CHEMOGENETIC & OPTOGENETIC METHODS FOR STUDYING THE ROLE OF THE NUCLEUS SOLITARY TRACT IN SATIATION

by

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LIST OF ABBREVIATIONS

NTS	Nucleus Solitary Tract
cNTS	Caudal Nucleus Solitary Tract
rNTS	Rostral Nucleus Solitary Tract
GI	Gastrointestinal
Dox	Doxycycline
CNO	Clozapine-N-Oxide
hM3Dq	Gq-coupled human M3 muscarinic receptor
CCK	Cholecystokinin K
PVN	Paraventricular Nucleus of the Hypothalamus
Ip	Intraperitoneal
AAV	Adeno-Associated Virus
pAAV	Plasmid Adeno-Associated Virus
CC	Central Canal
DVC	Dorsal Vagal Complex
GLP-1	glucagon-like peptide-1
TH	tyrosine hydroxylase
PrRP	prolactin- releasing peptide
tTA	Tetracycline Transcriptional Activator
ETV- 1	(a transcriptional factor)
PBN	Parabrachial Nucleus
CGRP	Calcitonin Gene Related Peptide
AGRP	Agouti-Related Peptide)
hM4Di	Gi-coupled human M4 muscarinic receptor
DREADD	Designer Receptor Exclusively Activated by Designer Drug
GABA	Gamma aminobutyric acid
$GABA_BR$	Gamma aminobutyric acid receptor
NMDA	N-methyl-D-aspartate
DBH	dopamine-beta-hydroxylase

ABSTRACT

Increased meal size on a western diet is a major contributor to development and maintenance of obesity. This also leads to decreased sensitivity to the satiating effects of the western diet. Excitation of cells during consumption of a meal in the caudal two-thirds of the nucleus solitary tract (cNTS) in the brainstem are thought to produce satiation and inhibit feeding. Currently, it is unknown how excitation of these cells inhibits feeding. A major obstacle has been the inability to selectively manipulate these cells without affecting intermixed cells that mediate other autonomic functions. We propose a novel approach using inducible, activity-dependent chemogenetics or optogenetics to test whether artificial excitation of cells in the caudal two-thirds of the nucleus solitary tract (cNTS) activated during satiation can reduce food intake and could contribute to preventing or reversing obesity in humans.

We tested four different mouse models with potential for answering this question: double transgenic mice with cFos-tTA & Tet-O-hM3Dq genes, a single transgenic cFos-tTA mouse with a virally delivered hM3Dq gene injected into the cNTS, a double transgenic mice with the TRAP2-tdTomato genes and double transgenic mice with c-Fos-tTA and ChEF genes. Evidence suggested that clozapine-N-oxide might activate satiation-related cells in the absence of the hM3Dq receptor and this should be taken into consideration for future experiments. All four models had promising aspects for studying feeding as well as serious limitations. These limitations will need to be considered when deciding to use any of these models to study any feeding behaviors, especially satiation.

INTRODUCTION

The Problem

Obesity is a major risk factor for a number of diseases such as Type II diabetes, cardiovascular disease and cancer. Not only is obesity a health concern, it is also an economic burden (Haslam & James, 2005) as obesity healthcare costs account for approximately 21% of all healthcare costs (Moriarty et al., 2012; Cawley et al., 2015). Obesity is often promoted and maintained by increased meal size due to the extra consumption of calories (Furnes, Zhao & Chen, 2009; Stromeyer & Smith, 1987) and leads to decreased sensitivity to the satiating effects of a western diet (Stewart et al., 2011 ; Swartz, Duca & Covasa, 2010). Meal size is also increased in dietary and genetically obese animal models (Farley et al., 2003; Rogers & Blundell, 1984; Becker & Grinker, 1977) as well as in humans (Brunstrom, Mitchell & Baguley, 2005; Raben et al., 1994; de Krom, 2007). Excitation of cells in the caudal two-thirds of the nucleus solitary tract (cNTS) in the brainstem that are excited during consumption of a meal are thought to produce satiation (Grill & Hayes, 2012). Currently, it is unknown how excitation of these cells inhibits feeding. A key barrier to study satiation has been the inability to selectively manipulate these cells without affecting intermixed cells that mediate other autonomic functions.

Obesity research has relied heavily on dietary and genetically obese animal models and these models have limitations. Genetic modification to exhibit some of the characteristics of obesity are permanent. The use of chemogenetics or optogenetics could allow for temporal and spatial resolution of gene expression within cells specifically activated during satiation.

Satiation

Development and maintenance of obesity are promoted by increased meal size. Meal size and meal frequency are influenced by a number of direct and indirect controls which can increase or decrease food intake (Smith, 1996; Smith, 1999; Smith, 2000). An example of a direct control of meal size is gut to brain signaling via the vagus nerve. Distention in the stomach cause by increased contents leads to the activation of gut mechanoreceptor signals and secretion of gut satiation hormones (Langhans & Geary, 2010). These signals are transmitted to the cNTS mainly by sensory vagus nerve fibers. All of these signals excite cNTS neurons to produce satiation

(Schwartz et al., 2000; Swartz & Moran, 1998). This is one of the major direct controls of food intake because gastric volume is a major rate-limiting factor in the consumption of food (Phillips & Powley, 1996). Other direct controls of food intake could also include activation of sensory receptors from the tongue to the end of the digestive system. For example, Fox et al., (2001) demonstrated a loss of vagal intraganglionic mechanoreceptors in the small intestine, which led to abnormal feeding. Mice showed longer meal duration on a solid diet and increased meal size when consuming a liquid diet (Fox et al., 2001). Direct controls of meal size also include positive feedback from taste which will promote feeding until enough negative feedback from the gut is sensed by the appropriate brain circuits. Direct controls are typically short-acting and indirect controls can modulate these direct controls over longer periods of time. Indirect controls of meal size can include stimuli that are rhythmic, metabolic, thermal, conditioned, cognitive, or ecological (Smith, 1996). To understand how indirect cues modulate satiation by acting on direct controls of meal size, it is first important to understand how direct controls of meal size function.

Satiation signals, or signals that contribute to meal termination, originate in the peripheral nervous system and are integrated into the central nervous system to influence meal size. For a signal to be considered a satiation signal, the signal must reduce food intake, act over a short period of time, and increase food intake if the signal is blocked (Smith, 1999). Satiation signals are controlled by biological mechanisms, whereas meal initiation can be initiated with environmental signals. There have been five major mechanisms of communication between the peripheral nervous system and the central nervous system proposed for relaying satiation signals. These proposed mechanisms include: vagal signaling, specific transporters, vascular endothelial cell signaling, direct signaling to the arcuate nucleus of the hypothalamus (ARC) and signaling to areas of the brain which do not have the blood brain barrier (Smith et al., 2009).

The vagus nerve provides information about the amount of food in the stomach and nutrient content in the upper intestine to the hindbrain and specifically, the NTS. The vagus nerve is stimulated as there is an increase in the amount of food in the stomach and intestine eventually inhibiting feeding through negative feedback to the brain (Schwartz et al., 2000; Schwartz & Moran, 1998). Cells within the rostral part of the NTS have been implicated in taste while the cNTS has been implicated in satiation (Grill & Hayes, 2012). Satiation has been demonstrated in rats where the brainstem has been separated from the hypothalamus and forebrain demonstrating

the brainstem is sufficient for satiation (Grill & Norgren, 1978; Seeley, Grill & Kaplan, 1994). This suggests there might be more than one brain circuit contributing to satiation.

The specific cells activated by negative vagal feedback to produce satiation signals in the cNTS have been previously quantified by counting cFos (a marker of early gene activation) expressing cells in rats and mice (Rinaman et al., 1998, Fox et al., 2013a). Mice were exposed to Ensure (a liquid meal replacement diet) or no food for one hour and then a thirty-minute waiting period (Fox et al., 2013a). In mice receiving no food, approximately 15 neurons expressed c-Fos, whereas Ensure fed mice, trained to eat a large meal, had approximately 175 neurons expressing c-Fos.

When nutrients enter the upper intestine and vagal sensory fibers are activated by these signals, cholecystokinin (CCK) is released from intestinal cells sending satiation signals to the NTS (Lorenz & Goldman, 1982; Crawley & Corwin, 1994; Smith, 1996; Schwartz et al., 2000). Peripheral injection of CCK increased c-Fos expression in the NTS to approximately 100 cells per section over the control mice with only approximately 30 labeled cells per section. (Wang et al., 1998). D'Agostinio et al., 2016, demonstrated approximately 175 CCK positive cells per section in the NTS in response to a large meal suggesting these cells might be some of the cells involved in satiation because these numbers are similar to the number of cells expressing cFos in response to a large meal. Obese mice are less sensitive to vagally-mediated stimuli (de Lartigue et al., 2012) and therefore, the inhibition of feeding by CCK is weaker in obese rats (Brenner & Ritter, 1995; de Lartigue et al., 2012). This suggests in obese animals, cNTS neurons are producing less than normal satiation, which is possibly leading to or maintaining the increased meal size.

Chemogenetic and optogenetic activation of all CCK neurons within the NTS decreased food intake in mice by approximately 40% over the one-hour test period (Roman, Derkach, & Palmiter, 2016). When CCK neurons in the NTS that project specifically to the paraventricular nucleus of the hypothalamus (PVN) are activated using a chemogenetic mouse model, food intake was decreased by about 40% over the course of 1.5 hours (Roman, Sloat, & Palmiter, 2017). This suggests that the CCK neurons important for satiation project to the PVN and this might be the first pathway activated to promote satiation (Roman, Sloat, & Palmiter, 2017).

The other main brain pathway, besides the vagal -> NTS -> PBN (parabrachial nucleus) pathway, implicated in satiation is the ARC -> PVN -> PBN. The ARC or the ventromedial hypothalamus are typically activated by satiation signals coming through the circulation (Banks &

Kastin, 1998; Ciofi, 2011). Molecules such as ghrelin, leptin, glucose and other molecules can activate these areas over a longer time course than signaling which occurs via the vagus (Adachi et al., 1984; Kojima et al., 1999). Administration of CCK to 24 hour fasted rats increased c-Fos expression within the NTS and the PVN but not in the ARC, ventromedial or dorsomedial nuclei of the hypothalamus demonstrating these brain areas do not become active in response to short-term satiation signals (Caquineau, Douglas, & Leng, 2010).

NTS Structure

The cNTS contains a heterogeneous population of neurons (Garfield et al., 2012; Hisadome et al., 2011; Rinaman, 2010). In addition to CCK positive neurons, as described above, two other types of neurons within the cNTS have been implicated with meal size control: noradrenergic A2 and glucagon-like peptide-1 (GLP-1) neurons. A2 neurons are activated within the cNTS in response to a satiating meal and GLP-1 neurons remain inactivated until rats have consumed nearly 5% of their body weight of Ensure (Kreisler, Davis & Rinaman, 2014).

A2 neurons are defined by positive immunolabeling for either tyrosine hydroxylase (TH), which is the rate limiting enzyme for dopamine synthesis and/or dopamine-beta-hydroxylase (DBH), which is the enzyme responsible for the conversion of dopamine to norepinephrine. The majority of A2 neurons are found in the cNTS and it is thought these A2 neurons projecting from the cNTS to the PVN are likely glutamatergic (Stornetta, Sevigny, & Guyenet, 2002). A2 neurons have diverse functions within the medulla including involvement in vagal sensory-motor reflex arcs, vagal motor outflow, satiation, stress responses, memory consolidation and many others (Browning & Travagli, 2010). A2 neurons release norepinephrine from their terminals and can release other signaling molecules. One signaling molecule of possible importance is prolactin-releasing peptide (PrRP). The majority of A2 neurons within the cNTS also express PrRP (Maruyama et al., 2001) and the A2 neurons that project from the cNTS to the PVN terminate on neurons containing the PrRP receptor (Uchida et al., 2010). Food intake decreases with intracerebroventricular injection of PrRP suggesting PrRP might be acting on the PVN to inhibit food intake (Lawrence et al., 2000) possibly via the A2 neurons. A2 neurons have also been shown to be activated when food intake is inhibited in rats (Rinaman et al., 1998). There have been many studies that demonstrate a loss of normal functioning A2 neurons prevents inhibition of food intake (Ahlskog & Hoebel, 1973; Lorden, Oltmans, & Margules, 1976; Myers & McCaleb, 1980;

Rinaman, 2003; Date et al., 2006) and therefore, the disruption or loss of NTS A2 signaling could contribute to increased meal size.

It is unknown specifically what cells in the NTS cause satiation and how this satiation is produced. It is also possible more than one cell type contributes to satiation such as A2 neurons and CCK neurons. This project will selectively target any cell types activated in the cNTS during satiation. Previously, only one cell type has been activated at a time in most studies. We will attempt to activate these cells directly, thus bypassing the peripheral nervous system and will essentially be able to mimic brain signaling activated by satiation signals coming from the vagus nerve to the cNTS. If these cells involved in satiation can be activated without actually consuming a meal, we may be able to inhibit the consumption of extra calories in large meals and begin to study how these neurons inhibit feeding. A better understanding of this could benefit development of treatments to prevent or reverse obesity.

Another neuron type within the NTS are the promelanocortin (POMC) expressing neurons. These neurons are integral in maintaining normal feeding by reducing appetite, increasing energy expenditure and metabolism rates. Zhan et al., 2013, demonstrated that NTS POMC neurons contribute to meal termination in response to satiety signals and not to long-term feeding behavior (Zhan et al., 2013).

The NTS makes connections with many areas of the brain that are involved in higher control of functions including: cardiorespiratory and metabolic homeostasis. These connections are often reciprocal and include both short and long-term functions. Some of these connections include: the dorsal motor nucleus of the vagus (Armstrong et al., 1988; Shapiro & Miselis, 1985a), the area postrema (Shapiro & Miselis, 1985b), the ventrolateral medulla (Ross, Ruggiero, & Reis 1985), the PBN (Joseph & Michael, 1988; Kawai et al., 1988; Herbert, Moga & Saper, 1990; Riche, Pommery & Menetrey, 1990), the locus coeruleus (Joseph & Michael, 1988; Thor & Helke, 1987), the nucleus ambiguus (Ross, Ruggiero, & Reis 1985; Cunningham & Sawchenko, 1989), the hypothalamus (Ricardo & Koh, 1978; Sawchenko & Swanson, 1982; Van Der Kooy et al., 1984; Luiten et al., 1985; Kannan & Yamashita, 1985; Ter Horst et al., 1989) and many others.

In the present set of studies, four mouse models were created and tested to attempt to investigate satiation cells within the NTS. These four models include: a double transgenic cFos-hM3Dq mouse model, a single transgenic c-Fos-tTA mouse with viral delivery of the hM3dq receptor (cFos-AAVhM3Dq), a TRAP2-Tomato mouse model and a optogenetic c-Fos-ChEF

mouse model. These models use inducible, activity-dependent systems to label satiation cells in response to a large meal. These labelled cells can be activated in the future to potentially decrease meal size and/or daily food intake that could contribute to preventing or reversing obesity in humans. If this excitation reduces meal size, it will confirm the role or these neurons in producing satiation, a first step in identifying and characterizing these neurons as a foundation for determining how they produce satiation. Each of these models are explained in the subsequent chapters and the experiments carried out to test the usability in satiation research are also described.

DOUBLE TRANSGENIC CFOS-HM3DQ MICE

Introduction

Highly active neurons can be identified by the cFos protein (Dragunow & Faull, 1989; Sagar, Sharp, & Curran, 1988). cFos is activated reliably in the NTS after receiving direct input from the sensory vagus nerve after consumption of a large meal (Rinaman et al., 1998; Zittel et al., 1999; Schwartz, 2000; Emond, Schwartz & Moran, 2001; Fox et al., 2013a). cFos activity can be linked to many different behaviors such as after consumption of a large meal in the nucleus solitary tract (Rinaman et al.,1998; Zittel et al., 1994; Phifer & Berthoud, 1998; Fox et al., 2013a). Several studies have used cFos expression to modulate expression of other genes involved in numerous types behaviors such as learning and memory. In the double transgenic mouse model, cFos-hM3Dq, when the cFos-tTA gene (the bacterial tetracycline-regulated transactivator (tTA)) is expressed, cFos in turn drives the expression of tTA to produce tetracycline. Tetracycline then activates the expression of the target gene, either a chemogenetic receptor or an optogenetic ion channel, in this case the Tet-O-hM3Dq gene causing the hM3Dq receptor to be expressed in neurons activated by cFos. The cFos-tTA gene has been used many times in different mouse models (Gossen & Bujard, 1992; Berens & Hillen, 2004). (See Figure 1).

The Tet-O-hM3Dq gene expresses the G-coupled protein receptor hM3Dq (Gq-coupled human M3 muscarinic designer receptors exclusively activated by designer drugs (DREADD)). Activation of this receptor leads to an increase in intracellular calcium release causing neuronal burst firing. This receptor has been used in many studies in many different neuron types including: ETV-1(a transcriptional factor) neurons in the subfornical area which increased drinking (Betley et al., 2015), PBN, CGRP (calcitonin gene related peptide) projection neurons which decreased feeding (Cai et al., 2014) and in AGRP (agouti-related peptide) neurons which increased feeding (Krashes et al., 2011). The hM4Di receptor is similar to the hM3Dq receptor in structure and is activated by clozapine-N-oxide (CNO). However, the activation of the hM4Di receptor results in presynaptic inhibition and neuronal silencing (Stachniak, Ghosh, & Sternson, 2014).

Behavioral analyses including: elevated plus maze, neurobehavioral screen, accelerating rotarod, prepulse inhibition of acoustic startle responses, buried food test for olfactory ability, and Morris water maze of hM3Dq mice and controls showed no significant differences (Alexander et

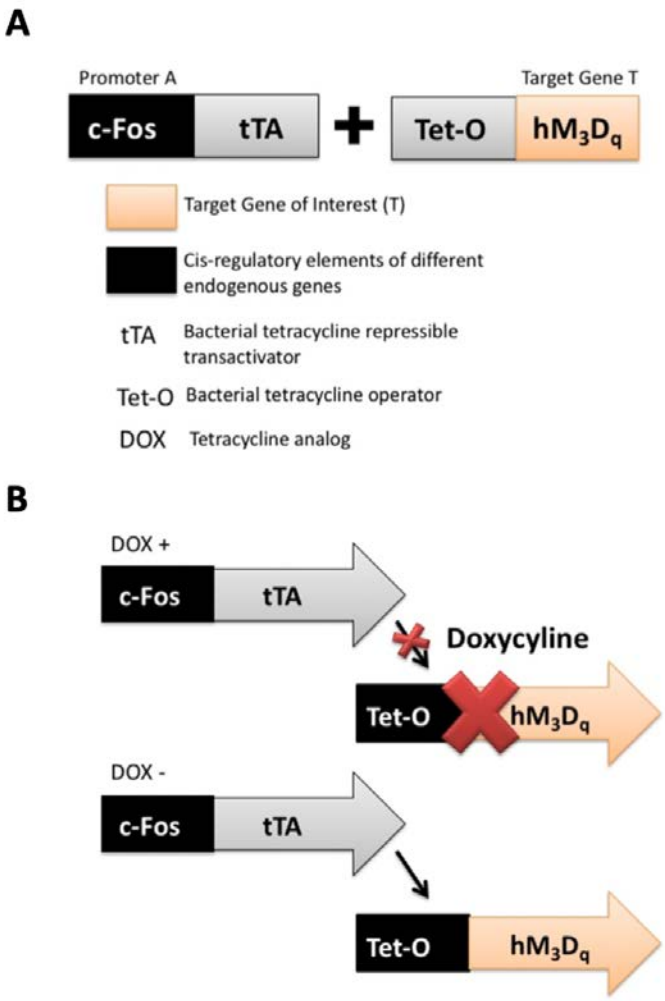


Figure 1. Schematic of cFos-tTA-hM3Dq gene expression. (A) The cis regulatory element, promoter A (cFos), drives the expression of the bacterial tetracycline-regulated trans activator (tTA). When this gene is expressed, it activates target gene T (Tet-O-hM3Dq). This target gene is only expressed under the control of the Tet-O operator in the presence of tetracycline. (B) Doxycycline (Dox) is a tetracycline analog which can turn gene T off or on. In the presence of Dox, gene T is blocked (off) and in the absence of Dox, gene T is expressed (on).

al., 2009). Demonstrating there are no changes in these behaviors is critical because these behaviors could ultimately lead to changes in feeding behaviors. Activation of neurons containing the hM3Dq receptor occurs starting within 5-10 minutes of administration of CNO, with peak activation around 45-50 minutes and does not return to baseline for several hours after ip injection (Alexander et al., 2009). Because of the relative short peak activation of CNO, if it is administered to mice expressing the hM3Dq receptor minutes before when feeding normally begins, the hM3Dq

receptor will be activated and this could possibly lead to partial or complete inhibition of food intake. Activation of these receptors is only temporary and Nakajima et al., 2016, demonstrated CNO injections were only effective in reducing food intake over a period of one day.

The hM3Dq receptor can be blocked by the administration of doxycycline (Dox) within the food. The hM3Dq receptor can be induced over time by removing Dox from the food once Dox is no longer in the system. This means the expression of the hM3Dq receptor is temporally inducible because Dox administration can be controlled by the experimenter. The hM3Dq receptor is also spatially inducible because of the cFos-tTA gene is only expressed in response to neuronal activation and therefore expressed within the NTS after a large meal. This means the Tet-O-hM3Dq gene is only expressed in cells where cFos was activated by the large meal in the absence of Dox. This also means that the large meal could cause cFos expression in other areas of the brain, and since these cells are activated by physiological satiation, they will be important to be able to activate later with this models.

When Dox is present in the mouse, the cFos promoter still drives the expression of tTA, however tTA cannot bind to the target promoter PTRE-tight and therefore PTRE-tight cannot drive the expression of the hM3Dq-mCherry. When Dox is absent in the mouse, tTA can bind and activate the target promoter PTRE-tight and thus activate hM3Dq-mCherry (Figure 1). This expression only occurs in neurons where tTA expression was produced by the cFos promoter due to the consumption of a large meal, due to excitation of those neurons. By keeping mice on Dox all the time except when they are eating a large meal, hM3Dq expression can be restricted to neurons highly excited during this meal.

Early research initially used very high doses of Dox to regulate gene expression, some as high as 2mg/mL of Dox in water (Mayford, 1996). A dose response study demonstrated a dose of 0.25ug/mL was sufficient to decrease expression by more than six times and a dose of 2.5ug/mL or greater was sufficient to reduce gene expression very close to zero (Chen et al., 1998). A more recent study investigated the differences between Dox administered in water and in chow. A small, but significant difference in administration of Dox in water or feed was observed at a low dose of Dox (Redelsperger et al., 2016). A dose of 150ug/mL in water and 200ppm in feed resulted in about a 100ng/mL or less difference in plasma concentration of Dox (Redelsperger et al., 2016). It should also be noted these animals were only on Dox for 6-14 days which is typically a shorter time frame than most studies performed. Typically, Dox is administered in chow for longer

periods of time. A month long dose of 40mg/kg Dox exposure mixed in chow was sufficient to block receptor expression in multiple studies (Reijmers et al., 2007; Matsuo & Mayford, 2008; Zhang et al., 2015). A dose of 1g/kg of Dox was enough to stop additional receptor expression after the experimental window off 40mg/kg Dox in chow (Reijmers et al., 2007; Zhang et al., 2015).

The aims of these experiments are firstly, to demonstrate Dox inhibits hM3Dq expression within the brain after one month of 40mg/kg Dox administration in chow. Secondly, to demonstrate the hM3Dq receptor is present after the large meal training in the NTS. Finally, the third experimental aim was to investigate and determine the timing of the removal of Dox from the mouse food to capture the highest amount of hM3Dq expression during the large meal training.

Methods

Mice

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

The cFos-tTA and the Tet-O-hM3Dq mouse strains were originally obtained from Jackson Laboratories (cFos-tTA also referred to as c-fos, TetTag, cfos-htTA, cfos-tTA/cfos-shEGFP; cat. no. 018306; Tet-O-hM3Dq also referred to as hM3Dq, TRE- hM3Dq; cat. no. 014093, JAX Laboratories, Bar Harbor, ME) and bred for several generations in our laboratory. Double transgenic cFos-tTA Tet-O- hM3Dq mice will be referred to as cFos-hM3Dq mice from this point on. Mice had ad libitum access to water and standard chow in pellet form (Teklad Rodent Diet 2018, Envigo, Madison, WI) and were bred and maintained at 22℃ on a 14:10 hr light/dark cycle to facilitate breeding with lights on at 0500 and off at 1900 prior to the experiment. Mice were 3 months old at the start of the meal training and were adapted to single housing for one week prior to meal training (Roman, Derkach, & Palmiter, 2016).

Generation of cFos-tTA and Tet-O-hM3Dq Mice

Each mouse line was created from two breeder pairs. Male mice were heterozygous for one of the gene mutations and bred with a female mouse from the same line that did not contain the mutation. This created a ratio of offspring of 1:1 with half of the offspring being heterozygous for the mutation and the other half being wild-type. Heterozygous mice from each of the two strains were then bred together to create mice with both cFos-tTA and Tet-O-hM3Dq mutations. This created offspring containing both the cFos-tTA and Tet-O-hM3Dq mutations, cFos-tTA mutation alone, Tet-O-hM3Dq mutation alone, and wild-type. Wild-type mice and mice expressing either cFos or hM3Dq were used as control mice throughout the experiment.

Genotyping

Mice were tail snipped at postnatal day 21 and tails were stored at -20℃ until genotyping. Briefly, tails were thawed to room temperature and using a DNA extraction kit (DNAeasy Blood and Tissue Kit, #69506, Qiagen, Hilden, Germany) tails were lysed and DNA was extracted. DNA samples were stored at 4℃ until polymerase chain reaction (PCR) was performed. PCR was performed for both cFos-tTA and Tet-O-hM3Dq genes using the primers and PCR programs provided by Jackson Laboratory and reagent protocols from our lab. Primers for the c-Fos-tTA transgene were forward transgene primer oIMR0872 5'-AAG TTC ATC TGC ACC ACC G-3' and reverse transgene primer oIMR1416 5'-TCC TTG AAG AAG ATG GTG CG-3' (IDT Forward catalog #198693093, Reverse catalog #198693094) in order to amplify a 173 bp cFos-tTA fragment. The PCR procedure was as follows: 95℃ 15 min; 94℃ for 3 min; 10 cycles of 94℃ for 20 sec, 65-58℃ (0.5℃ step-down over the 10 cycles) for 15 sec, 68℃ for 10 sec; 28 cycles of 94℃ for 15 sec, 60℃ for 15 sec, 72℃ for 10 sec; and 72℃ for 2 min. Primers for the Tet-O-hM3Dq transgene were forward transgene primer 13561 5'-CGT CAG ATC GCC TGG AGA-3' and reverse transgene primer 13562 5'-CGG TGG TAC CGT CTG GAG-3' (IDT Forward catalog #198713942, Reverse catalog #198713943) in order to amplify a 259 bp Tet-O-hM3Dq fragment. The PCR procedure was as follows: 95℃ 15 min; 94℃ for 2 min 20 sec; 10 cycles of 65℃ (1.5℃ step-down over the 10 cycles) for 15 sec, 68℃ for 15 sec; 28 cycles of 94℃ for 15 sec, 50℃ for 15 sec, 72℃ for 10 sec; and 72℃ for 2 min. Both PCR reactions also included two internal control primers: oIMR7338 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3' and oIMR7339 5'-GTA

GGT GGA AAT TCT AGC ATC ATC C-3' internal positive control which was 324 bp in size (IDT Forward catalog #198693091, Reverse catalog #198693092).

Justification for the Use of Both Sexes

Throughout the experiments both male and female mice were used. Generating transgenic mice is time consuming and it is much more practical to use both sexes. Previous work done by our lab has shown there were no differences in food intake or body weight between sexes when using the same large meal training paradigm and more importantly, there was no difference in cFos expression between males and females (Fox et al., 2013a; Fox et al., 2013b). Other studies using similar animal models also used both sexes (Alexander et al., 2009; Stachniak, Ghosh, & Sternson, 2014).

Diets

Mice were maintained on Teklad Repelleted Control 2018 diet (3.1 Kcal/g TD.150279, Envigo, Madison, WI). One month prior to testing, mice were transitioned to Teklad 2018 diet containing 40mg/kg Dox (TD. 120462 Envigo, Madison, WI). Day 1 of the experimental feeding mice were on the Teklad 2018 diet containing 40mg/kg Dox. Days 2-5 mice were switched to the Teklad Repelleted 2018 diet with no Dox to allow for hM3Dq expression during the experimental period. After the test diet exposure on day 5, mice received Teklad Repelleted 2018 diet containing a dose of 1g/kg of Dox at 5pm and continued to receive the diet until sacrifice (TD. 120658, Envigo, Madison, WI) to quickly inhibit expression of hM3Dq produced outside of the experimental timeframe.

Meal Training

The feeding protocol used for these experiments induces a voluntary consumption of a large meal to activate vagal sensory fibers. This protocol has been adapted from Rinaman et al., (1998) and has previously been used in our lab to elicit cFos expression (Fox et al., 2013a; Fox et al., 2012). On day 0, both groups were food-deprived starting at 5pm. On days 1-4, all mice were given Ensure (liquid Ensure Diet, Ensure Vanilla, 1.48 kcal/mL) at 9 am for one hour followed by 40 mg/kg Dox chow or chow for three hours from 3pm-6pm. This allowed for hM3Dq receptor

expression in mice taken off Dox (n = 40). On day 5 of the large meal training, all mice were fed Ensure. All mice were then switched to ad libitum 1 g/kg Dox chow at 5pm to rapidly stop hM3Dq receptor expression on day 5 and continued until sacrifice on day 6 (Figure 2A). Food intake and body weights are presented as averages $\pm$ the standard error of the mean.

Immunohistochemistry

At the end of day 6, after enough time for the hM3Dq receptor to express had passed, mice were given a lethal dose of Brevital Sodium (sodium methohexital; 100 mg/kg) and then perfused transcardially with saline at a rate of 4 mL/min for ten minutes and then with chilled 4% paraformaldehyde dissolved in 0.1M sodium phosphate buffered saline (PBS) kept on ice for thirty minutes. Brains were removed and stored at 4℃ in 4% paraformaldehyde in PBS overnight and then incubated in 30% sucrose PBS overnight at 4℃. Next, a block of brain containing the NTS was excised, frozen in OCT (Tissue Tek) using liquid nitrogen, and stored at -80℃ until frozen sectioning.

Brains were cross sectioned at 30 μm and immunostained for the hM3Dq receptor by staining for hemagglutinin tag on the receptor. Sections were stained using the free-floating technique with light agitation during for the entire immunostaining procedure. Sections were first washed 3 x 15 min in PBS, incubated in goat block (15% normal goat serum, 0.5% Triton X-100, 2% bovine serum albumin) for 1 hr. The hM3Dq receptor was immunostained using rabbit polyclonal anti-HA (1:450, Rockland Immunochemicals Inc., Limerick, Pennsylvania, 600-401-384) over 72 hr. Next, sections were washed 3 x 15 min in PBS and incubated in a Cy3 secondary antibody (1:600 Cy3-conjugated goat anti-rabbit, Jackson ImmunoResearch, 111-165- 144) in diluent for 2 hr at 4℃ in the dark. Sections were washed 3 x 15 min in PBS, mounted on slides with glycerol, and coverslips sealed with clear nail polish. Slides were stored at 4℃ in the dark until confocal imaging. An Olympus BX-DSU spinning disk confocal microscope was used to image each nucleus using a Hamamatsu 1394 ORCA-ERA SIN 660671 camera at a magnification of 100X.

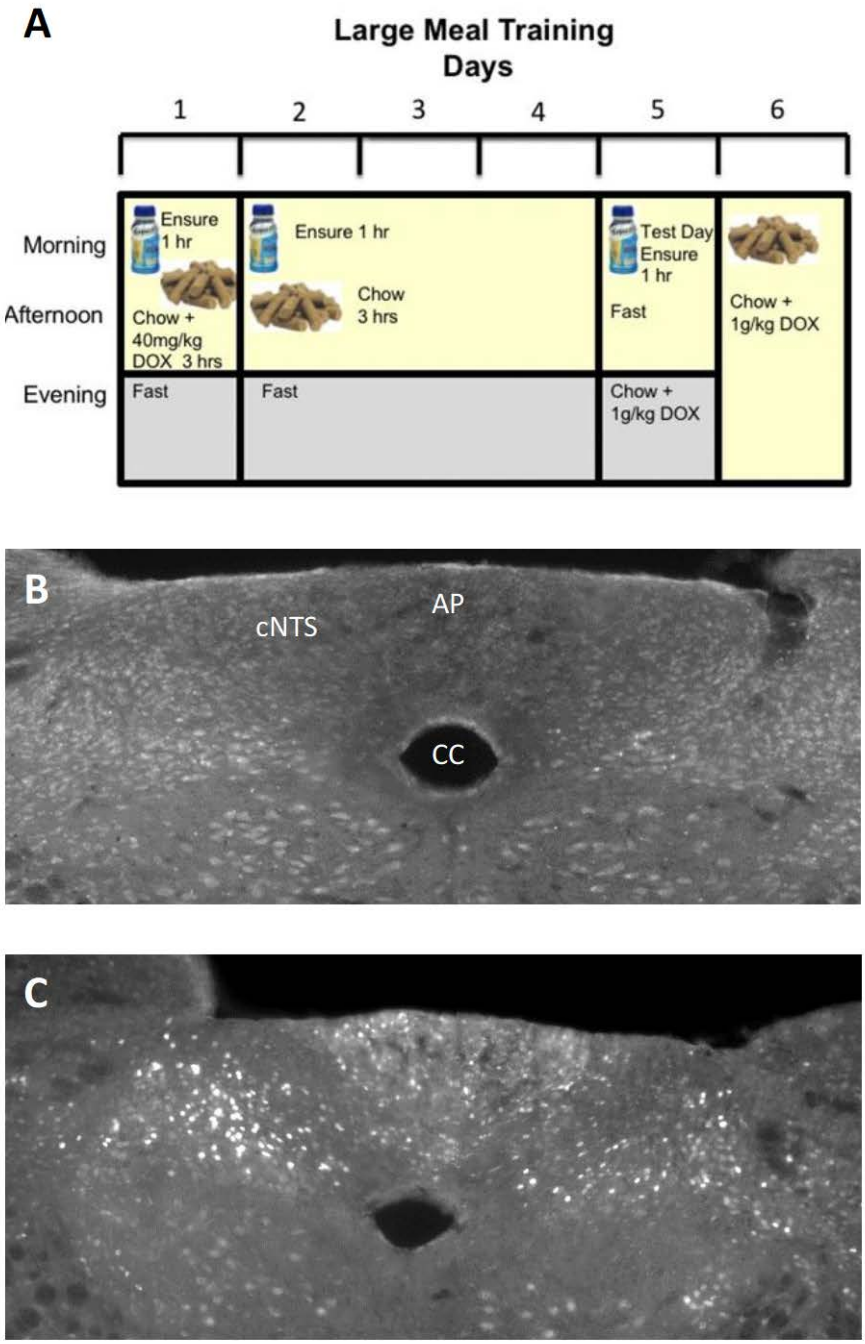


Figure 2. Large meal training. (A) Schematic of Large Meal Training. Day 1 mice will be fed Ensure for one hour from 9am-10am, followed by chow mixed with 40 mg/kg Dox for three hours from 3pm-6pm. Days 2-4 mice will be fed Ensure for one hour starting at 9am, followed by chow with no DOX for three hours from 3pm-6pm. On day 5, mice will be given Ensure for one hour starting at 9am. Mice will be fasted until 5pm when they will be given 1g/kg DOX + chow through day 6 is when mice will be sacrificed. (B) cFos expression in response to fasting. (C) cFos expression in response to large meal training. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

Results

First, mice were tested to demonstrate that 40mg/kg of Dox administered in chow for one month was sufficient to block hM3Dq expression. No expression of the hM3Dq receptor was observed in the NTS or AP. Other areas of the brain were observed including the hippocampus, amygdala and cortex and no hM3Dq receptor expression was visible.

Our lab has consistently shown that a large meal produces a 10X increase in endogenous cFos expression in the cNTS (Fox et al., 2013a; Fox et al., 2013b). We tested if activation of the cFos promoter that controls hM3Dq expression by consumption of a large meal can increase hM3Dq expression by a similar magnitude as endogenous cFos is expressed by its cFos promoter. The transgene cFos promoter is linked to the hM3Dq gene and to a gene that expresses green fluorescent protein (GFP). The large meal did in fact produce a similar magnitude increase in the number of cNTS neurons labeled with GFP as occurred for endogenous c-Fos (Figure 2C). Thus, most, if not all, of these GFP-positive neurons should also express hM3Dq receptors.

Next mice were put through the meal training paradigm and the 40mg/kg Dox was removed from their diets. Three different time periods were tested: 3, 4 and 5 days off Dox prior to the test meal. All three time points showed no hM3Dq receptor expression in the NTS or AP. Some labeled cells were present in the hypoglossal nucleus, which is adjacent to the dorsal vagal complex (Figure 3A). hM3Dq receptor expression was present in the amygdala, hippocampus, choroid plexus, claustrum and cortex (See Figure 3B-E). Levels of staining were not assessed in these areas because they were not areas directly related to satiation.

Discussion

Mouse body weight and meal size increased over the course of the large meal training. This suggests, cFos expression would be high within the cNTS and would promote hM3dq expression. However, no expression of the hM3Dq receptor was identified. There are a few possibilities why this could have occurred with the most probable being the tTA was not able to bind to the Tet-O and the hM3Dq receptor was not made. It could also be possible that the tTA was able to bind to the Tet-O but the DNA sequence containing part of the hM3Dq receptor was wound too tight for transcription.

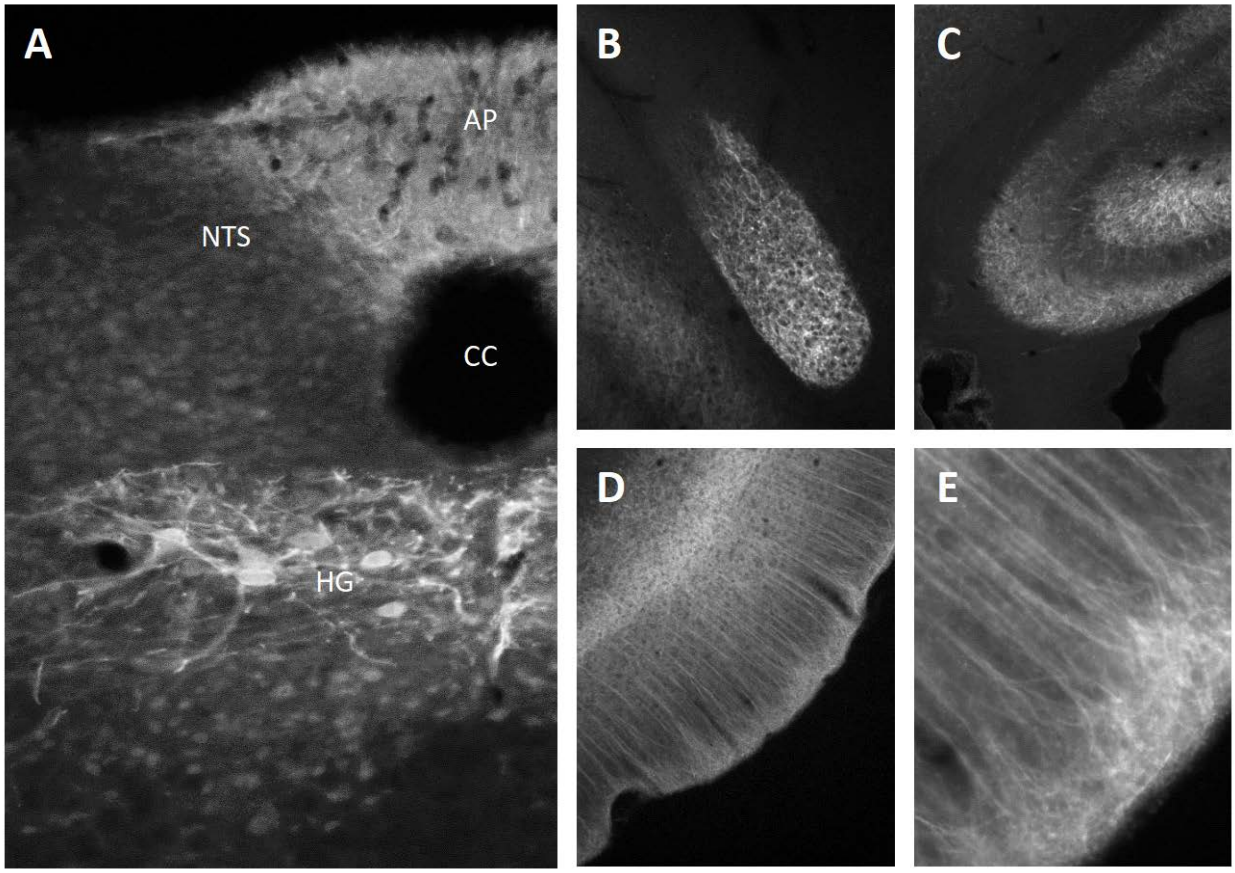


Figure 3. hM3Dq receptor expression in the cFos-tTA-Tet-O- hM3Dq mouse model. (A) hM3Dq receptor expression in the hypoglossal nucleus, (B) basolateral amygdala, (C) hippocampus, (D) cortex and (E) higher magnification of cortex to show axons. CC central canal, NTS nucleus solitary tract, AP area postrema, HG hypoglossal nucleus.

The main benefits for the use of this mouse model is that there would be no need for intracranial injections and no use of virus. This eliminates two time consuming steps, performing surgery and waiting for the virus to fully express after injection. It also significantly decreases the cost of the experiments since stereotaxic surgical equipment and the construction of viral vectors are relatively expensive.

The negatives associated with this mouse model seem to be higher than the benefits. The biggest negative is that there is no expression of the hM3Dq receptor in the dorsal vagal complex, specifically the NTS, AP and DMV. hM3dq receptor expression is not localized to one brain area and can be expressed in any cell that makes cFos. This creates a problem for artificial activation with CNO. CNO would activate all brain areas with the hM3Dq receptor. In the current model, if CNO was administered, activation would occur in the amygdala, hippocampus and cortex. This

could create issues while trying to study behavior. Activation of one brain area could effect another and modulate behavior and thus activation of one brain area could not be attributed to the behavior.

In order to get around the brain area specificity issue, a direct injection of CNO to the specific brain area could potentially prove promising. However, controls would be needed to demonstrate the spread of CNO within the brain and that CNO did not activate any cells without the hM3Dq receptor. CNO would need to be kept within the desired brain area and not diffuse to other areas where unwanted activation could occur. Another control needed would be a vehicle only injection to demonstrate the injection itself did not cause any activation within the desired brain area.

It cannot be for certain why the hM3Dq receptor expresses in some brain areas and not others. However, it is likely the hM3Dq gene inserted itself into the genome in an area that is heterochromatin within the NTS, meaning that the chromosome is tightly bound and gene expression within that area does not occur. In other cells, like cells in areas the hM3Dq receptors are expressed, the same area of the genome could be euchromatin, meaning that the area of the chromosome is very active in transcription and not tightly bound, which would allow for the transcription hM3Dq receptor in that particular cell. This is the most likely explanation for the expression pattern of the hM3Dq receptor in the cFos-hM3Dq mice.

This model could be used in the study of emotion, memory and other functions related to the amygdala. With high levels of expression in the amygdala, using this model could be an alternative to viral injections into the amygdala. It could also be used to study long-term memory or spatial navigation with respect to the hippocampus (Alexander et al., 2009). Expression levels of the hM3Dq receptor are high within the hippocampus. This mouse model could be used to study these areas where expression was present such as the cortex, choroid plexus and claustrum.

SINGLE TRANSGENIC CFOS-AAVHM3DQ MICE

Introduction

The chemogenetic system used for this mouse model (Alexander et al. 2009; Garner et al., 2012; Zhang et al., 2015) involves the injection of a virally delivered hM3Dq-mCherry transgene into the cNTS of cFos-tTA-GFP transgenic mice (referred to as cFos-AAVhM3Dq) to study satiation. This model, like the double transgenic cFos-hM3Dq model, also uses activity-dependent, inducible hM3Dq transgene expression that is selectively expressed only in cNTS cells activated during consumption of a large meal when there is no Dox present within the mouse. The main advantage of this model is that the hM3Dq gene is injected directly into the cNTS using a virus to deliver the hM3Dq transgene resulting in the hM3Dq expression only infecting the area of the brain where it was injected. Only highly activated cells in the cNTS, and not in other brain regions, will produce the hM3Dq receptor. This allows for systemic delivery of CNO to artificially excite cNTS cells that have hM3Dq receptors. Finally, cells that produce both hM3Dq and mCherry proteins can be easily visualized and quantified because both will be produced in the same cell.

The adeno-associated virus (AAV) is used to deliver the transgene because it is not known to cause disease in mice or humans and, more importantly, is unable to replicate within the body. There are also nine different serotypes of AAVs which have been previously identified. Each serotype has different properties as to what brain area it is most effective in, the spread and duration of expression. More specifically, serotypes1-4 have a decreased anatomical spread likely due to the increased viral particle size in comparison to serotypes 5-9 which are compact in size and have increased anatomical spread. (Aschauer, Kreuz & Rumpel, 2013). Infection rate of these viruses differs and have not been well studied. Serotypes typically used within the CNS are types 1, 2, 4, 5, 8 & 9. The serotype chosen to deliver the pAAV-PTRE-tight-hM3Dq-mCherry gene was a combination of serotype 1 & 2 based on Zhang et al., 2015.

Using the cFos-AAVhM3Dq mouse model, we aimed to demonstrate the hM3Dq receptor could be expressed within the NTS using a viral vector. To do this, we first demonstrated cFos gene expression within our model and then demonstrated hM3Dq receptor expression in mice that consumed a large meal. We also investigated the effects of Dox and CNO on body weight and

food intake. Next, we injected CNO systemically into cFos-AAVhM3Dq mice to artificially activate cells involved in large meal related satiation. The hypothesis was if we activated the hM3Dq receptor in the cNTS, then mice should experience satiation and possibly decrease food intake.

Methods

Mice

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

The cFos-tTA mouse strain was obtained from Jackson Laboratories (c-Fos-tTA also referred to as c-fos, TetTag, cfos-htTA, cfos-tTA/cfos-shEGFP; cat. no. 018306) and bred for several generations in our laboratory. Mice had ad libitum access to water and standard chow in pellet form (Teklad Rodent Diet 2018, Envigo, Madison, WI) and were maintained at 22℃ on a 14:10 hour light/dark cycle to facilitate breeding with lights on at 0500 and off at 1900 prior to the experiment. Mice were approximately 3 months old at the time of surgery. Mice were also adapted to single housing for one week prior to surgery.

Genotyping

Mice were tail snipped at postnatal day 21 and tails were stored at -20℃ until genotyping. Briefly, tails were thawed to room temperature and using a DNA extraction kit (DNAeasy Blood and Tissue Kit, #69506, Qiagen, Hilden, Germany) tails were lysed and DNA was extracted. DNA samples were stored at 4℃ until PCR was performed. PCR was performed for cFos-tTA gene using the primers and PCR program provided by Jackson Laboratory and reagent protocols from our lab. Primers for the cFos-tTA transgene were forward transgene primer oIMR0872 5’-AAG TTC ATC TGC ACC ACC G-3’ and reverse transgene primer oIMR1416 5’-TCC TTG AAG AAG ATG GTG CG-3’ (IDT Forward catalog #198693093, Reverse catalog #198693094) in order to amplify a 173 bp cFos-tTA fragment. The PCR procedure was as follows: 95℃ 15 min; 94℃

for 3 min; 10 cycles of 94℃ for 20 sec, 65-58℃ (0.5℃ step-down over the 10 cycles) for 15 sec, 68℃ for 10 sec; 28 cycles of 94℃ for 15 sec, 60℃ for 15 sec, 72℃ for 10 sec; and 72℃ for 2 min.

Diets

Mice were maintained on Teklad Repelleted Control 2018 diet (3.1 Kcal/g TD.150279, Envigo, Madison, WI). At the time of surgery (four weeks prior to testing), mice were transitioned to Teklad 2018 diet containing 40mg/kg Dox (TD. 120462 Envigo, Madison, WI). During the feeding training, mice received three different diets (explained in detail in Meal Training above): liquid Ensure Diet, (Ensure Vanilla, 1.48 kcal/mL), Teklad 2018 diet and Teklad 2018 diet containing 40mg/kg Dox. In experiments 3.2.9 and 3.2.10, Teklad Repelleted 2018 diet containing a dose of 1g/kg of Dox (TD. 120658, Envigo, Madison, WI) was also used.

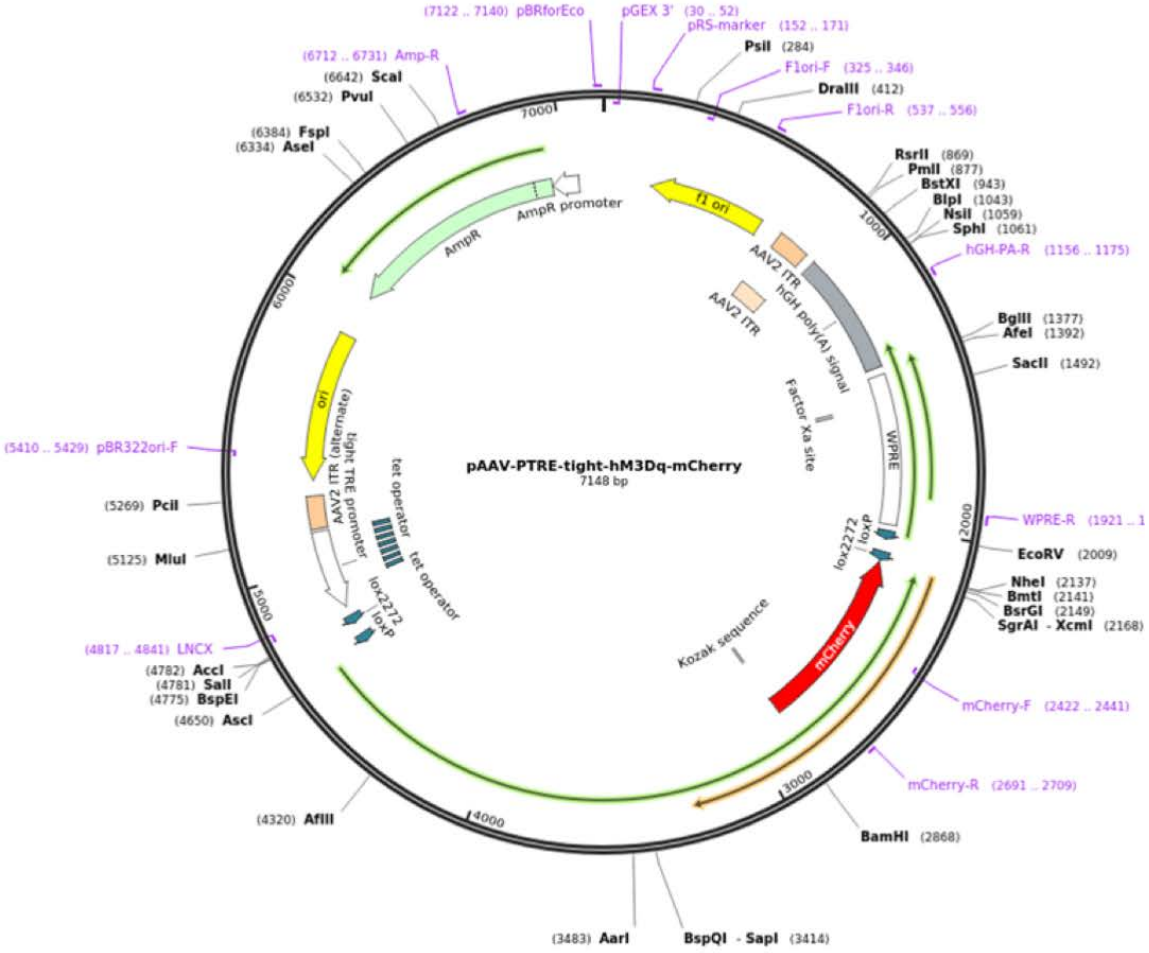
Generation and Stereotaxic Injection of AAV

The pAAV-PTRE-tight- hM3Dq-mCherry was was purchased from Addgene (Addgene Plasmid #66795, Cambridge, MA) (Figure 4A). Plasmids were grown up in lab and plasmid DNA was extracted using the Qiagen Plasmid Maxi Kit (Cat No/ID 12162) following the Qiagen Plasmid Mini, Midi and Maxi KITS – (EN) quick start protocol. Final concentrations of DNA were measured using a nanodrop spectrophotometer (Purdue Genomic Core Facility, Purdue University, West Lafayette, IN). Restriction digests were performed using restriction enzymes (BamHI (R0136S), SalI (R0138S), BglII (R0144S), PciI (R0655S), New England BioLabs, Ipswich, MA) to determine the correct DNA was amplified and extracted (Figure 4B). Restriction enzymes SalI and BamHI produced base pair fragments 5235 and 1913 base pairs in length. Restriction enzymes PciI and BamHI produced fragments 4747 & 2401 base pairs in length. BglII and SalI cut the plasmid into two fragments 3744 and 3404 base pairs in length and PciI and BglII cut the plasmid into two fragments 3892 and 3256 base pairs in length. Custom AAV were constructed containing plasmid DNA at the University of North Carolina Vector Core. The hM3Dq-mCherry transgene was packaged into a 1:1 mix of AAV1 & 2 serotype capsid proteins with AAV2 inverted terminal repeats (which allow for the synthesis of the transgenes).

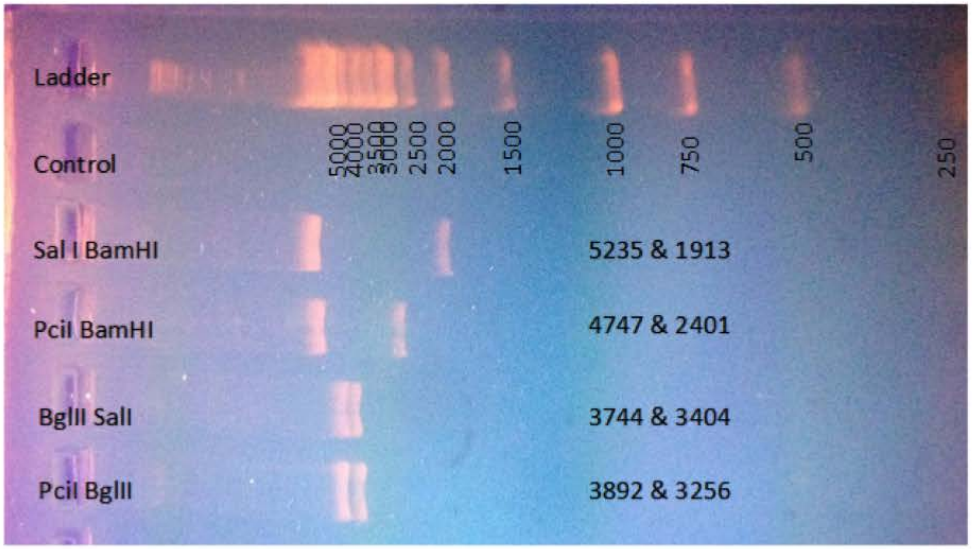
Briefly, mice were anesthetized with 5% isoflurane in O_2 by inhalation and mounted into a stereotaxic frame. Mice were maintained on 1.5-2% isoflurane during surgery. The head of the

Figure 4. Plasmid and restriction enzyme digest. (A) Diagram of plasmid containing the hM3Dq-mCherry genes (Addgene Plasmid Repository, 2019). (B) Sample digests from the plasmid containing the hM3Dq-mCherry genes. Lanes on the gel are labeled with the two restriction enzymes used to cut the plasmid and the lanes are also labeled with the expected base pair length of each fragment.

A


Created with SnapG
pAAV-PTRE-tight-hM3Dq-mCherry
7148 bp
(7122 .. 7140) pBRforEco
pGEX 3' (30 .. 52)
pRS-marker (152 .. 171)
PsiI (284)
F1ori-F (325 .. 346)
DraIII (412)
F1ori-R (537 .. 556)
RsrII (869)
PmlI (877)
BstXI (943)
BlpI (1043)
NsiI (1059)
SphI (1061)
hGH-PA-R (1156 .. 1175)
BglII (1377)
AfeI (1392)
SacII (1492)
WPRE-R (1921 .. 1
EcoRV (2009)
NheI (2137)
BmtI (2141)
BsrGI (2149)
SgrAI - XcmI (2168)
mCherry-F (2422 .. 2441)
mCherry-R (2691 .. 2709)
BamHI (2868)
(3483) AarI
BspQI - SapI (3414)
(4320) AflII
(4650) AscI
(4775) BspEI
(4781) SalI
(4782) AccI
(4817 .. 4841) LNCX
(5125) MluI
(5269) PciI
(5410 .. 5429) pBR322ori-F
(6334) AseI
(6384) FspI
(6532) PvuI
(6642) ScaI
(6712 .. 6731) Amp-R
AmpR promoter
AmpR
f1 ori
AAV2 ITR
hGH poly(A) signal
AAV2 ITR
Factor Xa site
WPRE
lox2272
loxP
mCherry
Kozak sequence
ori
bpht TRE (alternate)
AAV2 ITR
tet operator
tet operator
lox2272
loxP

B


Ladder
Control
5000
4000
3500
2500
2000
1500
1000
750
500
250
Sal I BamHI
5235 & 1913
PciI BamHI
4747 & 2401
BglII SalI
3744 & 3404
PciI BglII
3892 & 3256

mouse was shaved and cleaned with isopropyl ethanol. Ophthalmic ointment was applied to the eyes and the head of the mouse was fixed in a motorized stereotaxic apparatus (Neurostar). A midline incision over the skull was made to expose the bregma and lambda sutures. A 3% H_2O_2 solution was used to remove any remaining connective tissue and the skull was dried with cotton swabs. Bregma and lambda were marked and coordinates were stored using Neurostar Stereodrive software. Two drill holes were made at the most caudal 1/3 of the NTS using the following coordinates: bregma -6.8 mm and midline ± 0.35 mm. The AAVs were injected with a pulled glass micropipette into the drilled holes 4.03mm dorsal from the craniotomy. Four different injection volumes were used to determine the optimal injection size: 50nL, 100nL, 150nL and 500nL. Injections lasted for at least one minute and the micropipette was kept in place for at least five minutes before withdrawing. Mice injected with AAV were given four weeks to recover and allowed for the expression of viral transgenes before behavioral experiments (Zhang et al., 2015). These mice will be referred to as cFos-AAVhM3Dq from this point forward (Figure 5).

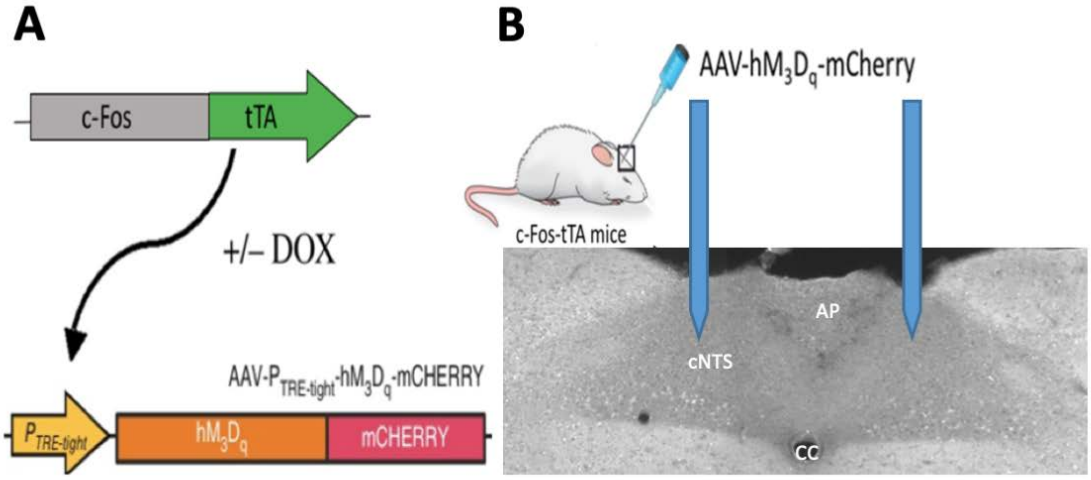


Figure 5. Schematic of viral delivery of AAV-hM3Dq-mCherry transgene. (A) Schematic of cFos-tTA mouse and AAV-hM3Dq viral gene expression. The cis regulatory element, promoter c-Fos, drives the expression of the bacterial tetracycline-regulated trans activator (tTA). When this gene is expressed, it activates hM3Dq-mCherry. This target gene is only expressed under the control of the PTRE operator in the presence of tetracycline. Dox is a tetracycline analog which can turn hM3Dq -mCherry off or on. In the presence of Dox, hM3Dq -mCherry is blocked (off) and in the absence of Dox, hM3Dq -mCherry is expressed (on). (B) Cross section through cNTS with blue arrows highlighting injection position. CC central canal, NTS nucleus solitary tract, AP area postrema.

Meal Training

Meal training procedures were as described above (Figure 2A). Forty-eight after the large meal, mice were sacrificed and the cNTS was immunostained for the hM3Dq receptor.

Doxycycline Testing

A subset of mice were placed on Dox for one month to demonstrate that 40mg/kg Dox was sufficient to block hM3Dq receptor expression throughout the hindbrain. Therefore, if one month of Dox exposure was long enough to inhibit hM3Dq receptor expression, then there would be no hM3Dq receptors seen throughout the hindbrain.

If Dox has no effect on food intake or body weight, then food intake and body weight should not be statistically different from when mice were fed chow versus 40mg/kg Dox containing chow. Mice were placed on powdered chow until stabilization criterion were met (described in Gilland & Fox, 2017). Mice were switched to powdered chow containing 40mg/kg Dox and was measured until stabilization criterion were met. The stabilization criterion was used so that food intake on days where the food was changed because the food intake amount could be compromised due to neophobia. Food intake and body weight averages from the chow and Dox chow were compared using a Student's *t*-test.

CNO Testing

If CNO has no effect on satiation and food intake, then CNO administration should not lead to cFos expression in the cNTS or changes in food intake. To test this hypothesis, CNO was administered to cFos-tTA only mice by intraperitoneal (ip) injection of 0.8mg/kg of CNO (C0832, Sigma-Aldrich, St. Louis, MO; dissolved in saline) or saline in order to determine if CNO had any effect on food intake. Food intake was measured for 5 days prior to the injection to establish a baseline. Food intake was also measured for five days after the administration of CNO or saline. Student's t-test was used to compare food intake between groups. In a separate set of mice, where the AAV-hM3Dq was not injected into the NTS, but rather into the cerebellum, 10 mice received CNO injections and two mice received saline injections. This was to test if CNO had any effect on cFos expression in the NTS. cFos immuno-positive cells were counted as in the Microscopy section. In another set of c-Fos-tTA mice not injected with virus, 6 received an injection of CNO

(0.8mg/kg) and 4 mice received a saline injection. 135min post injection (45min for CNO peak activation plus 90min for peak cFos activation) mice were sacrificed and processed as described in the Immunohistochemistry sections.

Immunohistochemistry

Immunohistochemistry procedures were similar as described above with a few minor changes. hM3Dq-mCherry was immunostained using the primary antibody DsRed (cat no. 632496, RRID:AB_10013483, Clontech, Palo Alto, CA) over 72 hr. Sections were stained with Cy3 as described above. Brains were also immunostained for endogenous cFos with a rabbit polyclonal c- Fos antibody (1:10,000; no. PC38, Calbio-chem, EMD Chemicals, Gibbstown, NJ) and a Cy2 secondary antibody (1:600 Cy2 AffiniPure goat anti-rabbit, Jackson ImmunoResearch, 111-225-144).

Microscopy

Microscopy and quantification procedures were carried out as described above with a few additions. The sections were imaged as described above and analyzed as in Gilland & Fox, 2017. Cells expressing cFos, mCherry and cells expressing both cFos and the mCherry were counted in the cNTS-AP. Three sections from each brain closet to plate numbers 93-94 from Paxinos & Franklin 2008, were counted and then averaged across the three sections. After images were taken, different color channels were selected with respect to the color of the secondary antibody, mCherry or the combination of both secondary and mCherry in order to automatically count cells expressing cFos, the mCherry and cells expressing both cFos and mCherry. A green color channel (510 nm) was selected for Cy2 in order to count only cFos expressing cells. A red color channel (610 nm) was selected to count cells expressing mCherry. A yellow color channel (560nm) was selected to count only cells expressing both cFos and mCherry. Counting procedures were carried out as in Gilland & Fox 2017. Briefly, images were converted to an 8-bit image and then inverted. Background was removed using a 50.0-pixel rolling-ball radius. Images were then converted to a binary image, and noise was removed using the Despeckle feature.

Brain sections were also assessed and characterized for the extent of the AAV infection in each mouse. Any mouse with questionable AAV infection, cell bodies labeled outside the cNTS

or possible expression in the rNTS was not included in the study. The border of the cNTS and AP was outlined based on The Mouse Brain Atlas (Paxinos & Franklin, 2008). A subset of sections from each mouse was also be stained with cresyl violet and used to determine borders of the cNTS within each mouse.

Inducibility of hM3Dq Receptors in cNTS Cells of cFos-AAVhM3Dq Mice

The pAAV-PTRE-tight- hM3Dq-mCherry virus was injected into cNTS of the cFos–tTA mice four weeks before testing (Zhang et al., 2015). Mice were placed on Dox mixed in pelleted chow (Dox 40mg/kg: Teklad Rodent Diet 2018, Envigo, Madison, WI) at the same time to prevent receptor expression before the start of the experiment. Four weeks after surgery mice were put through meal training, where half of the mice were switched to chow with no Dox on the first day of meal training, while the other half of the mice remained on Dox through out all days of the meal training. The hypothesis was if mice are on Dox for an extended period of time, no hM3Dq receptors will be expressed, however, taking the mice off Dox will temporarily allow for hM3Dq receptor expression.

Student's *t*-test was used to compare immunostained cells for the hM3Dq receptor and the mCherry protein in the 40mg/kg Dox and no Dox groups. The independent variable was if the mice were on 40mg/kg Dox or no Dox and the dependent variables were the number of immuno-positive hM3Dq cells. Immuo-positive cells were presented as averages $\pm$ standard error of the mean.

Demonstrate cNTS Cells That Have hM3Dq Receptors are Excited by Administration of the hM3Dq Receptor Ligand, CNO

cFos-AAVhM3Dq mice were treated the same as described above, except five hours after consuming the Ensure meal mice were given ad libitum access to 1 g/kg Dox chow in order to rapidly stop hM3Dq expression until day 7. Also different from the previous experiment, the next day half of the mice were injected ip with 0.8 mg/kg CNO, (Alexander et al., 2009; Gomez et al., 2017) (n = 27) and with saline (n = 7) and 135 minutes later all mice were sacrificed. The hypothesis was if the cells activated by a large meal were also activated by CNO, then cells expressing the hM3Dq receptor will also express endogenous cFos. On day 7, mice were given an

ip injection of CNO or vehicle. Ninety minutes after injection, mice were perfused and brainstem sections through the cNTS of all mice were immunostained for the hM3Dq receptor and for cFos.

Brains were sectioned and immunostained for endogenous cFos with a rabbit polyclonal cFos antibody (1:10,000; no. PC38, Calbio-chem, EMD Chemicals, Gibbstown, NJ) and a Cy2 secondary antibody (1:600 Cy2 AffiniPure goat anti-rabbit, Jackson ImmunoResearch, 111-225-144) using a modified protocol from to stain for cFos. The sections were imaged, analyzed and counted as described above.

A Student's *t*-test was used to test for differences in the amount of the large meal eaten by CNO- and saline-treated mice as well as both the number of cells stained for c-Fos in response to CNO vs. saline and the number mCherry positive cells in each group. The same test was used to compare the percentage of cells exhibiting both mCherry (and thus the hM3Dq receptor) and cFos staining between mice treated with CNO vs. vehicle. The hypothesis was if CNO activated cells with the hM3Dq receptor, then those same cells should also express cFos. Mice with inaccurate AAV injections were excluded from analysis.

Demonstrate Excitation of cNTS Cells That Express hM3Dq Receptors With CNO Reduces Meal Size

Procedures from the previous experiment were repeated, however, after ip injection of CNO or saline, mice were presented with food at the onset of the dark cycle and food intake was measured. For this experiment, cFos-AAVhM3Dq received a ip injection of CNO or saline 30 minutes prior to the start of the dark cycle so that activation of the cells by CNO were at peak activation by the start of the dark cycle. At the start of the dark cycle, mice were presented with Teklad 2018 chow (no Dox) and food intake was measured at 30min, 60min, 90min, 120min and 24hr. After 24 hr, mice were injected again with CNO and sacrificed 90 minutes later. This was to confirm and quantify cells expressing hM3Dq receptor, cFos or co-localization of both proteins as described above. The hypothesis was if hM3Dq receptors are activated by CNO in the cNTS and mice experience satiation, then food intake should be reduced.

Food intake was measured at 30min, 60min, 90min 120min and 24hr intervals and compared between groups using a one-way ANOVA and Tukey post hoc tests. A repeated measures ANOVA was used to compare the groups over the multiple time points. A Student's t-test was used to compare the percentage of cells exhibiting both mCherry (and thus the hM3Dq

receptor) and cFos staining between mice treated with CNO vs. vehicle as in the previous experiment. Mice with inaccurate AAV injections or failure to exhibit evidence of cFos activation by CNO were not included in analysis of food intake.

Results

Food Intake and Body Weight From Large Meal Training

Mice were put through the large meal training four weeks after injection of the virus. Body weight average for all mice (n = 32) on the first day of training was 27.64 + 0.77g and Ensure intake was 1.80 + 0.10g. On the day of the test meal, body weight was 28.68 + 0.66g and Ensure intake was 3.53 + 0.22g. (Body weight increase p=.31, no significance; Food intake increase p = .0001, significant). This demonstrates mice increased their meal size over the course of the meal training. All mice completed the requirements for meal training. None were dropped due to a significant decrease in body weight over the course of the meal training (Figure 6).

Dox Effects on Food Intake or Body Weight

During the period of stable food intake on powdered chow, the cFos and wild-type groups exhibited similar average daily food intake (cFos = 3.76 $\pm$ .07g, wild-type = 3.65 $\pm$ .11 p = 0.43) and body weight (cFos = 23.47 $\pm$ .98g, wild-type = 25.53 $\pm$ .89, p = 0.15; Fig). During the period of stable food intake on powdered chow containing 40mg/kg Dox, the c-Fos+ and wild type groups exhibited similar average daily food intake (cFos = 4.01 $\pm$ .11g, wild-type = 3.84 $\pm$ .14 p = 0.37) and body weight (cFos = 24.20 $\pm$ 1.04g, wild-type = 26.34 $\pm$ .98 p = 0.16). Comparisons of the stable food intake on chow and chow containing 40mg/kg of Dox showed no differences regardless of genotype in food intake (c-Fos p = .08; wild-type p = .30; or body weight (c-Fos p = .62; wild-type p =.55).

Inducibility of hM3Dq Receptors in cNTS Cells

Mice that underwent surgery were placed on 40mg/kg Dox immediately following surgery and maintained on Dox for one month. Mice showed no hM3dq receptor expression (n = 2) in the

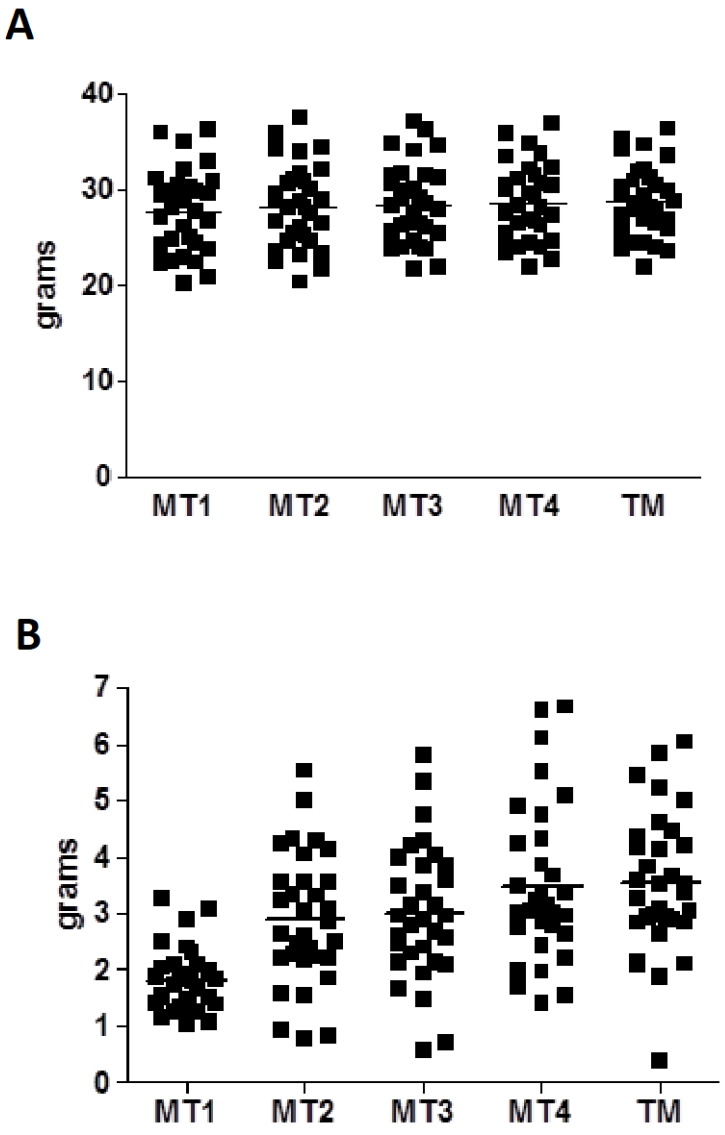


Figure 6. Body weight and food intake during cFos-AAVhM3Dq meal training. (A) Body weight in grams of cFos mice injected with AAV-hM3Dq across meal training. (B) Food intake in grams of cFos mice injected with AAV-hM3Dq across meal training. MT1-MT4 meal training day 1-4, TM test meal.

NTS, surrounding brain areas or along the injection tract in the cerebellum when maintained on Dox (Figure 7).

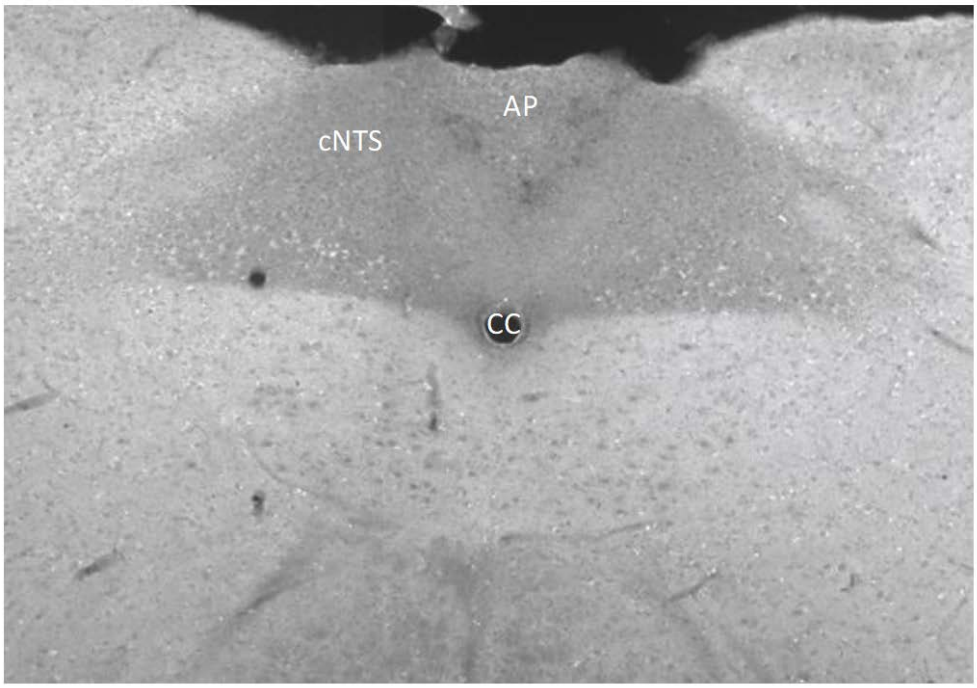


Figure 7. Dox blocks hM3Dq receptor expression in the cNTS, AP and throughout the entire hindbrain. Mice maintained on Dox for four-weeks blocks hM3Dq receptor in the cFos-AAVhM3Dq mouse model. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

Mice were taken off dox at three different time points during large meal training. The three different time periods were tested: the last 3 (n = 3), 4 (n = 5) and 5 (n = 31) days of the meal training and including the test meal off Dox. There were no observable differences between the three time points off Dox and experiments moving forward used 5 days off Dox to remain consistent with the literature (Cowansage et al., 2014; Roman, Derkach, & Palmiter, 2016).

Mice were sacrificed at 24 (n = 4), 48 (n = 30) (Figure 8 for example of staining) and 72 (n = 7) hours post meal training. Receptors were present at each of the three time points. There were no observable differences in expression of the receptors between the three time points, however, because the injections varied between mice, the uptake rate and expression of the virus could be different in different cell types. The most important result is that receptors are present as

early as 24 hours through at least 72 hours. This means experiments could be reliably carried out between 24-72 hours after the large test meal.

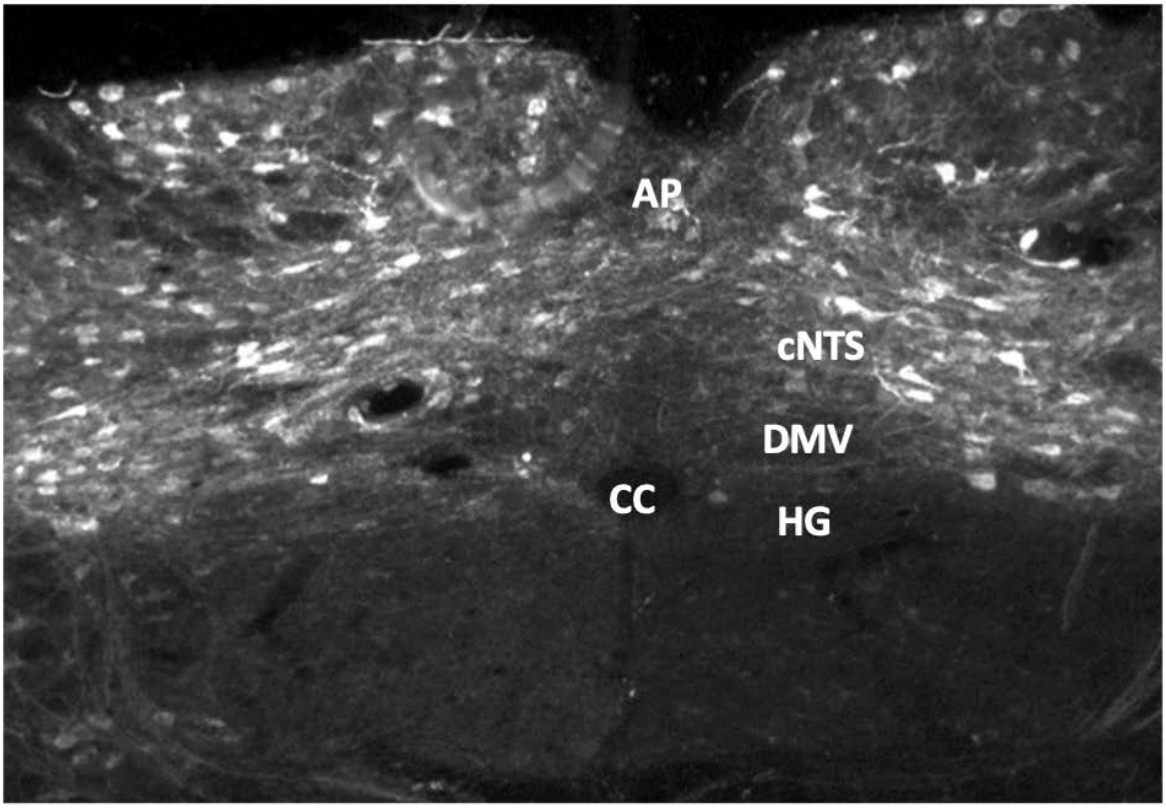


Figure 8. Representative hM3Dq label after viral injection and meal training. Expression is seen in the cNTS, AP. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema, DMV dorsal motor nucleus, HG hypoglossal nucleus.

Injection Volume

Four different injection volumes of virus were tested: 500nL, 150nL, 100nL and 50nL**.** 500nL injections (n = 5) were much too large and labeled almost the entire area of the hindbrain at the level of the NTS (Figure 9A). 150nL injections (n = 12) were still too large (Figure 9B). These injections labeled cells within the NTS as well as cells outside the NTS. Projections were visible going to the reticular formation from the NTS (Figure 9C) as well as projections going from the right NTS to the left NTS and visa versa. 100nL injections (n = 5) were observationally indistinguishable from 150nL injections. 50nL injections (n = 24) were too small and created

pressure/backflow issues where the majority of the injection went back up the injection tract or did not expel from the needle until it was brought back out of the brain.

CNO Results in Co-Expression of cFos and mCherry

In two cases, where the AAV injection was considered a hit (but spread to areas outside the NTS), and in one case where the injection was lateral to the NTS, the co-expression of cFos and the hM3Dq receptor was observed. Cells containing cFos and hM3Dq were observed within the NTS. There were also cells that expressed only cFos or only the hM3Dq receptor in response to an injection of CNO (Figure 10A). These cells were not quantified as the injections spread far outside of the NTS and minimal cells were cFos and hM3Dq positive. In the case where the AAV injection was lateral to the NTS, there were few double labeled cells lateral to the NTS. There were also many labeled cFos positive cells within the NTS in response to activation by CNO (Figure 10B). Cells along the injection tract in the cerebellum were also co-expressed cFos and the hM3Dq receptor when activated with CNO (Figure 10C).

CNO has no Effect on Daily Food Intake or Body Weight

There were no effects of CNO on daily food intake from an ip injection of 0.8 mg/kg CNO (n = 5) vs saline (n = 5) in wild-type mice. Food intake for saline mice averaged 3.62 $\pm$ 0.15 g/day for the five days prior to injection, 3.84 $\pm$ 0.16g the day after the injection and averaged 3.69 $\pm$ 0.10 g/day for the five days after injection. Food intake for CNO mice averaged 3.76 $\pm$ 0.16 g/day for the five days prior to injection, 3.67 $\pm$ 0.21 g the day after the injection and averaged 3.19 $\pm$ 0.19 g/day for the five days after injection. Each pair of values were compared using a Student's t-test and showed no significant differences (p = 0.27, p = 0.27 and p = 0.16 respectively). Nakajima et al., 2016 produced similar results and saw no effect of CNO on feeding in wildtype mice.

cNTS Cells are Excited by the Administration of CNO in the Presence and the Absence of the hM3dq Receptor

In mice where the hM3dq receptor was not expressed in the NTS (meaning the injection was considered a miss), cFos expression was quantified in the cNTS in response to CNO.

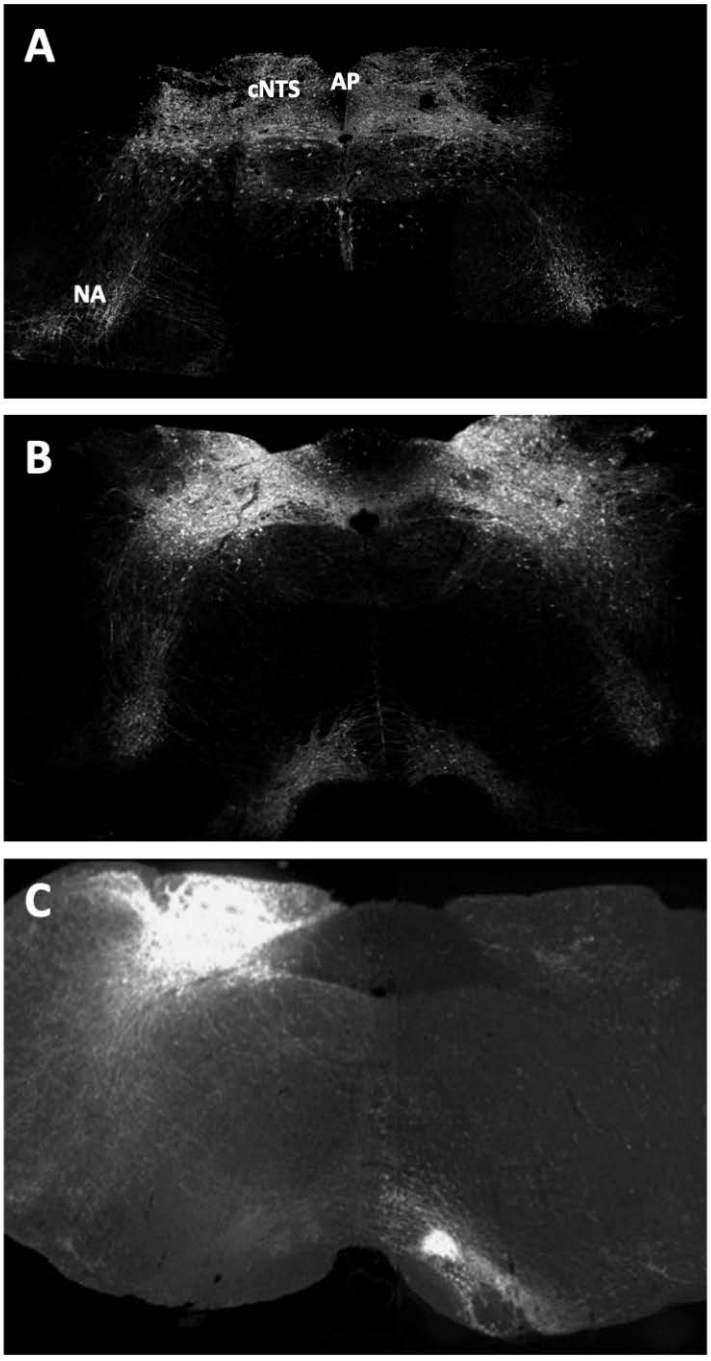


Figure 9. Viral injection size examples. Representative images of different injection sizes (A) 500uL bilateral injections, (B) 150 bilateral injections and (C) 150uL unilateral injection. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema, NA nucleus ambiguus.

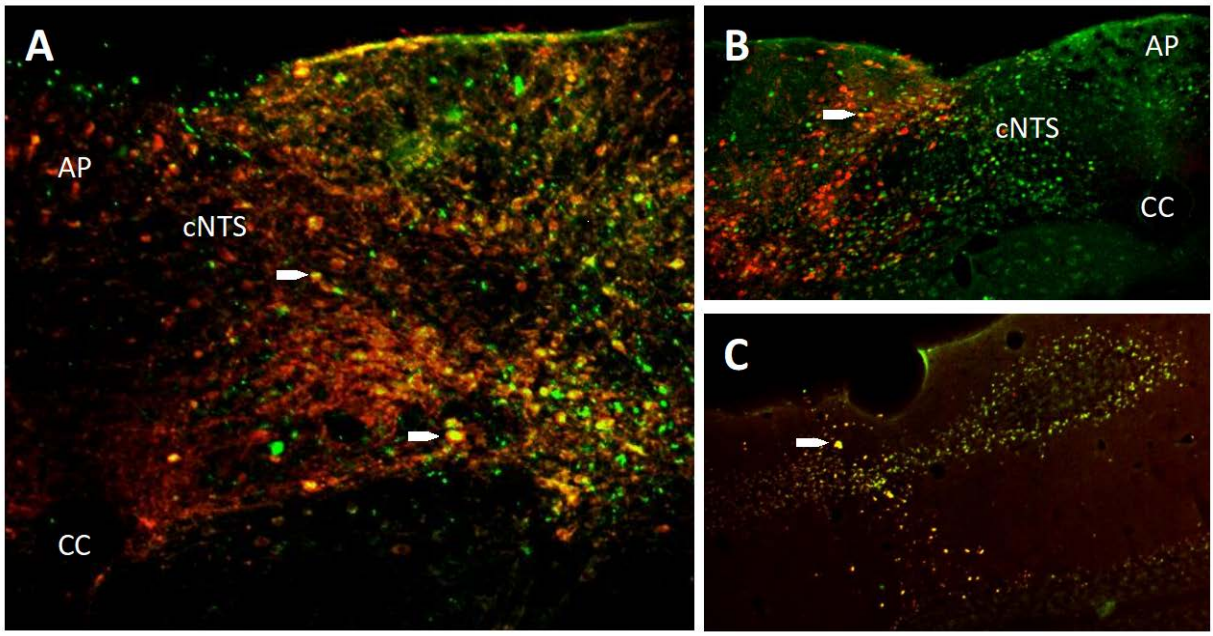


Figure 10. cFos-hM3Dq double labeling. (A) Image of double labeling in a cFos-AAVhM3Dq mouse with injection into the cNTS after administration of CNO. White arrow marks a cell with double label. (B) Image of double labeling in a cFos-AAVhM3Dq mouse with viral injection lateral to the cNTS. cFos label is seen within the cNTS and double label is seen laterally to the cNTS. White arrow marks cell with double label. (C) Image of double labeling in a cFos-AAVhM3Dq mouse along the injection tract within the cerebellum. White arrow marks an example of a double labelled cell. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

Meal training showed an increase in meal size from start of training to the test meal in both groups (significant: CNO group (n = 10): 1.64 $\pm$ .14g to 3.79 $\pm$ .32g p = .0001; trending: control (n = 2): 1.39 $\pm$ .13g to 4.41 $\pm$ 1.05g p = .10) and no significant changes in body weight (CNO group: 26.91 $\pm$ 1.30g to 28.62 $\pm$ 1.02g p=.31 control: 25.24 $\pm$ 2.91g to 25.72 $\pm$ 3.08g p = .92) (Figure 11A-B).

In mice injected with vehicle the number of cFos positive cells averaged over three sections in the cNTS were 25.5 $\pm$ 6.2 NTS cells and 5.5 $\pm$ 1.5 AP cells. In mice injected with 0.8mg/kg of CNO, the number of cFos positive cells averaged over three sections in the cNTS were 116.7 $\pm$ 12.4 NTS cells and 14.05 $\pm$ 2.7 AP cells). A Student's t-test revealed that there was significant cFos expression in the CNO treated mice in the cNTS compared to the vehicle treated mice (p = 0.01). There was no significant difference between the two groups in the AP (p = 0.30) (Figure 11C).

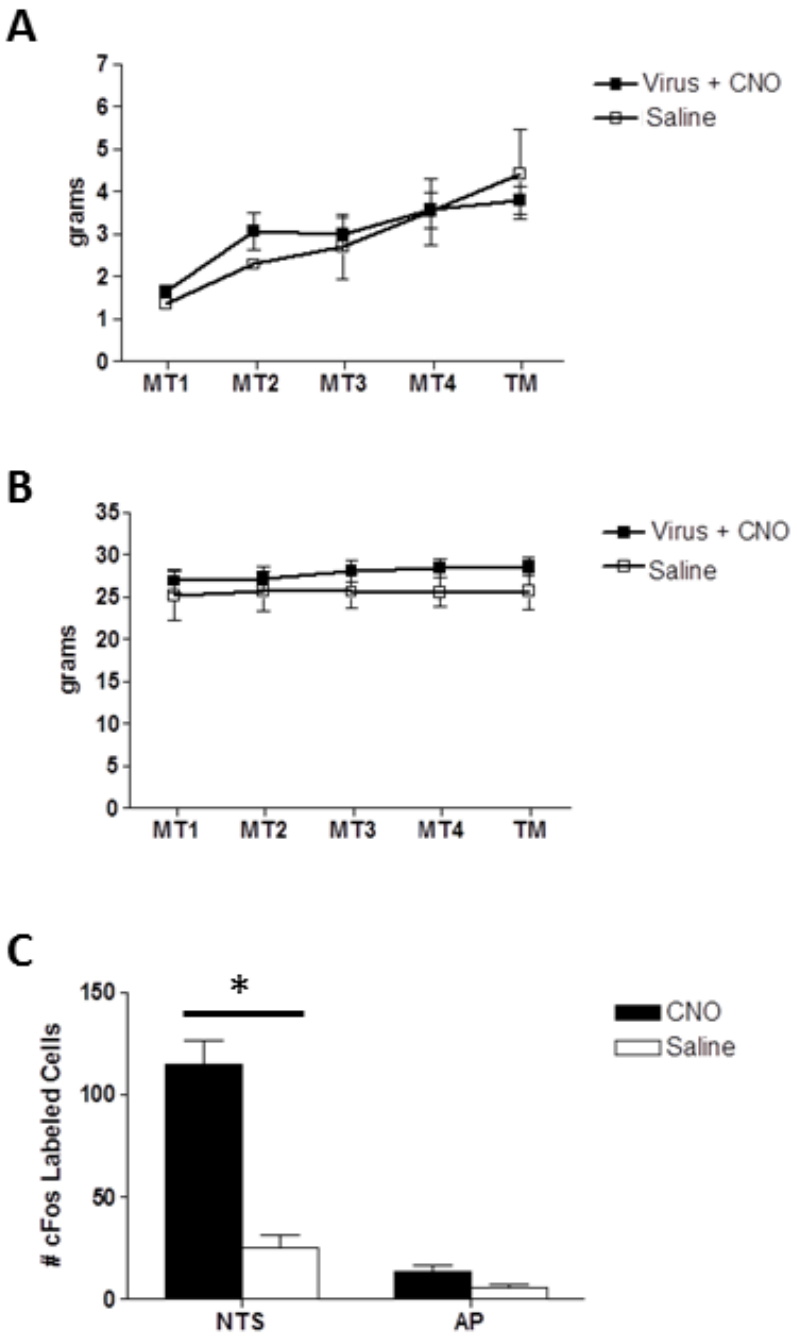


Figure 11. Meal training and cFos Expression for missed injections in cFos-AAVhM3Dq mice. (A) Body weight in grams of cFos mice injected with AAV-hM3Dq (n = 10) across meal training where virus was considered a miss. Control mice received vehicle treatment (n = 2). (B) Food intake in grams of cFos mice injected with AAV-hM3Dq across meal training where virus was considered a miss. (C) cFos expression within the cNTS in response to CNO or vehicle treatment in the absence of the hM3Dq receptor. MT1-MT4 meal training day 1-4, TM test meal.

This experiment was then followed up in cFos-tTA mice with no viral injection to confirm the injection of the hM3Dq receptor was not contributing to cFos expression in the cNTS in response to the CNO injection. Representative images of cFos expression in the NTS caudal to the area postrema, the NTS at the level of the area postrema (cNTS) and the rNTS (Figure 12A-C). In mice injected with 0.8mg/kg of CNO (n = 6), the number of cFos positive cells averaged over three sections in the cNTS were 62.28 $\pm$ 22.10 NTS cells, 26.72 $\pm$ 4.89 AP cells and 56.25 $\pm$ 9.52 DMV cells. Control mice injected with saline (n=4) showed cFos expression in the cNTS were 17.17 $\pm$ 8.81 NTS cells, 2.33 $\pm$ 1.47 AP cells and 25.25 $\pm$ 7.57 DMV cells (Figure 12D). Using a student's *t*-test to calculate significance, the difference in the increase of the cNTS cells in the CNO group was considered non-significant (p = .15), the increase in the number of the AP cells in the CNO group was considered to be a significant increase over the saline injection group (p = 0.004) and the increase in the DMV cells in the CNO group was considered a significant increase over the saline injection group (p = .05).

Projections From the NTS to Other Areas of the Brain

Cell bodies were present four weeks after injection and meal training in many areas of the hindbrain, with the majority being in and around the cNTS. Those areas include: the area postrema, solitary nucleus (commissural, medial, dorsomedial, dorsolateral, ventrolateral, medial, ventral, interstitial and lateral parts), the solitary tract, the parasolitary nucleus, the dorsal motor nucleus of the vagus, the intermedius nucleus of the medulla and the nucleus of Roller. Projections from the cell bodies to other areas of the brain include: the ambiguous nucleus, lateral reticular nucleus, intermediate reticular nucleus, inferior olive (beta subnucleus, cap of Kooy of the medial nucleus, dorsal nucleus, subnucleus B of medial nucleus and principle nucleus), raphe obscurus and the caudoventrolateral reticular nucleus (Figures 8 & 9).

Discussion

40mg/kg Dox was given for one-month post surgery and was enough Dox to block all hM3Dq expression in the hindbrain. This work confirmed previous findings where 40mg/kg Dox was also sufficient to block hM3Dq expression and confirmed hM3Dq expression inhibition under the experimental parameters of this study.

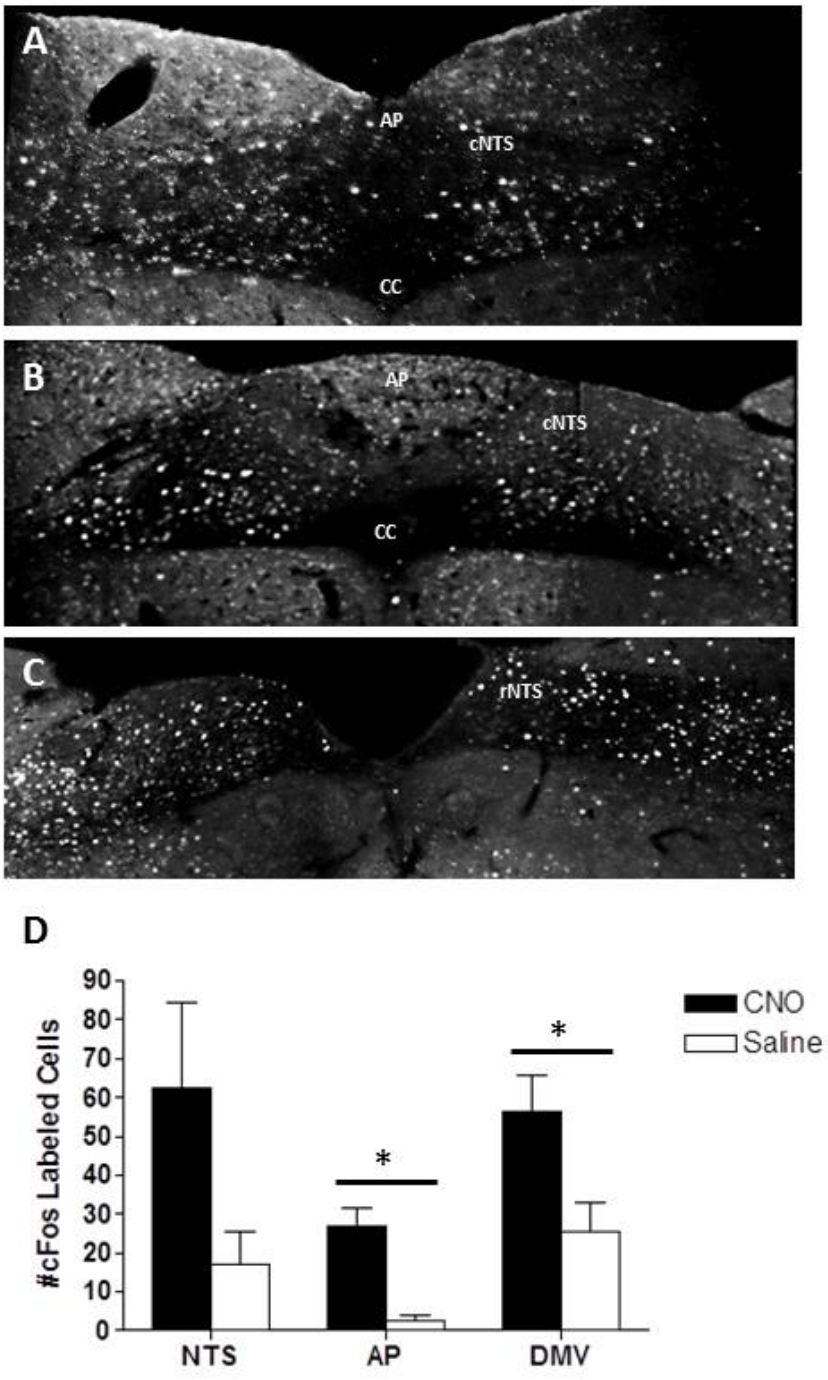


Figure 12. cFos expression at different levels of the NTS in response to CNO. (A) cFos expression at the level of the hindbrain caudal to the area postrema. (B) Example of cFos expression at the level of the hindbrain of the area postrema. (C) cFos expression at the level of the hindbrain rostral to the area postrema. (D) cFos expression within the cNTS in response to CNO (n = 6) or vehicle (n = 4) treatment in cFos-tTA mice. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

Dox did not effect food intake or body weight in wild-type mice. However, a counter balanced experiment should be performed where age matched animals receive dox first and are then switched to chow. The important finding here is that Dox does not decrease body weight or food intake.

The 5-day meal training was sufficient to induce hM3Dq expression. cFos expression occurred in response to the test meal on the last day of the meal training which in turn drove the expression of the hM3Dq receptor. The 5-day meal training paradigm produced similar food intake and body weight patterns as Rinaman et al., 1998 and Fox et al., 2013a resulting in similar cFos patterns. One issue with the virus spreading to areas outside of the NTS was that cFos expression occurs in all areas of the brain under various circumstances and so cFos expression resulting from neurons firing drove hM3Dq receptor expression in areas of the brain where the virus spread and along the injection tract.

The "hit rate" of injections into the NTS in general was very low due the small size of the NTS, variation of brains between animals and the NTS is subject to some movement while in the stereotaxic. While the skull was fixed in the stereotaxic, the brainstem was not fixed in place and allowed for some movement. Injections where the mouse head was able to be angled at 45˚ downward and injected without going through the skull have been previously successful with similar injection parameters to this study (D'Agostino et al., 2016). In the future, angling the head downward would increase tension on the brainstem and likely help to keep the brainstem in place.

Another issue with injecting a small volume into the NTS is backflow. This issue was due to either due to back pressure (a higher pressure in the brain than in the needle), back siphonage (pressure in the tract being lower than in the needle) or a combination of both. Based on the labeling pattern of the virus, it would appear both occurred. Back pressure appeared to be an issue while injecting, keeping the virus from exiting the needle. When the needle was withdrawn through the cerebellum, back siphonage became a problem and virus was pulled out of the needle into the brain. This means little to no virus was injected into the NTS and the majority of the virus ended up in the cerebellum or in the space between the medulla and the cerebellum. As a result of injection issues, not all experiments in this section were conducted.

Another issue with these studies was that the AAV expression rate and turnover rate is unknown. Some AAVs have been known to express for many months to years, however, cell turnover rate is the largest limiting factor to the time course of the expression of AAVs (Naso et

al., 2017). It will be important to determine the turnover rate for different cell types to best determine experimental parameters.

The biggest concern with this experiment was first observed in animals where the viral injection was considered a miss. CNO was administered to these animals and cFos expression was observed throughout the entire NTS in the absence of hM3Dq expression within the NTS. Because the mice were injected with virus, it had to be ruled out that there was still virus in the NTS that was below a detectable level that was responsible for the cFos expression. These observations were followed up with a small group of mice with no AAV-hM3Dq that received CNO or saline injections and cFos cells were counted within the cNTS. cFos cells were still present as in the mice with the missed viral injections, suggesting there was no virus in the NTS leading to cFos expression. Even though the NTS was not significantly different in the CNO vs saline group, the AP and DMV were both significantly different. This second experiment was conducted with a smaller group of animals and the sample size may not have been large enough to obtain statistical significance. It should also be noted that there was a large degree of variation in the number of cells in the NTS expressing cFos in response to CNO and this is why a larger sample size is necessary. Taking this data together with the food intake data in response to CNO administration, suggests that CNO itself may activate a subset of satiation cells, but that this activation does not modulate food intake over the course of the day. If CNO is reverted to clozapine, and clozapine binds to the serotonin receptor within the NTS to promote satiation, it would be possible CNO/clozapine could decrease food intake in the short-term. Based on the food intake results presented here, it could be hypothesized that short-term food intake (meal size, intermeal interval etc.) could be decreased but over the course of a 24-hour period, the decrease is compensated for and food intake remains the same (Biddinger & Fox 2014). In the future, it will be important to conduct a meal pattern analysis to determine if the activation of these satiation cells decrease meal size, even though they may not decrease daily food intake overall.

CNO-activated cFos in the NTS is a major confound that makes the interpretation of any successful injections questionable at best. If the hM3Dq is present in the NTS and CNO is administered to activate these cells, it will be impossible to tell if the activation is from the CNO binding with the hM3Dq receptor or if the CNO activates the cell directly.

Viral injection into the brain can allow for superior specificity over other transgenic models because receptors are not present within the brain from birth. It is unlikely these receptors have a

significant impact on development, however, it has not been demonstrated that these gene mutations have no effect on development. The experimenter also has control over the amount of the viral vector administered and thus some control over the amount of expression of the receptor. The spread of the virus could also be modulated somewhat by the serotype of the virus and the concentration of virus with respect to the volume of the injection. The virus is likely to spread further if the volume is greater.

The biggest downside to this method for the study of satiation and food intake is the reliance on CNO as the drug for activation. CNO is able to activate cells within the cNTS (and the rNTS) in the absence of the hM3Dq receptor, suggesting that CNO is likely reverted into clozapine. This is very problematic because it is unknown if the activation of satiation is due to the CNO or the activation of the hM3Dq receptor. Further research should investigate other compounds such as compound 21 or perlapine (Gomez et al., 2017) to activate DREADDs to avoid this issue in studying satiation in the future.

TRAP2-TOMATO MICE

Introduction

Targeted recombination in active populations 2 (TRAP2) mice are also an ideal candidate for studying satiation cells within the NTS. This system also uses cFos expression to drive the expression of tamoxifen-inducible CreER (Cre recombinase estrogen receptor), in addition to a transgenic Cre-dependent effector. We bred TRAP2 heterozygous mice to td-Tomato reporter mice (Figure 13). These mice will be referred to as TRAP2-Tomato mice from this point on.

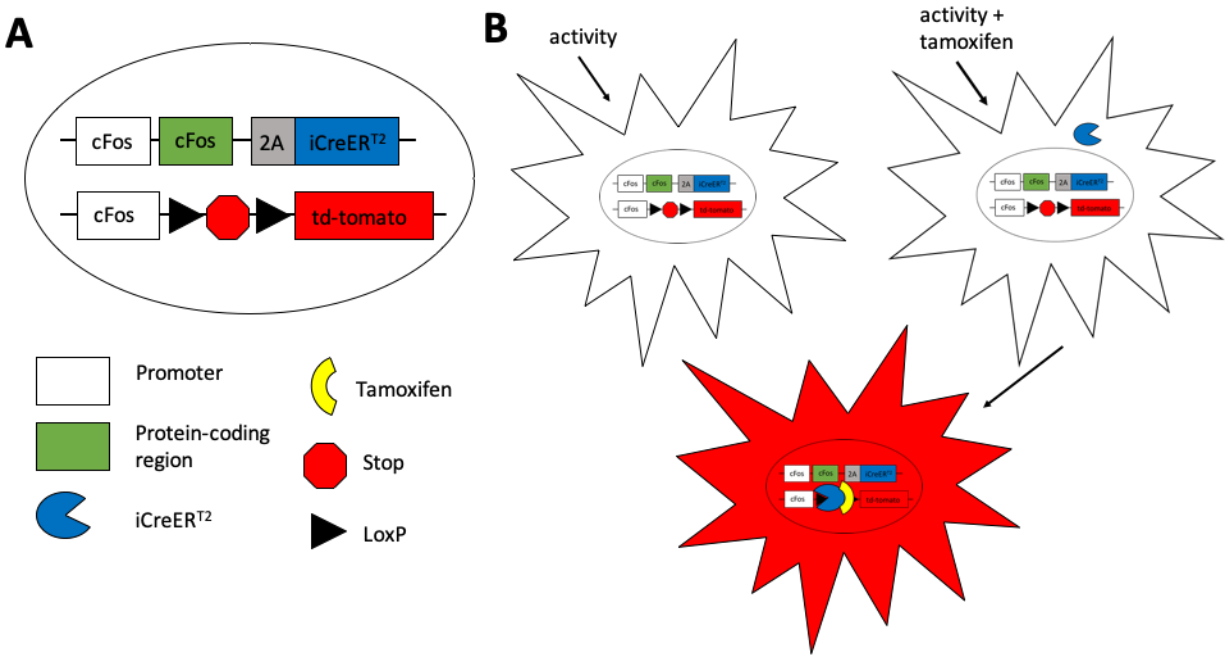


Figure 13. (A) Schematic of gene expression in TRAP2-Tomato mice. (B) cFos expression is induced by neuronal activity which allows for expression of CreERT2 in the presence of tamoxifen. If the neuronal activity is not in conjunction with tamoxifen, CreERT2 is not expressed and therefore the effector gene (tdTomato) is also not expressed. Tamoxifen allows for the expression of CreERT2 and in turn, CreERT2 then allows for the expression of the effector gene.

TRAP2 works differently than the tTA-Tet-O system because it uses tamoxifen-dependent recombinase CreER ^{T2} (Cre recombinase-estrogen receptor type 2) and can only undergo recombination in the presence of tamoxifen. When tamoxifen is present, CreER^{T2} expresses Cre

in cells expressing cFos and will then allow for the expression of tdTomato in a Cre-dependent manner. Cells that express the effector gene are often referred to as TRAPed cells. If tamoxifen is not present, activated cells will not express tdTomato because CreER ^{T2} is kept in the cytoplasm of the cell and no recombination can occur (Guenthner et al., 2013). If satiation cells were TRAPed, then they expressed fluorescent tomato protein.

Another possible benefit to this system is that it uses a modified estrogen receptor to express tdTomato. Estrogen receptors are expressed within the NTS naturally (where as the cFos-AAVhM3Dq mice express an artificial receptor) and therefore this system might be more likely to work within the cNTS.

Few studies have used these mice, as they have just become commercially available. These mice have been used to study thirst (Allen et al., 2017) and memory retrieval (DeNardo et al., 2019). We aimed to determine the usability of this model in the study of satiation by investigating the number of TRAPed cells in response to the large meal training paradigm used in the previous two models. We hypothesized if the number of TRAPed cells increased in the NTS and AP in response to large meal training and were similar to the number of cFos cells expressed in response to the large meal training, using TRAP2 mice to study the role of the NTS might be promising.

Methods

Mice

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

Original TRAP2 mice were obtained from the Liqun Luo Lab (Stanford University). TRAP2 and tdTomato mice were bred within our lab for several generations and maintained at 22℃ on a 14:10 hr light/dark cycle to facilitate breeding with lights on at 0500 and off at 1900 prior to the experiment. These mice are now commercially available from Jackson Laboratories (Fos$^{2A\text{-}iCreER}$ (TRAP2); cat. no. 030323). Heterozygous mice from the TRAP2 strain was crossed with a homozygous td-Tomato mice to create mice with both TRAP2 and tdTomato mutations.

This created offspring containing both the TRAP2 and tdTomato mutations or the tdTomato mutation alone. tdTomato mice and were used as control mice throughout the experiment.

Genotyping

Procedures were followed as described in previous sections. Primers for the iCre transgene were forward transgene primer iCre1 5'- GTGCAAGCTGAACAACAGGA -3' and reverse transgene primer iCre2 5'- ATCAGCATTCTCCCACCATC -3' (IDT Forward catalog #190240205, Reverse catalog #190240206) in order to amplify a 420 bp iCre fragment. The PCR procedure was as follows: 95℃ 15 min; 94℃ for 3 min; 31 cycles of 94℃ for 20 sec, 58℃ for 25 sec, 72℃ for 45 sec; 72℃ for 5 min, hold at 4℃.

Tamoxifen

40mg of tamoxifen (4-hydroxytamoxifen(4-OHT); cat. No. 7776-50MG, Sigma-Aldrich, St. Louis, MO) was added to a 15mL conical tube with 400uL of 100% ethanol and was then vortexed. Next 2.88mL of sunflower oil (cat. No. S5007-250ML, Sigma-Aldrich, St. Louis, MO) and 0.72mL of caster oil (cat. No. 259853-250ML, Sigma-Aldrich, St. Louis, MO) was added to the tamoxifen-ethanol mixture and then vortexed. The solution was placed on a shaker in an incubator at 37℃ for at least ten minutes and until any bubbles disappeared. Next, a homogenizer was used to get the 4-OHT into solution. The solution was placed back on the shaker in the incubator again for at least ten minutes and until the bubbles disappeared. The solution was then aliquoted out and stored at -20℃ until use. Tamoxifen was administered at a 50mg/kg dose.

Meal Training

Similar to the other experiments, on the night before the meal training began (day 0), all mice were food-deprived starting at 5pm. On the following day (day 1), all mice were given Ensure at 9am for one hour followed by standard lab chow for three hours from 3pm-6pm. This diet regimen was continued on days 2-4. On day 5 of the large meal training, at 3am, all mice received an injection of 50mg/kg tamoxifen. Mice were returned to chow at 5pm. Forty-eight after the large meal, mice were sacrificed and the cNTS was immunostained for TRAPed cells. Immuo-positive cells, food intake and body weights are presented as averages $\pm$ standard error of the mean.

Immunohistochemistry

Immunohistochemistry procedures were similar as described in a previous section with the use of DsRed as the primary antibody for td-Tomato (cat no. 632496, RRID:AB_10013483, Clontech, Palo Alto, CA) over 72 hr.

Microscopy

Microscopy and quantification procedures were carried out as described in previous sections except TRAP2-Tomato cells were counted in both the NTS and AP.

Results

The rate of mice having both the TRAP2 gene and the td-Tomato gene was surprisingly low. When mice were bred to have only one of the two genes, on average, mice would have those genes 50% of the time. However, it should be noted that when the TRAP2-Tomato mice were generated, mice only expressed both genes about 7% of the time.

Meal training resulted in a significant increase in meal size (TRAP2-Tomato mice: 1.59 $\pm$ 0.60g to 4.06 $\pm$ .42g, p = .04; tdTomato only mice: 2.09 $\pm$ .21g to 2.76 $\pm$ .18g p = .03) and body weights remained the same (TRAP2-Tomato mice: 25.23$\pm$ 1.66g to 24.47 $\pm$ 1.74 g, p = .77; tdTomato only mice: 23.40 $\pm$ 5.23g to 21.15 $\pm$ .88g p = .35) in the TRAP2-Tomato mice and tdTomato during the meal training (Figure 14).

TRAP2-Tomato mice expressed TRAPed cells in the cNTS and AP. TRAP2-Tomato mice (n = 4) expressed 100 $\pm$ 21.2 cNTS cells and 50 $\pm$ 3 AP cells vs wild-type littermates (n = 10) with no TRAPed cells (A representative image can be seen in Figure 14C). One TRAP2-Tomato mouse received meal training but no test meal on the final day to determine a baseline level of TRAP2-Tomato expression in the absence of satiation. That mouse expressed 21.33 $\pm$ .67 cNTS cells and 12 $\pm$ 1 AP cells averaged over three sections.

Discussion

Increased number of TRAPed cells in the area postrema in comparison to cells expressing c-Fos in the area postrema in response to a large meal could be due to the cells continuing to be TRAPed after peak c-Fos expression. These cells in the AP could be responding to the large meal

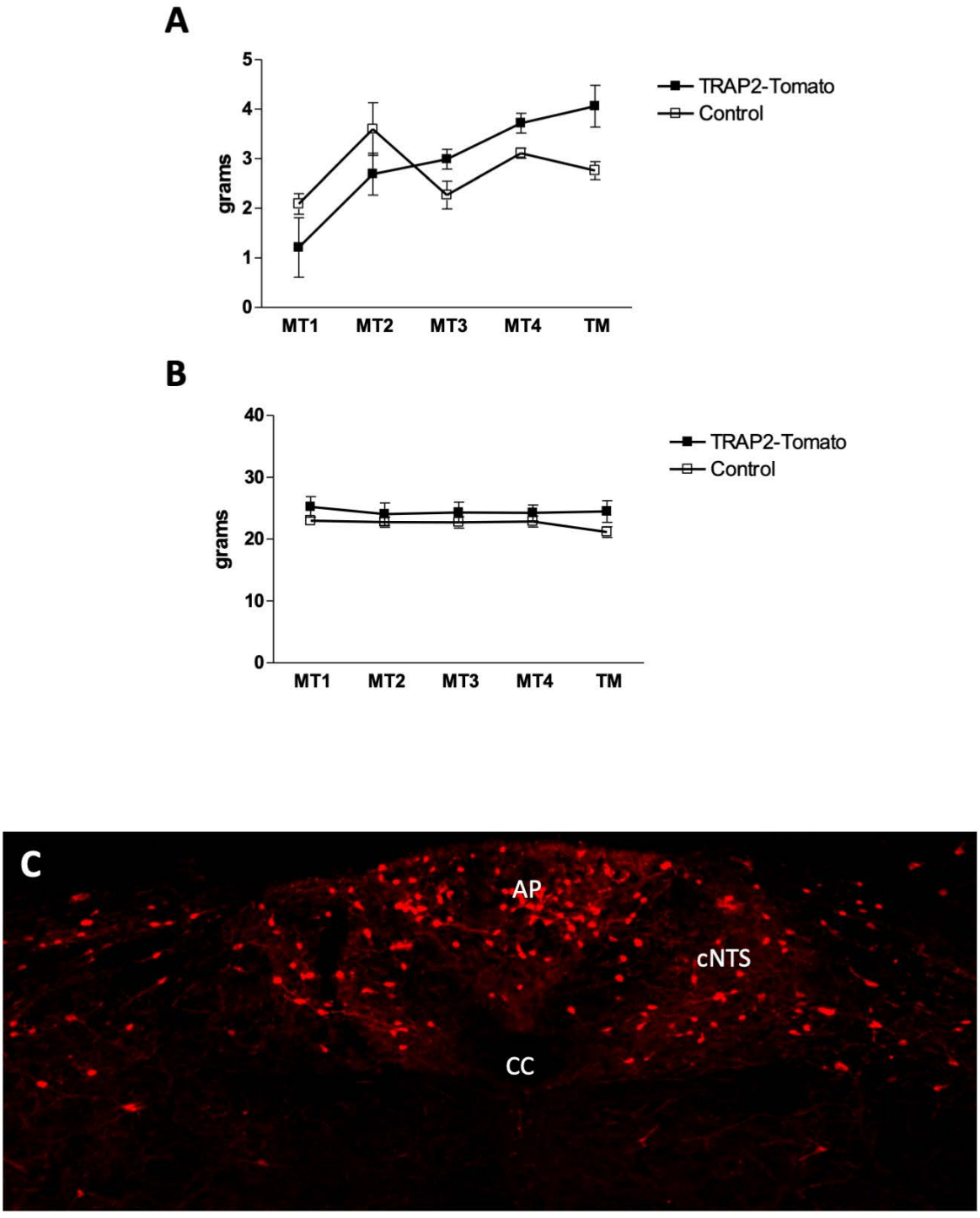


Figure 14. Meal training and TRAP2 expression for TRAP2-Tomato mice. (A) Food intake in grams of TRAP2-Tomato (n = 4) and control (n = 10) mice across meal training. (B) Body weight in grams of TRAP2-Tomato mice across meal training. MT1-MT4 meal training day 1-4, TM test meal. (C) TRAPed cell counts in TRAP2-Tomato and control mice in the cNTS and AP. (D) A representative image of TRAP2-Tomato label after meal training in TRAP2-Tomato mice. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

but over a longer period of time than what cells do in the NTS and therefore those cells would be missed in a traditional 90 minute Ensure feeding and then euthanizing the animal. In the TRAP2 model, mice would need to survive 72 hours post-large meal because eventually the TRAP2 mice would be bred with another cre expressing mouse line containing a receptor for activation of the cells. Because tamoxifen is active over the course of 72 hours, activation of the receptors could not occur until the majority of the tamoxifen is out of the system. This does not explain why less cells in the NTS are TRAPed when compared to c-Fos labeled cells. C-Fos expression is typically much higher in the NTS than the AP and it would be expected to see a greater number of TRAPed cells in the NTS.

Timing is also quite critical when TRAPing cells. The time in which the tamoxifen is administered in relationship to the behavior performed for TRAPing is the most important. Tamoxifen activity is at its highest between 0-12 hours after administration but remains at levels that can cause TRAPing over 3 days and within the system for longer. This is ideal for TRAPing cells over a longer time frame but could be problematic for shorter-term behaviors such as the consumption of one meal. Another critical timing issue is the amount of time the tamoxifen is within the system prior to the activation of the cells to be labeled. In this experiment, there was a six-hour period between tamoxifen administration and the test meal. This timing was selected because of previous work done with the labeling of cells in these mice (Allen et al., 2017). It is possible a shorter time period between tamoxifen administration and behavior could allow for more TRAPed cells. However, the timing of tamoxifen and behavior has not been thoroughly tested and therefore, the ideal timing has not yet been worked out. For these reasons, it will be critical to have the right control groups in future studies. A control where a mouse receives all of the same treatment except for the large meal will be necessary to tease apart off-target TRAPing.

Before pursuing this method further, it should be confirmed that the TRAPed cells are also satiation related cFos expressing cells. A double label experiment where cFos cells are TRAPed under the conditions above and then mice would be given an additional large meal at a later time after the tamoxifen has left the system to again induce cFos would be sufficient. The number of cFos, TRAPed and cFos/TRAPed cells could be compared to determine the effectiveness of the TRAPing of satiation-related cells in response to a large meal. Evidence provided by this study shows it is possible that not all of the cells related to satiation in the NTS are being TRAPed and extra cells might be TRAPed in the AP. It will be important to determine the percentage of cells

activated during satiation that are TRAPed in order to better understand how activation of these cells might reduce food intake. If only a small percentage of satiation-related cells can be activated, it might not be enough to induce satiation.

TRAP2 cells were observed throughout the medulla at low levels because cFos is expressed as a marker of neuronal activity and other neurons are always firing in the brain. So therefore, it makes sense that there would be some baseline level of TRAPed cells in the medulla in response to no meal training. However, this baseline level was not assessed due to the number of TRAP2-Tomato mice available for use. This will be an important consideration for designing experiments in the future and should be a reminder to be cautious with any interpretations of the experiments.

There are many chemogenetic and optogenetic possibilities that use cre recombinase that could be used in combination with the TRAP2 mouse. TRAP2 mice could be bred with an hM3dq transgenic mouse and cells could be TRAPed using meal paradigm. These cells would then express hM3dq receptor (instead of tomato) and then could be activated with CNO. There are also many cre-AAVs as well that could be injected into the NTS of a TRAP mouse and avoid having to create a double transgenic model. Similar models could also be created using an optogenetic transgenic mouse or virus.

OPTOGENETIC CFOS-CHEF MICE

Introduction

The cFos-tTA-Tet-O-ChEF mice work similarly to the cFos-tTA-Tet-O-hM3Dq mice, but instead of expressing the hM3Dq receptor, they express the channelrhodopsin variant, ChEF, which is activated by light instead of CNO. In these mice, when the cFos-tTA gene is expressed, it in turn promotes expression of the Tet-O-ChEF gene causing the ChEF ion channel to be expressed in the neurons that are activated by the cFos expression (Figure 15). These ion channels can then be activated by light. ChEF is a channelrhodopsin (ChR) variant that undergoes 44% less inactivation when continuously stimulated with blue light than channelrhodopsin 2 (ChR2). In contrast, ChEF ion channels close slower than ChR2 channels (Lin et al., 2009).

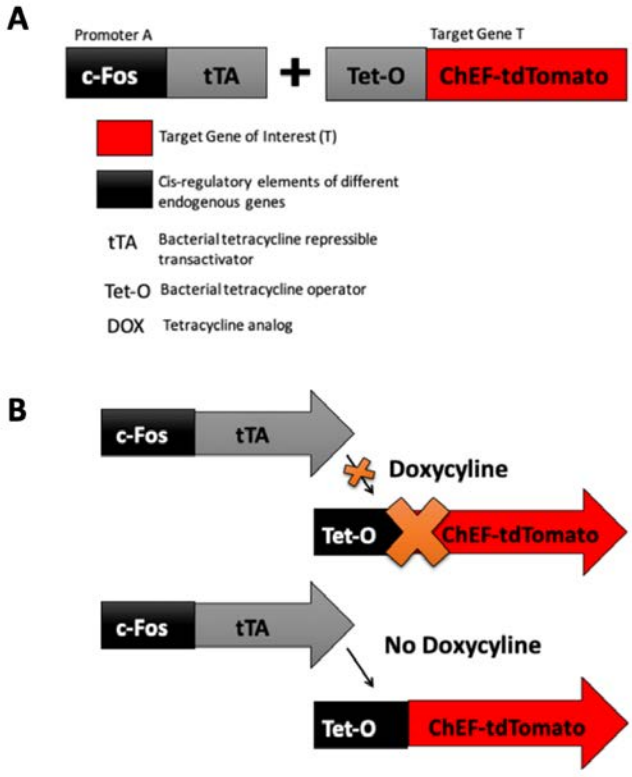


Figure 15. Schematic of cFos-ChEF gene expression. (A) The cis regulatory element, promoter A (cFos), drives the expression of the bacterial tetracycline-regulated trans activator (tTA). When this gene is expressed, it activates target gene T (Tet-O-ChEF). (B) This target gene is only expressed under the control of the Tet-O operator in the presence of tetracycline. Doxycycline (Dox) is a tetracycline analog which can turn gene T off or on. In the presence of Dox, gene T is blocked (off) and in the absence of Dox, gene T is expressed (on).

Optogenetics provides many potential benefits for studying satiation. These include better spatial and temporal resolution than other methods, such as chemogenetics. Spatial resolution, meaning the area of activation is improved because the stimulation is focused to that of the light source, rather than a ligand that could have off-target binding in undesired areas as in chemogenetics. The temporal resolution, or the timing of activation, of optogenetics is also much improved over chemogenetics because the light source can cause depolarization on the order of seconds (and possibly even smaller time scales) and are thus ideal for studying behaviors that can change rapidly. In addition, the frequency and intensity of the light stimulation is directly controlled by the experimenter and as long as the firing pattern is well established, the experimenter can effectively mimic physiological firing patterns. Unfortunately, in most optogenetic experiments, physiological firing patterns are not addressed and it is unknown if the stimulation is similar to the normal firing pattern associated with a behavior.

We hypothesized this mouse model might be the best for studying satiation within the NTS because of the improved spatial and temporal inducibility over chemogenetics. We aimed to demonstrate that ChEF expression could be inhibited by Dox and that ChEF was only expressed in the NTS in response to large meal training. The goal was to characterize the expression of ChEF in response to the large meal training before moving forward to activate the ChEF receptors with light.

Methods

Mice

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

cFos-tTA and Tet-O-ChEF-tdTomato mice obtained from the Mark Mayford Lab (Stanford University) and were bred in lab for several generations. The two single mouse strains were cross-bred to produced double transgenic cFos-tTA–Tet-O-ChEF heterozygous mice used in experiments.

Genotyping

Procedures were followed as described in previous sections. Primers for the cFos-tTA transgene were forward transgene primer FTU2 5'- CGTAGGAAGTCCATCCATTC -3' and reverse transgene primer test6 5'- CTTAGCGCAGAAGTCATGC -3' (IDT Forward catalog #166464100, Reverse catalog #166464101) in order to amplify a 263bp c-Fos-tTA fragment. Primers for the ChEF transgene were forward transgene primer GFU2 5'- ACAAGTTCAGCGTGTCCGG -3' and reverse transgene primer GFD2 5'- ACCATGTGATCGCGCTTCTC -3' (IDT Forward catalog # 166469102, Reverse catalog #166469103) in order to amplify a 581 bp ChEF fragment. Primers for the internal control transgene were forward transgene primer tsh-1 5'- TCCTCAAAGATGCTCATTAG -3' and reverse transgene primer tsh-2 5'- GTAACTCACTCATGCAAAGT -3' (IDT Forward catalog #166469098, Reverse catalog #166469099) in order to amplify a 383bp internal control fragment. The PCR procedure was as follows: 95℃ 15 min; 94℃ for 5 min; 35 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 1 min; 72℃ for 7 min, hold at 4℃.

Meal Training, Immunohistochemistry and Microscopy

The same procedures for meal training, immunohistochemistry and microscopy as in the three previous models of mice. DsRed was used as the primary antibody and Cy3 was used as the secondary antibody. Food intake and body weights are presented as averages $\pm$ standard error of the mean. Group sizes were: cFos-ChEF, meal training with test meal (n=6); wild-type, meal training with test meal (n = 3).

Results

We have previously received brains of these mice from Mark Mayford and looked in brain areas related to feeding and saw some ChEF expression throughout the hindbrain (Figure 16). However, these mice did not receive scheduled meal training and thus we were not able to determine expression of ChEF for certain within the NTS.

Meal training resulted in an increase in meal size when the first day of meal training was compared to the test meal size (significant increase, cFos-ChEF mice: 1.83 $\pm$ .25g to 5.57 $\pm$ .34g, p = .0001; trending increase, wild-type mice: 1.95 $\pm$ 0.18g to 3.61 $\pm$ .76g p = .10) and body weights

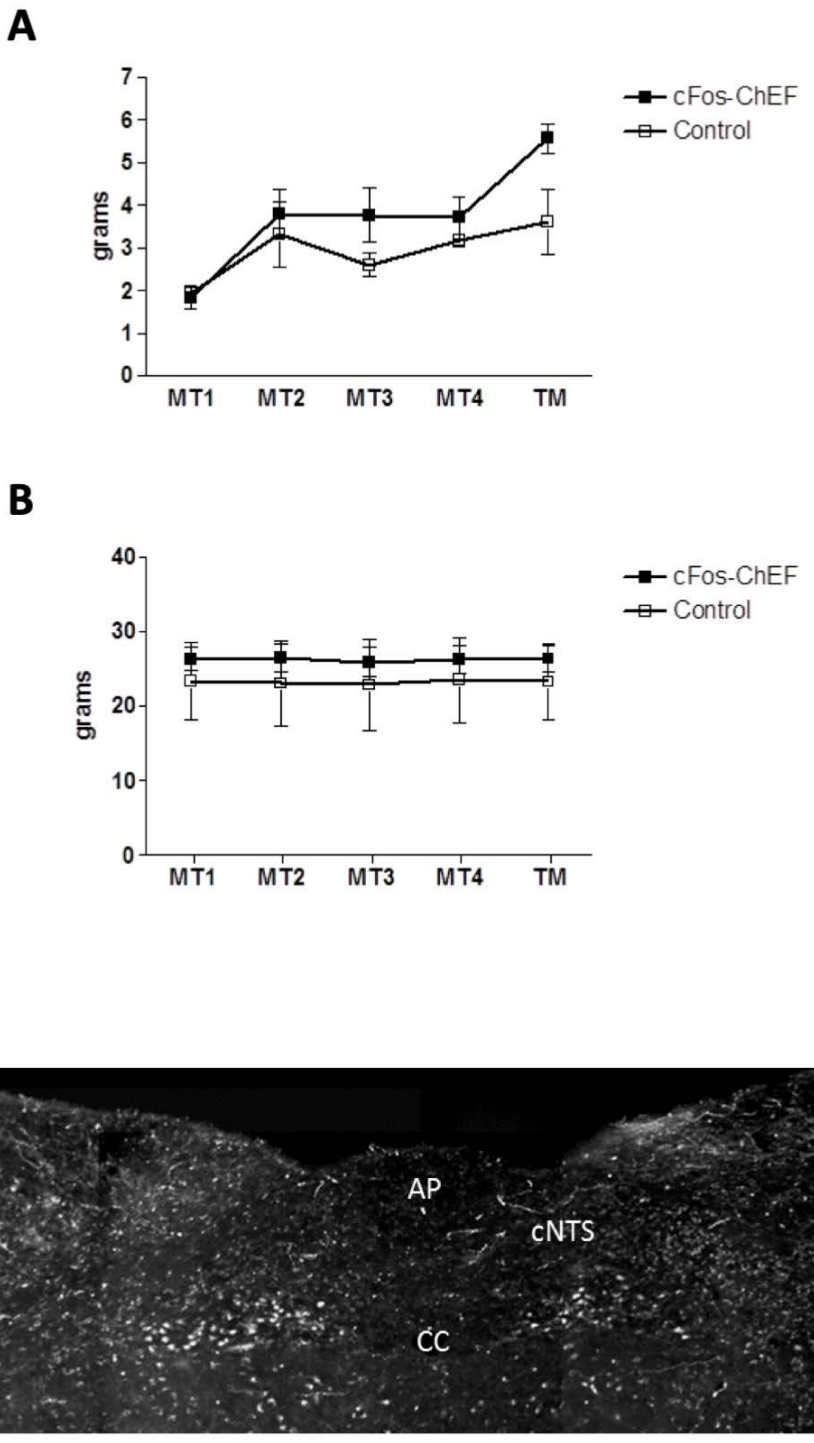


Figure 16. Meal training and ChEF expression for cFos-ChEF mice. (A) Body weight in grams of cFos-ChEF and control mice across meal training. (B) Food intake in grams of cFos-ChEF and control mice across meal training. (C) Image of ChEF expression without meal training in the absence of Dox. CC central canal, cNTS caudal nucleus solitary tract, AP area postrema.

remained the same (cFos-ChEF: 26.36 $\pm$ 1.62g to 26.40 $\pm$ 1.71 g, p = .99; wild-type: 23.40 $\pm$ 5.23g to 23.27 $\pm$ 5.07g p = .99) in the cFos-ChEF and wild-type control mice during the meal training (Figure 16).

After mice were placed through meal training (cFos-ChEF n = 6; control n = 3). In all cases, there was no stain above the level of controls and no statistical analysis was run.

Discussion

One issue with the cFos-ChEF mouse line is the reliability of breeding. These mice do not breed as frequently as other strains of mice and often pups do not survive. Pups are also often smaller than the average size mouse pup and are excluded from breeding and analysis. The body weights prior to meal training of these mice were significantly different, but these mice were all littermates. It is not ideal that their body weights differed, however, because these mice were hard to breed and due to the issue of no ChEF labeling in these mice, the sample size remained small. If further experiments were conducted, the sample size would be increased to where the body weights would not be different from each other.

The most probable cause for the lack of ChEF expression in this mouse model is due to gene silencing. It is likely either the cFos-tTA or Tet-O-ChEF gene integrated into an area of a chromosome that was silenced. Transcriptional activity tends to keep the chromatin locus from being silenced and thus would allow for gene expression. It is possible that by placing the mice on Dox for an extended period of time would lead to silencing because transcription of the ChEF ion channel would not occur and the chromatin locus could be silenced due to inactivation. It has been reported that these mice are susceptible to gene silencing and that this silencing is heritable (K. Cowansage, personal communication). Another hypothesis is that c-Fos-tTA expression in early development activates ChEF expression in neurons and promotes gene-silencing (Zhu et al., 2007).

An alternative for this model, to potentially avoid gene-silencing, would be to use the cFos-tTA mouse with an AAV vector to deliver the ChEF ion channel. The viral vector is a potentially a better choice because it is not integrated into the chromosomes but is episomal and thus not subject to chromosomal remodeling. The AAV-ChEF would also not be injected until the desired period in the mouse's life and would not effect the mouse during development. However, similar issues as in the cFos-AAVhM3Dq model would have to be addressed.

As a result of gene silencing, all of the positives and negatives could not fully be assessed, however it is likely that this model would have similar positives to the cFos-hM3Dq model, but better specificity due to the use of a light to activate the ion channels. Off target activation outside of the hindbrain would not be an issue, but if expression of the ion channels were present in areas of the brain near the NTS, activation by the light might still occur. This would need to be further evaluated within the model. One downside to using the light to activate the ion channels would be having to have a light fixed to the mouse. Because the NTS is at the base of the skull, the light might cause issues with movement for the mouse, or the movement of the mouse might cause the light to damage the brain.

CONCLUSION

All four methods described have benefits and limitations to studying satiation or other behaviors. In future experiments, it will be important to carefully assess the research question in order to determine the best chemogenetic or optogenetic method to appropriately study the research questions. Some of the methods described could potentially be useful when studying satiation and some may not. The double transgenic cFos-hM3Dq mouse would not be appropriate for studying satiation as there are no cells labeled in the NTS or AP. The other three models tested, have potential for studying satiation, but could also potentially be improved upon to do so. It is also important to keep in mind the limitation of these models in order to not over interpret results.

Considerations for Use of CNO

Originally, CNO was thought to be a pharmacologically inert ligand that is able to cross the blood-brain barrier and does not lead to any pathologies (Alexander et al., 2009). CNO has even been successfully administered to humans (Jann, Lam & Chang., 1994). CNO has been used to activate the hM3Dq receptor in two major ways through ip injection and microinjections directly into the brain. Firstly, and most commonly, CNO has been injected into rodents through ip injection. Typical concentrations of CNO injected ip are between 0.1 mg/kg (low dose) and 1.0 mg/kg (high dose) (Gomez et al., 2017). Other studies have microinjected CNO into the brain at ranges from 0.3uM-300uM with 50-100nL microinjected at a time (Stachniak, Ghosh, & Sternson, 2014) and limit the hM3Dq receptor activity to the area where the microinjection was administered.

The mechanism in which CNO acts upon DREADDS in vivo was recently elucidated, which raised serious concern with the use of CNO for chemogenetic experiments. CNO administered systemically does not cross the blood brain barrier and is first converted into clozapine in order to cross the blood brain barrier (Gomez et al., 2017). Clozapine then acts on DREADDS in order to produce behavioral responses. Clozapine facilitates the binding of GABA to the $GABA_BR$ which leads to inhibition of neurotransmission (Wu et al., 2011). Clozapine also leads to an increase in the release L-glutamate due to NMDA receptor activation (Tanahashi et al., 2012). Clozapine also has a very high affinity for the DREADD receptors hM3Dq and hM4di as

well as other receptors within the brain. Cells with one of these two DREADDs showed less free clozapine than those cells without either DREADD suggesting that the DREADD receptors outcompete the $GABA_BR$ or NMDA receptor. This demonstrated small doses of clozapine (at 0.01mg/kg), rather than the large doses CNO (1mg/kg) used in experiments should not be used in order to avoid unwanted binding of clozapine to off target receptors (Gomez et al., 2017).

The first structure-activity relationship study was conducted on the hM3Dq receptor and CNO. Compound 21 was identified and selectively targets the hM3Dq receptor at a significantly higher rate than compared with clozapine. Perlapine also binds to the hM3Dq receptor with at least a 10,000-fold more selective for the hM3Dq receptor than the native human M_3 receptor (Chen et al., 2015). More studies will need to be conducted in order to determine how compound 21 and perlapine work in vivo on the hM3Dq receptor. Compound 21 is likely not metabolized into clozapine and is likely to have equivalent potency (Roth, 2016, unpublished data). Perlapine must be tested in animals that do not express DREADDs and determine an appropriate dose that does not cause off-target activity (for example, how CNO has off-target activity in cNTS cells (Roth, 2016). A body of literature will also need to be developed in order to switch from using CNO or clozapine to compound 21 or perlapine.

Clozapine, CNO and compound 21 are all able to activate DREADD receptors within the brain (Jendryka et al., 2019). CNO is back-converted to clozapine in both mice and rats at levels high enough for DREADD activation within the nervous system. In addition, clozapine can also be forward-converted into CNO, however, the concentration of CNO after the forward-conversion is thought to be too low to have behavioral effects (Jendryka et al., 2019). CNO back-conversion to clozapine can cause behavioral effects as mice cannot fully discriminate between clozapine and CNO in a drug discrimination test (Manvich et al., 2018) further supporting the need for appropriate controls for off-target effects when using these drugs.

Our CNO doses were relatively small compared to studies demonstrating conversion of CNO to clozapine which were between 1-20mg/kg (Manvich et al., 2018; Jendryka et al., 2019). It is possible with such low doses of CNO, that there would be minimal off-target effects of clozapine, however because cFos expression is activated at lower doses of CNO (0.8mg/kg), it is possible that CNO is being metabolized into clozapine and that clozapine is activating cFos expression and not CNO activating the hM3Dq receptor. This is supported by the c-Fos activation in the NTS following administration of CNO in the absence of the hM3Dq receptor.

Mimicking Physiological Satiation

Large meal training previously used by our lab and others has been thought to label satiation-related cells within the cNTS. However, satiation-related cells should be looked at under normal conditions and not just under the large meal training paradigm. Mice typically consume 80% of their calories within the first two hours of the dark cycle (Bake et al., 2013). Using the large meal training paradigm in conjunction with a liquid diet, mice eat more and cFos expression is higher in response to the liquid diet (Rinaman et al., 1998; Edmund, Swartz & Moran, 2001). It would be important to know how accurately this paradigm mimics the satiation experience by mice feeding in the two hours after the dark cycle starts

Activation of cells using this method could activate cells involved in malaise. By labeling those cells and activating them later with CNO, it could be that a decrease in food intake could be a result of malaise.

Recommendations for Future Studies

The Tet-on system, where mice must be placed on Dox to express the target gene, is likely better than a Tet-Off system, where mice must be taken off Dox to express the target gene, for studying a feeding behavior because meal duration and intermeal interval occurs over a relatively short period of time. By placing the mice on Dox prior to a large meal, it would be possible to better target the timeframe when satiation occurs. This would eliminate the need for a period of time taking mice off Dox for it to washout of their system and would eliminate some of the non-feeding specific expression that occurs in the Tet-Off 5 days off Dox model. In food intake research, a method that works on a quicker time frame is necessary and having to be off dox for 3-5 days to see hM3Dq expression or having tamoxifen in system for 3 days is just not an ideal method, as it is not necessarily specific to the test meal. It will also be important to assess the use of these two systems with respect to the research question the experimenters are investigating to determine the appropriateness of using either system.

The use of episomal expression via the delivery of a gene virus prevents gene silencing because the gene is not inserted into the chromosome and the gene does not rely on the cell's replication mechanisms for expression. In general, the experimenter has no control over where genes are inserted in the chromosomes of a transgenic mouse model and will not know if the genes

will be able to be expressed until the experiments are conducted. With episomal expression, these variables can be avoided. However, episomal expression does have some negative attributes. As demonstrated by this research, it is very hard to determine how far the virus will spread (as seen in Figure 9).

TRAP2 has benefits over the other three models because TRAP2 also labels cFos cells within the AP, in addition to the NTS, whereas the other models did not. cFos expression in the AP is important as it is present in response to a large meal and activation of both NTS and AP cells might be necessary for satiation as well. By improving the timing of the tamoxifen administration and the relationship of the test meal to the tamoxifen administration would likely improve the number of TRAPed cells and more directly mimic physiological satiation.

More controls will be necessary if future experiments. Originally, these controls would have been conducted in this study, however, due to the discovery of limitations within these experiments, some of those controls were not run. For example, one control that is critical to have is a "cage control" mouse. This mouse would receive all of the same handling during the large meal training, but would not receive the meal training. Any hM3Dq receptor expression in mice in this group would serve as a level of baseline expression which would need to be subtracted when looking at mice that went through large meal training. This would account for receptor expression which was not due to the large meal training.

In the future, alternative models for testing would include: using the Tet-On system with cFos-tTA mice and Tet-On-hM3Dq mice, cFos-tTA mice with AAV-ChEF and TRAP2 with cre-hM3Dq or ChEF mice. TRAP2 could also be used in conjunction with an hM3Dq or ChEF virus. Each of these models will have its own benefits and setbacks. These will need to be addressed based on the research questions being asked. It is possible when using the Tet-On system, mice will undergo gene silencing as they did in the Tet-Off system. Any models that use virus will not be susceptible to gene silencing as the virus expresses its DNA episomally. However, delivering a virus to only the NTS and not surrounding areas is problematic and difficult to control. TRAP2 mice might be the next best solution, but a more thorough analysis of the cells TRAPed in the mouse model selected will need to be conducted to demonstrate the percentage of TRAPed cells in comparison to cFos cells.

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