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Incubation of feeding behavior is regulated by neuromedin U receptor 2 in the paraventricular nucleus of the hypothalamus

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Abstract

A diet of energy-dense food, characterized mainly as a high-fat diet, leads to a persistent excessive consumption defined as overeating. According to the National Institute of Health, more than 2 in 3 adults in the United States are overweight or obese, straining our healthcare system with epidemic proportions. Diets that include abstaining from high-fat foods, ironically, result in an increase in motivation and craving for said high-fat foods, defined as an incubation effect because the behavior aids in developing overeating. Previously, we have shown that modulation of neuromedin U receptor 2 (NMUR2) in the paraventricular nucleus of the hypothalamus (PVN) results in increased food intake and motivation for energy-dense foods. Here, we continue our focus on NMUR2 in the PVN, but in relation to the incubation effect on craving for high-fat food. We employed a model for incubation of craving by having rats abstain from high-fat foods for 30 days before undergoing intake of fatty food on fixed ratio and progressive ratio schedules of reinforcement, and then assess their response to reactivity to cues. Using this model, we compared the feeding behaviors of rats that underwent an mRNA knockdown of the NMUR2 in the PVN to controls after both underwent a 30-day abstinence from high-fat foods. Our results show knockdown of NMUR2 in the PVN blocks the incubation of feeding behavior for food-related cues and high-fat foods.

1. Introduction

High-fat diets are preferred over those lower in fat [1-4]. Brain regions associated with this preference include the hypothalamus and nucleus accumbens, areas associated with abstinence-induced changes in intake and motivated behavior, making high-fat consumption highly reinforcing [5-7]. When available *ad libitum*, a high-fat diet causes overeating and obesity, which is comorbid with some of the leading causes of preventable death, including heart disease, stroke, and certain types of cancer [8]. As such, we focused on a high-fat diet, with 45% of kcal from fat, which is obesogenic by causing increased adiposity [9]. A

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common diet plan is to abstain from high-fat foods for extended periods in an effort to exert behavioral self-control and lose body weight [10-14]. However, motivation for food is elevated after extended abstinence periods, with longer abstinence increasing the strength of the incubation effect [15]. Human studies have demonstrated that incubation behavior associated with high-fat food, in which food is taken, valued, and sought after at higher rates following a period of abstinence, underlies excess caloric intake, and is a key factor in the maintenance of obesity [16-19].

Rats too demonstrate incubation of feeding behaviors associated with overconsumption of high-fat food. Using an operant-response paradigm, reinforcement (the probability to shape future behavior), motivation (underlying reinforcement efficacy), and cue reactivity (associated with craving-like behaviors) can be quantified through number of lever presses toward acquisition of a high-fat food reinforcer or food-associated cues. The degree of incubation related to these behaviors can be measured by comparing lever presses prior to forced-abstinence to numbers immediately following the abstention from high-fat foods.

Though behavioral paradigms have confirmed the incubation of feeding behaviors, crucially, the associated mechanisms that leads to overeating remains understudied. In turn, we examined the neuroanatomical structures and signaling mechanisms potentially mediating incubation behaviors. Previous research has shown intracerebroventricular administration of the endogenous anorectic peptide NMU in rats acts as a central controller for food intake, with changes in hypothalamic NMU levels in conjunction with fasting [20]. Additionally, in female rats on a high-fat diet, a global knockout of the predominant central nervous system NMU receptor, the G protein-coupled NMUR2, was protective against weight loss [21]. This receptor has been implicated in regulating the preference for high-fat food over leaner diets, strengthening its role as a regulator of high-fat food intake [3]. Furthermore, studies focusing on hypothalamic NMUR2 have shown that NMU administration within the PVN regulates food intake [22, 23]. Though NMUR2 knockdown has been linked to food reinforcement, food preference, and changes in body weight [3], the role of NMUR2 in mediating incubation of feeding behaviors is unknown. Here, we use an operant-response paradigm to investigate the incubation of craving-type behavior for high-fat food in rats following knockdown of NMUR2 in the PVN.

2. METHODS

2.1 Subjects

Male Sprague-Dawley rats (n=28; Harlan, Inc., Houston, TX) weighing 225-250 grams (at experiment start) were used for all experiments. Animals were single-housed and maintained on a diet of standard rat chow (Teklad Mouse/Rat Diet 7912, Harlan Laboratories, Inc., Indianapolis, IN, USA), ad libitum, in home cages. Animals were weighed prior to dark cycle start (18:00). Colony environment was maintained at 71°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 Animal* (Eighth Edition), and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

2.2 Operant Conditioning

Operant conditioning was performed adhering to established procedure [24]. Between 13:00 and 17:00, rats were maintained in standard rat operant chambers incorporating a two-lever system (Med Associates, Georgia, VT). Responding on the active lever resulted in delivery of a high-fat food pellet (45% energy from fat, 45mg, Bioserv F06162, Frenchtown, NJ) into a small metal food tray and the blinking of a white cue light at one Hz for five seconds. A second, inactive lever was present in the chamber to distinguish reinforcer-seeking behavior from a response inducing generalized changes in activity. Responses on the inactive lever, which did not deliver pellets, were recorded.

We employed the fixed ratio (FR), progressive ratio (PR), and cue reactivity (CR) measures of feeding behavior within operant chambers to investigate incubation effects. An FR schedule of reinforcement measures the probability of the high-fat food to reinforce future behavior, such as lever pressing, independent of increasing effort. For example, a fixed requirement of five lever presses (FR5) is required for the delivery of each high-fat reinforcer throughout the session. In a PR schedule, an increasing number of lever presses is required to obtain each subsequent high-fat food reinforcer. For example, one lever press delivers the first reinforcer, two lever presses for the second reinforcer, four lever presses for the third reinforcer, etc. Eventually, the animal will reach its “breakpoint” by failing to achieve the required number of lever presses for the next reinforcer, thereby providing a quantitative measure of reinforcement efficacy [25, 26] used as a model for motivated behavior. Finally, CR to the high-fat food reinforcer measures the animal’s willingness to work solely for the reinforcer-associated cues in the absence of the reinforcer itself. Animals are trained to lever press for high-fat food pellets in the presence of specific light and sound cues. Once conditioned to the cues associated with the pellet reward, the rats are tested on their lever-pressing behavior to the cues alone as a reward (the pellet is withheld) on both FR and PR schedules. During periods of abstinence, studies have well-established that the lever pressing of rats reflect an incubation of craving behavior [27, 28]. The CR data provide an insight into the relative reinforcement value of the high-fat food pellets, independent of any direct effects from consumption [29].

Rats first were trained in half-hour sessions (one session per day) on an FR1 schedule, where a single response on the active lever is needed to receive a pellet and cue delivery. Once the responses on the active lever exceeded 85% for three consecutive days, animals were advanced to an FR3 and then an FR5 schedule, which required three and five correct responses for pellet delivery, respectively. After animals reached the FR5 criterion, they were moved to a PR schedule, where progressive responses (1,2,4,6,9,12,15,20,25,32,40,50,62,77,95) on the active lever were required to earn each successive high-fat food pellet and cue delivery. Responses on the active, reinforcement lever within a 60-minute test session were quantified, together with the number of reinforcer deliveries. Responses on the second, inactive lever were quantified also to control for behavioral interference. Test values represented three-day averages (Stage 1).

Following training on the FR and PR tasks, animals underwent surgery as described in Section 2.4, (creating NMUR2-knockdown and control groups) and given a 7-day recovery

period, during which they were fed a regular diet. While home-caged, animals were handled daily, but not given access to the operant chambers or the high-fat food. (Stage 2).

The animals were given an additional post-surgery period (seven days) to ensure for full viral expression, while undergoing the second training phase to stabilize the FR and PR schedules, but with high-fat food as the reinforcer for active lever presses. Upon stabilization, rats were tested for their pre-abstinence FR response (Stage 3).

Post initial testing, all rats underwent 30 days of abstinence from high-fat foods before being tested a second time on an FR schedule for their post-abstinence response (Stage 4). Following a brief interval, rats repeated Stages 3-4, but were tested on a PR schedule. For CR, rats were trained on an FR/PR schedule (Stage 3). Once stabilized, the animals continued on to the Stage 4 pre-abstinence testing, whereby the rats were placed in operant chambers initially set to an FR1 schedule, when active-lever presses resulted in cue delivery alone (no pellet). Cue deliveries for a single active-lever press included the light cue, the sound of the pellet dispenser, and the sound of the pellet dropping into the food tray. No pellets were delivered, regardless of responses. All rats received a 60-minute cue test in the operant chambers before entering a 30-day period of forced-abstinence from high-fat food, during which they were confined to their home cages, kept on a standard diet, and were not exposed to the cues, the high-fat food pellets, or the operant chambers. Proceeding the forced abstinence, a second cue-reactivity test was administered, after which, rats were allowed to reestablish responding first on an FR1 schedule, then FR3, FR5, and PR schedules (Stage 5). The data accumulated from the FR, PR, and CR schedules represent the effects of abstinence from high-fat food on the reinforcement, motivation, and cue reactivity due to our viral vector inserts, representing NMUR2 knockdown compared to controls (Figure 1A).

2.3 Virus design and production

We employed adeno-associated virus (AAV)-mediated RNAi to knock down NMUR2. The AAV was engineered to express either a small hairpin RNA (shRNA) designed to knock down NMUR2 (shNMUR2) or a control non-silencing hairpin (shCTRL). Hairpin RNAs were previously designed and validated as targeting NMUR2 rat mRNA [3]. Briefly, a 24-nucleotide sequence was identified within the mRNA sequence (Ensembl transcript ID ENSRNOT00000018967), using predefined search algorithms [30], and synthesized (Sigma-Aldrich, Saint Louis, MO, USA) as previously described [3]. A pAAV-shRNA and a plasmid already containing shCTRL were donated by Dr. Ralph DiLeone, PhD (Yale University). 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 AAV serotype 2 RNAi viruses were produced via a triple-transfection, helper-free method and purified as previously described [3, 30].

2.4 Surgical administration of NMUR2-specific virus

We administered AAV-shNMUR2 or AAV-shCTRL virus directly to the PVN of adult rats resulting in NMUR2 knockdown (shNMUR2^{PVN}) and control (shCTRL^{PVN}) animals, respectively. Viral administration was performed adhering to established procedure [3]. To

knock down expression of NMUR2 in the PVN, we stereotactically injected 2 μ L/side of AAV-shNMUR2 or AAV-shCTRL into each rat, based on the methods of Benzon et al. (67). Groups were counterbalanced based on operant-training activity to ensure the average initial active-lever presses and reinforcers earned by both treatment groups were similar. Coordinates of the PVN were adjusted for a 10° outside angle and were set at A/P -0.18, M/L +0.15, D/V -0.82 from the bregma. Injections were delivered at a rate of 0.2 μ L/30 seconds over a period of 5 minutes. After 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. [3]. Surgeries were performed between 09:00 and 17:00, after which, the rats recovered in home cages for 7 days plus an additional 7 days while undergoing operant-chamber testing, to ensure for full viral expression.

2.5 Euthanasia and targeting analysis

After completing the full series of operant tasks, the rats were euthanized and viral targeting was analyzed as previously described [3]. The 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 with 20% glycerol and 0.01% sodium azide, and sliced into 40 μ m sections. The sections were examined for green fluorescence to confirm the expression of the virus in the tissue. Injection sites were noted to allow 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.

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 repeated measures ANOVA with Sidak post-hoc analysis to account for the multiple treatment groups and time periods under consideration.

3. RESULTS

3.1 Training and acquisition

Targeted depletion of NMUR2 within the PVN has been demonstrated to increase *ad libitum* intake of high-fat foods and to increase preference for foods rich in fats [3]. Here, we expand upon this work, training rats to lever-press in operant chambers on both FR and PR schedules of reinforcement to clarify the role of NMUR2 signaling in the PVN on food reinforcement and food-associated cues. Rats were trained on an FR1, FR3, FR5, and PR schedule, allowing all animals time to successfully acquire and stabilize responding. After training and stabilization on each successive response schedule, rats received shRNA-mediated depletion of NMUR2 (shNMUR2^{PVN}), or infusions of a scrambled shRNA (shCTRL^{PVN}) bilaterally into the PVN (Figure 1A). Viral targeting data were confirmed after the operant-response experiments. Bilateral infusion sites in the PVN for each animal were confirmed, and the extent of viral fluorescence was used to ensure that infection was within the intended structure (Figure 1B).

We compared body weight in shCTRL^{PVN} and shNMUR2^{PVN} animals over the course of the study (Figure 2). Both groups showed no significant difference in body weight throughout the study, to include immediately following viral vector insert and after periods of forced abstinence (n=13-14, $p>0.05$ by repeated-measures ANOVA). Though our previous data show PVN knockdown of NMUR2 leads to increased body weight over time while on a high-fat diet [3], animals in this study were not maintained on a high-fat diet, resulting in no change in body weight, which is consistent with previous studies [3].

To rule out confounding effects of shNMUR2 creating gross behavioral interference in lever-pressing activity, acquisition rates for the operant tasks were monitored and compared across treatment groups on both active- and inactive-lever pressing. Average responding for shCTRL^{PVN} and shNMUR2^{PVN} animals was measured daily, with a minimum of 3 days required for behavioral patterns to be considered stable (with $<15\%$ day-to-day variability). 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 3).

3.2 Effects of PVN NMUR2 depletion on the incubation of reinforcement behavior on an FR schedule

After abstinence, FR operant conditioning (measuring reinforcement) was suppressed in shNMUR2^{PVN} relative to control (Figure 4). Prior to the abstinence period, rats in both groups displayed no significant differences in active-lever presses (230 lever presses for both shCTRL^{PVN} and shNMUR2^{PVN}; Figure 4A) and did not have significant differences in high-fat food pellet reinforcers (44 reinforcers for shCTRL^{PVN} and 41 reinforcers for shNMUR2^{PVN}) (Figure 4B) on an FR5 schedule, indicating that NMUR2 knockdown following the full expression of virus had no significant effect on the high-fat food intake. After 30 days of forced abstinence from high-fat food, an incubation effect was found in shCTRL^{PVN} rats, showing significantly increased active-lever presses (average of 375 lever presses across three sessions, n=13-14; $*p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis) (Figure 4A) and number of reinforcers obtained (73 reinforcements, n=13-14; $*p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis) (Figure 4B) on an FR schedule over their pre-abstinence baseline. This increase is consistent with previous work demonstrating incubation of intake behavior after forced abstinence [28, 31-33]. In contrast, shNMUR2^{PVN} rats did not display a significant incubation effect nor change in number of active-lever presses (average of 250 lever presses across three sessions, n=13-14; $p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis) (Figure 4A) or number of reinforcers obtained (51 reinforcers, n=13-14; $*p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis) (Figure 4B) over their pre-abstinence responses. Compared to shCTRL^{PVN} rats, shNMUR2^{PVN} rats displayed a significantly lower level of active-lever presses and obtained significantly fewer reinforcers after 30 days forced abstinence (n=13-14; $*p<0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis) (Figure 4A-B). Moreover, no significant difference in inactive-lever presses in shCTRL^{PVN} or shNMUR2^{PVN} animals was observed (n=13-14; $p>0.05$ by repeated-measures ANOVA) before or after abstinence (Figure 4C).

3.3 Effects of PVN NMUR2 depletion on the incubation of reinforcement behavior on a PR schedule

The propensity for consuming a particular substance is influenced by the motivation for a reinforcer [34, 35], as measured by the PR schedule. A clear effect of PVN NMUR2 knockdown is visible on the incubation of reinforcement behavior associated with high-fat food while on a PR schedule. Prior to the 30-day abstinence period, shNMUR2^{PVN} rats did not differ significantly from shCTRL^{PVN} animals on active-lever presses throughout the PR task (80 lever presses for both shCTRL^{PVN} and shNMUR2^{PVN}, pre-abstinence) (Figure 5A). However, following abstinence, a significant decrease in the number of active-lever presses versus shCTRL^{PVN} was observed (average of 133 lever presses across three sessions for shCTRL^{PVN} vs an average of 105 lever presses across three sessions for shNMUR2^{PVN}; $p < 0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, $n = 13-14$ per group) (Figure 5A). Pre-abstinence, no significant difference in the number of reinforcers earned was found between shCTRL^{PVN} and shNMUR2^{PVN} groups (6.5 reinforcers), but after abstinence shNMUR2 received significantly fewer high-fat food reinforcers (6.9 reinforcers, $n = 13-14$; $p < 0.05$ by repeated-measures ANOVA) than shCTRL^{PVN} rats (8.0 reinforcers) (Figure 5B). Additionally, no changes in the number of inactive-lever presses were observed (Figure 5C). Thus, the incubation of reinforcement behavior for high-fat food on a PR schedule was significantly attenuated in shNMUR2^{PVN} animals.

3.4 Effects of PVN NMUR2 depletion on the incubation of CR

Animals expressing shNMUR2 in the PVN, having been trained in operant chambers to lever-press for pellets of high-fat food, showed a significant decrease in CR compared to shCTRL^{PVN} animals (90 lever presses for shCTRL^{PVN}, 105 lever presses for shNMUR2^{PVN}; $p < 0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, $n = 14$ per group) (Figure 6A). This behavior continued through a 30-day abstinence period. While shCTRL^{PVN} animals showed the expected significant increase in lever-pressing in response to food-associated cues after the abstinence period (125 lever presses; $p < 0.05$ by multiple comparisons ANOVA with Sidak post-hoc analysis, $n = 14$ per group) (Figure 6A), no such pattern was found in shNMUR2^{PVN} animals (98 lever presses), which also failed to demonstrate a significant incubation effect over time. Moreover, a significant difference was found between shNMUR2^{PVN} and shCTRL^{PVN} cue deliveries earned before abstinence, with shNMUR2^{PVN} rats receiving significantly fewer deliveries of cues associated with high-fat food both before abstinence (49 cue presentations earned for shCTRL^{PVN} vs 40 cue presentations earned for shNMUR2) and after abstinence (61 cue presentations earned for shCTRL vs 50 cue presentations earned for shNMUR2^{PVN}; $p < 0.05$ by repeated-measures ANOVA, $n = 13-14$) (Figure 6B). No significant effect was found in responses on the inactive lever across treatment groups, either before or after the abstinence period (Figure 6C). These data support our hypothesis that NMUR2 depletion in the PVN induces a lasting decrease in cue reactivity, which is resistant to abstinence-induced incubation of craving-like behavior.

4. DISCUSSION

Previous studies indicated feeding behaviors incubate during abstinence of high-fat food [36]. Here, we identify PVN NMUR2 as a receptor that mediates this behavioral effect. We

tested this hypothesis by knocking down expression of NMUR2 in the PVN of adult rats, followed by measuring FR, PR, and CR operant responding both before and after a 30-day period of forced-abstinence from high-fat food. We observed a clear decrease in the incubation of FR responsiveness in shNMUR2^{PVN} animals, which was not attributable to changes in general activity. Importantly, no initial effect was found on FR responding; however, after 30 days of forced abstinence, the shCTRL^{PVN} group significantly increased its responding for pellets of high-fat food, while shNMUR2^{PVN} animals did not display a significant increase over pre-abstinence levels. After abstinence, shNMUR2^{PVN} animals responded significantly less for high-fat food than did shCTRL^{PVN} animals. Though our previous studies demonstrated PVN NMUR2 knockdown in rats was associated with an increase in binge-type food consumption and preference for high-fat food (Benzon, Johnson et al. 2014), these previous findings are not necessarily incongruent with our current data showing the same NMUR2 knockdown blocks the incubation of feeding behavior for high-fat foods. In particular, the neurocircuitry associated with binge-type eating differs from the neurocircuitry associated with the incubation of craving. Binge-type food intake is a behavior that is thought to be regulated by the mesolimbic dopamine system [37], whereas little is known regarding the neurocircuitry of the incubation of feeding behavior. Comparatively, the incubation of craving for drugs of abuse involves circuitry related to the prefrontal cortex (PFC) [38-40]. Although the PFC is likely involved in the incubation of feeding behavior similar to its role in the incubation of craving for drugs of abuse, how the PVN and PFC are interconnected is unclear. Possibly, the PFC modulates the PVN via an intermediate brain region such as the amygdala [41] or the bed nucleus of the stria terminalis [42], but future studies are needed to elucidate the nature of the interaction between the PFC and PVN. Nevertheless, we ultimately suspect the mechanistic involvement of the PFC in the ability of PVN NMUR2 to influence incubation of craving for high-fat food.

Depletion of PVN NMUR2 blunted the reinforcement efficacy of incubation of high-fat food, as measured on a PR schedule following a forced-abstinence period. Similar to the FR data, no initial effect was seen on responding within a PR schedule of reinforcement. However, after forced abstinence, shNMUR2^{PVN} animals displayed no significant increase in incubation of behavior as indicated by their lack of increase in responding for high-fat food. Additionally, no effect was observed on inactive-lever presses. This suggests the observed effects on the incubation of reinforcement efficacy are unlikely to be a result of impaired or increased physical activity or associative capabilities.

In contrast to the effects seen with schedules of reinforcement for high-fat food, we demonstrated that knockdown of NMUR2 in the PVN resulted in decreased reactivity to cues for high-fat food before abstinence, as well as the post-abstinence impairment in incubation of craving-like behavior. As food-associated cues have been shown previously to induce craving-like behavior for high-fat foods [36, 43-46], the inhibition of cue reactivity prior to the abstinence period among PVN shNMUR2 animals is indicative of a decrease in craving-like behavior. This decrease remained even after the 30-day period of forced-abstinence, showing no incubation effect (i.e., no increase in lever pressing after 30 days) associated with cue reactivity. Animal responses were not mitigated by confounds of activity, as measured with inactive-lever presses. Our data show NMUR2 expression within the PVN regulated reactivity to cues both pre- and post-abstinence.

Importantly, NMUR2 depletion did not significantly affect body weight at any point in the study. These data agree with our previous work on *ad libitum* access to standard or high-fat diets in PVN-specific NMUR2 knockdown animals [3], which indicate significant weight gain occurs only in those animals maintained on a high-fat diet for extended periods, and not at all in rats fed a standard rodent diet. Here, our model focused on the onset of post-abstinence overconsumption of food described as behavioral incubation, rather than protracted binge-type eating of a high-fat diet. Had continued *ad libitum* access been provided to the high-fat diet, then we anticipate that the rats would have gained body weight, similar to what was described in our previous studies [3].

Defined as a high-fat diet, the reinforcers we used contain 45% fat by calories. This well-established diet [47, 48] is the standard for diet-induced obesity studies in animals but has not previously been studied in the context of the incubation of feeding behavior. Nevertheless, these high-fat diets often include sucrose in addition to the dietary fat. In these studies, the high-fat reinforcers also consisted of 17% sucrose by calories, replicating the standard (i.e., Research Diets D12451). Previous findings indicate the incubation of specific feeding behaviors is observed for sucrose reinforcers too [28, 36, 49, 50]. Moreover, those studies show similar increases in CR for sucrose-paired cues, both in operant and binge-like models [28, 51-53]. Other studies demonstrated [50] even a mere eight days of abstinence from sucrose can induce hyper-reactivity to subthreshold doses of amphetamine. Our data build on these findings, demonstrating the incubation of feeding behavior is observed also with a high-fat diet, a food type more closely associated with obesity in rodents. Together, these data indicate that NMUR2 signaling in the PVN mediates reactivity to food-associated cues and the incubation of craving for food. Overall, these effects support exploration into NMUR2's larger role in maintaining energy homeostasis through modulation of these complex feeding behaviors.

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Figure 1: Behavioral timeline and targeting. **A)** Timeline showing the order of testing and treatment for shCTRL^{PVN} and shNMUR2^{PVN} animals **B)** Coronal representations of the viral target area for animals receiving AAV-shCTRL, left, or AAV-shNMUR2, right, into the PVN. X and O represent injection sites for AAV-shCTRL and AAV-shNMUR2 viruses, respectively.

Figure 2:

Animal body weights throughout the study. shCTRL^{PVN} and shNMUR2^{PVN} animals were fed a standard rodent diet and received. Body weights were assessed throughout the study, to include 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 3:

Acquisition and maintenance of operant responding. Operant responding for pellets of high-fat food was quantified on FR 1, 3, and 5 schedules as well as on a PR schedule. Treatment groups were balanced by responses 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 shCTRL^{PVN} or shNMUR2^{PVN} rats on acquisition or maintenance of operant responding.

Figure 4:

Knockdown of NMUR2 in the PVN blunted incubation of food intake. **A)** shNMUR2^{PVN} animals did not display a difference in FR responding from control (AAV-shCTRL^{PVN}) animals following full viral expression. However, after a 30-day forced abstinence period, shCTRL^{PVN} animals significantly increased responding over their pre-abstinence baseline, and responded significantly more than shNMUR2 animals. **B)** shNMUR2 did not alter earned high-fat reinforcers on an FR schedule prior to abstinence. Following forced abstinence, shCTRL^{PVN} animals significantly increased reinforcers earned over both their pre-abstinence baseline and shCTRL^{PVN} animals. **C)** No significant difference in inactive-lever responding was found across treatment groups before or after abstinence. * indicates $p < 0.05$.

Figure 5:

Knockdown of NMUR2 in the PVN blunted incubation of food motivation. **A)** shNMUR2^{PVN} animals did not display a significant difference in PR responding versus shCTRL^{PVN} animals following full viral expression. However, after a 30-day forced abstinence period, shCTRL^{PVN} animals responded significantly more than prior to abstinence, as well as significantly more than shNMUR2^{PVN} animals. **B)** shNMUR2^{PVN} did not alter the number of high-fat reinforcers earned on a PR schedule following full viral expression. Following forced abstinence, shCTRL^{PVN} animals showed a significant increase of reinforcers earned over their pre-abstinence baseline and earned significantly more reinforcers than shNMUR2^{PVN} animals. **C)** No significant difference in inactive lever responding was found across treatment groups before or after abstinence. * indicates $p < 0.05$.

Figure 6:

Knockdown of NMUR2 in the PVN decreased responding for high-fat diet associated cues.

A) shNMUR2^{PVN} animals had significantly lower CR responding versus shCTRL^{PVN} animals following full viral expression. After a 30-day forced abstinence period, shCTRL^{PVN} animals responded significantly more than prior to abstinence, as well as significantly more than shNMUR2^{PVN} animals. **B)** shNMUR2 decreased the number of high-fat associated cues earned on a CR schedule following full viral expression both before and after abstinence. **C)** No significant difference in inactive lever responding was found across treatment groups before or after abstinence. * indicates $p < 0.05$.