 in the rodent CNS, starting with regions implicated in feeding and reinforcement. By correlating expression levels with behavioral data, we should be able to reinforce the findings produced by our pharmacological and genetic manipulations, and to offer a platform for their modification *in vivo* through the use of two-component viral knockdowns, such as the Cre-Lox system. Additionally, we have begun testing small molecule agonists for the receptor, using compounds synthesized by Dr. Zhou, here at UTMB. The current compounds have met with moderate success, limited by compound specificity, in initial feeding tests. We intend to continue our collaboration with Dr. Zhou, screening potential NMUR2 regulators in our new NMUR2-expressing cell line, and examining their ability to regulate motivation for, and consumption of high-fat food in rodents.

Characterizing not only the symptoms, but the underlying mechanisms of diseases and disorders is an essential component of successfully understanding and treatment. Obesity, in particular, is well-characterized in terms of its symptoms and comorbidities, but the underlying mechanisms leading to, and maintaining the condition are poorly understood. Thus, we sought to identify novel circuitry for a relatively unknown neuropeptide signal, having established its role in mediating the motivated consumption of high-fat food, and the propensity of individuals to relapse, or break their dieting regimes. We demonstrate that NMU, a regulator of high-fat food consumption, acts in CNS structures classically associated with both food, such as the PVN, and reinforcement, such as the DRN. We clarify that endogenously, these regions should be innervated by NMU-releasing projections from the LH, and that the PVN, in particular, then activates a pathway to the NAcSh, a major center for reinforcement signaling. In addition, we establish that this pathway is not only driven by NMUR2, but may produce its effects through the regulation of ENK, a known regulator of high-fat food consumption and motivation, and its release into the NAcSh. Specifically, it may do so

through activation of the mu opioid receptor in the dorsomedial NAcSh, which appears to receive the projections from the PVN.

We now hope to confirm the release of ENK from the PVN-NAc pathway, in response to NMU treatment, by means of microdialysis. We have established a collaboration with Dr. Mark Emmett, a world expert at the microdialysis of enkephalin, here at UTMB. This should allow us to clarify if the pathway is releasing ENK into the NAcSh. Additionally, we would seek to confirm that the actions of the PVN-NAcSh pathway on regulating motivated behavior are driven through mu opioid receptor agonism in the NAcSh; enkephalin has been shown to bind both the mu and delta opioid receptors[63, 69, 110, 154, 156], and both signaling pathways have been implicated in reinforcement and consumption behaviors. The work of the Kelley lab suggests that, at least in the NAc, mu opioid receptor agonism, but not delta opioid receptor agonism, induces significant effects on food consumption and reinforcement[154]. Additionally, we seek to clarify the timeline of behavioral effects, particularly for the PVN-NAcSh pathway, and PVN NMU. Our work supports the idea that the PVN-NAcSh pathway may act through ENK, but the differences in ad libitum feeding behavior are considerable. Moreover, we have not yet established a time course for the effects of PVN NMU, or PVN-NAcSh pathway activation. If the effects are indeed regulated by ENK signaling in the NAcSh, we would expect to see compensatory spikes in motivation at later timepoints. These experiments would allow us to expand upon the reinforcement signaling mechanisms of NMUR2, and tie it to the existing literature on feeding and motivated behavior.

Elucidating and regulating the mechanisms of reinforcement and craving is thought to be essential in successfully treating obesity, and compounds that have been shown to alter reinforcement, such as Lorcaserin[42, 171, 172, 178], have been somewhat successful in regulating obesogenic behavior. We show that neuromedin U acts through previously unknown pathways to link feeding and reinforcement behaviors, and demonstrate its association to known regulators of food intake (Figure 6.1). Therefore, we are confident that investigation of hypothalamo-accumbal NMUR2 signaling, as a

mechanism underlying motivation and craving behaviors, will lead to a clearer understanding of obesity.

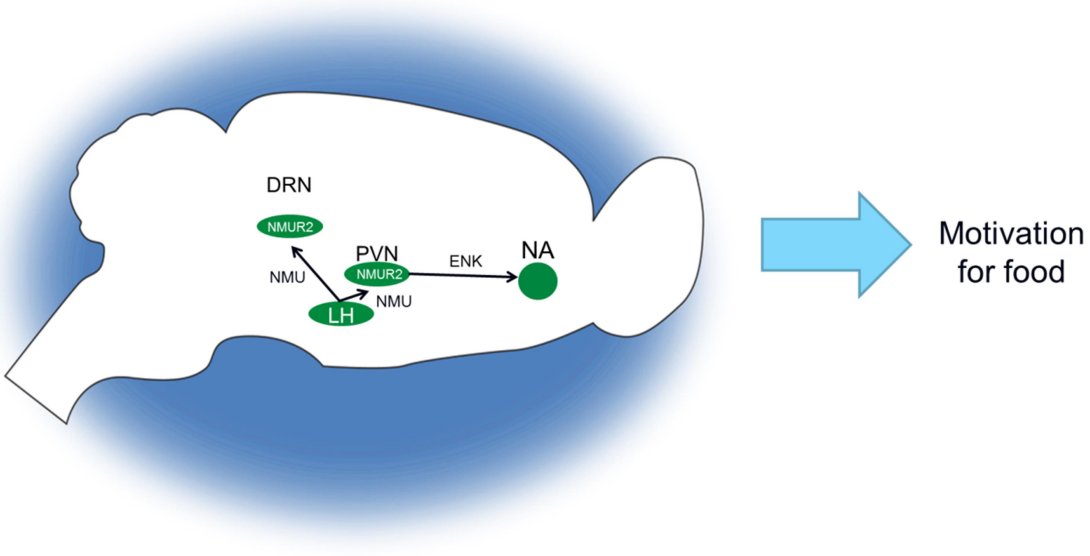

Figure 6.1. Proposed mechanism by which NMU-NMUR2 signaling endogenously regulates motivation for high-fat food.

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VITA

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