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# Proopiomelanocortin projections to the nucleus accumbens modulate acquisition and maintenance of operant palatable pellet administration in mice

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## Abstract

Obesity is a crisis in the United States, producing many co-morbid diseases that can drastically decrease quality of life. While diet is a major focus for therapeutic intervention, the need to understand underlying appetitive neurocircuitry persists. Proopiomelanocortin (POMC) peptides are well-known for their anorexigenic activity, but also mediate reward and learning. The nucleus accumbens (NAcc) is best known for its role in reward-based learning, but the contribution of POMC projections to NAcc on feeding are controversial since the two major POMC-derived peptides (β-endorphin and α-MSH) have opposite effects on food intake. Our objective was to determine the effect of stimulating POMC projections in the NAcc on acquisition and maintenance of operant self-administration of a palatable food. Adult POMC-Cre mice were microinjected into the NAcc with a Cre-dependent retrograde adeno-associated viral vector expressing Gq Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Mice were trained to self-administer palatable 20-mg pellets in daily operant sessions. Acquisition of self-administration (fixed ratio 30) and baseline self-administration were measured in daily sessions, with mice receiving injections of either JHU37152 (DREADD agonist) or saline (i.p.) 15 min prior to the sessions. POMC stimulation (JHU injection) before training sessions produced a significant increase in rate of acquisition and accuracy compared to the saline treated group, with no significant effect on rewards earned. Removal of POMC stimulation before sessions initially reduced consumption with a gradual increase in responding for reinforcer over 3 days of saline injections. Reinstatement of POMC stimulation (JHU) before the session resulted in a significant decrease in responding and rewards earned. These results suggest a complex role of POMC peptides within the NAcc that increase reward learning for a novel palatable food while decreasing consumption of the reinforcer following experience with it.

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## INTRODUCTION

Obesity has become pervasive in Westernized society and is a significant risk factor for developing cardiovascular disease, type II diabetes, and cancer (1). The primary physiological driver of feeding is the metabolic need to maintain a neutral energy balance; however, rewarding properties of the food itself can lead to eating beyond metabolic need (2). While diet and metabolism remain the primary foci of therapeutic interventions for the treatment of obesity, a greater understanding of underlying neurocircuitry that governs appetitive reward circuitry is essential due to the current abundance of energy dense and palatable foods.

Neuronal release of proopiomelanocortin (POMC) peptides in the brain are part of the primitive and critical appetitive circuitry that governs central consumptive behaviors (3,4). POMC neurons project to the nucleus accumbens (NAcc)(5–7), which is considered to be a key reward center of the brain (8,9). Previous studies have shown the importance of the NAcc on reward-learning (10), however, the role of POMC projections to this region remains understudied. POMC is a pro-peptide that cleaves to form two distinct neuropeptides that are implicated in feeding, α-melanocyte stimulating hormone (α-MSH) and the endogenous μ-opioid receptor agonist β-endorphin (β-End), that have opposing effects on feeding (3,11). α-MSH is a melanocortin product of POMC that acts centrally and specifically within the NAcc to induce anorexia, and pharmacological studies have demonstrated that melanocortin-activity in the NAcc is required for reward learning (12–19). The POMC peptide β-End is an endogenous opioid that acts in the NAcc to facilitate both natural and drug reward, however β-End and μ-opioid receptor agonists are orexigenic when injected into the NAcc (20–29). These discordant effects of POMC peptides that are presumably co-released (3,11) on feeding have not been resolved by previous work which has largely relied on injections of supra-physiological doses of these peptides (30–32).

The objective of this study was to determine the effects of stimulating POMC neuron projections to the NAcc on reward-based learning and consumption of a palatable food in mice that are not food deprived or restricted. To accomplish this goal, we combined a genetic mouse model (POMC-Cre) with microinjection of Cre-dependent retrograde viral vector (AAV-DIO-Gq-DREADD) into the NAcc to allow selective chemogenetic (Designer Receptors Exclusively Activated by a Designer Drug; DREADD) activation of POMC projections to the NAcc. This intervention provided spaciotemporal control of POMC neuronal stimulation which was used to assess the effects of co-release of POMC peptides on acquisition and consumption of palatable pellets within an operant behavioral apparatus.

## METHODS

### Animals:

Adult, male (n = 13) and female (n = 11), POMC-Cre mice (Jackson Laboratory, strain 005965) (33) were used for all studies and were tested between 2–5 months of age. Mice were group-housed in standard clear plastic shoebox containers with ad libitum food (PicoLab Rodent Diet 20 #5053), water, and environmental enrichment (red igloo, nesting material, sticks, or wood blocks). The housing room was maintained on a reverse 12-h light/dark cycle with lights off at 10 am and on at 10 pm each day, and mice were acclimated to the vivarium for at least 4 days before beginning an experiment. All procedures were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee and were consistent with the best practices in *The Guide for Care and Use of Laboratory Animals*.

### Surgery:

Mice were anesthetized with isoflurane (Isothesia NDC 11695-6776-2, Henry Schein, OH) at 1.8–2.3% for stereotaxic surgery. Stereotaxic coordinates for NAcc and identification of POMC-expressing cells that project to NAcc were identified in adult mice (n = 4) by microinjection of 200 nL of red retrobeads (Lumafluor, Inc) bilaterally in the NAcc. Placement and localization of injection into NAcc and identification of projections to NAcc were conducted two weeks after retrobead injection. Mice were positioned into a stereotaxic frame (Kopf, Model 1900), and the surface of the skull was exposed through an incision on the dorsal surface of the skull. Bregma was located, and holes were drilled at the proper coordinates (M/L = +/−1.33 mm; A/P = +2.48 mm, relative to Bregma). To avoid the injectate entering the ventricle, injectors made from 33 g hypodermic stainless-steel tubing (304SS Regular Wall; Component Supplies, TN) was lowered (D/V = −5.000 relative to skull surface at Bregma) at a 15-degree angle lateral from perpendicular so that the tip of the injector ended near the NAcc core. Injections were made using a syringe pump fitted with a 10-µl Hamilton syringe connected to the 33g injector via PE20 tubing. For the DREADD study, mice received bilateral microinjections of the Cre-dependent retrograde DREADD AAV-hSyn-DIO-hM3D(Gq)-mCherry (a gift from Bryan Roth; Addgene viral prep #44361-AAVrg; http://n2t.net/addgene:44361; RRID:Addgene_44361) (34) into the NAcc. Injections were 200 nL in volume, and were administered at a rate of 0.2µl/min. Injectors were left in place for 5 minutes per injection to allow for diffusion, and the injector was retracted over a 2-minute time period. Following injection, the exposed skull was covered with Durelon cement (3M EPSE, St. Paul, MN). Before removal from anesthesia, mice were administered ketoprofen (7.5 mg/kg; NDC 0338-0049-41, Baxter Healthcare, IL) subcutaneously. The mice were checked regularly after surgery to monitor body weight, check incision site, and habituate the mice to handling. Mice recovered for 2 weeks prior to their first operant session. For three days prior to the beginning of training, mice were sham injected to habituate them to handling and injections.

### Operant Training:

Approximately 14-days after surgery, mice were introduced to the modular mouse operant chambers (Lafayette Instruments, Lafayette, IN). Each chamber was individually housed

within a sound cancelling exterior cabinet, accompanied by a fan to mask external noise. One wall of the chamber housed 2 nose-poke holes with a pellet receptacle centered between them. Opposite of that wall, mice had ad libitum access to a metal sipper nozzle connected to an inverted conical tube containing water. The feeder attached to the receptacle released a single 20-mg chocolate flavored fortified chow pellet (Cat. No. F05301, Bio Serv, NJ) upon completion of the assigned fixed ratio (FR) nose-poke response. The side of the correct nose-poke for each mouse was counterbalanced across chambers to avoid bias from a side preference. Sessions began at 11 am, 1 hour after the dark cycle began. Mice were trained to a fixed ratio (FR) value of 30, beginning with an FR5 and increasing by increments of 5 across sessions. Mice were advanced to a higher FR ratio if they performed with an accuracy of 80% or higher in the previous session. If the prior session activity was between 60 – 79% accurate they remained at the same FR value, and if accuracy was below 60% the FR was reduced by an increment of 5. Acquisition of operant self-administration was defined as completing a session with 90% or greater accuracy with no more than a 5% decrease in accuracy the following day. Performance for three days prior to reversal treatment at an FR30 was averaged to establish a baseline value (BL).

### Experimental Design:

To determine the effect of NAcc POMC stimulation on acquisition of operant self-administration of palatable pellets in non-food restricted mice, mice were divided into either saline (n=11) or JHU37152-dihydrochloride (n=13; JHU; 0.3 mg/kg in sterile saline; Hello Bio, Princeton, NJ) (35) treatment groups. Mice received injections of saline or JHU daily (i.p. injection), 15-minutes prior to the 3-hour operant session. Upon establishing baseline intake, the effect of discontinuing POMC stimulation on intake was examined by moving the JHU treated mice to saline injections before the operant sessions for 3 days before reinstating POMC stimulation via JHU injection for one day (Figure 1A).

### Perfusion and Tissue Preparation:

Mice were anesthetized with 2, 2, 2-tribromoethanol (Sigma-Aldrich) prior to perfusion. Once completely anesthetized, mice were transcardially perfused with 15 mL of 10% sucrose in 1X Phosphate Buffered Saline (PBS), immediately followed by 4% paraformaldehyde in 1X PBS. Brains were collected and post-fixed for 24 hours in 4% PFA, followed by 24–48 hours in 30% sucrose in 1X PBS. Once tissues were cryoprotected, brains were embedded into OCT in tissue blocks and flash frozen using methyl-butane and dry ice. Brains were stored at −20° C overnight prior to sectioning. Brains were sectioned into 20-micron coronal slices using a cryostat machine (Cryostar NX50 Cryostat, Thermo Scientific, MA). Sections were placed into 24-well plates with 1X PBS for storage until immunostaining.

### Immunohistochemistry:

Immediately after brains were sectioned, free-floating slices were hand selected for the levels of rostral to caudal hypothalamus. Within a 12-well plate, the tissue was blocked using 2% Normal Donkey Serum (NDS) in 1X PBS. After a 30-minute blocking period and two 15-minute washes, primary antibody mix for adrenocorticotropin hormone (ACTH; 1:750; National Hormone and Pituitary Program, NIDDK, Dr. AF Parlow) and mCherry

(1:1000; Cat. No. ab205402, Abcam, Waltham, MA) in 1X PBS + 0.3% triton-X and 2% NDS was applied. Primary antibodies incubated for 24 hours at 4° C followed by four washes in 1X PBS. Secondary antibodies for rabbit (1:750; Cat. No. 711-545-152, Jackson ImmunoResearch, PA) and chicken (1:200; Cat. No. 703-295-155, Jackson ImmunoResearch, PA) were diluted in 1X PBS were then added to the well-plates and incubated at room temperature for 2-hours before washing the sections at least 4 times in 1X PBS. After washes were complete, slices were mounted onto gelatin-coated Superfrost slides (Cat. No. 12-550-143, Fisher Scientific) and light-protected using Invitrogen Prolong Diamond antifade mountant (P36961, ThermoFisher Scientific). Slides sat overnight prior to imaging to reduce photo-bleaching.

### Imaging:

Slides were imaged using a Leica M205-MFC 2D/3D THUNDER large specimen imaging system microscope (Leica Microsystems, Buffalo Grove, IL) with filters appropriate for the fluorophores used. Images were obtained using LAS X software (Leica Microsystems, Buffalo Grove, IL) and saved for later analysis. Proper placement of the AAV injection and confirmation of transduction was made by comparing coronal sections with mCherry fluorescence to a mouse brain atlas.

### Statistical Analysis:

Prism software (GraphPad, CA) was used to complete statistical analyses of acquired data. Comparisons among groups were completed with unpaired Student's *t* test, one-way ANOVA (treatment) or two-way ANOVA (treatment x sex) with post-hoc Fisher test when appropriate. All results are presented as mean ± SEM. Data was defined as significant if the *p*-value was less than 0.05.

## RESULTS

### Localization of microinjections to NAcc and demonstration of POMC projections from arcuate nucleus to NAcc.

We verified the localization of our bilateral stereotaxic coordinates for NAcc with injection of red Retrobeads. Images of these brains demonstrated accurate and precise injections that remained localized to the NAcc and did not enter the ventricle due to the 15° angle of injection (Figure 1B). Immunofluorescence conducted on coronal sections through the arcuate nucleus showed cell bodies with red Retrobeads from the NAcc injection that colocalized with staining for ACTH, indicative of POMC-producing neurons (Figure 1C). In addition, coronal sections from mice injected with the AAV-hSyn-DIO-hM3D(Gq)-mCherry were examined after euthanasia to verify bilateral placement of the viral vector injections were within the NAcc. Placement of these injections, noted by presence of mCherry, were bilateral and contained within the NAcc for all mice injected (Figure 1D).

### Effect of DREADD stimulation of POMC projections to NAcc on acquisition of self-administration of palatable food pellets

Daily injections of the DREADD agonist JHU were administered to determine if stimulation of POMC projections in the NAcc contribute to acquiring palatable food self-administration

in mice that were not food restricted in the home cage. Of the 26 mice that began training, 2 saline treated male mice failed to acquire self-administration of the pellets and 2 additional JHU mice experienced malfunctions in the operant chambers and were excluded from the study. On day 1 of training there was no significant effect of JHU on nose pokes made in the hole associated with food pellet delivery, suggesting no initial difference between groups on food-seeking ($p = 0.8257$, t (23) = 0.2227; Figure 2A). However, over the training sessions daily JHU treatment significantly decreased the number of days to acquire the behavior as compared to the saline treated group (main effect of treatment $p = 0.015$; F (1, 20) = 7.084; Figure 2B). Furthermore, this increase in learning behavior occurred independent of sex (main effect $p = 0.7198$, F (1, 20) = 0.1324; interaction (sex x treatment) $p = 0.5594$, F (1, 20) = 0.3523; Figure 2C). Once the behavior was acquired, mice were subjected to three baseline days at an FR30. We observed that during the last three baseline days, the JHU dominant treatment group displayed significantly higher accuracy than the saline-treated group ($p = 0.0267$, t (67) = 2.266; Figure 2D) with no difference in number of correct side nose pokes ($p = 0.15$, t(21) = 1.49; data not shown).

### Effect of removing and then re-initiation of chemogenetic stimulation of POMC projections to NAcc on self-administration of palatable food pellets

Mice that acquired the behavior at an FR30 with >90% accuracy were subjected to a 3-day period of saline injection instead of JHU before their daily session to examine the effect of releasing POMC stimulation on self-administration (Figure 3A). Mice who learned self-administration under POMC stimulation by JHU displayed a decrease on the first day of saline treatment ($p = 0.035$, t (10) = 2.441; Figure 3B). Over 3 days of saline injections before the daily session mice showed a steady increase in correct-active nose pokes for the palatable pellets that failed to reach statistical significance ($p = 0.097$, t (7) = 1.92 (Figure 3C). Following 3 days of saline injection, mice were returned to JHU injection before the session for one day (Figure 4A). Every mouse that returned to chemogenetic stimulation of POMC projections showed decrease in responding for food when returned to JHU ($p = 0.01$, t (7) = 3.48; Figure 4B). These effects appeared to be independent of sex, although this was not fully powered to detect sex differences (Figure 4C).

## DISCUSSION

POMC is a pro-peptide that is cleaved to form multiple neuropeptides that are presumably co-released upon stimulation of POMC neurons at projection sites. While contributions of individual POMC peptides on appetitive behaviors and reward learning have been thoroughly investigated previously through pharmacological (13,14,16,20,21,23,24,26,28,29,32,36–41) and genetic models (4,25,42–47), examinations of co-release of POMC peptides through stimulating POMC projections are largely absent from the literature. Here we report that stimulation of NAcc POMC projections during learning of operant self-administration of palatable pellets in non-food-deprived mice hastens acquisition of reward learning, independent of number of pellets earned.

Previous studies examining the effect of co-administration of μ-opioid and melanocortin receptor agonists demonstrate that pretreatment with β-End is able to block the anorexic

effects of α-MSH (30) and that pretreatment with a melanocortin receptor agonist (MT-II) can decrease β-End or μ-opioid receptor agonist-induced feeding (31,32). These effects, however, followed ventricular injection of one or all of these compounds and thus effects induced in a specific brain region at physiological concentrations cannot be interpreted.

Chemogenetic stimulation affords a region-specific way to examine the co-release of POMC peptides even after peripheral administration of the DREADD agonist. Previous studies using this chemogenetic approach have focused primarily on the effect of stimulation or inhibition of POMC neurons in the hypothalamus for effects on feeding behaviors (5,6,48–50), demonstrating that hypothalamic POMC activity modulates feeding behaviors without specificity for projection regions. Using chemogenetics, Klawonn and colleagues (51) elegantly demonstrated that stimulation of POMC projections to the ventral striatum is sufficient to condition a learned aversion that is likely driven by melanocortin activation of MC4R on medium spiny neurons. However, previous studies have established various, often very small, “hot” and “cold” spots in the area of the ventral striatum and NAcc that have drastically different effects on reward (20,21). Our findings demonstrate increased reward learning, not an aversion, following stimulation of POMC projections to the NAcc and are consistent with these previous findings, establishing small zones for increased feeding and reward consistent with the role of μ-opioid receptor activation.

Two previous studies have examined the role of μ-opioid receptor activation on reward learning (38,52) and failed to see any increase of learning for food reward in rats injected into the NAcc with μ-opioid receptor agonist. Both studies, however, involved habituation to the operant chamber and the reinforcer (non-contingent presentation) prior to treatment with the μ-opioid receptor agonist which may have negatively affected the ability to switch learning tasks for the reinforcer. This is supported by the non-significant difference in food receptacle entries (similar to non-contingent presentation) in the μ-opioid receptor agonist treated rats, even while lever pressing for the reward was decreased relative to vehicle controls (38). In contrast, our mice were naïve to both the palatable reinforcer and the operant chamber until the POMC stimulation was begun and thus the reward learning was a discrete and novel reward learning task. These differences in methodology may account for the increased acquisition for the reward that we observed in our study.

While stimulation of POMC NAcc projections during learning operant self-administration appeared to have no effect on the amount of palatable pellets earned or consumed, when the stimulation was ceased there was an initial decrease in responding that increased over 3 days to levels above baseline intake, suggesting that removal of the NAcc POMC stimulation resulted in a modest increase in feeding that could be seen after 3 days of self-administration with no chemogenetic POMC stimulation (Figure 5). This is consistent with a removal of melanocortin stimulation inducing an increase in feeding, and a reestablished anorexic response when chemogenetic POMC stimulation was resumed. We believe that these results support a dominant role for β-End during reward learning that shifts to α-MSH anorexic-dominating effects after the behavior is learned. It should be noted that the mice in our study were not food restricted, although access to the palatable food was restricted to the daily 3-hour session. In addition, our studies were conducted in the dark portion of the light-cycle

to maximize behavior, and thus normal feeding and circadian rhythmicity may contribute to our effects.

These studies did not assess motivation to receive the reward. Future studies should examine motivation via progressive ratio to determine if NAcc POMC stimulation increases the perceived valuation of the palatable pellet compared to a saline treated group. In addition, pharmacological blockade of μ-opioid receptors or melanocortin receptors in the NAcc combined with chemogenetic stimulation could help to determine the contribution of these two receptors to the increased reward learning seen here.

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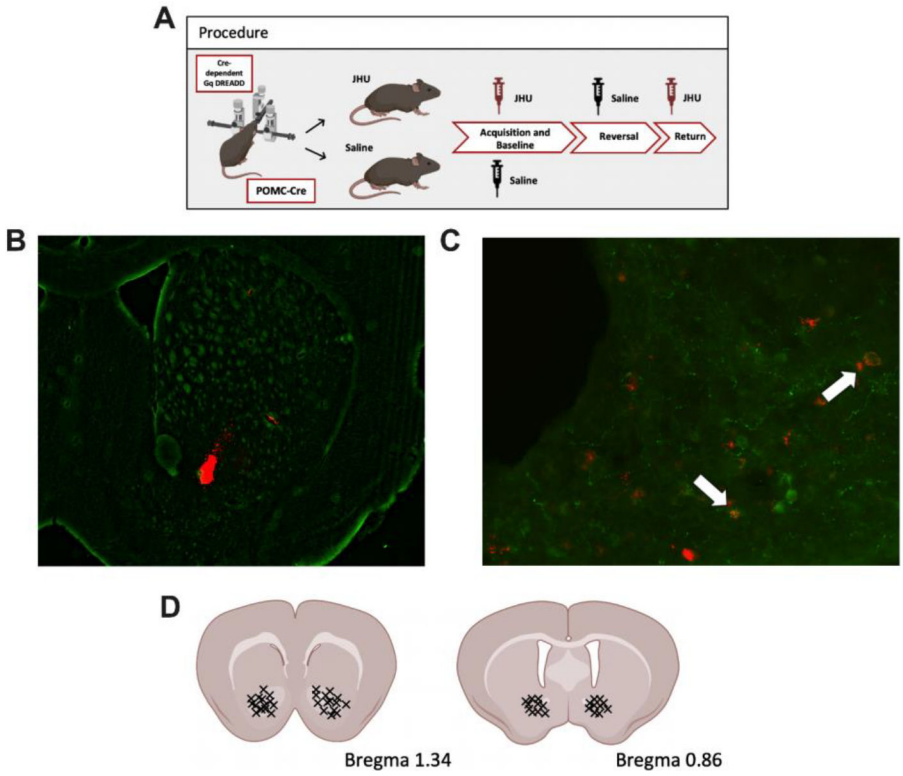


**Figure 1. Experimental Design and Validation of Microinjections**

The experimental study design outlines the surgical interventions, group division and the sequence of i.p. injections (A). Red fluorescence from Retrobeads microinjected into NAcc (coronal section) showing location of our coordinates and the avoidance of the ventricle with our angled approach (B). A coronal section of mouse brain shows arcuate nucleus with ACTH-immunofluorescent cells (green) and neurons with projections to NAcc (red, Retrobeads). White arrows indicate ACTH-immunofluorescent (POMC) cells that project to NAcc with colocalization of ACTH and Retrobeads (C). The bilateral placements of AAV-DREADD viral injections are represented on coronal brain sections with distance from Bregma noted (D).

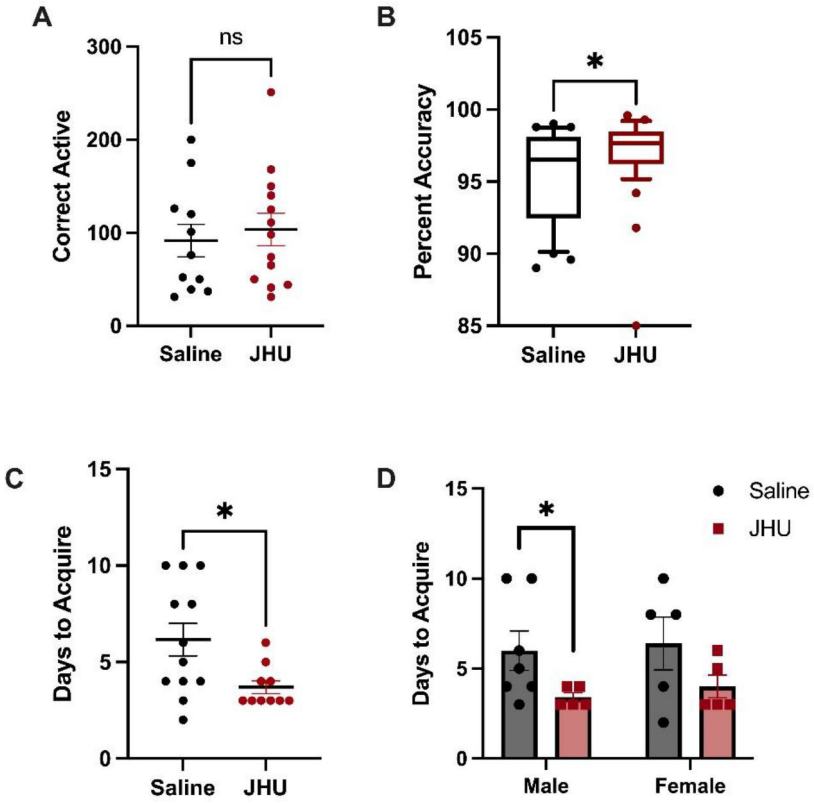


**Figure 2. Effects of Chronic JHU Administration on Behavior Acquisition.**

Bilateral AAV-DREADD injected, ad libitum fed mice were given daily IP injections of either JHU or saline 15-minutes prior to entering their respective operant chambers. Correct active nose pokes on operant Day 1 showed no significant difference between treatment groups (A; P = 0.8257). Mice receiving JHU showed significantly higher daily accuracy following acquisition (B; *P = 0.0018). Acquisition of the task was defined as days to achieve session accuracy (correct nose poke versus total nose pokes) of greater than 90%. Mice chronically exposed to JHU required significantly fewer days to acquire (*P = 0.0105) than those administered saline (C). This trend was observed independent of sex (D) but was only statistically significant in males *P = 0.0403.

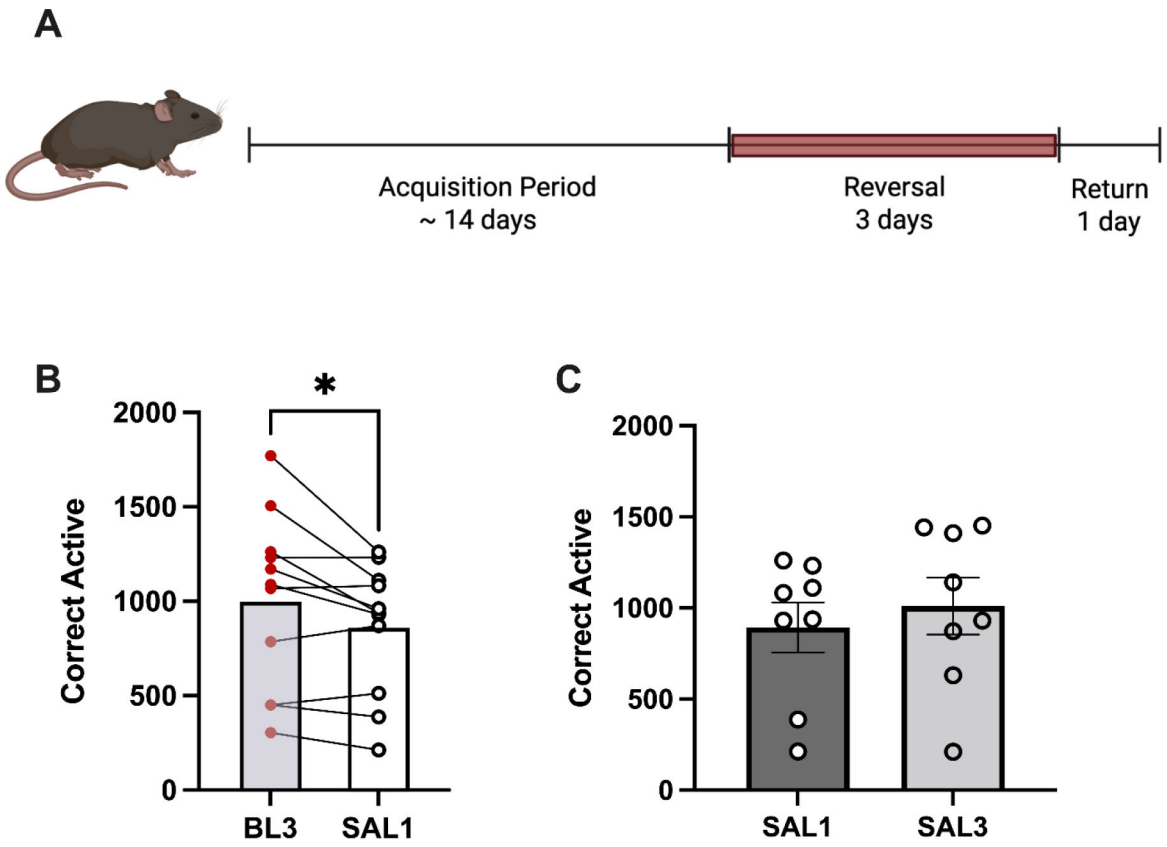


**Figure 3. Appetitive Activity During the Reversal Period.**

Mice previously treated with JHU were given 1–3 days of saline reversal where they received i.p. injections of saline (A). All mice had acquired the behavior (accuracy >90%, FR30) for three days prior to the reversal, and continued at an FR30 throughout. Saline day 1 activity (correct active nose pokes) was compared to baseline day 3 activity to determine percent change in self-administration behavior when POMC stimulation via Gq DREADD was discontinued (B; $*P = 0.035$). Over the 3 days of saline injection before the session, mice exhibited a steady but not statistically significant increase in nose pokes (C; $P = 0.097$). Three mice only received saline for one day and were not included in the analysis on panel C or in the remainder of the study.

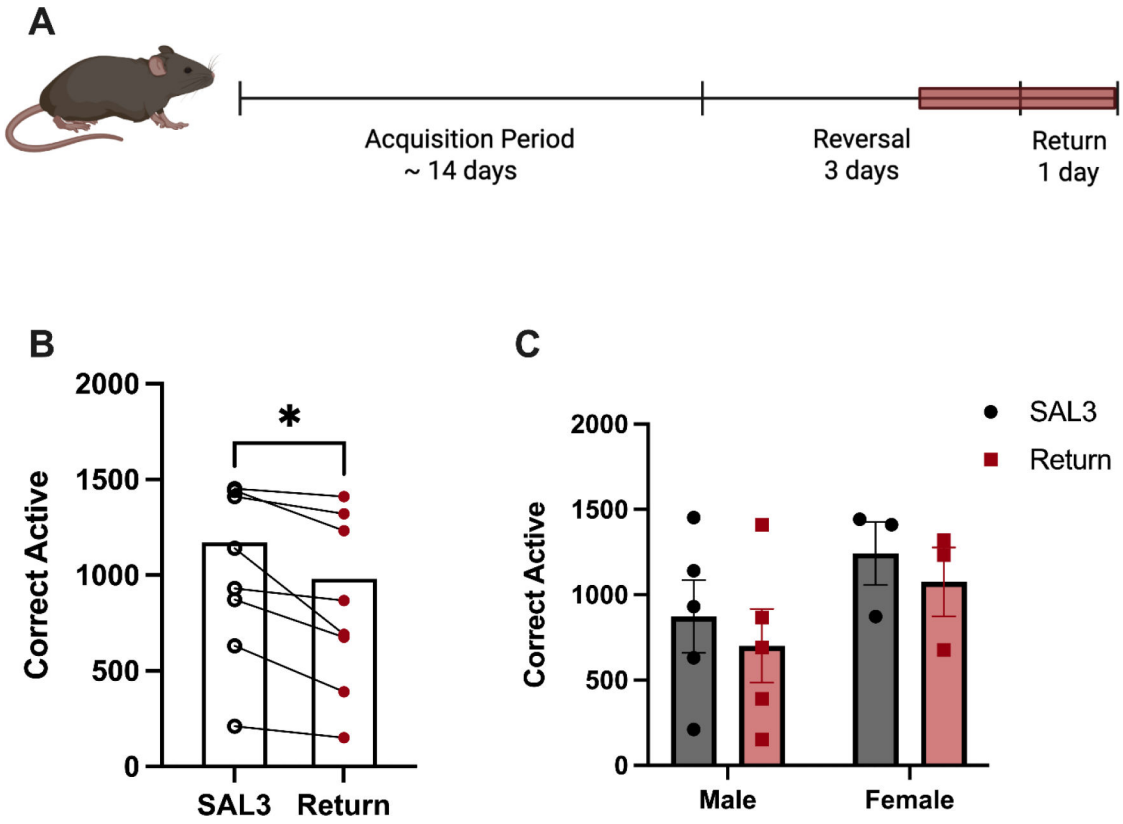


**Figure 4. Appetitive Activity Upon Return to Original Treatment.**

Mice completed a single day return to an i.p. injection of JHU administered before the daily operant session to determine the effect of re-stimulation of POMC to NAcc on self-administration behavior (A). Every mouse returning to JHU displayed a decrease in correct active nose pokes compared to reversal/saline day 3 (B; $*P = 0.01$). This trend appeared to be independent of sex (C).

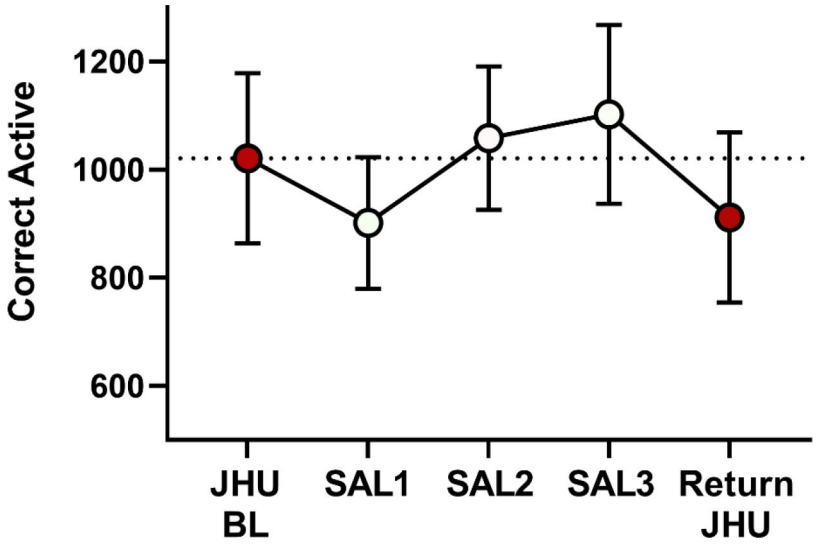


**Figure 5. Overview of operant responding for palatable pellets.**

Operant responding for palatable pellets decreased following discontinuation of chemogenetic POMC stimulation, but then rose over 3 days of saline (white circles, SAL1–3) before decreasing again with POMC stimulation (red, Return to JHU).