Mechanisms of Motivation in the Nucleus Accumbens

by

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Dedication

To my loving parents, Karen and Roland Cole.

You encouraged me to be inquisitive and gave me the tools to be who I need to be.

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Table of Contents

List of Figures

Abstract

The nucleus accumbens (NAc) is a primary brain target of mesolimbic dopamine projections, and is well known to be important to motivation for rewards. Precisely how NAc neural systems generate motivation remains unclear. Here, I aimed to tease apart 1) the different roles of the two main neural populations/systems in NAc in generating reward motivation and 2) the relative roles of neuronal excitations versus neuronal inhibitions in NAc in generating intense motivation for reward. I also aimed to 3) expand understanding of the role of NAc mesolimbic dopamine projections to include motivation for social rewards, such as making a social partner potentially more attractive to interact with, beyond the motivation for physical sensory rewards (food, drugs, etc.) that has been traditionally studied.

First, I examined the role of the two main subpopulations of neurons in NAc (medium spiny neurons): NAc D1 neurons (i.e., containing D1-type dopamine receptors, and which form a 'direct pathway' for anatomical outputs to midbrain) versus D2 neurons (i.e., with D2 receptors for dopamine, and forming only 'indirect' pathways to forebrain targets). D1-direct neurons have been proposed to be reward-related or "go" neurons, whereas D2-indirect neurons have been thought to cause aversion or "stop" signals. I used newly-developed optogenetic techniques in transgenic mice, which now allow these two populations to be selectively excited in ways that were impossible before. I stimulated these two distinct subpopulations one at a time, using viral

vectors targeting either D1 neurons in D1 Cre mice or D2 neurons in D2 Cre mice. In line with expectations, I found that laser-light stimulation of D1 neurons in the NAc was potently rewarding, and that mice receiving D1 stimulation would avidly work for laser depolarization and actively seek out locations paired with laser reward. Surprisingly and in contrast to standard hypotheses, I found laser-light stimulation of D2 neurons was also rewarding, and that mice would work for D2 stimulation, though at weaker levels than D1 levels.

Next, I tested the relative roles of NAc neuronal hyperpolarization vs depolarization in generating intense motivation. A major hypothesis is NAc neuronal hyperpolarization (inhibition) generates motivation by releasing targets from constant suppression (disinhibition). I directly tested whether NAc neuronal inhibition is *necessary* for drug microinjections (glutamate blockade) to induce intense reward motivation by *reversing* neuronal hyperpolarization with optogenetic laser-induced depolarization at the same NAc site. My results confirmed that NAc hyperpolarization was necessary for intense motivation to eat.

Further, to test more directly whether NAc hyperpolarization is *sufficient* to enhance eating, I directly used inhibitory optogenetic laser techniques to hyperpolarize neurons without drugs. I found that in direct laser inhibition of NAc neurons generated intense reward motivation to enhance food intake, confirming that NAc inhibition is *sufficient* to produce intense motivation.

In a final pilot dopamine experiment, I have examined how enhancement of dopamine release within the NAc can increase motivation for social exploration. I discovered that pairing laser activation of dopamine neurons to NA in TH Cre rats with encountering a social partner made that partner suddenly more attractive to pursue and interact with.

Taken together, these studies illuminate key neural mechanisms through which the NAc produces reward motivation. These findings highlight how particular neural systems and neuronal states generate intense motivations for brain stimulation, food and social rewards.

Chapter 1

General Introduction

The nucleus accumbens as a limbic-motor interface

Motivation serves to guide organisms in navigating the barrage of environmental stimuli in order to take advantage of opportunities and resources, such as sex and food, and to avoid recurrent threats over the course of evolutionary history (Nesse, 1990). However, as brain systems and concordant adaptive psychological faculties arose a technical issue was presented: how do modular neural systems integrate to yield an adaptive, motivated response? The nucleus accumbens (NAc) is thought to be one such motivational integrator of convergent internal homeostatic, sensory, and executive control signals to serve as a "sensory sentinel", and gate the expression of motivation via tonic inhibition of downstream effector sites (Kelley et al., 2005; Berridge et al., 2010; Castro et al., 2015). The nucleus accumbens (NAc) is important to appetitive motivation for diverse rewards, which range from food, sex, addictive drugs and brain self-stimulation in animals and humans (Rolls, 1975; Mogenson et al., 1980; Kelley et al., 2005; Vuust and Kringelbach, 2010; Salimpoor et al., 2011; Hernandez et al., 2012; Saunders et al., 2013; Castro and Berridge, 2014; Mueller et al., 2015; Zatorre, 2015; Volkow et al., 2016).

Additionally, increasing evidence demonstrates the NAc to play a role in avoidant/aversive motivation and negative motivational states, such as fear, pain, and disgust (Filibeck et al., 1988; Reynolds and Berridge, 2001, 2002; Carlezon and Thomas, 2009; Richard and Berridge, 2011b).

How does the accumbens talk to other areas?

Mogenson and colleagues (1980) first proposed the NAc as an interface between limbic regions controlling motivation and emotion and through manipulation of lower motor effector sites which translate these psychological elements into action (Mogenson et al., 1980). The NAc has since been compartmentalized into core and shell components based on function, chemistry, morphology, and afferent and efferent connectivity (Zaborszky et al., 1985; Groenewegen et al., 1987; Zahm and Heimer, 1990; Heimer et al., 1991; Voorn et al., 1994; Meredith et al., 1996; Kelley and Swanson, 1997; Meredith et al., 2008). The NAc receives input from several cortical regions, which each contributing specific forms of information in general motivations. The NAc receives glutamatergic input from hippocampus (predominantly ventral subiculum) which conveys information for spatial navigation, maintaining associations between environmental stimuli, and the processing of novel stimuli (Floresco et al., 1997; Ito et al., 2008; Mannella et al., 2013). Inputs from the basolateral amygdala (BLA) are necessary for establishing valence and value of stimuli (Shiflett and Balleine, 2010; Fernando et al., 2013). The NAc also receives inputs from prefrontal cortical (PFC) areas necessary for attentional control and adaptation of behaviors across contexts (Christakou et al., 2004). Additionally, projecting afferent cortical neurons display a topographical organization through NAc subregions and projections from different cortical regions often connect on adjacent or same NAc neurons (O'Donnell and Grace, 1994; O'Donnell et al., 1999; Floresco et al., 2001; Shiflett and Balleine, 2010; Britt et al., 2012).

The direct interaction of these cortical sites with each other can ultimately shift how the NAc responds. For instance, the BLA is required for ventral hippocampus stimulation of the NAc both due to interactions of cortical afferents within the NAc or through cortico-cortical signaling and subsequent alteration of NAc activity (Gill and Grace, 2011).

How does the accumbens talk to other brain regions?

One point of debate exists over the psychological roles of specific NAc *cell populations* and *projections*, and how these projections may differentially effect *downstream* targets. Cannonical striatum primarily contains two populations of neurons: one population of GABAergic medium spiny neurons (MSNs) which expresses D1 dopamine receptors and possesses a 'direct' midbrain projection to dopamine neuron regions, such as ventral tegmental area (VTA) or substantia nigra pars compacta (SNc), and is suggested to code for "go" or reward seeking signaling, whereas a separate population of MSNs expresses D2 dopamine receptors and projects 'indirectly' back to midbrain, first synapsing on to forebrain targets, such as ventral pallidum (VP) and lateral hypothalamus (LH), is thought to mediate "no-go" or signal punishment (Graybiel, 2000; Humphries and Prescott, 2010). These distinct neurons/projections have been thought to mimic that of dorsal striatum, which has highly segregated D1-direct vs D2-indirect neuron populations. Optogenetic study within the last few years has shown two populations of receptors and their respective pathway differences differentially support these "go" and "no-go" through selective optogenetic manipulation of D1-reward neurons and D2-avoidance (Kravitz et al., 2012). Some of the evidence that suggests that NAc follows a similar D1/ "direct"-D2/ "indirect" dichotomy comes from studies utilizing optogenetic modulation of drug reward (Lobo et al., 2010; Koo et al., 2014a), and pharmacological modulation of either D1

receptors (Wakabayashi et al., 2004; Hamlin et al., 2006; Richard and Berridge, 2011a) or D2 receptors (Filibeck et al., 1988; Lex and Hauber, 2008; Liao, 2008; Faure et al., 2010; Richard and Berridge, 2011b; Porter-Stransky et al., 2013) during food/ drug intake and modulation negative valence, such as defensive behaviors.

However, this distinction within the NAc is not absolute: D1/'direct' versus D2/'indirect' segregation is diminished in NAc compared to the neostriatum as NAc D1 MSNs also send up to 50% projections 'indirectly' to targets in ventral pallidum and lateral hypothalamus. These D1-"*indirect*" projections are capable of modulating the seeking and intake of food or drug reward (Heimer et al., 1991; Humphries and Prescott, 2010; Kupchik et al., 2015; Larson et al., 2015; O'Connor et al., 2015). Additionally, some pharmacological studies have also linked NAc D2 receptor activation to reward (Bachtell et al., 2005; Bari and Pierce, 2005). Thus, differences between dorsal striatum and the nucleus accumbens may exist in terms of functionality, and the roles of D1 & D2 neurons may not be so simple.

A Second NAc Motivational Dichotomy: The Rostrocaudal Gradient

In our lab, manipulations of amino acid neurotransmission in particular locations of NAc medial shell, can produce bivalent and intense affective and motivated states of opposite valence. Such as desire (Reynolds and Berridge, 2001, 2002, 2003; Richard and Berridge, 2011b). In particular, microinjections of either a GABA-A agonist or glutamate AMPA antagonist within NAc shell serve to potentiate either intense appetitive behaviors (e.g., food intake, establishment of conditioned place preference) or intense fearful behaviors (e.g., anti-predator behaviors such as defensive treading, distress calls and defensive biting of a human experimenter's hand, conditioned place avoidance) or both together (Reynolds and Berridge, 2001, 2008; Richard et

al., 2013). GABA/glutamatergic manipulations at rostral sites in NAc medial shell typically produce appetitive behaviors, whereas the same neurochemical manipulations at caudal sites in NAc shell instead produce fearful or defensive behaviors. Sites in the middle of NAc can often elicit a mixture of appetitive and fearful behaviors from the same rat during the same 1-hour test. These intense motivations are generated along a rostrocaudal "keyboard-type" pattern induced by localized disruptions via microinjections of DNQX or muscimol (Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008; Reynolds and Berridge, 2008; Richard and Berridge, 2011b, 2013). One possible common psychological explanation for the production of the motivated states of desire and dread may be intense, but differently valenced, motivational salience that becomes attributed to particular sensory percepts (i.e., the sight of food a pellet becomes more salient and attractive after rostral shell microinjections; the sight of light reflecting off glittering surfaces or of objects in the room beyond becomes more salient, but is perceived as threatening after caudal shell microinjections). A possible common neurobiological explanation is that a GABA agonist or glutamate antagonist microinjection induces a relative inhibition of GABAergic MSNs within NAc shell, which then disinhibit distinct downstream projections to targets such as LH, VP, or (VTA) from the tonic suppression that is usually exerted by NAc GABAergic projections (Mogenson et al., 1983; Zahm and Heimer, 1990; Heimer et al., 1991; Lu et al., 1998; Usuda et al., 1998; Zhou et al., 2003; Humphries and Prescott, 2010).

Excitation vs Inhibition Generation of Motivation

A second debate exists over whether accumbens-mediated motivation is encoded via *inhibition* or *excitation*. One popular hypothesis is that the hyperpolarization of MSNs in NAc is

the primary mechanism for generating appetitive motivation (Carlezon and Wise, 1996; Cheer et al., 2005; Roitman et al., 2005; Taha and Fields, 2006; Meredith et al., 2008; Roitman et al., 2008; Wheeler et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010). Central to this hypothesis is that inhibition of NAc projection neurons releases downstream neurons in target structures from chronic GABAergic suppression, and consequently disinhibit those target neurons into states of relative depolarization. This hypothesis is supported by findings that neural excitations in downstream targets, such as VP, LH, or VTA occur during reward events (Ljungberg et al., 1991; Baldo et al., 2004; Stratford, 2005; Bromberg-Martin and Hikosaka, 2009; Tindell et al., 2009; Smith et al., 2011). Others have shown that GABA-A stimulation of food intake, at least in rostral shell sites, requires VP and LH recruitment, as pharmacological inhibition or lesion of VP or LH attenuates the NAc-induced increase in eating (Stratford and Kelley, 1999; Stratford and Wirtshafter, 2012; Urstadt et al., 2013b; Urstadt et al., 2013a). Further, GABA stimulation produces decreased VP Fos and lowers cocaine CPP (Wang et al., 2014). Additionally, the NAc inhibition hypothesis fits the desire-dread 'keyboard' effects of inhibitory drug microinjections, such as muscimol (a GABA agonist which should hyperpolarize NAc neurons) or DNQX (a glutamate AMPA antagonist which should induce relative NAc inhibition by preventing glutamatergic depolarization). It also has been suggested to apply to other drugs such as opioid agonists, on the presumption that those drugs have generally inhibitory effects (Kelley et al., 2005; Baldo and Kelley, 2007; Carlezon and Thomas, 2009).

Further support comes from electrophysiological reports that NAc neurons are most likely to show inhibitions of firing evoked by drug or sweet rewards (Peoples and West, 1996; Chang et al., 1997; Janak et al., 1999; Nicola et al., 2004a; Roitman et al., 2005; Roitman et al., 2010). Conversely, aversive tastes of bitter quinine evoke excitatory increases in firing (Roitman

et al., 2005). Additionally, NAc neurons switch from reductions in firing to increases in response to a sweet taste that has become disgusting following acquisition of a Pavlovian taste aversion, and neuronal inhibition to the taste of food is augmented by physiological hunger that makes the taste more rewarding (Hollander et al., 2002; Wheeler et al., 2008; Roitman et al., 2010). Similarly, physiological states of salt depletion cause the normally aversive taste of hypertonic NaCl to become palatable, switching NAc neuronal responses from excitation to inhibition. Furthermore, thirst states are also seen to augment the inhibition of firing to the taste of water (Hollander et al., 2002; Loriaux et al., 2011).

Yet, beyond this evidence for NAc neuronal inhibition in reward, other evidence exists that rather confusingly points toward an opposite conclusion: NAc neuronal *excitation* also may mediate motivation and reward. For example, electrophysiological studies by Roitman, Carelli, and colleagues reported that approximately 30% of NAc core and shell neurons increased in firing in response to sweet rewards (Roitman et al., 2005; Wheeler et al., 2008; Roitman et al., 2010). Taha and Fields (2005) reported that nearly 75% of shell and core neurons in NAc showed increases in firing elicited by sucrose rewards, with highest firing to the most concentrated sucrose solution. Additionally, several other electrophysiological studies report that approximately 30% to 50% of NAc shell and core neurons increase firing during anticipation or during instrumental actions aimed at obtaining food, water or cocaine rewards (Carelli, 2000; Carelli et al., 2000; Hollander et al., 2002; Nicola et al., 2004b).

A second line of evidence for NAc excitation in reward comes from several decades of studies on NAc electrode self-stimulation in rodents. That is, rats will work to activate depolarizing electrodes in NAc sites, implying that excitation of some NAc neurons is sufficient as a reward (Rolls, 1971; Phillips and Fibiger, 1978; Mogenson et al., 1979; Van Ree and Otte,

1980; Phillips, 1984). Similarly, human deep brain self-stimulation has been reported for patients who have had electrode sites that likely included NAc (Rolls, 1971; Heath, 1972; Phillips, 1984; Heath, 1996). However, the exact effects of electrodes on nearby neurons is admittedly complex, and has been suggested to involve neuronal disruption as well as neuronal stimulation (Ranck, 1975).

Contemporary optogenetic techniques allow for neuron specific stimulation, ensuring that neuronal depolarization is the neurobiological mechanism of an observed behavioral effect. Recent optogenetic studies have shown that direct excitatory depolarization of neurons in NAc, via laser activation of channelrhodopsin-2 photoreceptors (ChR2) supports self-stimulation (Britt et al., 2012). ChR2 stimulation of NAc shell neurons has also been shown to potentiate a cocaine-induced conditioned or morphine place preference (CPP), suggesting that depolarization of NAc neurons can also enhance drug reward (Lobo et al., 2010).

Beyond direct excitation of intrinsic neurons of NAc, a final line of support for NAc excitation in reward is evidence that there are reward effects of stimulating excitatory glutamatergic inputs to NAc, especially from prefrontal cortex, (Britt et al., 2012) basolateral amygdala, and hippocampus (Will et al., 2004; Ambroggi et al., 2008; Britt et al., 2012). For example, Ambroggi and colleagues (2008) reported that glutamatergic inputs from the BLA to NAc were required for cue-triggered seeking of sucrose reward. Others have reported that optogenetic excitation of glutamatergic projections from prefrontal cortex, BLA, or ventral hippocampus to NAc produces self-stimulation conditioned place preference effects (Stuber et al., 2011; Britt et al., 2012). These observations suggest that glutamate release from those structures excites NAc neurons to contribute to reward processes.

Chapter Outline

In the subsequent chapters, I first attempted to test the hypothesis that activation of D1 neurons mediates positive/reward behaviors and D2 activation mediates negative/avoidant/punishment. In Chapter 2, I address whether D1- "direct" pathway vs D2- "indirect" pathway neurons have opposing roles, similar to that of dorsal striatum. Using transgenic mice to selectively optogenetically stimulate either D1 or D2 neurons, I found evidence supporting rewarding roles for both populations of neurons, but also D2 was capable of producing avoidance in a location-based paradigm. Secondly, I tested the hypothesis that NAc inhibition is a key signal in NAc motivation. In Chapter 3, I addressed whether DNQX-mediated motivation requires localized inhibition of NAc neurons. Here, I utilized optogenetic excitation to combat microinjections of DNQX within the NAc shell of rats. In Chapter 4, I present two individual pilot experiments: First, I attempt to answer whether direct inhibition of NAc neurons is sufficient to induce appetitive motivation for food. Here, I utilized optogenetic inhibition NAc inhibition across multiple timescales to see if I could recreate the rostrocaudal gradient. Second, I attempted to see whether activation of VTA dopamine neurons can enhance the incentive salience of social partners. I utilized transgenic rats to selectively target dopamine neurons of the VTA which project to the nucleus accumbens, and activated neurons upon contact with social partners. In Chapter 5, I conclude with a general discussion of how D1 and D2 activity can both support reward roles. Further, I discuss potential means by which excitation and inhibition can both be viable mechanisms of motivation production and gating in the NAc.

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Chapter 2

Optogenetic Self-stimulation in Nucleus Accumbens: D1 Incentive vs D2 Ambivalence

Introduction

The nucleus accumbens (NAc) is important to appetitive motivation for diverse rewards, which range from food, sex, addictive drugs and brain self-stimulation in animals and humans, to more abstract rewards such as music at least in humans (Rolls, 1975; Mogenson et al., 1980; Kelley et al., 2005; Vuust and Kringelbach, 2010; Salimpoor et al., 2011; Hernandez et al., 2012; Saunders et al., 2013; Castro and Berridge, 2014; Mueller et al., 2015; Zatorre, 2015; Koob and Volkow, 2016).

Within the NAc are distinct subpopulations of GABAergic medium spiny neurons (MSNs), which differ in their expression of dopamine receptors (D1-type versus D2-type), and in connectivity to other structures. From NAc, D1 MSNs project 'directly' to the midbrain ventral tegmental area (VTA), whereas NAc D2 MSNs instead project only 'indirectly' to targets such as ventral pallidum (VP) and lateral hypothalamus (LH) in basal forebrain. To that extent, NAc connectivity resembles that of neostriatum, where D1 MSNs project 'directly' to midbrain targets such as substantia nigra, while D2 MSNs project 'indirectly' to forebrain targets such as globus pallidus (Graybiel, 2000; Humphries and Prescott, 2010). However, NAc D1 MSNs also send 'indirect' projections to VP and LH, similarly to NAc D2 MSNs, which dilutes the NAc

distinction between ‘direct’ versus ‘indirect’ outputs(Humphries and Prescott, 2010; Kupchik et al., 2015; Larson et al., 2015; O'Connor et al., 2015). In addition, a third group of up to 30% of MSNs in NAc shell are reported to co-express both D1 and D2 receptors on the same neuron, which also likely project to indirect VP and LH targets (Bertran-Gonzalez et al., 2008; Perreault et al., 2011b). Finally, acetylcholine neurons in NAc and neostriatum may also express D2 receptors (Brene et al., 1990; Le Moine et al., 1990).

What are the respective NAc roles of D1 neurons vs D2 neurons in reward motivation? In dorsal neostriatum, D1 MSN excitation is reported to support optogenetic laser self-stimulation in mice, which instrumentally work to turn on illumination, whereas D2 neuronal stimulation is avoided (Kravitz et al., 2012). In NAc, substantial evidence also supports a role for D1 MSNs in positive motivation for reward. For example, optogenetic D1 MSN stimulation in NAc enhances drug-induced conditioned place preferences (Lobo et al., 2010; Koo et al., 2014a). Similarly, NAc pharmacological D1 receptor stimulation in D1 MSNs promotes incentive motivation to pursue or consume food or drug rewards (Wakabayashi et al., 2004; Hamlin et al., 2006; Schmidt et al., 2006).

In NAc, D2 receptor activation has been often oppositely linked to suppression of appetitive motivation or reward, such as measured by conditioned place preference (Lobo et al., 2010; Koo et al., 2014a) and even to generation of negatively-valenced avoidance or defensive behaviors, including fearful anti-predator responses (Lex and Hauber, 2008; Liao, 2008; Faure et al., 2010; Richard and Berridge, 2011b; Porter-Stransky et al., 2013). However, other studies have indicated positive appetitive motivation functions for D2 neurons in NAc (Song et al., 2013; Trifilieff et al., 2013; Steinberg et al., 2014; Soares-Cunha et al., 2016).

Here we aimed to more directly compare motivation roles of D1 neurons vs D2 neurons in NAc in optogenetic self-stimulation that selectively excites either one or the other NAc population. We used two self-stimulation tasks to compare D1-Cre versus D2-Cre transgenic mice that could earn laser excitations of Cre-targeted channelrhodopsin (ChR2) expressed in either D1 or D2 types of NAc neurons. Our findings indicate that D1 MSN excitation supports rapid, robust, and intense NAc laser self-stimulation, in both an active response spout-touch task and a relatively passive place-based self-administration task. By comparison, NAc excitation of D2 neurons produced weak positive self-stimulation in the active-touch task, but eventually became mildly avoided for most ChR2-expressing D2-Cre mice in the place-based task.

Methods

Overview

Two independent self-stimulation procedures were used to allow mice to earn brief NAc laser stimulations as reward. First, a spout-touch self-stimulation procedure allowed mice to earn brief 1-sec laser pulses in NAc each time they touched a particular metal spout (which we will call laser-spout) that protruded into the chamber. Another spout was also present, but earned nothing when touched, and served as a control stimulus for comparison. Second, a separate place-based self-stimulation task allowed mice to earn series of laser pulses by entering a particular corner of a 4-corner chamber and remaining there; this task was based on the original Olds and Milner procedure that discovered deep brain self-stimulation when a rat went to a particular location to activate its electrode (Olds and Milner, 1954). After the location task, some mice were also retested on the spout task to reconfirm their initial results.

Subjects

BAC transgenic mice on a C57Bl/6 background (n= 59) were obtained from NINDS/GENSAT (www.gensat.org) from Rockefeller University/NIH/NIMH, and maintained on a 12-hour reverse light-dark cycle with food and water *ad libitum*. These included 33 D1-Cre mice (12 male, 21 females; strain: B6.FVB(Cg)-Tg(Drd1a-cre)EY262Gsat/Mmucd), and 26 D2-Cre mice (12 male, 14 females; strain: B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmucd).

D1-Cre females and males, and D2-Cre females and males were randomly assigned to receive NAc infection with either a channelrhodopsin virus (AAV5-DIO-ChR2-EYFP) to be an optogenetic group, or an optically-inactive virus to be an EYFP-only control group that lacked the ChR2 gene (AAV5-DIO-EYFP). This created four Cre/Virus groups for all following experiments 1) ChR2 D1-Cre [n=14 total (4 male, 10 female)]; 2) ChR2 D2-Cre [n=14total (7 male, 7 female)]; 3) EYFP D1-Cre control [n=19 total: (8 male, 11 female)]; 4) EYFP D2-Cre control [n=12 total: (5 male, 7 female)]. Male and female mice were housed separately, and test chambers were cleaned after each mouse was tested to avoid pheromone contamination. All animal protocols were in accordance with the National Institutes of Health Guide for Care and use of Laboratory Animals and approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Viral Vectors

A DIO Cre-dependent ChR2 Adeno-associated virus (AAV) was used to infect Cre-expressing cells (vectors-double loxP-flanked inverted (DIO) - channelrhodopsin 2 (ChR2) - enhanced yellow fluorescent protein (EYFP) (AAV5-DIO-ChR2-EYFP; purchased from the

University of North Carolina Vector Core with MTA by courtesy of Karl Deisseroth and Stanford University).

Surgery

Mice were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance), placed in a stereotaxic instrument (David Kopf Instruments), and the skull surface was exposed. Bilateral microinjections of virus (0.5 μl per side; 0.1 μl/min) into NAc were targeted at the medial shell and medial core. Either ChR2 virus (AAV5-DIO-ChR2-EYFP) or an optically-inactive control virus (AAV5-DIO-EYFP) (0.5 μl) was delivered via 28-gauge syringe over 5 min, and left unmoved for 10 minutes to allow for viral diffusion. Stereotaxic coordinates for virus microinjections centered around AP +1.42 to +1.32; ML +/- 1.5; DV – 4.78; injectors were angled at 12.29 lateral degrees to avoid ventricles and permit space for bilateral fiber implants. NAc sites were staggered slightly across individual mice to nearly fill the entire medial shell as a group, and include some penetrations in core (AP coordinates range from +1.42 to +1.32), but within a mouse bilateral sites were kept as symmetrically identical as possible.

In the same surgery, optic fibers (6 mm long; 0.220μm core; confirmed to exceed 85% light efficiency prior to surgery) were bilaterally implanted in NAc approximately 0.3 mm above each site of virus injection (AP + 1.42 to +1.32; ML +/- 1.5; DV – 4.48; 12.29 degree lateral angle). Optic fibers were anchored to the skull using surgical screws and dental acrylic. Mice were allowed at least 4 weeks after surgery for incubation and virus expression before behavioral tests began.

Experiment 1: Active-response Laser Self-Administration: Spout-touch tasks

NAc self-stimulation was tested first using a spout-touch self-administration task, in which active touches of a designated empty metal drinking-spout could earn phasic 1-sec illuminations of NAc laser. Optic fibers were attached through an FC/PC adaptor to a 473 nm blue DPSS laser (OEM Laser Systems). Two empty metal spouts (lickometer touch-capacitive detectors) protruded through the wall of the 8x10x5cm chamber (MedAssociates Inc.), placed approximately 5 cm apart.

Active touches of one arbitrarily-designated spout (laser spout) delivered a brief 1-sec bin of laser illumination activated by an Arduino control board (Arduino Hardware), and accompanied by a distinctive auditory cue that served as a sensory label for the laser-delivering spout (either white noise or tone, 1 sec). Touching the other control spout produced no laser, but did produce the different auditory cue as a distinctive marker, and touches on it served merely as a control measure of generalization, exploration and general motor activity (no-laser spout).

In an initial screening wave of mice (n = 6 D1 ChR2; n=5 D2 ChR2; n=7 D1 EYFP & n=6 D2 EYFP), we first compared the relative effectiveness of constant laser versus pulsed 25 Hz laser in 1-sec illumination bins during 30 min sessions. Both types of laser stimulation were compared at three different laser intensities: 0.1, 1.0 and 10 mW. The same spout location delivered laser on all days (paired either with white noise or tone). The other spout remained the control inactive spout on all days (paired with the other sound; spout/noise-tone assignments were balanced across mice). Optic fiber light transmission at the end of the output optic cable was confirmed each day (Laser Check Photometer, Clairvoyance Inc.), and cranial fiber implants had been tested for 85% efficacy prior to surgery. Individual D1 ChR2 mice or D2 ChR2 mice were excluded from being considered self-stimulators if they failed to meet both criteria of 1) at

least 1 session of 20 contacts on the laser-delivering spout and 2) a 2:1 ratio of laser-paired versus non-laser paired contacts on at least one test day.

Constant 1-sec illumination vs pulsed 25 Hz stimulation: Pulsed laser stimulation at a particular frequency (e.g. 25 Hz) is often used in optogenetic studies to drive neuronal firing at the same frequency. By contrast, constant laser illumination at low-intensity (e.g., 1-2 mW) has been suggested to avoid driving neuronal firing at any particular artificial frequency, and instead to promote striatal endogenous firing patterns (e.g., 10% – 30 %) without significantly altering patterns of natural wave-form potentials (Kravitz et al., 2010 & 2013). To compare efficacy of pulsed vs constant NAc stimulation, mice received 1-sec bins at each of the 3 illumination intensities of either constant or pulsed laser (order of pulsed/constant conditions was counterbalanced across mice).

Dose-response comparison (0.1, 1, 10 mW intensities): To compare the relative effectiveness of different intensities of laser illumination, laser intensity was changed consecutively between 3 sessions at each of the 3 intensity levels: 0.1mW, 1mW, 10mW (either at constant illumination or at 25 Hz, both 1-sec duration; order counter-balanced across mice).

Location-tracking 9-day groups: The initial screening test results identified the middle 1.0 mW intensity and the constant-illumination 1-sec parameters as suitable for producing moderate levels of NAc self-stimulation. However, screening results also showed within-group variability for each condition that was higher than optimal statistical comparison, leading us to follow up with a more extended 9-day test with a single laser setting. This extended test with constant-illumination, 1.0 mW intensity, and 1-sec bin parameters was intended to stabilize self-stimulation rates and potentially reduce variability (n = 9 D1 ChR2 mice; n=9 D2 ChR2; n=6 D1

EYFP & n=6 D2 EYFP). Additionally, we wished to assess whether self-stimulations truly motivated in the sense of being instrumental actions that were directed flexibly and specifically aimed at obtaining NAc ChR2 laser excitations, or instead if spout-touches were merely being stamped-in rigid stimulus-response habits or simply repeated as a mere motor reaction to an immediately prior NAc stimulation. Therefore, during the 9-daily sessions we shifted the location of the laser spout three times (a shift every 3 days) to test whether mice would flexibly track the source of NAc laser excitations as spouts were moved). The location of the two spouts were fixed across the first three days of testing (Days 1-3; active/inactive locations were counter-balanced across mice). On Day 4, the active laser spout and inactive spout were both moved to new positions on the opposite wall, and then kept stable over Days 4-6. On Day 7, both spouts were moved back to the original wall, but reversed from their original positions on days 1-3, so that the laser spout now occupied the former no-laser spout position and vice versa, and kept in their new positions over Days 7-9. This presented the mouse with a 3rd new location for the active spout, which was exactly opposite to its original location, see Figure 2.1A.

Laser-extinction test: Finally, on the 10th day, an extinction session with no laser was given to further assess if self-stimulation behavior became habitual or aimed at conditioned reinforcement, or instead remained flexible and dependent on NAc laser activation. In the extinction session, the laser reinforcement was discontinued, though touching each spout still produced its associated auditory cue.

Experiment 2: Location-based self-stimulation task

A second location-based self-stimulation task was conducted subsequently, which allowed NAc laser stimulations to be earned more passively by simply entering or remaining in a particular corner location in a 4-corner chamber. The center of the 90x90x106cm chamber was occluded by a large cylinder (20cm diameter plexiglass), so the mouse could circumnavigate only along the outer periphery and among the four corners of the chamber, and the floor of the chamber also contained bedding. Within the laser-delivering corner, any movement triggered an infrared motion detector that delivered a 1-sec laser constant illumination (1mW, constant) per movement during a 30-min session. Each corner had its own motion detector (Visonic), and MATLAB software was utilized to compile entries and time spent within each of four corners. One corner was arbitrarily designated as laser-delivering each day (corner assignment balanced across mice; corner changed each day for a particular mouse). Laser stimulations were earned on entry and by every further movement detected while the mouse remained within that corner. Laser immediately ceased when the mouse left the designated corner. Entries and time spent in the laser-corner was monitored. Entries and time spent in the other three corners were also monitored but did not produce laser illuminations. On the second test day, the active corner designation was shifted to the corner opposite the Day 1 laser-corner. On the third day, the active corner designation was arbitrarily shifted to a new location in one of the remaining two corners.

One reason this location-based procedure was added is that it can assess laser *avoidance* of the laser-corner, as well as preference, as avoidance would be evident by a mouse's not entering or more quickly leaving the laser-delivering corner compared to alternative corners. Behavior was also videotaped each day for subsequent video analysis, and scored for seconds duration engaged in burrowing (submerging head and using bilateral forepaw movements to throw bedding backwards), defensive treading (throwing bedding forward via alternating

unilateral forepaw movements), rearing (elevating body and head together on hind paws so that forepaws rose >1cm above floor), locomotion (seconds of continuous forward movement), and immobility.

Histological analyses of virus expression, local Fos Plumes, and distant Fos activations

Immediately before euthanasia, a standardized dose of laser stimulations was passively delivered to all mice in order to 1) generate local Fos plumes of neuronal Fos activation immediately surrounding the fiber tip in NAc (Robinson et al., 2014; Warlow et al., 2017), and 2) to potentially recruit distant Fos activations in other brain structures that would reflect functional connectivity patterns for D1 vs D2 circuitry. Beginning 90 minutes prior to euthanasia and perfusion, each mouse was put into a self-administration spout chamber and given 1s bursts of 1mW laser stimulation every 10 seconds for 90 min. Laser stimulation was not contingent on any behavior prior to perfusion as to ensure equal laser exposure for D1 Cre and D2 Cre mice. All mice were then deeply anesthetized with an overdose of sodium pentobarbital, transcardially perfused, and brains were removed and analyzed for Fos plumes as described previously (Reynolds and Berridge, 2008; Richard and Berridge, 2011b; Warlow et al., 2017) and for distant Fos recruited in other limbic structures (Castro and Berridge, 2017). Briefly, brains were stored in 4% paraformaldehyde for 1-day post-perfusion, and then soaked in a 30% sucrose solution for 2 days prior to slicing. Brain slices were processed for Fos immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat AlexaFluor 488 (Invitrogen). Brain slices were mounted, dried for 24 hours, and cover slipped using Prolong Gold antifade solution (Invitrogen). Fos plumes, or local neurons expressing Fos (AlexaFluor 488) surrounding an optic fiber tip, were counted using a grid with 8 arms emanating from the

fiber tip, each arm containing consecutive 50-micron boxes, similar to Fos plume analyses described previously (Reynolds and Berridge, 2008; Castro and Berridge, 2014; Robinson et al., 2014). Virus infection was also measured by placing a similar grid overlay, and measuring EYFP fluorescence in 50 micron increments until levels fell to baseline levels (always within 1mm radius from the fiber tip and virus center).

Statistics

Non-parametric Friedman's ANOVA was used for initial within-subject repeated measures, and Kruskal-Wallis ANOVAs for between-subject comparisons. If initial analyses were significant, additional Mann-Whitney or Wilcoxon tests were used as appropriate for subsequent paired comparisons. For all analyses, the significance level was set at $p = .05$, two-tailed. Effect sizes for pairwise comparisons were calculated using the following formula: $r = z/\sqrt{(N1+N2)}$. Average self-stimulation rates of the two control groups, D1 EYFP and D2 EYFP, were statistically similar (D1 EYFP: 9 (SEM± 4); D2 EYFP: 13 (SEM±3); Kruskal-Wallis: $X^2=2.01$, $p=.156$) and so were combined into a single EYFP control group for comparisons.

Results

Initial Screening: dose-response curves for constant vs pulsed laser at 0.1, 1.0 and 10 mW

D1 NAc self-stimulation: For NAc laser illumination at the lowest 0.1mW intensity, D1 ChR2 mice self-stimulated at >300 responses per 30-minute session on their constant or pulsed laser spout compared to only ~20 responses on the non-laser spout (D1 ChR2 Laser vs Non-laser 25Hz 0.1mW: Wilcoxon, 2.023, $p=0.043$, $r=0.83$; Fig. 2.1). Pulsed 25 Hz bins and constant illumination for 1 sec bins produced similar levels of D1 self-stimulation at this 0.1 mW

intensity, and did not differ (D1 ChR2 constant vs 25 Hz 0.1mW, Z=0.365, p=.715, r=0.15). By contrast, inactive virus control D1 EYFP mice, which received only the EYFP virus, failed to self-stimulate at all for NAc laser illuminations, touching both spouts equally and fewer than 15 times each, and so were significantly below D1 ChR2 mice that had optogenetic virus (D1 ChR2 vs EYFP: Mann-Whitney U, Z=2.553, p=0.11, r=0.58).

At the middle intensity of 1mW stimulation, D1 ChR2 mouse responses rose to >1200 touches on the laser spout compared to merely 8 touches for the non-laser spout (D1 ChR2 Laser vs Non-laser 25Hz 1mW: Wilcoxon, 2.023, p=0.043, r=0.83; Fig. 2.1). Again, 1-sec illuminations of either constant laser or pulsed 25 Hz laser produced similar levels of self-stimulation (D1 ChR2 constant vs 25 Hz 1mW, Z=0.524, p=0.60, r=0.21). D1 Cre mice always self-stimulated more than inactive virus control D1 Cre mice with EYFP virus, which touched both spouts equally and less than 10 times (D1 ChR2 vs EYFP: Mann-Whitney U, Z=2.553, p=0.11, r=0.58)

Finally, at the highest 10mW 25Hz, D1 ChR2 mice self-stimulated >1500 times on the laser spout vs merely 14 times on the non-laser spout (D1 ChR2 Laser vs Non-laser 25Hz 10mW: Wilcoxon, 1.826, p=0.068, r=0.745). By contrast, control EYFP mice with inactive-virus failed to self-stimulate, and were much lower than ChR2 mice (D1 ChR2 vs EYFP: Mann-Whitney U, Z=2.873, p=0.004, r=0.66) Again, constant laser and pulsed 25 Hz laser illuminations did not differ in self-stimulation efficacy at this highest laser intensity (D1 ChR2 constant vs 25 Hz 10mW, Z=0.365, p=0.715, r=0.15). Overall, then, there was a clear dose-response effect of laser illumination intensity for NAc self-stimulation of D1 MSNs. However, despite theoretical expectations that constant illumination might have different consequences

from pulsed 25 Hz stimulation, for NAc effects here, our results suggest that these two forms of laser illumination may still often produce similar behavioral patterns of D1self-stimulation.

D2 NAc self-stimulation:

At the lowest 0.1mW intensity, D2 ChR2 mice also self-stimulated, at least at moderate levels, making approximately 50 to 150 touches on the laser-spout vs only 15 touches on the non-laser spout (D2 ChR2 mice vs Non-laser 25Hz 1mW: Wilcoxon Z=1.604 p=0.109, r=0.50; Fig 2.1). Inactive virus control D2 Cre mice, which received only the EYFP virus, failed to self-stimulate for NAc laser illuminations, touching each spout not more than a few times (D2 ChR2 vs EYFP: Mann-Whitney U, Z=1.173, p=0.241, r=0.28). At this lowest intensity of 1-sec bins for D2 mice with ChR2 virus, constant illumination appeared somewhat more effective at supporting NAc D2 self-stimulation, reaching nearly 150 responses, compared to only about 50 at responses at the 25 Hz pulsed condition (D2 ChR2 Laser 0.1mW constant vs 25Hz: Wilcoxon, Z=2.023, p=.043, r=0.64).

At the middle laser intensity of 1mW, D2 ChR2 mice self-stimulated their laser spout 200 - 900 times, compared to <15 touches on the control spout, reaching a 65:1 ratio (D2 ChR2 mice Laser vs Non-laser 25Hz 1mW: Wilcoxon=1.753, p=0.08). D2 ChR2 mice also touched the laser spout 50 times more than did EYFP control D2 Cre mice with inactive virus (D2ChR2 vs EYFP: Mann-Whitney U, Z=2.301, p=0.021, r=0.54). At 1 mW intensity, the pulsed 25 Hz frequency trended towards higher self-stimulation rates of 980 touches compared to 215 for constant illumination, though that difference did not quite reach statistical significance, likely due to high variation at the 25Hz stimulation (D2 ChR2 Laser 1mW constant vs 25Hz, Wilcoxon, Z=1.753, p=0.080, r=0.55).

Finally, at the highest 10mW intensity, D2 ChR2 mice self-stimulated at rates of 500 - 1000 responses on the laser-spout vs merely 9 on the non-laser spout (D2 ChR2 mice vs Non-laser 25Hz 10mW: Wilcoxon Z=1.342, p=0.180, r=0.42). Inactive virus control D2 mice failed to self-stimulate for NAc illumination and were far below ChR2 mice (D2 ChR2 vs EYFP: Mann-Whitney U, Z=1.364, p=0.172, r=032). Comparing pulsed to constant laser conditions, the pulsed stimulation again trended toward stronger self-stimulation at rates of >1800 touches for 25 Hz frequency compared to ~500 touches for constant illumination at 10 mW, but this also difference failed to reach significance and higher variation was observed within the 25Hz group (D2 ChR2 Laser 10mW 1 Hz vs 25Hz, Wilcoxon, Z=1.345, p=0.180, r=0.42).

D1 vs D2 self-stimulation in initial screening:

Overall, D1 mice tended to self-stimulate for ChR2 laser in NAc at three times higher rates than D2 mice, at least for constant laser illuminations at all intensities, though given within-group high variance no paired differences reached statistical significance (Kruskal Wallis, X2=0.33, p=0.856; 0.1mW laser intensity Wilcoxon, Z=0.823, p=.41; 1mW laser intensity, Wilcoxon Z=0.183, p=0.855; 10mW laser intensity, Wilcoxon Z=0.548, p=.584). For 25-Hz pulsed laser condition, D1 mice similarly self-stimulated at least three times more than D2 mice for the lowest 0.1 mW intensity (again still not significant: Wilcoxon, Z=1.10, p=.269), and at more nearly comparable rates for higher 1.0 and 10 mW intensities.

Extended 9-day & moving spout test at 1.0 mW constant illumination Given that results from initial screening above had quite high within-group variability, a more extended 9-day access paradigm was designed that used a single constant-illumination laser setting at the middle 1 mW

intensity, to try to achieve more stable levels of self-stimulation (below). The location of the spout was shifted on Day 4 to a new location, and shifted again on Day 7 to a third location.

D1 9-day spout self-stimulation: Overall across 9 days of testing, D1 ChR2 mice strongly self-stimulated on their laser-paired spout, achieving >500 laser-spout touches per 30 min session on average, compared to only 18 touches on the non-laser spout (D1 ChR2 Laser vs. non-laser: Wilcoxon, Z=-6.541, p<.0001, r=.72; D1 ChR2 vs Control Laser-preference: Mann-Whitney U, Z=-7.437, p<.0001, r=.54; Fig 2.2). Females and males showed similar levels of D1 ChR2 NAc self-stimulation on all days, and there was no sex difference in magnitude of laser preference (Mann-Whitney U, Z=.76, p=.443, r=.10). By contrast, D1 EYFP-control mice with NAc inactive-virus that lacked ChR2 touched fewer than 10 times on either the laser-spout or an alternative spout, with no difference between spouts, and at only $1/50^{th}$ of the self-stimulation level for D1 ChR2 mice (EYFP Preference: Wilcoxon, Z=1.023, p=.306, r=.03; D1 ChR2 vs EYFP Control: Mann Whitney U, Z=2.110, p=0.34, r=.46; Figure 2.2B). Lack of self-stimulation by inactive-virus controls confirms that mice were not simply self-stimulating for visual light or heat of intracranial laser, but rather that activating neuronal D1 ChR2 photoreceptors was essential for high levels of D1 NAc self-stimulation.

NAc D1 self-stimulation was rapidly acquired within a few minutes on the first day of the spout task, reaching statistical significance by the 16^{th} minute (Laser vs Non-laser spout, 16^{th} min-30^{th}; Friedman's, X^2=4.654, p=.031; 2A). In total on Day 1, D1 ChR2 mice reached >400 contacts on their laser-spout, but only 26 touches on the control spout (Wilcoxon, Z=2.492, p=.013, r=.83).

On subsequent Days 2 and 3 (with spouts kept in same positions as Day 1), D1 mice continued to self-stimulate NAc at high levels: always >400 stimulations for every mouse per 30-

min session, and over 1,000 laser-spout touches for a few individuals (Day 2: Wilcoxon, Z=2.490, p=.013, r=83; Day 3: Wilcoxon, Z=1.960, p=0.05, r=.65). By comparison, EYFP mice touched the laser spout fewer than 14 times each on Day 2 (Wilcoxon, Z=2.110, p=.034, r=.46) and on Day 3 (Day 3 Wilcoxon, Z=2.635, p=.007, r=.57)

On Day 4 the locations of laser-delivering spout and control spout were both moved to the opposite wall. D1 ChR2 mice immediately moved to their new laser spout location and began self-stimulating within the first minute of Day 4, reaching statistical preference by the 11th minute (Friedman's, X^2=5.188, p=.023; Fig. 2.3A), and >500 contacts on the laser spout for the entire session vs <25 contacts on the control spout (Wilcoxon, Z=1.836, p=.066, r=0.612; Fig. 2.3A). By comparison, control EYFP mice failed to touch either spout more than 13 times on Day 4, and remained far lower than D1 ChR2 mice (Mann-Whitney U, Z=2.171, p=0.030, r=0.47). D1 ChR2 mice continued to self-stimulate NAc at levels of at least several hundred laser pulses per session, far above EYFP mice that touched either spout equally and only <20 times (Day 5 Z=2.101, p=.036, r=0.45; Day 6: Z=2.813, p=.005, r=0.61)

On Day 7 the locations of laser spout and control spout were again switched to the original wall, but now in reverse positions from Days 1-3, so that the laser-delivering spout now occupied the initial location of the inactive spout, and vice versa. D1 ChR2 mice again followed their laser-delivering spout to its new location nearly within the first minute on Day 7, reaching by the 3rd minute significantly more touches on the laser-spout than the non-laser spout (Friedman, X^2=4.571, p=.033; Fig 2.3A), and making over 350 self-stimulations for the entire session (versus 13 touches on their now-inactive spout; a >30:1 ratio; Wilcoxon, Z=2.192, p=0.028, r=0.73). By contrast, inactive-virus control mice again hardly touched either spout, each < 10 times in the session (Mann-Whitney U, Z=2.398, p=.015, r=0.52). Similarly, on

subsequent Days 8 and 9, with spout positions the same as on Day 7, D1 ChR2 mice continued to self-stimulate NAc at several hundred laser pulses per session (Day 8: Wilcoxon, Z=1.482, p=.138, r=0.49; Day 9: Wilcoxon, Z=-2.31, p=.021, r=0.77). By contrast, inactive-virus control mice touched fewer than 25 times on the laser spout on Days 8 and 9, remaining far below D1 ChR2 mice (Day 8: Mann-Whitney U, Z=2.293, p=.023, r=0.50; Day 9: Mann Whitney U, Z=3.490, p<.0001, r=0.76).

Across all 9 test days, we did note a slight trend for inactive-virus EYFP control D1 mice to mildly prefer their laser spout over the alternative spout by nearly 2:1, but this failed to reach significance on any day (9+4 on laser spout, 4+3 on non-laser spout). D2-EYFP controls also showed a slight 3:2 bias toward laser (SEM13±4 laser spout vs 11± 2 non-laser), though also non-significant. Previously, rodents have been reported to work for a visual light stimulus even without any ChR2-induced brain activation (Ikemoto and Bonci, 2014), so it is conceivable that EYFP mice might have a mild preference for a visual light or intracranial heat stimulus. However, it is clear that visual light alone could not have motivated the high rates of NAc self-stimulation for either D1 ChR2 mice or D2 ChR2 mice.

D2 mice in 9-day spout self-stimulation:

Overall, D2 ChR2 mice also positively self-stimulated on the spout-touch task, though at relatively modest rates of about 60 laser illuminations per session, yet still significantly above EYFP control rates of roughly 10 illuminations (Mann-Whitney U, Z=6.88, p<.0001, r=0.50). D2 ChR2 females and D2 ChR2 males showed similar levels of laser-spout contacts, with no difference between the sexes (Days 1-9, D2 Male vs Female; Mann-Whitney U, Z=.767, p=.443,

r=0.09). Nearly all D2 ChR2 mice touched their laser-delivering spout at least 400% more often than the non-laser spout (Wilcoxon, Z=6.193, p<.0001, r=0.69).

D2 ChR2 self-stimulation was relatively slower to emerge on Day 1 than in D1 mice: D2 ChR2 mice had made only 5 laser-spout touches by the 15th minute (a level the D1 ChR2 mice had reached within their first 5 min; Fig. 2.3B), and took 25 min to become statistically elevated over the non-laser spout (Laser vs non-laser cumulative response: Friedman's X^2=4.596, p=.032; D2 ChR2 laser vs control spout: Wilcoxon, Z=2.10, p=0.036, r=0.70; D2 ChR2 vs EYFP: Mann-Whitney U, Z=1.996, p=0.046, r=0.44). However, once achieved, the D2 ChR2 self-stimulation rate remained stable at 40–70 NAc laser-spout contacts per session on subsequent Days 2 and 3, while inactive spout contacts remained < 10 per session (6:1 ratio; Day 2 Laser vs on-laser contacts: Wilcoxon, Z=2.524, p=.012, r=0.84; Day 3: Wilcoxon, Z=2.073, p=0.038, r=0.69; Day 2 D2 ChR2 vs EYFP: Mann-Whitney U, Z=2.147, p=0.032, r=0.47; Day 3 D2 ChR2 vs EYFP: Mann-Whitney U, Z=2.068, p=0.039, r=0.45).

D2 ChR2 Mice Slowly Track Spout Shifts in Location: On Day 4, when both spouts were shifted to the opposite wall, D2 ChR2 mice initially failed to track the active laser spout to its new location on that day, making ~30 or so laser spout contacts vs 20 control-spout contacts (D2 ChR2 Laser vs on-Laser: Wilcoxon, Z=1.820, p=0.069, r=0.61; D2 ChR2 vs EYFP: Mann-Whitney U, Z=1.71, p=0.87, r=0.37). However, they began to track on Day 5, and by Day 6 were again self-stimulating at about 70 NAc laser-spout contacts vs 18 on control-spouts a > 3:1 ratio over 18 contacts on control spout (Wilcoxon, Z=2.31, p=0.021, r=0.77), while EYFP-controls remained at 7-15 contacts at both spouts (D2 ChR2 vs EYFP Control, Day 6; Mann-Whitney U, Z=2.563, p=0.010, r=0.56).

On Day 7 the laser spout was moved to a third location (original wall, but laser and control spouts were reversed in position from their Day 1-3 locations). This time, D2 ChR2 mice did successfully track the laser spout to its new location on the same day, reaching significant self-stimulation levels by the 8th minute of Day 7 (Fig 2.3B. Cumulative Minute by Minute Laser v Non-responses: Friedman's X^2=4.587, p=.032), and earning nearly 60 NAc self-stimulations in the session, compared to only 20 touches on the control spout (Day 7 Session D2 ChR2 Laser vs Non-laser: Wilcoxon, Z=2.312, p=0.21, r=0.77). On subsequent Days 8 and 9, D2 ChR2 mice continued to self-stimulate at levels of 60-80 illuminations per session (Day 8: Wilcoxon, Z=2.134, p=0.33, r=0.711; Day 9: Wilcoxon, Z=2.380, p=0.17, r=0.79). By contrast, inactive-virus control EYFP mice showed no preference for either spout and remained low on both spouts on all days (12 touches ± 3 on laser-spout to 11 ± 2 on alternative spout; D2-ChR2 vs. D2-EYFP Day 7-9: Kruskal-Wallis, X^2=18.91, p<.0001).

Self-stimulation immediately declines during laser extinction:

On Day 10, the laser was discontinued, and only the Pavlovian auditory cues were earned by spout touch (Fig 2.2D). In laser extinction, D1 ChR2 mice quickly declined within a few minutes of the laser-extinction session, and in total made only 10% of their previous day's level of touches on the former laser-spout in the 30-min session (Wilcoxon, Z=2.201, p=.028,r=0.59). Similarly, D2 ChR2 mice also immediately declined within minutes to <15-20% of their previous day's level in touches on the formerly-active spout, and no longer differed from their inactive-virus control D2 counterparts in total contacts (D2 ChR2 vs EYFP Extinction: Mann-Whitney U, Z=.20, p=.328, r=0.26).

Explicit D1 vs D2 Comparison for NAc self-stimulation on 9-day spout-touch task: Overall, contrasting D1 mice to D2 mice, D1 ChR2 levels self-stimulation levels were nearly an order of magnitude higher than D2 ChR2 levels (>700%; Mann-Whitney U, z=2.627, p=.009, r=0.21). D1 ChR2 levels reliably achieved >500 NAc self-stimulations per daily session, and a few D1 ChR2 individuals exceeded 2000 self-stimulations per session. By comparison, D2 ChR2 levels remained at about 60 self-stimulations per session on average (Mann-Whitney U, Z=2.627, p=0.009, r=0.21; Fig 2.2), and the top few D2 individuals reached a maximum of only 100 to 200 per session. For the non-laser spout, both D1 ChR2 and D2 ChR2 mice touched at equivalent rates of about 15-20 times per session (Mann-Whitney U, Z=0.505, p=0.614, r=0.04). This divergent pattern for the laser spout suggests that D1 levels of NAc ChR2 self-stimulation were reliably much higher than D2 levels. Further, similar rates of touching the non-laser spout implies the difference in self-stimulation was clearly not due to simple differences in general activity or spout interest between D1-Cre mice and D2-Cre mice, but rather reflected true differences in appetitive motivation for selective NAc excitation.

Passive Location-Based Self-Stimulation: Only D1 ChR2 mice seek out Laser Location

In the separate location-based self-stimulation task, entering or simply moving while remaining in the designated laser-corner triggered an infrared motion detector that delivered 1-sec constant (constant) 1 mW laser pulses to NAc. In the square doughnut-shaped chamber, mice typically ran almost continuously in a single direction around the periphery during the session as if on a running track (usually in counter-clockwise direction), with occasional brief 1-3 sec pauses in corners. Mice were restricted to the periphery because the center of the chamber was blocked by a cylinder. The laser-designated corner remained constant throughout the entire first

session, but then was switched to a different corner the next day, and switched again to a third new corner on the 3rd day.

D1NAc ChR2 mice: strong preference: Across the 3 days of testing, D1 ChR2 mice preferred and followed their NAc laser-delivering corner as it moved: reliably pausing and spending about 150% more time in that corner than in any other corner (Friedman One Way, X^2=9.643, p=.022; Fig 2.4). D1 ChR2 mice triggered about 180s of cumulative NAc laser stimulations per session overall, and reached that level as early as Day 1. By comparison EYFP inactive-virus D1 control mice EYFP D1 mice essentially distributed their time equally across all four corners, and received significantly less at only about two-thirds of that laser stimulation (120 sec; Kruskal-Wallis, X^2=5.549, p=.018).

In general, comparing individual D1 ChR2 performance on place-based versus spout-based self-administration tasks, there was a significant positive individual correlation between self-stimulation on the two separate tasks: individuals with higher numbers of total spout touches tended to also spend more time in their laser corner (Spearman's rho=0.649, p<0.0001). Therefor the individuals that earned the highest total amounts of laser stimulation on the spout task also tended to earn higher amounts in the place-based task, whereas other individuals earned lower total duration amounts of NAc laser on both tasks (Spearman's rho=0.596, p=0.001).

D2 NAc ChR2 mice rarely prefer, and instead ignore or eventually avoid: D2 ChR2 mice as a group did not detectably prefer the NAc laser-delivering corner on any day (; Days 1-3 2 time spent: Friedman's, X^2=4.911, p=.178; Fig 2.4). Instead on the first and second day, all D2 ChR2 mice spent equal time in all four corners, and made roughly equal numbers of entries and

movements in each corner (Day 1 Time: Friedman, X^2=4.333, p=.228; Day 1 motion detector triggers: Kruskal-Wallis, X^2=.4.067, p=.254; Day 2: Time Spent: Kruskal-Wallis, X^2=0.733, p=.865; motion detector triggers: X^2=3.267, p=.352).

However, on Days 2 and 3, individual differences appeared to emerge among several D2 ChR2 mice. For example, while a few mice continued to ignore their laser corner, one D2 ChR2 mouse spent approximately 140% more time in its laser corner than any other corners on Day 2. However, the largest subgroup of D2 ChR2 mice began to significantly avoid their laser-delivering corner on Day 2, spending less time in it than in any other corner (Friedman's, X^2=13.667, p=.003; Fig 2.4C). Similarly, on Day 3, this same group continued to avoid the laser corner as it moved to a new location, compared to all other corners avoided (Friedman's, X^2=9.944, p=.019). For the avoiding subgroup, pairwise comparisons of individual pairs of corners for the group confirmed that those D2 ChR2 mice spent less time in their laser corner than in at least 2 other corners on Day 3 (Wilcoxon, Z's=2.3 to 2.7, p's<0.017). One of these mice reliably paused prior to entry to apparently avoid entering the laser corner, while the other D2 ChR2 mice in this group appeared instead to escape the laser corner more rapidly after entry (often within 1-2 seconds of laser onset). This laser-avoiding group of D2 ChR2 mice also spent less time in the laser corner than EYFP control mice on Days 3 (Kruskal-Wallis, X^2=4.854, p = 0.028), which spent equal time in all four corners (EYFP Corner Time Friedman's, X^2=6.051, p=.109).

We noted that D2 mice which avoided their laser corner on Days 2 & 3 had previously shown reliable positive self-stimulation on the earlier spout task. We therefore returned these mice to the spout-task for retesting. Results showed that these mice still positively self-stimulated by making several dozen to >100 contacts on the laser spout. Thus, there was no

correlation between time spent in the laser-corner in the place-based task and spout-touch self-stimulation in the spout task (Spearman's rho=-0.048, p=0.812).

In terms of general locomotion, all D1 ChR2 and D2 ChR2 mice showed similarly high levels of running, spending about 90% of time in chamber in motion (D1 vs D2 ChR2 Time Locomotion; Kruskal Wallis, X^2=.308, p=.579), as did also EYFP D1 and D2 control mice (D1 vs Control Time Locomotion: X^2=426, p=.670; D2 vs Control Time Locomotion: Kruskal Wallis, X^2=2.515, p=.113).

Histological NAc sites, local NAc Fos plumes and distant Fos in recruited structures

NAc Self-Stimulation Sites: D1 ChR2 sites for optic fiber tips were clustered mostly in the medial shell of NAc (n=8) (Figure 2.5A). An additional set of D1 ChR2 mice had tips in medial NAc core (n=4), and a few had sites on the border between core and medial shell (n=3). The relative intensities of self-stimulation produced at different sites is shown in Figure 2.5, but shell and core were essentially comparable for D1 self-stimulation (medial shell = ~1000±560 laser spout touches; with two D1 ChR2 mice >4500; core group= ~1400±1149 overall, with one individual >4500). We could not detect any systematic anatomical differences in self-stimulation rates in core vs shell, nor in rostral vs caudal placements within a subregion.

D2 ChR2 sites were similarly clustered in NAc medial shell (n=7) or medial core (n=5), or on the core-shell border (n=2). D2 ChR2 shell and core sites again had comparable levels of self-stimulation (D2 ChR2 shell = 180±67 for group, with highest individual at >500; core= ~200±50 and highest individual at 400; Figure 2.5B). Again, we did not detect systematic anatomical differences in D2 ChR2 self-stimulation rates across NAc sites in core or shell.

Local Fos plumes in NAc

Laser illumination in D1 ChR2 mice and D2 ChR2 mice produced local Fos plumes of elevated Fos expression immediately surrounding the optic fiber tips, which were approximately 0.3 to 0.9 mm in outer diameter, depending on level of Fos elevation (laser stimulation was given immediately prior to perfusion; Figure 2.6). Fos plumes were typically centered immediately beneath the optic fiber tip. D1 ChR2 Fos plumes contained an inner 0.32 mm diameter center of intense >200% Fos elevation (e.g., 2 times above the control levels of EYFP D1 mice that also received laser illumination), surrounded by a larger 0.6 mm diameter middle plume of more moderate >150% Fos elevation, and finally by a still-larger 0.9 mm outer plume of mild >125% elevation (Kruskal-Wallis, $Z=9.39$, $p<.0001$, $r=0.19$). This outer plume diameter of approximately 0.90 mm was used to set the largest diameter of D1 ChR2 symbols in NAc self-stimulation maps, with concentric circles showing the inner >200% and middle >150% zones (Fig 2.6). This is based on the logic that detectable Fos plumes reveal the size of the zone in which laser/ChR2 combination objectively alters neuronal function, even if we do not know the precise threshold of Fos elevation within the 125%-200% range that is most relevant to mapping motivation function.

In D2 ChR2 mice, illuminated local Fos plumes produced by laser illumination similarly contained an outer ~1.0.mm- diameter plume of mild >125% Fos elevation, which contained a middle 0.30 mm diameter plume of moderate >150% Fos elevation, and an inner plume 0.02 mm-diameter of intense >200% Fos elevation (compared to baselines in inactive-virus EYFP control D2 mice that also received laser before perfusion; (Kruskal-Wallis, $Z=3.790$, $p<.0001$, $r=0.08$). No D2 ChR2 plumes reached the most intense >250% elevation seen in D1 ChR2

plume centers, not even in the inner D2 center (which only reached >200%). However, outer >125 plumes were more similar for D1 and D2 laser plumes. Thus, the same outer plume 0.90mm outer diameter of >125% elevation was used to maximally size D2 map symbols, with inner and middle symbols of 0.2 and 0.3 mm diameters (Fig 2.6).

Taken as groups, both the D1 ChR2 and D2 ChR2 Fos plumes filled over 90% of the entire medial shell, and at least the most medial portion of core. EYFP/ChR2 virus infection in NAc of all mice typically was about 2 mm in diameter, with a few individuals reaching nearly 3 mm. The observation that Fos plumes were smaller than virus infection suggests that laser illumination altered only a portion of infected neurons, namely chiefly those within 0.3 mm radius of the optic fiber tip.

NAc D1/D2 stimulation recruits similar distant Fos activation in other brain structures

Laser stimulation in NAc also recruited distant Fos increases in several other limbic brain structures, in both D1 ChR2 mice (n=6) and D2 ChR2 mice (n=5) (Figure 2.7). Distant Fos was measured in 'indirect' and 'direct' NAc output targets including ventral pallidum, lateral hypothalamus, and ventral tegmental area; in substantia nigra; basolateral nucleus and central nucleus of amygdala; subiculum of hippocampus; and in orbitofrontal, prelimbic, infralimbic, and insula regions of cortex. For baseline comparisons, we measured levels in two control groups: 1) inactive-virus EYFP D1/D2 mice that similarly received laser before perfusion (n=13; 7 D1 + 6 D2), and 2) normal unoperated D1 and D2 mice that never received any surgery (n=6).

Distant elevations were produced by ChR2 NAc stimulations in both D1 ChR2 and D2 ChR2 mice, compared to similarly illuminated EYFP control mice with inactive virus. For example, in ventral pallidum, illuminated D1 ChR2 mice had elevations >270% above

illuminated EYFP controls, both in rostral and caudal subregions of ventral pallidum (Kruskal Wallis, X^2=4.60, p=0.032), raising them a full order of magnitude above unoperated naive mice (Kruskal Wallis X^2=7.533, p=0.006). Similarly, illuminated D2 ChR2 mice were >250% above EYFP controls in ventral pallidum, both in rostral and caudal subregions (Kruskal Wallis, X^2=8.45, p=0.004). This results in raising D2 ChR2 mice to an order of magnitude above unoperated naïve control levels (Kruskal Wallis X^2=8.22, p=0.004).

In lateral hypothalamus, another indirect output target of NAc, both D1 ChR2 and D2 ChR2 stimulations again recruited similar >200% Fos elevations above EYFP control levels, and nearly 300% above unoperated control levels (D1 vs naïve Mann-Whitney U, 2.22p=0.026, r=0.62; D2 ChR2 vs naïve Mann-Whitney U, Z==1.87, p=0.061, r=0.54). In the 'direct output' target of ventral tegmentum, absolute Fos levels were much lower for all groups. Here, both D1 ChR2 and D2 ChR2 stimulations induced trends toward roughly 200% elevations in ventral tegmentum above EYFP controls (though only 50% above unoperated naïve controls), those these optogenetic elevations did not reach statistical significance.

In basolateral amygdala, D1 ChR2 laser stimulation of NAc induced Fos elevation of >250% above similarly-illuminated EYFP control levels (Mann Whitney U, Z2.07, p=0.039, r=0.47), and >270% above unoperated control EYFP mice (Mann Whitney U, Z2.07, p=0.038, r=0.576). D2 ChR2 stimulation similarly induced a marginal trend toward elevated Fos in basolateral amygdala >280% above EYFP control levels (Mann-Whitney U, Z=1.83, p=0.067, r=0.53), and similarly above unoperated naïve levels for basolateral amygdala (Mann-Whitney U, Z=1.802, p=0.072, r=0.52). Possibly also recruited were roughly 200% increases in prefrontal cortex (prelimbic, infralimbic regions) and hippocampal ventral subiculum especially in D2 ChR2 mice, though not statistically significant with our group sizes.

Finally, comparing just the two baseline control groups (EYFP mice versus unoperated mice), NAc- illuminated EYFP mice typically had moderately higher Fos levels than normal unoperated mice in several structures, and much higher levels in one structure (Figure 2.7). Most notably, in caudal ventral pallidum, EYFP mice had Fos levels 600% higher in than unoperated control mice (Mann-Whitney U, Z=2.093, p=0.036, r=0.47). This pattern suggests that mere laser heat/light in NAc and/or virus infection in NAc by itself may rather powerfully recruit Fos in this 'indirect' output target even without ChR2 opsin gene at the NAc site. Though not statistically significant, EYFP mice had up to 50% elevation in most other structures sampled over unoperated mice. However, we stress that all the D1 and D2 Fos elevations caused by ChR2 illumination described above were always assessed statistically by comparison to the higher EYFP control baselines, to conservatively identify optogenetic recruitments of distant Fos activation.

Discussion

Our results showed that optogenetic excitation of D1 neurons in NAc shell and core reliably supported strong appetitive self-stimulation seeking in both spout-touch and location-based tasks. D1 Cre mice made at least several hundred touches, and sometimes over four thousand, on a spout to earn ChR2 laser stimulations in NAc in each half-hour session (i.e., equivalent to 2.5 spout touches per sec). D1 mice also nearly instantly tracked their ChR2-stimulating laser spout when it moved, shifting within a minute or so to its new location. Similarly, in the location-based task, D1 mice reliably spent more time in their ChR2-stimulating laser corner, earning significant NAc self-stimulation, and successfully followed their laser-corner as it moved each day.

By comparison, D2 Cre mice in the spout-touch task displayed lower, yet still clearly positive, levels of NAc ChR2 self-stimulation behavior. D2 ChR2 mice made at least several dozen laser-spout touches per session to stimulate NAc D2 neurons, and in a few cases earned hundreds of NAc illuminations. Though much slower than D1 mice to acquire and track initially when their laser-spout moved, D2 ChR2 mice also did eventually succeed in following to its new location the first time it shifted, and on their final shift D2 mice followed adeptly within 10 min.

For both D1 and D2 mice in the spout-touch self-stimulation task, the mW intensity of laser illumination in NAc (0.1, 1, 10 mW) was roughly proportional to the magnitude of ChR2 self-stimulation behavior. Also for both D1 and D2 mice, either constant laser illumination or 25Hz pulses appeared sufficient and even roughly comparable at most intensities in supporting self-stimulation when either was delivered in 1-sec bins.

However, when tested in the separate location-based task, D2 ChR2 mice initially ignored their laser-delivering corner (unlike D1 ChR2 mice), and on subsequent days were at least as likely to avoid or escape from their laser-corner than to prefer its location. These D2 laser-avoiders included some individuals that had previously shown positive self-stimulation in the spout task, suggesting that the motivational valence of their NAc D2 ChR2 stimulation had in fact flipped from one situation to another.

What explains the reversal of valence from positive to negative for D2 ChR2 stimulation in NAc? One answer might be the relatively longer duration of laser stimulation in the location task than in the spout task. D2 ChR2 mice rarely received more than a single 1-sec NAc laser stimulation at a time in the spout task, whereas in the location task two to four consecutive 1-sec bins of laser stimulation were commonly received while remaining in the laser-corner. Longer D2 ChR2 laser stimulations in NAc in the location task may conceivably have accumulated a

negative motivational impact. Preference for shorter over longer durations would be similar to recently reported preferences for shorter optogenetic bins for self-stimulation of glutamatergic neurons in ventral tegmentum under 5 sec duration (Yoo et al., 2016), as well as for shorter durations of electrical self-stimulation in lateral hypothalamus (<2 sec durations) and related regions described decades ago (Valenstein and Valenstein, 1964). A second contributing factor could be stimulus/response differences between tasks. Active touch on a spout allowed stimulation to be more instrumentally controlled by active responses, and allowed attribution of laser to that response or to the discrete localized cue-stimulus of the laser spout. By contrast, the location corner presented a broader spatial context associated with laser, which could even be encountered inadvertently while running through the chamber periphery, and so more diffuse in stimulus and perhaps not as readily controlled by the mouse's active response. Finally, in principle it is also possible that order effects or additional virus incubation could have played a role, as the location-based task was run at least a week after the initial spout-touch task. However, we do not believe order or time lapse contributed much to valence reversal, because when D2 ChR2 individuals were retested on the spout task again after their location task, they reverted to positive self-stimulation.

Reversals of valence for D2 ChR2 stimulation in NAc suggest that the D2 NAc motivational role is not as fixed as D1 positive role, but rather D2 can be relatively plastic in motivational valence. That is, D2 NAc stimulation can take on either positive or negative motivational valence depending situational factors, whereas D1 NAc stimulation remains more robustly positive across situations.

Comparison to other NAc results on D1 vs D2 roles

Our finding that D1 MSNs in NAc supported strong appetitive self-stimulation behavior here is consistent with many previous reports of D1 NAc participation in appetitive motivation. For example, D1 ChR2 stimulation in NAc enhances acquisition of drug reward (Lobo et al., 2010; Koo et al., 2014b), and D1 pharmacological stimulation similarly amplifies incentive motivation to obtain or consume food, sex, drugs and other rewards (Schmidt et al., 2006; Lex and Hauber, 2008; Liao, 2008; Richard and Berridge, 2011b; Porter-Stransky et al., 2013). Similarly, in dorsomedial neostriatum, optogenetic excitation of D1 MSNs supports robust self-stimulation on a laser-spout task (Kravitz et al., 2012).

Conversely, a negative-avoidance role for NAc D2 neuronal stimulation for some individuals in our location task is consistent with the report that optogenetic D2 ChR2 stimulation in dorsomedial neostriatum is also avoided by mice (Kravitz et al., 2012). An escape-avoidance role for D2 excitation in NAc is also consistent with many reports that either optogenetic D2 NAc stimulation or neurochemical D2 receptor activation can suppress motivation to seek rewards (Lobo et al., 2010; Koo et al., 2014b; Carvalho Poyraz et al., 2016; Volkow et al., 2016), or even induce negatively-valenced fearful threat reactions such as conditioned freezing or unconditioned anti-predator and escape behaviors (Richard and Berridge, 2011b; De Bundel et al., 2016).

An anti-appetitive or aversive role for D2 NAc excitation is also relevant to the 'appetitive-NAc-inhibition' hypothesis of reward, which posits hyperpolarization of MSNs in NAc to be the primary mechanism for appetitive motivation (Meredith et al., 2008; Roitman et al., 2008; Carlezon and Thomas, 2009). By this hypothesis, NAc inhibition halts GABA release from NAc output projection axons, and so disinhibits downstream targets into relative excitation in hypothalamus, VP and VTA to mediate motivation for rewards. While a strong version of this

hypothesis might predict that both D1 and D2 NAc inhbitions would be more effective at generating appetitive motivation than NAc excitations, a more moderate version might hold that NAc D2 inhibition, but not exitation, would generate appetitive motivation, even if D1 neuronal excitation participated in reward. That modification would accommodate evidence that at least a subset of neurons in NAc are often excited during reward-related events or pusuit, and that D1 pharmacological and optogenetic stimulations can promote reward seeking behavior (Taha and Fields, 2005; Lobo et al., 2010; Kravitz et al., 2012; Koo et al., 2014b). By that view, pharmacological D2 stimulations that promote reward seeking, such as D2/D3 agonist medication induction of addictive-like motivations in Parkinson's patients with Dopamine Dysregulation Syndrome (O'Sullivan et al., 2009), would be seen as medication-induced G_i G-protein receptor-induced neuronal inhibitions of D2 neurons, which releases appetitive motivation. Similarly, virally-mediated increase in NAc D2 receptor expression is reported to promote incentive motivation to obtain food reward (Trifilieff et al., 2013). Conversely, pharmacological D2 receptor blockade, which has long been known to reduce appetitive motivation for rewards(Wise, 1985; Bachtell et al., 2005; Bari and Pierce, 2005; Heidbreder et al., 2005; Bernal et al., 2008; Liao, 2008; Nunes et al., 2013; Xi et al., 2013; Larson et al., 2015), would be viewed as disinhibiting D2 neurons into relative excitation by blocking the same G_i G-protein receptors. Thus, either strong or moderate hypotheses that endorse 'appetitive-NAc-inhibition' views might predict that D2 NAc neuronal excitations should oppose, rather than enhance, appetitive motivation.

By contrast, our results indicate that direct neuronal excitation of D2 neurons in NAc is sufficient to generate positive self-stimulation at least under some circumstances, as in our spout-touch task. Our finding of an appetitive D2 role in NAc for ChR2 self-stimulation is also

consistent with some other studies. Most relevant here, optogenetic stimulation of D2 neurons in NAc also has been reported to amplify appetitive motivation, expressed as breakpoint effort to obtain food reward (Soares-Cunha et al., 2016). Further, inhibition of D2 neurons in lateral neostriatum is reported to reduce motivation for reward similarly to inhibition of D1 neurons (Natsubori et al., 2017). Perhaps most relevant to self-stimulation *per se*, even optogenetic excitation of D2 neurons in lateral neostriatum has similarly been reported to support self-stimulation behavior (Vicente et al., 2016), although we note those authors interpreted their D2-reinforced response as rather sensorimotor, and suggested it to reflect a stimulus-response habit rather than an instrumental or goal-directed action. However, in our case, we note that flexibility of our D2 ChR2 mice in pursuing their laser spout when it moved to new locations (following within 10 min for its final shift) rules out a simple stimulus-response habit interpretation, which would be expected to produce more rigid preservation of responding and behavioral insensitivity to the shift in outcome value.

Given that NAc D2 excitation can contribute to appetitive motivation, how can this positive role be explained? One potential explanations for why excitation of D2-expressing neurons in NAc might contribute to appetitive motivation similarly to D1 neurons is the anatomical overlap in their output projections, and seen here in functional connectivity patterns reflected in distant Fos recruitment. NAc D2 MSNs and D1 MSNs both send 'indirect' output projections targets to nearly the same sites in ventral pallidum and lateral hypothalamus (Humphries and Prescott, 2010; Kupchik et al., 2015; O'Connor et al., 2015). Further, we observed here that D1 stimulation and D2 stimulation in NAc produced quite similar patterns of functional connectivity, as reflected by overlapping recruitment of Fos activations in ventral pallidum, lateral hypothalamus, ventral tegmentum, amygdala, hippocampal subiculum, and

medial prefrontal cortex. Overall, there is about an 85% overlap in D1 and D2 Fos production across these sites. Functional overlap in recruited circuitry may explain why D1 ChR2 and D2 ChR2 stimulations in NAc both produced positive motivated behavior in the spout-touch task here. If so, much less overlap in circuitry recruitment might be expected for D1 vs D2 excitation in the dorsolateral region of neostriatum, where D1 is reported to support optogenetic self-stimulation but D2 is avoided (Kravitz et al., 2012).

Second, up to 1/3 of neurons in NAc shell have been suggested to express both D1 and D2 receptors together on the same cell (Bertran-Gonzalez et al., 2008; Humphries and Prescott, 2010; Perreault et al., 2011a). A D1/D2 co-expressing subpopulation would likely have been activated by laser in both D1 Chr2 mice and D2 ChR2 mice, again potentially contributing to overlap in functional connectivity and in behavioral effects. Finally, we note that D2 receptors are expressed also by >80% of acetylcholine striatal interneurons (Brene et al., 1990; Le Moine et al., 1990), which also would have been excited by NAc ChR2 stimulation in D2 Cre mice here. NAc acetylcholine neurons contribute to appetitive motivation (Witten et al., 2010; Castro et al., 2016; Collins et al., 2016), and so laser excitation of NAc acetylcholine neurons could conceivably have contributed to our D2 self-stimulation effects. Presumably acetylcholine neurons also were stimulated by investigators of other striatal regions where D2 ChR2 excitation apparently does not support self-stimulation, such as dorsolateral neostriatum (Kravitz et al., 2012), as well as for NAc in our location-based task, but this possibility still remains open.

In conclusion, our findings indicate that D2 neuronal excitation can support moderate appetitive motivation to self-stimulate in NAc, at least under some conditions. By comparison, D1 MSN excitation in NAc supports far more intense appetitive self-stimulation behavior, and does so reliably across multiple situations, including some where D2 neuronal excitation fails.

Beyond D2 being less intense than D1 in NAc for appetitive motivation, D2 NAc excitation also appears capable of flexible shifts in motivational valence from positive to neutral, or even from positive to negative, in the same individuals. This suggests that D2 NAc roles in motivation may be relatively ambivalent and plastic by comparison to D1 roles. These results underline the complexity of D1 versus D2 neuronal contributions to motivation in NAc, and add to evidence that NAc D1 and D2 neurons play distinct, yet potentially overlapping, roles.

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Figures

Figure 2.1. Dose-response screening of 0.1, 1.0, and 10 mW intensities at constant illumination vs 25 Hz pulses. Touches on a laser-spout produced either 0.1mW, 1mW or 10mW levels of laser intensity at either constant illumination (A) or 25Hz laser light pulses (B) delivered in 1-sec bins during 6 daily 30-min sessions in the initial screening test (all conditions counter balanced; n = 6 D1 ChR2, 5 D2 ChR2, and 15 EYFP control mice for 0.1 & 1.0 mW intensities; 4 D1 ChR2 and 3 D2 ChR2 10mW intensity). NAc ChR2 self-stimulation levels were roughly proportional to mW laser intensities, both for D1 and for D2 mice. However, self-stimulation levels were much lower for D2 mice than for D1 mice, especially under constant illumination conditions. Data shown are mean ±SEM; comparison bars and asterisks denote statistical differences between conditions with alpha set to $p<0.05$.

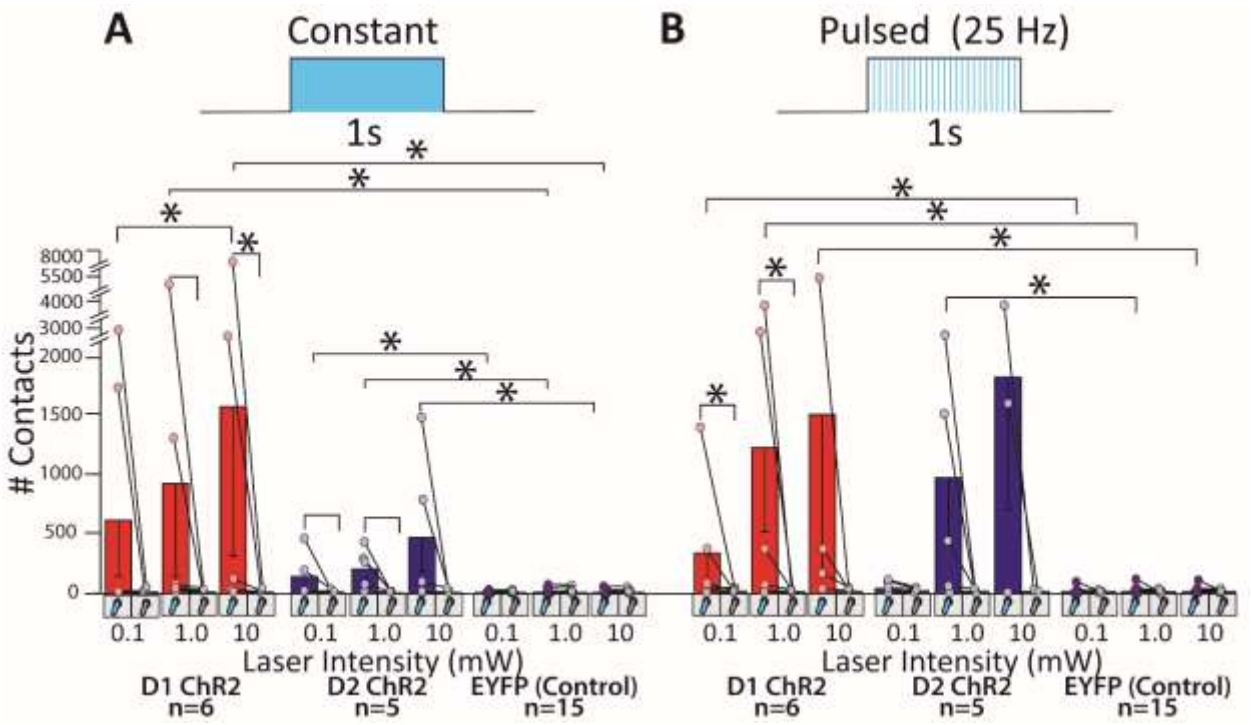


Figure 2.2. Spout self-stimulation totals for extended 9-day spout-task (1 mW, constant illumination, 1s): strong D1 and weak D2 self-stimulation. A) Locations in chamber are shown for laser-spout and non-laser spout, with novel position shifts on Day 4 and Day 7. B) Self-stimulation totals for D1 mice (red bar-laser spout) or D2 (blue bar- laser spout) mice with ChR2 and EYFP control mice (purple histogram-laser spout; gray = non laser inactive spout for all mice). Bars show mean contacts per day for D1, D2 and EYFP groups, across all 9 days, and connected dots show individual mouse average contacts on laser-spout vs non-laser spout (linear y-axis; 9 D1 ChR2 mice, 9 D2 ChR2 mice, and 12 EYFP D1/D2 control mice; pie charts show percentage of laser vs non-laser spout**).** D1 mice developed robust self-stimulation and bias for the laser-paired spout, making >500 times on average and at a ~27:1 ratio for laser-spout vs non-laser spout. D2 mice also developed preferences for the laser-paired spout (blue) though more weakly, self-stimulating 60 times on average per session at a ~ 12:1 ratio for laser-spout vs non-laser spout. Mice receiving either D1 or D2 depolarization self-stimulated at rates higher than inactive-EFYP viral controls (see left; purple), reaching 7700% and 850% above control spout contacts. **C)** Logarithmic totals. Log y-axis more clearly reveals differences for D2 vs EYFP groups between laser-spout and non-laser spout. **D)** Extinction (no-laser) test on Day 10 (compared to preceding Day 9 with laser)**.** No laser was earned by either spout contact, and only auditory cues were delivered (laser extinction/removed) (D1=9 mice; D2=9 mice; EYFP=12 mice), animals received a 10^{th} session, where. Both D1 and D2 mice virtually ceased responding when laser stimulation of ChR2 was discontinued. Data shown are mean ±SEM; * $p<0.05$.

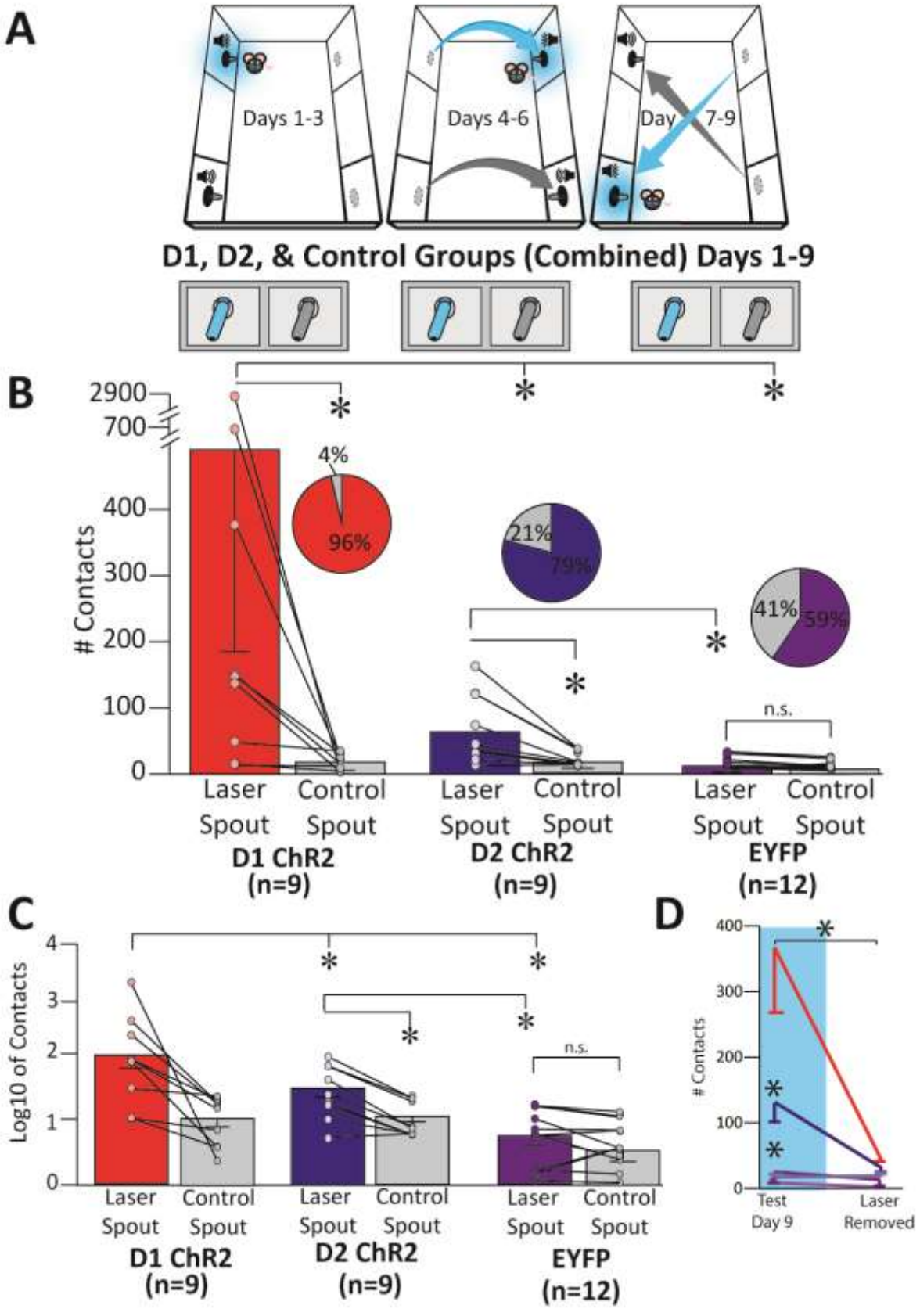

A
Days 1-3
Days 4-6
Day 7-9
D1, D2, & Control Groups (Combined) Days 1-9
B
Contacts
2900
700
400
300
200
100
0
4%
96%
21%
79%
41%
59%
n.s.
Laser Spout
Control Spout
D1 ChR2 (n=9)
D2 ChR2 (n=9)
EYFP (n=12)
C
Log10 of Contacts
4
3
2
1
0
n.s.
D1 ChR2 (n=9)
D2 ChR2 (n=9)
EYFP (n=12)
D
Contacts
400
300
200
100
0
Test Day 9
Laser Removed

Figure 2.3. Tracking new positions: Minute-by-minute spout contacts by ChR2 mice on Day 1, Day 4 and Day 7. Touches per minute are shown by descending horizontal bars for each day (**A**: D1 mice at left side, n=9; **B**: D2 mice at right side, n=9). Bars projecting left from 0 vertical axes show laser-spout contacts per min (red for D1 mice; blue for D2 mice). Bars projecting right from vertical axis show non-laser spout contacts for same min (gray for all mice). Cumulative touches on within the day are shown by 2-line graphs at left of bar graph for each day, together with time-point at which touches on laser vs non-laser spouts became statistically different on that day. Pie charts show the percentage of laser spout contacts vs non-laser spout contacts per day On Day 1, the position of laser-spout and non-laser spout are new, and D1 mice begin to self-stimulate NAc ChR2 within first two minutes, while D2 mice take about 10 minutes to begin. On Day 4, with new positions on opposite wall, D1 mice again begin within two minutes, while D2 mice take about 10 min to begin. On Day 7, with a third new position for each spout (reversed from Day 1), both D1 and D2 mice begin to self-stimulate within first minute. Data shown are mean ±SEM; * $p<0.05$.

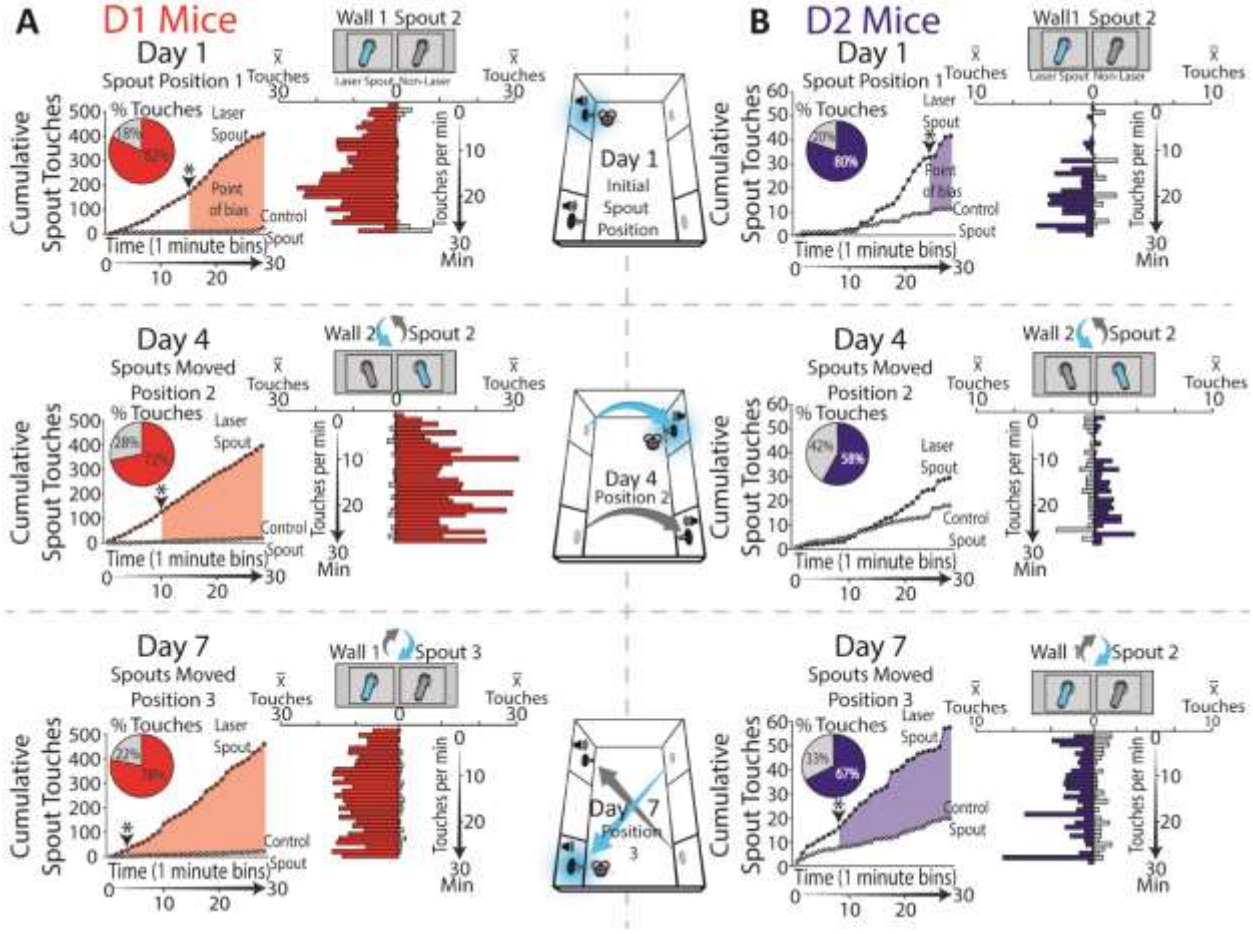


Figure 2.4. Location based NAc ChR2 task: D1 self-stimulation, D2 gradual subset avoidance. A square chamber, with center occluded, allowed mice to earn NAc laser stimulations by entering each day's laser-designated corner, where an infrared motion detector triggered a 1mW, 1s, constant pulse of laser upon entry and again upon each subsequent detected movement in that corner (30-min session). The laser-designated corner moved each day for three days. **A) Time spent:** D1 mice (n=9) significantly spent more time in their laser corner each day than in other corner. By contrast, D2 ChR2 (n=9) mice showed no preference for any corner overall. **B) Number of corner detector triggers:** D1 mice earned an average of approximately 180 ChR2 laser bins via their laser-corner, whereas D2 mice and inactive-virus control mice received only two-thirds of that amount, respectively. **C)** D2 gradual avoiders: By Day 3, a subset of D2 ChR2 mice (n=7/9 mice) mostly showed avoidance of laser-paired locations, spending only 19% of time in the laser corner **D) Correlation between spout-touch vs place-based self-administration.** D1 ChR2 spout self-stimulation is correlated with stronger preference for laser-paired locations. By contrast, D2 mice show no correlation, as individuals with high self-administration in spout-task become likely to avoid laser-corner in the location-based task by Day 3. **E) Locomotion:** D1, D2, and inactive-virus control mice all showed similar amounts of time in locomotion indicating that differences in corner preference a were not simply due to differences in motor effects. Data shown are mean ±SEM; * $p<0.05$.

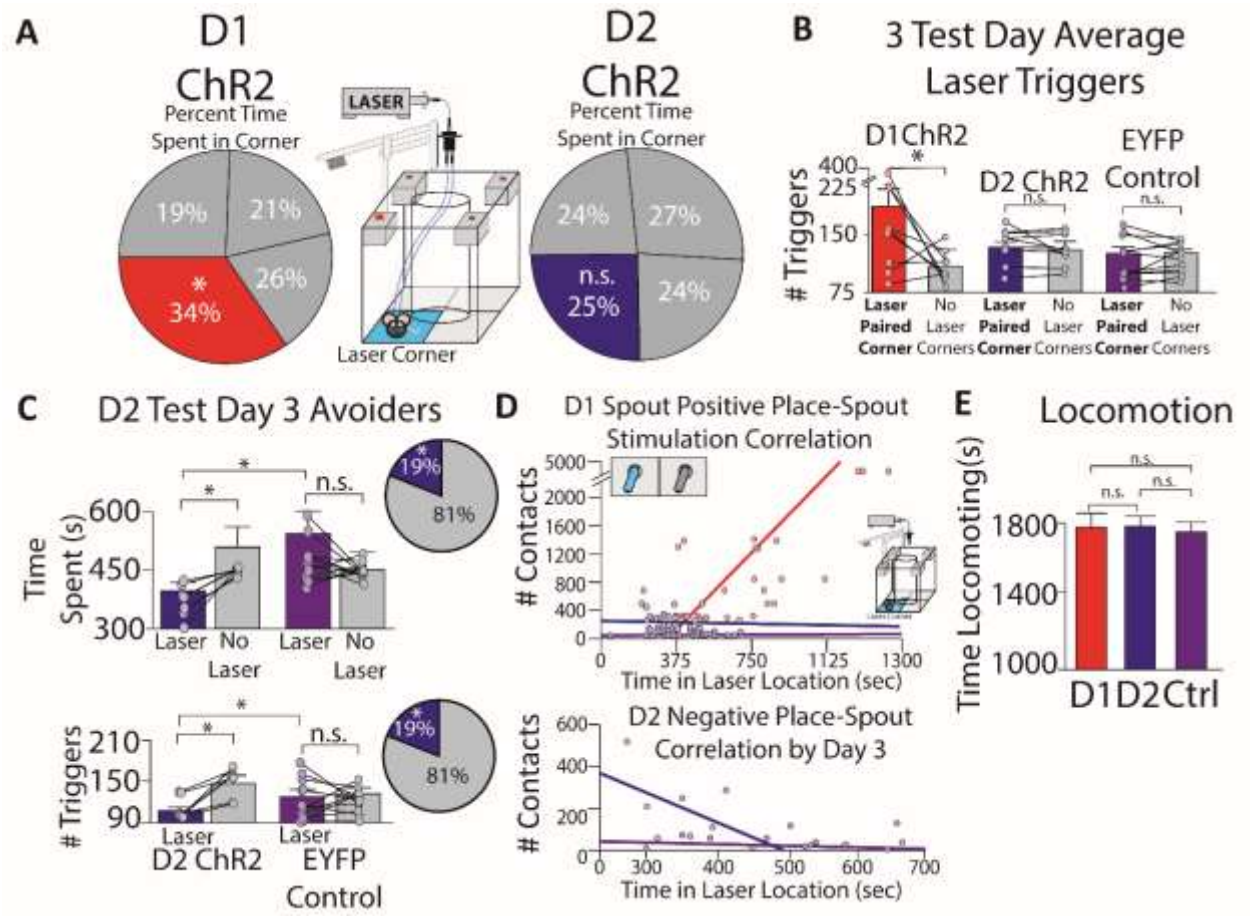


Figure 2.5. Anatomical sites in NAc: shell and core sites support self-stimulation. Circle symbol locations show individual D1 (A) or D2 (B) sites in coronal, sagittal, and horizontal planes. Circle colors show the level of self-stimulation supported at each site (measured in the same mouse). Symbol sizes show the mean diameters of concentric Fos plumes (produced by laser illumination of ChR2 prior to perfusion). D1 2 Fiber optic placements per animal for D1 mice (n=17) or D2 (n=17) mice mapped on to **A)** D1 sites were within NAc medial shell or core which supported similar levels of 500 to 4000 self-stimulations per spout session. **B)** D2 ChR2 sites (n=14) were similarly within either NAc shell or NAc core, and generally supported self-stimulation at levels between showed moderate rates of 50 to 400 self-stimulations per session.

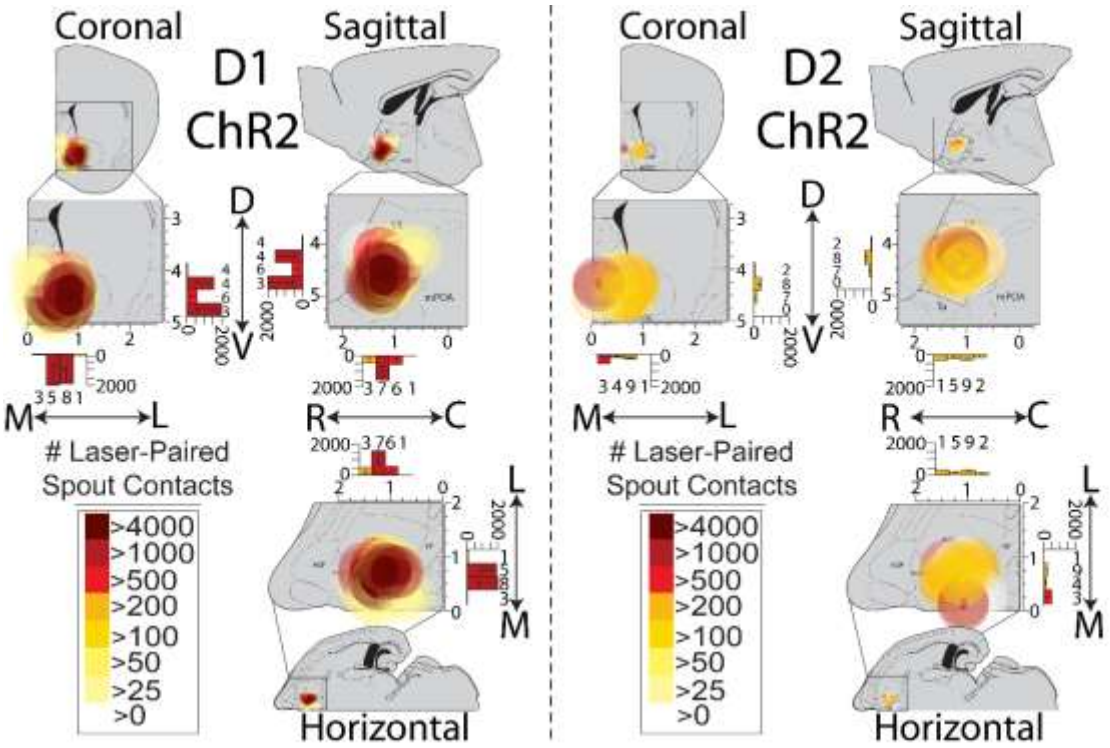


Figure 2.6. Laser-induced Fos plumes and virus expression in NAc. Top A: Overlay o immunohistochemistry-labeled Fos protein expression (AlexaFluor488; red), virus expression **(**enhanced yellow florescent protein; EYFP; green), and calculated Fos plume boundaries (% Fos elevation induced by laser + ChR2 over EYFP baselines from **B)**: D1 mice are in left column; D2 mice are in right column receiving laser stimulation) Squares emanate along radial arms from the center of the fiber tip. Each square represents a 100μm x 100μm area. All images are obtained from mice that received ChR2 virus microinjections and that received laser stimulations immediately before euthanasia. **B)** Virus spread alone: D1 mean diameter of 1.12mm (SEM±.11mm), and D2 diameter of 0.80mm (SEM±.11mm). **C)** Individual plume boundaries, assessed by different intensities of laser+ChR2 Fos elevation above EYFP control baselines (>125%; >150%; >200%indicated by colors of dashed lines). **D)** Mean diameters of Fos plume intensity zones. D1 ChR2 stimulation produced a 0.32mm inner plume reaching 200% elevation of Fos above EYFP control, with a larger 0.60mm diameter middle plume of >150% elevation of Fos, surrounded by outer >125% elevation in Fos 0.96mm in diameter. D2 ChR2 plumes had lower intensity centers of >200% Fos elevation with 0.04mm diameter, a larger 0.30mm diameter middle plume of >150% enhancement, and an outer 1.0mm plume of >125% Fos enhancement.

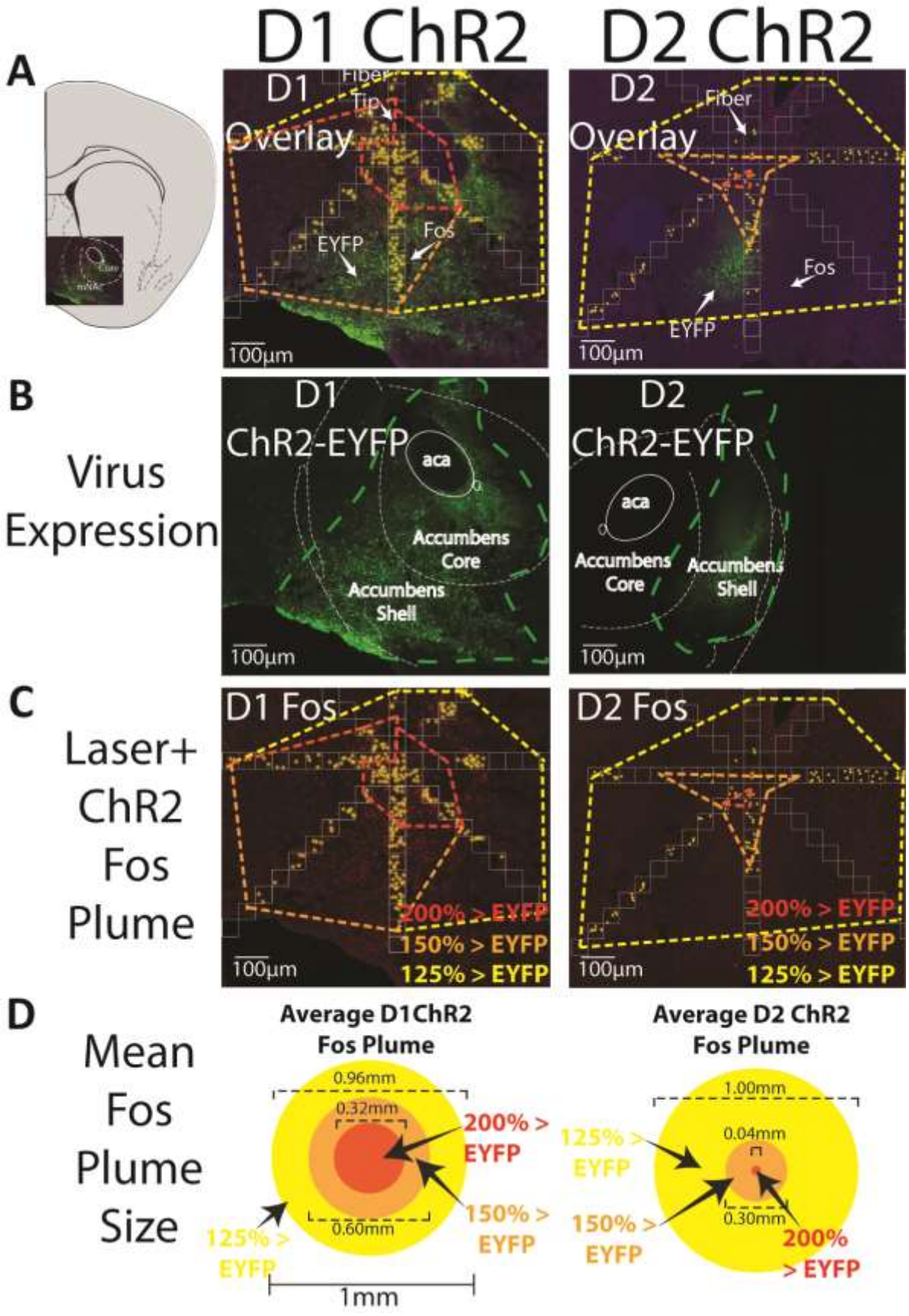
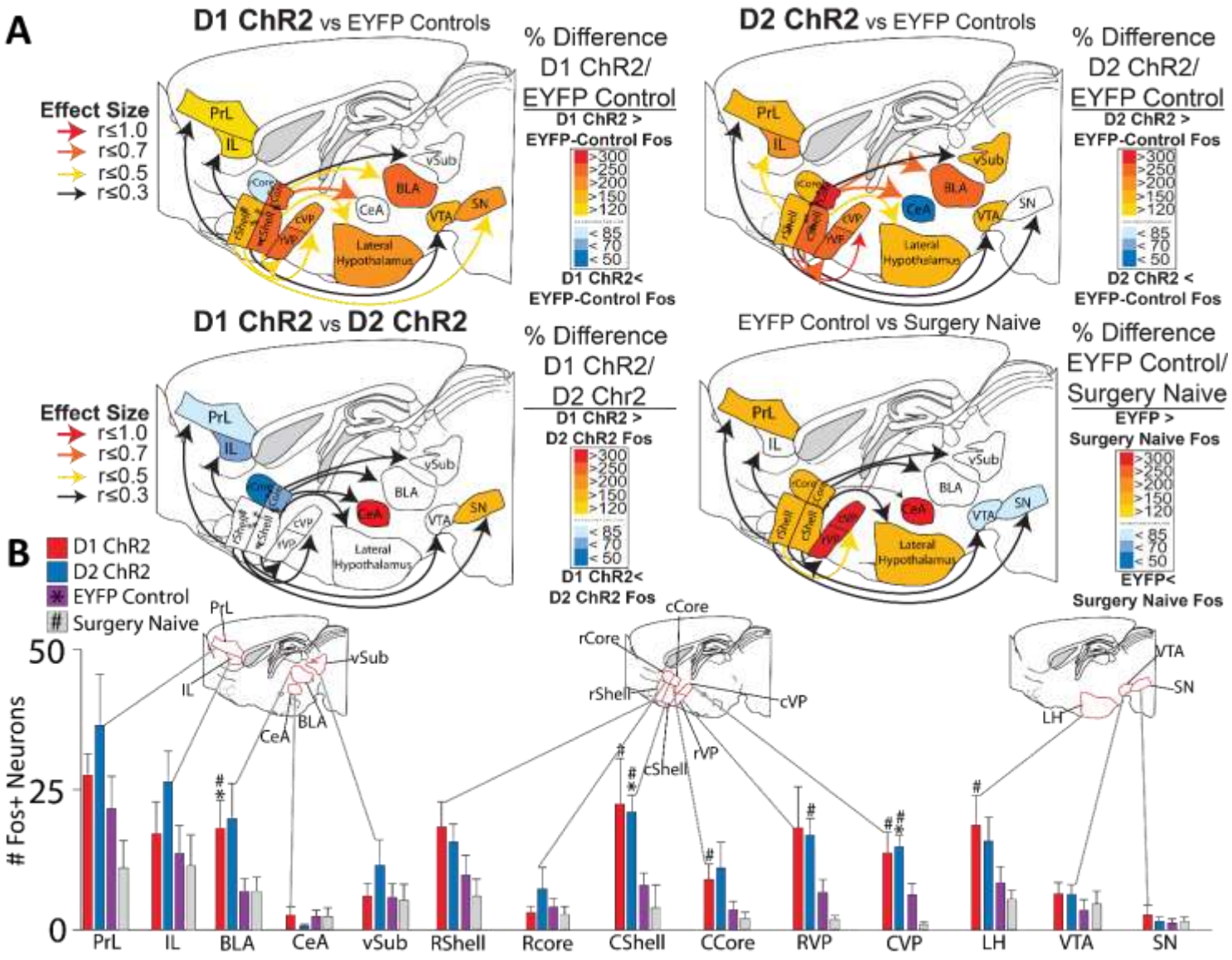

D1 ChR2
D2 ChR2
A
D1
Overlay
Fiber
Tip
EYFP
Fos
100µm
D2
Overlay
Fiber
Fos
EYFP
100µm
B
Virus
Expression
D1
ChR2-EYFP
aca
Accumbens
Core
Accumbens
Shell
100µm
D2
ChR2-EYFP
aca
Accumbens
Core
Accumbens
Shell
100µm
C
Laser+
ChR2
Fos
Plume
D1 Fos
200% > EYFP
150% > EYFP
125% > EYFP
100µm
D2 Fos
200% > EYFP
150% > EYFP
125% > EYFP
100µm
D
Mean
Fos
Plume
Size
Average D1ChR2
Fos Plume
0.96mm
0.32mm
0.60mm
200% >
EYFP
150% >
EYFP
125% >
EYFP
1mm
Average D2 ChR2
Fos Plume
1.00mm
0.04mm
0.30mm
125% >
EYFP
150% >
EYFP
200%
> EYFP

Figure 2.7. D1 vs D2 maps of distant Fos recruitment in limbic structures. A) Sagittal maps show relative Fos elevations in each structure induced by NAc laser stimulation in D1 ChR2 mice compared to EYFP control mice (top left), D2 ChR2 mice compared to EYFP controls (top right), and a direct contrast between D1 ChR2 mice and D2 ChR2 mice (bottom left). A baseline comparison map shows elevation of Fos in EYFP mice over unoperated naïve D1/D2 control mice. Similar patterns of ChR2 elevation were produced in D1 and D2 mice (compared to EYFP), and the only chief differences being that D1 mice had higher elevation in central nucleus of amygdala and in substantia nigra, whereas D2 mice had higher laser-induced elevations in medial prefrontal cortex. In each map, arrow size denotes effect size of Fos change (assessed using the formula r=Z/sqrt (N) and color of structure denotes percentage change in Fos in that structure. **B)** Bar histograms showing mean (+SEM) absolute levels of Fos expression for each group/structure. Neurons were counted in three 600umx800um core samples in each indicated structure or subregion. Red bars =D1 ChR2 mice; blue=D2 ChR2 mice; Purple =EYFP mice; gray = unoperated/surgically-naïve control mice. *=different from EYFP control at p=0.05. #=different from surgery naïve controls at p=0.05. Brain region abbreviations are: Prelimbic cortex (PrL), Infralimbic cortex (IL), Basolateral Amygdala (BLA), central nucleus of Amygdala (CeA), ventral subiculum (vSub), rostral NAc Shell (rShell), rostral NAc core (rCore), caudal half of NAc medial shell (cShell), caudal NAc Core (cCore), rostral ventral pallidum (rVP), caudal Ventral pallidum (cVP), lateral hypothalamus (LH), ventral tegmental area (VTA), substantia nigra (SN).


A
D1 ChR2 vs EYFP Controls
% Difference D1 ChR2/ EYFP Control
D1 ChR2 > EYFP-Control Fos
D1 ChR2< EYFP-Control Fos
D2 ChR2 vs EYFP Controls
% Difference D2 ChR2/ EYFP Control
D2 ChR2 > EYFP-Control Fos
D2 ChR2 < EYFP-Control Fos
Effect Size
r≤1.0
r≤0.7
r≤0.5
r≤0.3
D1 ChR2 vs D2 ChR2
% Difference D1 ChR2/ D2 Chr2
D1 ChR2 > D2 ChR2 Fos
D1 ChR2< D2 ChR2 Fos
EYFP Control vs Surgery Naive
% Difference EYFP Control/ Surgery Naive
EYFP > Surgery Naive Fos
EYFP< Surgery Naive Fos
PrL
IL
vSub
BLA
CeA
VTA
SN
Lateral Hypothalamus
B
D1 ChR2
D2 ChR2
EYFP Control
Surgery Naive
Fos+ Neurons
50
25
0
PrL
IL
BLA
CeA
vSub
RShell
Rcore
CShell
CCore
RVP
CVP
LH
VTA
SN

Chapter 3

Optogenetic Stimulation Reverses DNQX-Motivation

Introduction

Manipulations of amino acid neurotransmission via the AMPA-glutamate antagonist DNQX or the GABA-A agonist muscimol in locations of NAc medial shell can produce bivalent and intense affective and motivated states of opposite valence, such as desire (i.e., positively valenced in the sense that microinjections produce positive place preference and appetitive eating behavior) versus dread (i.e., negatively valenced in the sense that microinjections produce negative place avoidance, fearful vocalization and defensive treading and biting) or both together (Reynolds and Berridge, 2001, 2002, 2008; Faure et al., 2010; Richard and Berridge, 2011b; Richard et al., 2013). Microinjections of muscimol generate these appetitive vs avoidant/fearful motivations along a rostrocaudal axis, with microinfusions in rostral regions producing desire, those at caudal sites producing fear, with moderate levels of both in intermediate sites. Additionally, there are distinct profiles of this GABA activation or glutamate blockade. The motivated behaviors generated by DNQX-glutamate blockade in this rostrocaudal gradient, or "keyboard," can be environmentally retuned and modulated by psychological factors, such as environmental ambience (Reynolds and Berridge, 2008; Richard and Berridge, 2011b), and by neurobiological factors, such as inputs from prefrontal cortex (Richard and Berridge, 2013)

providing further indication of overlapping psychological and physiological processes between distinct valences of motivation. Further, DNQX-microinjections require D1 dopamine activity at the same NAc site for both feeding and fear, but only D2 dopamine for fear (Faure et al., 2010; Richard and Berridge, 2013). However, GABA activation fits a slightly different profile: 1) muscimol microinfusions resists environmental retuning and are not dependent on endogenous local dopamine (Richard and Berridge, 2011b). Furthermore, GABA activation of muscimol produces enhancement of hedonic reactions to sucrose solutions at rostral sites, and suppression of pleasure and increased disgust reactions in caudal site, which DNQX-glutamate blockade does not (Reynolds and Berridge, 2002; Faure et al., 2010). A proposed neurobiological explanation is that a GABA agonist or glutamate antagonist microinjection commonly induce *inhibition* of GABAergic medium spiny neurons (MSNs) within NAc shell, whereas DNQX blockade of glutamate merely blocks excitations (producing relative inhibition, but not absolute inhibition below resting potential). Both disinhibit to different degrees downstream projections to targets such as LH, VP, or ventral tegmental area (VTA) from the tonic suppression that is usually exerted by NAc GABAergic projections (Mogenson et al., 1983; Zahm and Heimer, 1990; Heimer et al., 1991; Lu et al., 1998; Usuda et al., 1998; Zhou et al., 2003; Humphries and Prescott, 2010). Others have shown that GABA-A stimulation of food intake, in at least rostral shell sites, requires VP and LH recruitment, as pharmacological inhibition or lesion of VP or LH attenuates the NAc-induced increase in eating (Stratford and Kelley, 1999; Stratford and Wirtshafter, 2012; Urstadt et al., 2013b; Urstadt et al., 2013a). However, the distinct profiles of DNQX and muscimol suggests an alternative to specific receptor-based properties may be responsible for the generation of these motivations.

Despite the considerable work over the last 20 years examining DNQX and muscimol induced motivation, it is not clear whether NAc neuronal inhibition is the key mechanism by which intense fear and feeding behaviors are generated, or whether it is due to alternative receptor-based mechanisms. Here, I tested whether DNQX-induced motivation *requires neuronal hyperpolarization* by combating DNQX-microinjections with optogenetic, ChR2 excitation to induce relative depolarization, and thus potentially reverse the ability of DNQX microinjections to cause motivated behavior.

Methods

Subjects

Twenty-two male Sprague Dawley rats (fiber optics aligned and cannulae within NAc shell = 9; fiber optics misaligned and cannulae within NAc shell = 5; cannulae not within NAc shell = 8); 300-500 g and at least 3 months of age at time of testing) were housed in pairs or groups of three on a 12:12-hour reverse light/dark cycle at ~21°C with *ad libitum* access to food (Purina Rat Chow) and water. Statistical analysis was performed using data from fourteen of twenty-two rats based on histological placements within the NAc shell. Eight rats were excluded from statistical analysis because their cannula placements were not within the NAc shell. Subjects were procured from both an in-house breeding colony and research model services (Envigo, Cambridgeshire, UK).. Animals with fiber optics greater than 1.0 mm away from the site of injection (fiber optics misaligned) but had cannulae within the NAc shell were excluded in the experimental analysis, but were categorized as anatomical control subjects. All experimental procedures were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of

Michigan and carried out in accordance with the guidelines on animal care and use by the National Institutes of Health.

Surgery

Cranial cannulation and fiber implantation.

All rats were anesthetized using isoflurane (5% induction, maintenance at 1-2%) and pretreated with atropine (.05 mg/kg) to prevent respiratory distress. Rats additionally received injections of carprofen (5.0 mg/kg) for analgesia and cefazolin (75 mg/kg) to prevent infection, after induction with isoflurane. Rats were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA), with the mouth bar set to 5.0 mm above intra-aural zero so that the cannulae can be inserted at an angle to avoid puncturing the lateral ventricles. Bilateral stainless steel cannulae (14 mm, 23-gauge) and fiber implants approximately 8-9 mm in length were aimed at predetermined sites throughout the medial shell (rostral, intermediate, and caudal sites) or at anatomical control sites. Once the cannulae were inserted at 5.0 mm above intra-aural zero, .5 μL of AAV5-hSYN-ChR2-YeFP virus (UNC Vector Core, Chapel Hill, NC, USA) was injected on each side at a rate of .1 μL per minute. Virus was allowed to diffuse for 10 minutes after the 5-minute microinjection. After the virus diffusion, fiber implants were inserted in a flat-skull position at a 16.4° lateral angle for precise stimulation of the injection target sites.

Anatomical Coordinates

The coordinates used for each rat were symmetrical, but placements within the experimental group were staggered in the anterior-posterior plane to eventually map the medial shell. Cannula placements were aimed at coordinates antero-posterior (AP) +2.4 to +3.0, medio-lateral (ML) ±

.8 to 1.0 mm and dorsoventral (DV) +5.6 mm in comparison to bregma. Fiber implants were aimed at coordinates antero-posterior (AP) +2.5 to +1.0, medio-lateral (ML) ± 3.0 to ± 3.2 and dorsoventral (DV) -6.5 to -7.0 mm in comparison to bregma. Cannula and fiber implants were secured to the skull with four surgical screws and standard dental acrylic. Stainless steel stylets (28-gauge) were inserted into the cannulae to prevent infection and clotting.

Handling and Habituation

All rats were monitored post-operation for 7 days, during which they received daily triple antibiotic ointment around the skullcap. On the first day of the recovery period, subjects were administered the same dose of carprofen that they received during surgery for pain relief. To allow for adequate ChR2 expression, the virus was given at least 4 weeks to incubate before testing. Before habituation, rats were handled for at least 15 minutes each for 2-3 days. Habituation involved the rat being in the testing apparatus with cob bedding and *ad libitum* access to food and water for 1 hour so that they would become familiar with the testing environment. Optogenetic cables were attached to the fiber implants for all subjects during habituation. Laser power supplies were turned on during these sessions for consistent ambient sound, but laser output was turned off. On the fourth day of habituation, rats received a mock microinjection of vehicle solution and had optogenetic cables attached to the fiber implants with no laser output.

Intracranial Microinjections

Drug microinjections were administered bilaterally, except in the cases when the fiber implants had broken off or been pulled out of the skullcaps. In these cases, a unilateral microinjection on

the same side as the remaining fiber implant was performed. Drug microinjections of .5-μL-per-side were spaced 48 hours apart on test days to ensure that there were no lingering drug effects. Rats received either the AMPA antagonist DNQX (Sigma-Aldrich Corporation, St. Louis, MO, USA) or vehicle (50% DMSO and 50% .15M saline) according to the assigned condition. The dose of DNQX was 220 ng/.5 μL per side based on previous studies in our laboratory which attempted to counter DNQX effects through excitation of prefrontal cortical regions (Richard and Berridge, 2013), suggesting that DNQX could be reversed by excitatory inputs at this dose.

The microinjection was set at a rate of 0.3 μL/minute using a microinjection syringe pump (Hamilton Company, Reno, NV, USA) attached via PE-20 tubing to stainless steel injectors (16 mm, 29-gauge). The injector tips extended 2.0 mm beyond the end of the cannulae (7.6 mm on the dorsoventral plane). Before the microinjection, rats were individually taken out of their home cages and the experimenter removed the stylet protecting the cannulae to start drug administration. After the microinjection was complete, the injectors were left in place ~1 minute to allow for drug diffusion. The stainless-steel stylet was replaced after microinjections were performed bilaterally and the rat was then immediately put into the testing chamber.

Optogenetic Laser Stimulation

For all conditions, all rats had two fiber optic cables attached to their fiber implants (one if the rats only had one fiber optic, see above section). Laser power supplies were turned on for all conditions (the no laser condition had no output) to control for the sound of the fans, though laser was activated only during particular conditions. Laser illumination was programmed to elicit 5 s of 25 Hz (5 ms ON, 25 ms OFF) blue (473 nm) laser stimulation with 15 s in between the laser turning on. Fiber optic output was measured to be > 85% efficiency, and laser intensity was

between the ranges of 8-10 mW. Laser was presented continuously in this fashion for the duration of the testing session.

Behavioral Food Intake Testing

All animals were subject to four test days with four different conditions counterbalanced for order: (i) standard (vehicle microinjection, no laser), (ii) laser condition (vehicle microinjection), (iii) DNQX condition (no laser), and (iv) test condition (DNQX microinjection, laser stimulation). Each testing session lasted 1 hour. The rats were placed in a transparent testing chamber (25.5 x 46 x 46 cm) with at least 3 cm of bedding, a water cup taped to the bottom on one side, and a pre-weighed amount of rat chow (25-30 g of Purina rat chow). The testing apparatus was in a conventional laboratory setting with normal, ambient illumination. A video camera was placed in front of each testing apparatus to record all sessions. At the end of the testing session, experimenters used a standardized approach to retrieve the animal from the apparatus utilizing a stereotyped movement and grabbing pattern. Experimenters took three slow steps from the door to the side of the testing chamber (3 s), reached inside the testing apparatus, stroked the side of the animal (1 s), and slowly lifted the animal out of the chamber (1 s) within a 5 s interval. Experimenters noted any bite attempts, distress calls, escape attempts, or defensive treading directed at the experimenter during the retrieval process.

Behavioral Coding of Video-recorded Behaviors

All sessions on test days were video-recorded for offline analysis. During the test days, experimenters retrieved the rats from the testing apparatus using the rehearsed procedure. Offline observers scored the duration (measured in seconds) of spontaneously emitted behaviors during

the recorded sessions for each of the following: eating (actively chewing and swallowing food), drinking, defensive treading/treading-burrowing behavior, grooming, escape behaviors (subject tries to move away from the experimenter's reach), rearing (forepaws are lifted at least 3 cm off the floor), and immobile/sleeping. Discrete events include: cage crosses (counted when an animal moves at least half the length of the testing chamber in one bout of movement), sniffing and carrying food (counted as each second that the animal is engaged in behavior), bouts of eating (counted as the number of times subject initiated eating), and distress vocalizations.

Histology

Following the last behavioral testing session, rats were sacrificed with an overdose of sodium pentobarbital (0.8 mL). Brains were extracted and fixed in 4% paraformaldehyde for 1-2 days, then placed in 25% sucrose solution for 2 days. To assess microinjection, fiber implant, and virus expression sites, brains were sliced at 40 μm on a cryostat (Leica Biosystems, Buffalo Grove, IL, USA). Skullcaps were also removed after the transcardial perfusion to assess general proximity of fiber implants to cranial cannula placements (see Fig 3.1 for example). Sites were mapped onto coronal slices from a rat brain atlas (Paxinos and Watson, 2007), and positions were extrapolated and transferred onto a sagittal slice. Intended and confirmed cannulation and fiber implant sites were mapped using a color-coded system on Adobe Illustrator to express the percent decrease of food intake from the DNQX-no laser condition to the vehicle-laser condition.

Statistical Analysis

To test the initial hypothesis, we tested the effect of drug microinjection and laser stimulation on behaviors of interest using a three-factor repeated-measures between-subjects ANOVA (drug x

fiber alignment x anatomical placements anterior to bregma). Follow up planned comparisons utilizing interactions of placement by drug/laser conditions and main effects of drug/laser conditions were compared utilizing planned testing of two-way ANOVAs, one-way ANOVA, and pair-wise post hoc comparisons between vehicle, drug, and drug by laser conditions. For statistical analysis, rostral or caudal classification is determined by previous studies. These have indicated that AP coordinates > 1.7 mm ahead of bregma were placed in the rostral group and < 1.7 mm ahead of bregma in the caudal group. When significant main effects prompted additional statistical analysis performed with one-way ANOVA and post hoc comparison with the Tukey test. Data was considered statistically significant if $p<.05$.

Results

Nine animals were confirmed to have cannulae placements targeting rostral NAc shell, and six rats had cannulae in caudal sites. Of the nine rostral animals, six also had fiber optics that were aligned with its ipsilateral microinjection cannula (meaning, that they were within 1 mm of the cannula placement on both sides). Three of the caudal cannulae placement rats had aligned fiber optics. In sum, nine rats had cannulae within the NAc shell and aligned fiber optics, while five animals had fiber optics that were misaligned. Subsequently, statistical analysis was performed on these groups separately to determine the effect of alignment, as well as drug microinjection and laser conditions, on behaviors.

DNQX-mediated glutamate blockade increases food intake

Overall, the drug and laser test conditions affected observed behaviors in subjects (one-way ANOVA effect of condition: $F_{18,81} = 3.054$; $p = .0001$). In particular, food intake, time eating,

and locomotion were different across drug and laser conditions (one-way ANOVA effect of condition x food intake: $F_{3,30}$ = 15.473, p = .001; one-way ANOVA effect of condition x time spent eating: $F_{3,30}$ = 9.327, p = .0001; one-way ANOVA effect of condition x locomotion: $F_{3,30}$ = 4.203; p = .014, Figures 3.2 & 3.4). Consistent with previous findings, DNQX administration alone in the rostral NAc shell increased food intake compared to vehicle microinjection alone (Reynolds and Berridge, 2003, 2008; Faure et al., 2010; Richard and Berridge, 2011b; Richard et al., 2013) see Figure 3.2). DNQX-glutamate blockade greatly increased eating >300% relative to animals given a microinjection vehicle solution (pairwise comparison DNQX/no laser vs vehicle/no laser on food intake: t = 2.874, p = .001). Animals consumed approximately 1.5g± .6g of chow when administered vehicle, compared to an average of 5.50± .6 during the DNQX test session (pairwise comparison DNQX/no laser x vehicle/no laser on food intake: t = 4.031, p = .001; see Figure 3.2).

Optogenetic laser stimulation reverses DNQX-mediated food intake

Combining ChR2 excitation with DNQX microinjection reversed drug-induced increases in eating behavior from elevated drug-alone levels. Amount of rat chow consumed during a DNQX with laser stimulation was reduced from DNQX alone. However, this laser-blockade of DNQX effect was only observed in animals who had fibers within 1mm of the microinjection site. In animals with aligned fibers the increase in eating evoked by NAc glutamate blockade was markedly decreased (pairwise comparison DNQX/laser x DNQX/no laser on food intake: t = 2.943, p = .001; see Figure 3.2 and 3.3), but not in rats that had >1 mm separation between their microinjection cannula and optic fiber. Rats with aligned fiber/cannula placements consumed 5.5 ± .6 grams of rat chow on average under the influence of DNQX alone, but this mean intake was

profoundly reduced to 2.6± .75 grams with laser stimulation added to DNQX (pairwise comparison fibers aligned-DNQX/laser vs DNQX/no laser on food intake: t = 2.943, p = .001). There was no difference in food intake between DNQX with laser and the control vehicle microinjection condition, suggesting that the laser stimulation suppressed DNQX-amplified food intake back statistically to baseline levels when fiber optics were aligned (pairwise comparison vehicle/no laser x DNQX/laser on food intake: t = 1.088, p = .189). By contrast, in rats who had fiber optics not aligned to the microinjector tip, DNQX produced food intake of approximately 3.2 grams (SEM± .7 grams) and the addition of ChR2 stimulation produced no change in food intake (pairwise comparison fibers not aligned-DNQX/laser vs DNQX/no laser on food intake: t = 0.836, p=.232). Thus, localized excitation of neurons immediately impacted by a DNQX microinjection, rather than general excitation within the NAc nearby, is necessary to counter increases in eating produced by localized DNQX-inhibitions. It should be noted that rats in fiber aligned group ate approximately 5.5g on average vs 3.5g in non-aligned animals, though no statistical differences were seen between the two groups (pairwise comparison aligned vs non-aligned: t=2.0, p=0.062). However, despite consumption differences produced by DNQX, laser stimulation suppressed food intake only in the aligned group and did not alter DNQX action on ingestive behavior for non-aligned animals. Interestingly, laser stimulation on its own was not sufficient to suppress chow consumption below vehicle and produced similar levels of food intake to vehicle microinjection (pairwise comparisons of food intake; aligned-vehicle/no laser x vehicle/laser: t = .121, p = .815; fibers not aligned-vehicle/no laser vs vehicle/laser: t=.268, p=0.702).

Glutamate blockade increases time spent eating

DNQX microinjections also produced an increase in the duration of eating throughout the hour-long session relative to vehicle or laser-stimulation alone (pairwise comparison fibers aligned-vehicle/no laser vs DNQX/no laser on duration of eating: $t = 258.542$, $p = .003$; pairwise comparison vehicle/laser vs DNQX/no laser on duration of eating: $t = 255.083$, $p = .003$; see Figure 3.2). DNQX eating was blunted by approximately 40% during ChR2 laser-stimulation, though the average time spent eating was still somewhat above vehicle (pairwise comparison fiber aligned-vehicle/no laser x DNQX/laser on duration of eating: $t = 217.375$, $p = .008$). This suggests that the laser reversal of DNQX-induced eating is substantial yet only partial, at least when measured by time spent eating rather than by grams of food consumed.

Alignment gates laser reversal of DNQX-induced effect on time spent eating

Analysis by subgroup confirmed that only rats with aligned fiber optics showed the laser-stimulation decrease in the duration of eating behaviors during a test session with DNQX compared to a DNQX administration alone (pairwise comparison DNQX/no laser x DNQX/laser on duration of eating: $t = 203.083$, $p = .04$; see Fig. 3.2). By contrast, rats with misaligned fibers showed no significant change in eating behavior (pairwise comparison fibers not aligned-DNQX vs DNQX/no laser on duration of eating: $t = 120.750$, $p = .218$), again demonstrating a requirement of local depolarization at the site of glutamate blockade.

In terms of the number of individual eating bouts, among fiber aligned animals, glutamate blockade increased the number of eating bouts (pairwise comparison vehicle/no laser x DNQX/no laser on number of eating bouts: $t = 8.083$, $p = .018$; Fig. 3.3) and laser stimulation produced a trend for lower on DNQX-mediated eating (pairwise comparison DNQX/no laser vs

DNQX/laser on number of eating bouts: t = 6.750, p = .085), and no differences were observed in the number of eating bouts between DNQX+laser stimulation and either vehicle or laser alone.

No effect of anatomical placement on observed behavior in subjects

In this cohort of rats, the entire NAc shell zone of appetitive motivation appeared expanded to include caudal sites as well as rostral sites, an effect which our lab has previously seen only when rats were tested in a comfortable home-cage environment. However, more sites were rostral than caudal, and further probing of the caudal is expected to yield defensive/fearful behavior. Here rostral-caudal anatomical placement did not appear to impact DNQX or laser effects on food intake, time spent eating, eating bouts, locomotion, or defensive reactions (two-way ANOVA test condition on food intake x anatomical placement: $F_{3,30}$ = 1.282, p = .299; two-way ANOVA test condition on time spent eating x anatomical placement: $F_{3,30}$ = .911, p = .447; two- way ANOVA test condition on eating bouts x anatomical placement: $F_{3,30}$ = 1.791, p = .170; two- way ANOVA test condition on locomotion x anatomical placement: $F_{3,30}$ = 2.163, p = .113; two- way ANOVA test condition on defensive reactions x anatomical placement: $F_{3,30}$ = 2.019; p = .132; see Figures 3.2 & 3.3). No differences were seen in DNQX enhanced food intake between rostral (average 4.6g+/- 1.9) and caudal (average of 5.0+/-2) halves of the NAc (One-Way ANOVA, $F_{1,14}$=0.156, p=.699).

No increases in defensive behaviors with DNQX administration

Almost no rats elicited any defensive behaviors under any drug or laser condition, and only in the form of distress vocalizations upon removal from the test chamber (i.e., no bite attempts or bites), and no differences were observed between test conditions (one-way ANOVA test

condition on defensive reactions: $F_{3,30}$ = 1.250; p = .309). Rats under the influence of DNQX did not elicit more defensive behaviors compared to vehicle levels (pairwise comparison vehicle/no laser vs DNQX/no laser on defensive reactions: t = .208, p = .152; see Figure 3.4), nor under DNQX+laser conditions (pairwise comparison vehicle/no laser vs DNQX/laser: t=0.125, p=0.290). Further, no differences were found following DNQX microinjections in rostral vs caudal subregions (One-Way ANOVA, DNQX/no laser rostral vs caudal: F1,14=0.101, p=0.756) nor in DNQX+laser conditions (One-Way ANOVA, DNQX/laser rostral vs caudal: F1,13=0.42, p=0.841). Additionally, we did not observe defensive treading under any conditions for any period of time.

No increases in locomotion with DNQX administration

Initial analyses indicated differences between drug and laser conditions (one-way ANOVA condition on locomotion: $F_{3,30}$ = 4.203; p = .014; Fig 3.4). Upon subsequent analysis, this effect appears to be primarily driven by animals with misaligned fibers. Specifically, in animals with fibers greater than 1mm away from the microinjection site, laser alone induced average locomotion of about 10 minutes of the 60-minute session which though statistically different from baseline, animals receiving only laser depolarization showed about 200% levels of locomotion compared to vehicle animals and about 300% higher than combination DNQX+laser (pairwise comparison, t=388, p=0.046). Further, analyzing in rats in which fibers were aligned no shift in locomotion was found (one-way ANOVA condition on locomotion: $F_{3,21}$ = 237; p = .870). Pair-wise comparisons further demonstrated that there were no other significant relationships between conditions for locomotion. This implies that there may be something about

the misalignment of fiber optics that contributed to large increases in time of locomotion during test conditions.

Discussion

Consistent with previous findings, DNQX administration robustly increased consumption and engagement in feeding behaviors during the testing session (Reynolds and Berridge, 2003, 2008; Richard and Berridge, 2011, 2013). Food intake, duration of eating, and number of eating bouts were all increased greatly by glutamate blockade. Through examination of NAc-gated motivation using optogenetics and pharmacological methods, we found that laser stimulation of medial shell targets can locally counteract the effects of DNQX microinjections through cellular depolarization. This indicates that motivations produced in the NAc by decreases in glutamatergic-AMPA caused by DNQX microinjections are mediated by neural inhibition of MSNs, as hypothesized by the inhibition hypothesis of drug-induced NAc motivation generation. That is, the intense appetitive motivation induced by DNQX microinjection requires local hyperpolarization as a key mechanism.

No observed rostrocaudal gradient effect

However, we were not able to establish a rostrocaudal gradient that was reported in previous studies (Reynolds and Berridge, 2002, 2003, 2008; Richard and Berridge, 2011, 2013). This may be in part due to relatively small sample sizes across the NAc shell, especially in caudal shell. The lack of a rostrocaudal gradient could also be due to a few reasons: 1) differences in rats from previous years, either genetic or in environmental conditions. For example, environmental enrichment in housing conditions, such as the addition of toys or nesting, has increased in recent

years, which conceivably could have some effects on emotional reactivity similar to testing in a home-cage environment. 2) The addition of the optogenetic cables attached to the fiber optic implants could have been a distraction for the animals during the testing sessions, preventing fearfully salient stimuli from being acted upon or noticed over other sensory stimuli in the testing environment. Previous studies did not use optogenetic techniques or methods in which animals were fitted with a cranial tether, so this has not been an issue (Reynolds and Berridge, 2003, 2008; Richard and Berridge, 2011, 2013). 3) Though unlikely, with the addition of lasers for optogenetic stimulation, fans for the laser power supply were left online across all conditions. The ambient "white noise" produced by the power supplies may muffle sounds from experimenters throughout the session, which may otherwise startle or alert the rats. 4) Another possibility is the lack of a cage top for the testing chamber. Past microinjection studies have utilized a standard transparent cage with a metal cage top. However, the present study could not allow for this experimental design due to the attachment of optogenetic cables. For the animal to have free range within the testing chamber, the test chamber was fitted with a wide opening at the top to allow for the optogenetic cables to move in along with test animals. Typically, animal treading has been directed to front portions of testing cages, to experimenters present throughout testing, and towards the entrance/exist through which animals are entered and removed from test chambers (Richard &Berridge, 2011) or to a direct threat, such as a shock prod (Reynolds &Berridge 2001). In previous studies, experimenters approaching or opening the stainless-steel cage lid could have been seen as a physical invasion of the rat's enclosure. Wild California ground squirrels have been observed to defensively tread by kicking sand and dirt at snakes and other predators attempting to enter their burrow (Cross and Owings, 1978). However, in our studies, 1) an open top and 2) taller testing chamber may produce a different suite of

spatial/contextual-dependent behaviors. Therefore, making the testing chamber more "burrow-like" and enclosed could potentially evoke more defensive behaviors in our subjects.

In one of our pilot studies, we have used the same testing room as the previous studies in our laboratory that have also investigated the localized amino acid disruptions within the NAc shell (Reynolds and Berridge, 2003, 2008; Richard and Berridge, 2011, 2013). In these investigations, we attempted to replicate these former experiments by using additional light and noise stimulation, and on occasion observed that animals evoked more fear responses. On one occasion, experimenter experienced multiple bite attempts while retrieving a rat, whereas our present study rats were relatively calm during the end of the testing session. However, it should be noted that this experimenter in this instance did not follow our typical approach, instead producing rapid/jittery movements in an attempt to retrieve test rats. Thus, there might be something unique about the experimental setup that was used in previous studies that permitted the behavioral manifestation of fearful salience. It also should be noted that in this study DNQX-enhancement of food intake occurred even at caudal regions. While feeding can occur at more caudal locations, in other studies it was generally observed to produce about 1/3 to 1/2 that of what is observed by rostral DNQX microinjections (Reynolds and Berridge, 2008; Richard and Berridge, 2011)

Laser stimulation in NAc shell partly blocked drug-induced increases in appetitive motivation

One popular hypothesis is that the hyperpolarization of MSNs in NAc is the primary mechanism for generating appetitive motivation (Carlezon and Wise, 1996; Cheer et al., 2005; Roitman et al., 2005; Taha and Fields, 2006; Meredith et al., 2008; Roitman et al., 2008; Wheeler et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010). The inhibition of NAc projection

neurons is viewed by this hypothesis to release downstream neurons in target structures from chronic GABAergic suppression, and consequently disinhibit those target neurons into states of excitation. This hypothesis is supported by findings that neural excitations in downstream targets, such as VP, LH, or VTA occur during reward events (Ljungberg et al., 1991; Baldo et al., 2004; Stratford, 2005; Bromberg-Martin and Hikosaka, 2009; Tindell et al., 2009; Smith et al., 2011). Furthermore, the NAc inhibition hypothesis fits the desire-dread 'keyboard' effects of inhibitory drug microinjections, such as muscimol (a GABA agonist which should hyperpolarize NAc neurons) or DNQX (a glutamate AMPA antagonist which should induce relative NAc inhibition by preventing glutamatergic depolarization). It also has been suggested to apply to other drugs such as opioid agonists, on the presumption that those drugs have generally inhibitory effects (Kelley et al., 2005; Baldo and Kelley, 2007; Carlezon and Thomas, 2009).

Here, this hypothesis was supported by significant reductions in food intake, duration of eating, and number of eating bouts when ChR2 laser stimulation reversed appetitive motivation generated by DNQX microinjection. Furthermore, such counteraction of DNQX-mediated motivation was only demonstrated when fibers within 1mm of drug infusion sites, demonstrating that local inhibition through drug microinfusion requires localized depolarization of NAc cells, and that general stimulation of the NAc shell is unable to counter these regionally-specific effects.

Seemingly counter to this chapter's findings, there is evidence to support a hypothesis of neuronal excitation in the NAc shell, including the evidence presented in Chapter 2. Beyond that, older evidence from electrode self-stimulation studies demonstrate that cellular depolarization of these regions can inherently be rewarding (Rolls, 1971; Mogenson et al., 1979; Van Ree and Otte, 1980; Phillips, 1984). Recent optogenetic studies of D1 cells have demonstrated that

stimulation of cells in the NAc shell can enhance the rewarding properties of morphine and cocaine (Lobo et al., 2010; Koo et al., 2014a). Furthermore, stimulation of excitatory glutamatergic terminals from BLA, vSub, and PFC have been shown to induce conditioned place preference and occasionally self-stimulation behavior, demonstrating that excitation of NAc cells can produce enhanced motivation and be inherently rewarding (Britt et al., 2012). Further, Ambroggi and colleagues (2008) found that excitatory BLA input was necessary for cue-triggered seeking of sucrose reward.

However, two recent optogenetic studies have found that neuronal depolarization of the NAc can lower motivation for food (O'Connor et al., 2015) and that activation of glutamatergic inputs to the NAc can stop motivation for food or ethanol (Millan et al., 2017). The activation of D1 MSNs can stop the consumption of a fatty solution, and the inhibition of D1 terminals within the LH actually enhances consumption. Moreover, Millan and colleagues (2017) found that high-stimulation of BLA terminals within the NAc suppressed approach behavior and consumption of both food and ethanol reward. In our hands, laser stimulation alone did not inhibit food intake, though this may represent a floor effect, as animals in this study were fed *ad libitum.* It would be of interest to see if food restricted animals would stop food intake upon laser depolarization in accordance with the hyperpolarization hypothesis.

Thus, taking Chapter 2 and these results together, it appears both excitation and inhibition can produce motivated states in certain situations. One possibility is that striatal cells gate motivation through bimodal states, such that they contain relative “up” and “down” states that may shift in response to cortical inputs (O'Donnell and Grace, 1995; O'Donnell et al., 1999). Additionally, receptor subtypes can change their mode of action as a function of cell-potential state. D1 receptors have been shown to promote cellular inhibition in hyperpolarized cells, but

potentiate action potentials in depolarized states (Surmeier et al., 2007) which may be responsible for the dopamine dependent valence shifts that occur in different environments following DNQX-microinfusion (Richard &Berridge, 2011). Another possibility is that the receptors and cells influenced across various lines of study generate very different modes and signaling profiles than those found in this study. In the present study, 1) general neuronal cell bodies were stimulated to 2) counteract AMPA-kainate receptors. Other studies of excitation-based low motivation mentioned above either target discrete populations of neuronal cell bodies or non-receptor specifically enhance glutamatergic yield within the NAc. It may be that D1 or D2 MSN activation may differentially counter DNQX microinjections, or not at all. Activation of fast spiking interneurons (FSI) may produce inhibition of surrounding neurons and enhance DNQX passive inhibition. Yu et al., (2017) found 1) that BLA-NAc stimulation caused more rapid activation of FSIs than MSNs, and that the activation of FSIs in turn caused a feed forward inhibition of local MSNs. It may be that general excitation or inhibition are actually activating specific groups of anatomically or electrically connected neuronal ensembles (Pennartz et al., 1994; O'Donnell et al., 1999), and that the selective activity of these ensembles produces shifts in communication that prevent interpretations of "just excitation" or "just inhibition" as the as the primary mechanism for NAc-mediated motivation.

GABA-mediated inhibition and gating of motivation

An additional avenue navigate is determining whether ChR2 stimulation is capable of challenging GABA stimulation, which is hypothesized to also inhibit NAc neurons (Reynolds and Berridge, 2001, 2002; Richard and Berridge, 2011, 2013). Preliminarily, GABA enhancement of motivated behaviors does appear to be counteracted by ChR2 stimulation,

though findings are highly variable. Considering that previous studies from our lab have shown that muscimol produces higher feeding and fear, it may take greater levels of stimulation to counter the direct hyperpolarizing effects of GABA activation (Richard &Berridge, 2011). Furthermore, the fact that GABA activation is so inflexible in its nature may indicate a different inhibitory profile from that of DNQX. Muscimol injections into the NAc are strongly hyperpolarizing, though they do not completely abolish all activity (Kiyatkin and Rebec, 1999). Perhaps the lack of excitation produced by DNQX does not quite match the hyperpolarization induced by GABA activation. Thus, from two major transmitter systems the common mechanism of cellular inhibition appears to be necessary to release motivated behavior. Further, there may be interactions between neurotransmitter systems in the NAc shell, such as glutamate and dopamine, which contribute to the production of bivalent motivations (Carlezon and Thomas, 2009; Castro et al., 2015; Lammel et al., 2014; Surmeier et al., 2007). Therefore, different combinations of neurotransmitter signals can generate varying motivational states within the NAc, such as appetitive and fearful states.

In conclusion, I directly combatted DNQX-microinjections into the NAc shell with optogenetic excitation. In support of the inhibition hypothesis of NAc motivation, I found that optogenetic excitation reversed DNQX eating. Further, only in animals who had fiber tips positioned within 1mm of the microinjection site was I able to get a reduction in food intake; that is, local inhibitory microinjections were only combatted by localized depolarizations, rather than general NAc stimulation. Interestingly, ChR2 laser stimulation of NAc had no discernable effect by itself on any behavior. Together, these findings give credence to the NAc inhibition hypothesis for DNQX microinjection induction of intense appetitive motivation.

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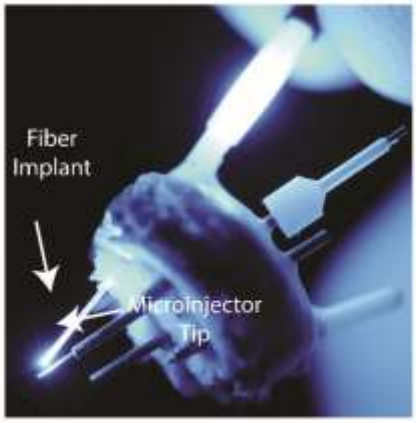
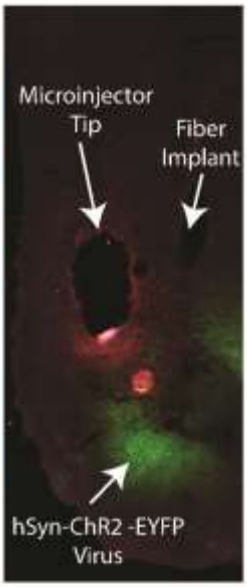
Figures

Figure 3.1. Representative skull cap and viral expression. (Top) Sagittal schematic of our tandem drug and optogenetic stimulation procedure with virus infused at the same site as microinjection. Under sagittal section are stimulation parameters for the project. **(Middle left)** Skullcap showing cannula and fiber alignment. (Middle Right) Coronal slice showing viral spread, cannula placement, and fiber. **(Bottom)** 4 conditions of our microinjection and stimulation paradigm





DNQX+ChR2 Testing Conditions (1hr counterbalanced)

No Laser + Vehicle	Laser + Vehicle
No Laser + DNQX	Laser + DNQX

Figure 3.2. ChR2 Stimulation Reverses DNQX Induced Food Intake. Animals receiving DNQX microinjections ate about 5.5 grams of rat chow on average. During laser stimulation conditions, animals with fibers aligned had approximately 50% reduction in food intake, whereas animals who did not have fibers in alignment did not show any difference from DNQX alone. The duration of time eating followed similar trends, with animals with aligned fibers showing a about a 40% decrease in time spent eating. Only DNQX conditions produced more eating bouts. Data for all animals in NAc is located in the first column. Animals with fibers white bars=no laser vehicle, grey bars= laser alone, green bars=DNQX alone, and blue bars=DNQX+laser. Comparisons with values beneath. *p<.05

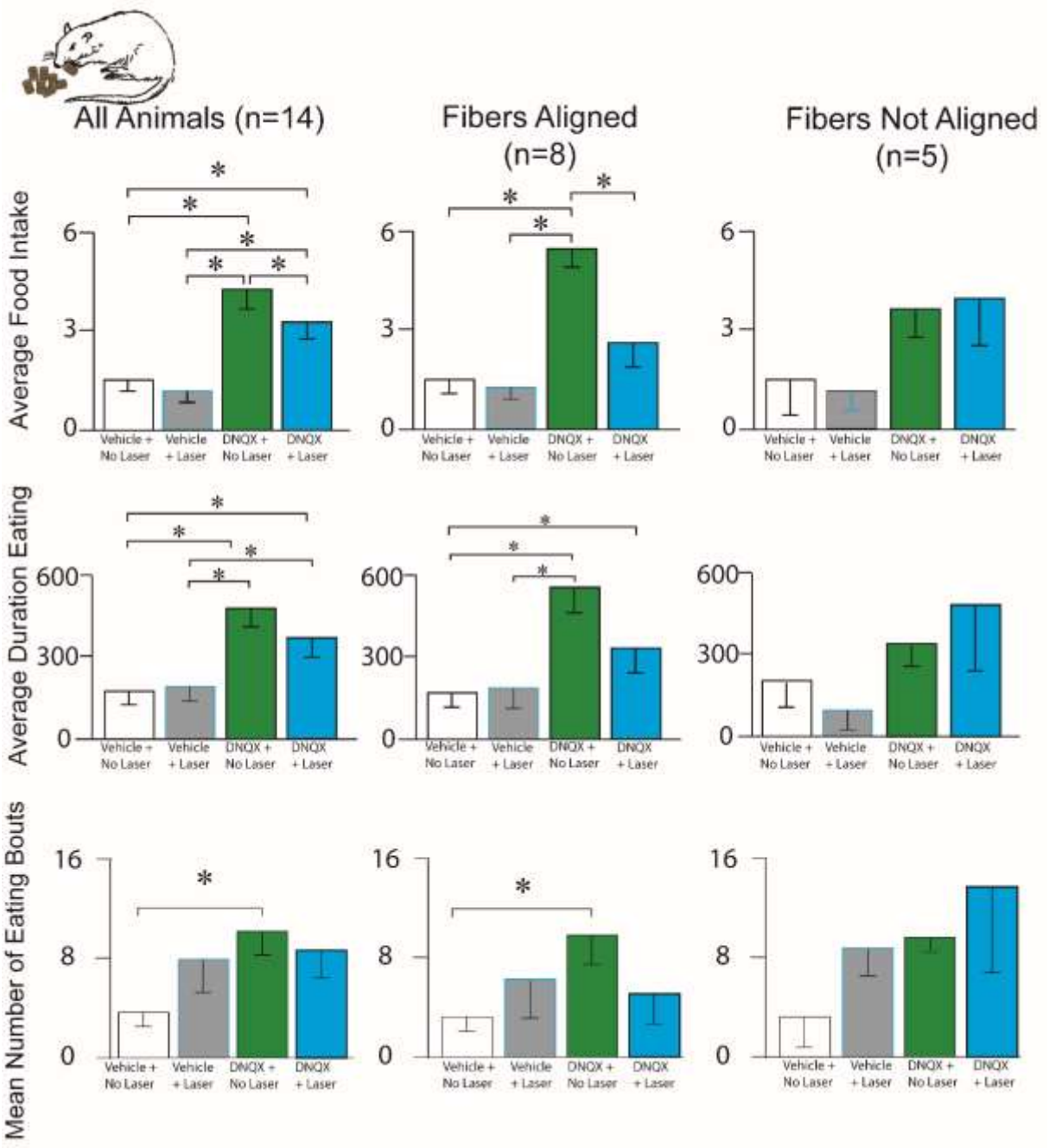


Figure 3.3. Sagittal placements of drug and laser effects of appetitive motivation. (Left) % Food intake change from baseline induced by DNQX microinjection mapped to individuals' cannula placement. **(Middle)** Percentage shift induced by laser-stimulation from DNQX conditions. Animals with aligned fibers shoed about a 50% drop in food intake on average. **(Right)** Animals without aligned fibers did not show major shifts in food intake.

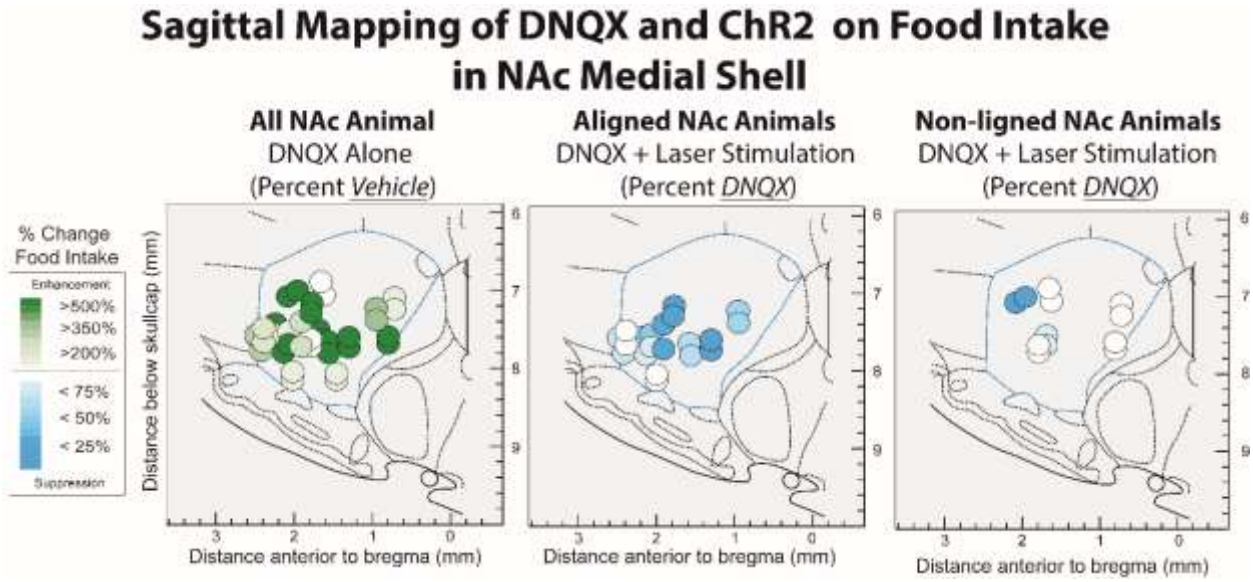
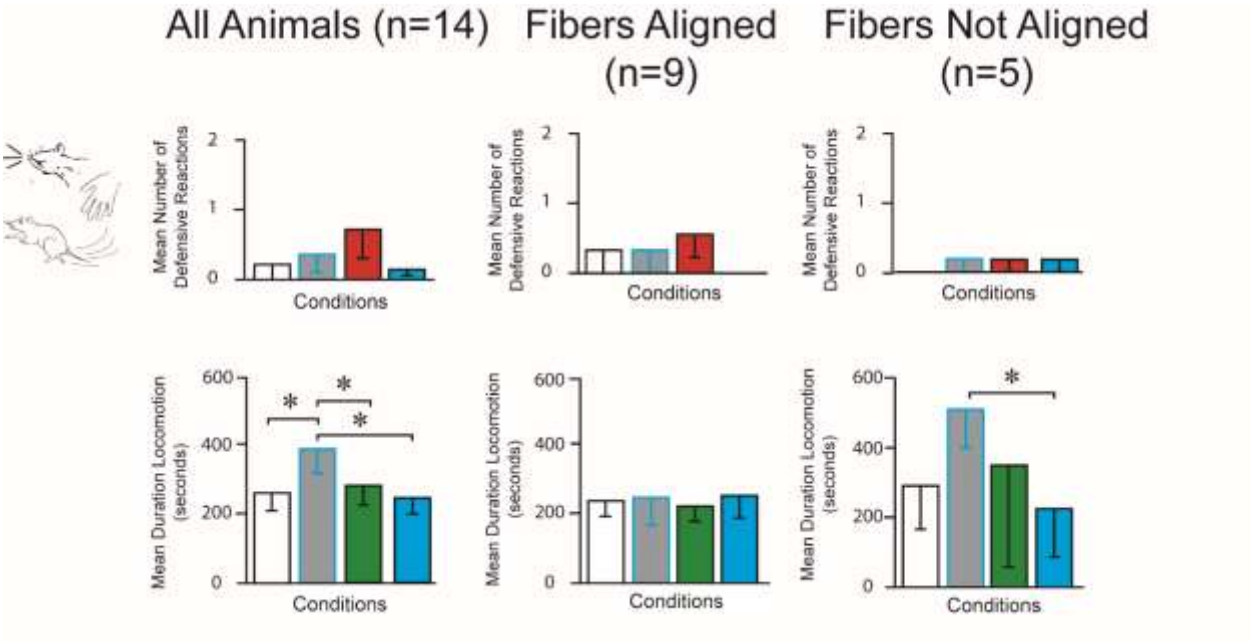


Figure 3.4. Locomotion and defensive reactions. (Top) no differences in defensive reactions were observed for any condition. Few only 3 animals made distress calls under DNQX microinjection. (Bottom) No major effects of locomotion were observed for any conditions upon separation into fiber aligned and non-aligned rats. Interestingly, animals in with fibers not aligned showed about 200% higher locomotion than when DNQX was on board.


All Animals (n=14)
Fibers Aligned (n=9)
Fibers Not Aligned (n=5)
Mean Number of Defensive Reactions
Mean Duration Locomotion (seconds)
Conditions
0
1
2
200
400
600
*

Chapter 4

Pilot Projects

Pilot Project 1: Direct Inhibition of the Nucleus Accumbens Shell Potentiates Eating

Introduction

Excitation vs inhibition

The dominant hypothesis of NAc mediated motivation states that inhibition of NAc neurons generates motivation, by releasing regions downstream of the NAc from tonic, GABAergic inhibition (Cheer et al., 2005; Roitman et al., 2005; Taha and Fields, 2006; Wheeler et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010). Proponents of the hypothesis hold that inhibition of the NAc medial shell generates motivation by disrupting these GABAergic projections, thus disinhibiting the NAc's target structures into states of excitation (Carlezon and Wise, 1996; Cheer et al., 2005; Roitman et al., 2005; Taha and Fields, 2006; Meredith et al., 2008; Roitman et al., 2008; Wheeler et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010). Numerous studies have shown that micro infusions of inhibitory GABA-A agonists stimulate of food intake, in at least rostral shell sites, requires VP and LH recruitment, as pharmacological inhibition or lesion of VP or LH attenuates the NAc-induced increase in eating (Stratford and Kelley, 1999; Stratford and Wirtshafter, 2012; Urstadt et al., 2013b; Urstadt et al., 2013a). Further, over the last 20 years studies from our lab and others have shown that drug microinfusions of the GABA-A agonist, muscimol, or the AMPA-kainate antagonist, DNQX, are both capable of enhancing motivation for food reward (Reynolds and Berridge, 2002, 2003, 2008; Richard and Berridge, 2011b; Richard et al., 2013). A body of electrophysiological

evidence focusing on NAc inhibition lends support for this hyperpolarization hypothesis (Peoples and West, 1996; Janak et al., 1999; Roitman et al., 2005; Loriaux et al., 2011). For example, during sucrose consumption, up to 75% of sample NAc neurons show decreased firing rates during (Roitman et al., 2005) whereas consumption of bitter, aversive quinine is associated with increased activity in NAc neurons. NAc inhibition also seems to encode and track the valence of stimuli, and salt consumption is associated with decreased NAc activity in salt-depleted animals, but with increased NAc activity in salt-sated animals (Loriaux et al., 2011) NAc inhibition is also seen in drug reward, similar pauses in NAc neuron firing are seen during cocaine and ethanol administration (Janak et al., 1999; Peoples and West, 1996). Further, this hypothesis is supported by findings that neural excitations in downstream targets, such as VP, LH, or VTA occur during reward events (Ljungberg et al., 1991; Baldo et al., 2004; Stratford, 2005; Tindell et al., 2009; Smith et al., 2011).

The depolarization hypothesis of accumbens-generated motivation.

Paradoxically, many studies also show evidence for excitation-mediated motivation, more consistent with my NAc ChR2 self-stimulation results in Chapter 2 (Brit et al., 2012; Carelli, 2000; Carelli et al., 2000; Hollander et al., Mogenson et al., 1979; 2002; Nicola et al., 2004a; Nicola et al., 2004b; Rolls, 1971; Taha and Fields, 2005). For example, Carelli et al. (2000) found that over 40% of sample neurons in the NAc exhibited increased firing during instrumental responding for food, water, or cocaine, and a number of other studies have observed similar trends (Hollander et al., 2002; Nicola et al., 2004a, b); Hollander et al., 2002; Nicola et al., 2004a; Nicola et al., 2004b). In another study, over 70% of cells that altered their firing rates during instrumental responding for increasingly palatable sucrose solutions showed *increases* in

firing (Taha and Fields, 2005). Moreover, activation theoretically excitatory glutamatergic inputs to the NAc are sufficient to generate self-stimulation or seeking of locations paired with stimulation of PFC, hippocampal, or BLA (Britt et al., 2012).

Direct Inhibition of NAc Medial Shell

In Chapter 3, the hyperpolarization hypothesis was supported by my own findings, where I showed that ChR2 depolarization reversed enhancement of food intake induced by DNQX microinjection, supporting a necessary cause hypothesis, at least for drug-induced motivation. Here in Chapter 4, I tested whether optogenetic inhibition by itself via halorhodopsin optogenetic inhibition was sufficient to induce increases in food motivation. I report that this NAc neuronal inhibition did indeed produce increases in food intake, at least in some individual rats. Interestingly, some individuals also continued eating at elevated rates even during laser off periods, demonstrating a lingering effect of exposure to inhibitory laser. Finally, I note that overall variance in mean amounts of consumption was much higher in animals receiving optogenetic inhibition rats than in inactive-viral controls, indicating inhibition may induce highly variable degrees of motivation and patterns of eating behavior even within an individual. The following results provide a case-study analysis of rats that showed elevations in eating during laser sessions, and demonstrates that direct NAc inhibition may be a sufficient mechanism of appetitive motivation production.

Methods

Subjects

Twenty male Sprague-Dawley rats were run in four waves (of n=4, n=6, n=5, and n=5, respectively). All rats were at least two months old and at least 250g at time of surgery. Rats were pair-housed at 21° C on a reverse 12hr light/dark cycle and had *ad libitum* access to food and water. In the case of odd-numbered waves, rats were housed in threes. The Committee on the Use and Care of Animals at the University of Michigan approved all experimental procedures.

Surgery

Virus infusion.

Rats were first anesthetized using isoflurane gas and then injected with the respiratory aid atropine (0.05mg/kg), antibiotic cefazolin (0.05mg/kg, i.p.), and analgesic carprofen (0.1mg/kg, s.c.). After securing animals on a stereotaxic apparatus (David Kopf Instruments), 0.5µl infusions of halorhodopsin virus (AAV5-hSyn-NpHR3.0-EYFP) (n=18) or an inactive control virus (AAV5-hSyn-EYFP) (n=2) were administered bilaterally into the rostral NAc medial shell (AP, +1.7; ML, ±3.0; DV, -7.56).

Fiber optics and skullcaps.

Fiber optic implants comprised optic fibers of diameter 230µm inserted into 9mm-long zirconia ferrules. Implants were inserted just above the viral infusion sites (D/V: -7.24) and secured to the skull with dental acrylic such that approximately 0.5cm of ferrule protruded from the skull cap. Carprofen (0.1mg/kg, s.c.) was administered 24 and 48 hours following surgery. Animals were given at least three weeks' recovery time before testing began.

Food Intake Testing

Food intake sessions lasted 1hr unless otherwise noted. Each rat was designated a 43x23x42cm test chamber containing approximately 3cm of bedding. A small water dish was placed in the front left corner of the chamber and ~25g of rat chow (Purina) in the front right corner. Animals had free access to this food and water for the duration of each session. Optic cables connected a laser of wavelength 593nm (Shanghai Lasers) to skullcaps. Laser output from each of the two cables was approximately 10-12mw at the fiber tip. Following testing, remaining chow was collected and weighed. Additionally, offline video scoring was performed following test session.

With the exception of Wave I, each wave began food intake testing with a single habituation day in which rats were placed in the test chamber for 1hr with free access to food while the laser remained off. As the pattern of inhibition-specific behavior varied day-by-day, the specific order of sessions varied by wave of animals to test and prevent lingering inhibition or enhancement of eating from day to day (all conditions for all waves given in Table 1). At some point during testing, each wave underwent a standardized, six-day cycle of alternating laser (L) and non-laser (NL) test days (counterbalanced) (L1, NL1, L2, NL2, L3, NL3). On "laser days", rats received two 15min blocks of constant "laserON" each preceded by 15min of "laserOFF" (i.e., 15minOFF/15min_constantON/ 15minOFF/15min_constantON;"standard laser protocol") to see if eating was bound to laser-inhibition within session. During non-laser days, the laser remained off with all other conditions held constant. For consistency, the laser power units were left on, though not firing, for ambient noise and an experimenter entered the test room every 15 minutes as would on laser days.

Animal Selection Criterion

Five halorhodopsin (NpHR) rats (9618 and 9619 from Wave I, 9788 and 9790 from Wave II, and 10129 from Wave IV) met criteria for further analysis as NAc inhibition eaters based on at least a 15% net increase in food intake across the six-day laserON/laserOFF cycle. Four of these rats showed increased eating on laser sessions, compared to baseline measured in non-laser sessions; a fifth showed an escalation in eating across the six-day cycle, regardless of laser/non-laser. All other animals either showed a less than 5% change from non-laser eating. Given our low N and the variable nature of NpHR behavior, we chose to do a case study analysis of these selected inhibition eaters, comparing them to inactive-viral controls (10098 and 10099 from Wave IV).

Testing Protocols by Wave

As the pattern of inhibition-specific behavior varied day-by-day, the specific order of sessions varied by wave of animals to test and prevent lingering inhibition or enhancement of eating from day to day (all conditions for all waves given in Table 1). Immediately below are the protocols and rationales for each wave and test day, which are also addressed along with findings within *Results***.** A condensed account of test conditions can be found in Table 1. Further, test days during the week were varied, to prevent possible interaction between interaction with husbandry staff or cage changes from affecting food intake. No general pattern was observed for behavior vs day.

Wave I. Rats 9618 and 9619. The first wave of rats began testing with a six-day laserON/laserOFF cycle (TD1-TD6). On test days 7-9, rats 9618 and 9619 underwent non-laser testing to extinguish the appetitive effect of laser exposure seen during the initial six-day cycle.

Rats were exposed to standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON) on test days 10 and 11, followed by a further six days (TD12-17) of extinction, to first reinstate any laser-induced eating effect and then extinguish it a second time.

On TD18-27, we probed the extent to which feeding behavior is discretely bound to laser exposure. We began this exploration by turning the laser on and off at random intervals on TD18. On TD19, rats were given a 15min baseline, followed by 15min during which the laser was turned off when rats began eating and on again when rats stopped eating to test whether laser inhibition is sufficient to induce an eating state; this test day ended with a 15min laserOFF period, for a total session duration of 45min. TD20 was also 45min in duration, with 15min of constant laser independent of rat behavior, preceded and followed by 15min laserOFF periods (15minOFF/15min_constantON/15minOFF). On test days 21 and 22, rats underwent standard laser protocol (15minOFF/15min_constantON/15minOFF/15min_constantON). Test days 23 and 24 both comprised a 15min baseline, 15min laserON, 15min laserOFF, and a final 15min laserON period. However, instead of constant laser during these laserON periods, rats received 10sec bouts of laser stimulation followed by 30s of no laser on TD23, and 3min bouts of laser followed by 3min of no laser on TD24 (15minOFF/15min_cycledON/15minOFF/ 15min_cycledON). Our aim here was to determine if lower levels of NAc inhibition might be optimal for eating induction.

Wave II. Rats 9788and 9790. We were interested to see if we could gradually increase eating behavior in Wave II. After an initial habituation day (TD1), we slowly increased laser duration across six test days (TD2-7) until reaching standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON). Following this, both rats

underwent the standard six-day alternating laserON/laserOFF cycle (TD8-13) (each 1hr; laser sessions L1, L2, and L3 comprising 15minOFF/ 15min_constantON/15minOFF/15min_constantON).

Wave III. No rats from Wave III met stated criterion.

Wave IV. Rat 10129. Due to time limitations, tests of this wave were less exploratory in approach and were limited to a habituation day (TD1) followed by a six-day alternating laserON/OFF cycle (TD2-7) (each 1hr; laser sessions L1, L2, and L3 comprising 15minOFF/ 15min_constantON/15minOFF/15min_constantON).

Offline Video Scoring

All sessions on test days were video-recorded for offline analysis. During the test days, experimenters retrieved the rats from the testing apparatus using the rehearsed procedure. The behavioral ethogram distinguishes discrete events, such as cage crosses and food carrying, and states such as grooming and eating.

Offline observers scored the duration of spontaneously emitted behaviors (measured in 1 second increments) during the recorded sessions for each of the following: eating (actively chewing and swallowing food), drinking, defensive treading/treading-burrowing behavior, grooming, escape behaviors (subject tries to move away from the experimenter's reach), rearing (forepaws are lifted at least 3 cm off the floor), and immobile/sleeping. Discrete events include: cage crosses (counted when an animal moves at least half the length of the testing chamber in one bout of movement), sniffing and carrying food (counted as each second that the animal is

engaged in behavior), bouts of eating (counted as the number of times subject initiated eating), and distress vocalizations.

Histology

30 minutes following the last behavioral testing session, rats were sacrificed with an overdose of sodium pentobarbital (.8 mL). Brains were extracted and fixed in 4% paraformaldehyde for 1-2 days, then placed in 25% sucrose solution for 2 days. To assess fiber implant and virus expression sites, brains were sliced at 40 μm on a cryostat (Leica Biosystems, Buffalo Grove, IL, USA. Sites were mapped onto coronal slices from a rat brain atlas (Paxinos and Watson, 2007).

Results

Five selected rats were classified as NAc inhibition eaters after expressing NpHR chloride ion channels. Our five selected NpHR rats are 9618 and 9619 from Wave I, 9788 and 9790 from Wave II, and 10129 from Wave IV. These were chosen based on heightened eating across the six-day alternating laserON/laserON cycle. Though the schedule of inhibition varied across waves, all rats were exposed to a six-day alternating laserON/laserOFF cycle (L1, NL1, L2, NL2, L3, NL3) administered at least once during testing for direct comparison. The three laser sessions within this six-day cycle (L1, L2, and L3) lasted 1hr and comprised 15min laserOFF baseline, followed by 15min constant laser, 15min laserOFF, and a final 15min laser period (15minOFF/15min_constantON/15minOFF/15min_constantON). Non-laser sessions (NL1, NL2, and NL3) ran 1hr and involved no laser exposure. The individual profiles of each rat are described below. Note: For a summary of the order of test conditions, please see Table 1. Rationales for testing conditions by day are described within results below.

Wave I (Fig. 4.1)

Rat 9618. During the initial six-day laserON/laserOFF cycle, Rat 9618 ate >200% more during laser sessions than during non-laser sessions, eating an average of 5.2g per each of the first three laser days and 2.4g per non-laser day. 9618 spent 24% of its time eating during laser sessions and only 12% during non-laser sessions. 9618 spent minimal time drinking, interacting with environment, locomoting, rearing, grooming, and burrowing, with these behaviors each accounting for nearly or less than 10% of the rat's total behavior across both laser and non-laser days (Fig. 4.1c). 9618 also showed more general activity on laser days than non-laser days, spending 34% of its time immobile or sleeping during laser sessions versus 42% of time during non-laser sessions. Of the rats tested 9618eating was unique in that within each laser session (L1, L2, and L3), over 80% took place while the laser was on, a demonstrating temporally specific inhibition-bound eating (Fig. 4.1b).

Since 9618's eating was largely synchronized to laser inhibition, this rat was put under non-laser extinction testing for three days (TD7-9) to extinguish the appetitive effect of laser exposure seen during the initial six-day cycle. 9618 ate on average 1.4g per extinction day, or just 30% of laser-induced eating.

Following extinction, 9618 underwent two laser inhibition sessions to determine if elevation of eating could be repeated. 9618 first underwent standard laser protocol (15minOFF/ 15min_constantON/15minOFF/15min_constantON) on TD10, during which it ate 9.0g; 72% of this eating occurred while the laser was on. On TD11, we used a lesser amount of laser stimulation within a 1hr session to determine if we could induce a milder effect on eating behavior, running a 30min laserOFF baseline followed by 15min of laser stimulation and a final

15min of no laser (30minOFF/15min_constantON/15minOFF). The rat ate 4.7g under this modified laser condition, almost exactly half what it had consumed under the standard laser protocol on TD10.

To ensure that the decrease in eating during extinction was caused by lack of laser inhibition and not an artifact of test day, we conducted a second series of non-laser sessions spanning TD12-17. Elevated eating lingered for the first few sessions of this second extinction block, and dropped off to 1.9g by TD17. On average, 9618 reduced its food intake by 0.6g per extinction session. While there seemed an immediate extinguishing effect during the first extinction period spanning TD7-9, the decline in food consumption during the second period was quite gradual; 9618's eating lingered at an elevated rate for the first two sessions of this second extinction block. However, after six consecutive non-laser trials, its feeding dropped to rates consistent with those seen during the first extinction period.

TD18 began a final and particularly exploratory series of sessions aimed at eliciting temporally synchronized laser-bound eating within a session. 9618 first underwent a probe session (TD18), during which laser inhibition alternated on and off at pseudo-random intervals, and ate 3.9g. To determine if laser exposure could more directly shape the rat's behavior, 9618 then underwent a session (TD19) involving a single 15min block during which the laser was turned on when the rat stopped eating and turned back off when the rat began eating (15minOFF/ 15minON/OFF [with eating]/15minOFF). This was done to determine if inhibitory laser exposure could reactivate the rat's appetitive motivation such that it would begin eating again within moments of re-exposure. However, 9618 ate 3.2g during TD19 and no such time-locked eating was observed.

Spanning the final five test day (TD20-24), sessions vacillated between the standard inhibition protocol that had previously produced increases in food intake and novel variations on these parameters. Specifically, 9618 underwent five consecutive test days (TD20-24) involving varied amounts of laser exposure, including two standard laser sessions (each 15minOFF/ 15min_constantON/15minOFF/15min_constantON) and three sessions comprising variations on this standard laser protocol. On TD20, the animal underwent a laser session totaling 45min (15minOFF/ 15min_constantON/15minOFF). This was followed by two standard laser days on TD21 and 22. On TD23, 9618 underwent a 15minOFF/15min_cycledON/15minOFF/ 15min_cycledON session, with the ON trials comprising a 10secON/30secOFF cycle instead of constant inhibition for the full 15min. TD24 also comprised non-standard laser exposure in 15min blocks, with laser cycling 3minON/3minOFF. 9618's food consumption fluctuated greatly during this last block of sessions, ranging from 5.2g to 0.0g across these five sessions. There was no discernable effect on this rat's behavior during these five varied laser test days. This indicates that in our hands these lower levels of inhibition are not sufficient to impact eating, or that these cycles may produce different signaling patterns than do 15min of constant inhibition.

Rat 9619. 9619's testing conditions were identical to those of 9618, beginning with a six-day alternating laserON/OFF cycle (TD1-6). 9619 ate an average of 4.3g and spent 20% of its time eating during laser sessions (TD1, 3, and 5), and ate an average of 3.3g with 15% of its time spent eating during non-laser sessions (TD2, 4, and 6). Drinking, interacting with environment, rearing, grooming, and burrowing contributed minimally to the rat's overall behavior (Fig. 4.1c). 9619, like 9618, showed less activity during non-laser session (38% of total time inactive) than during laser sessions (26% of total time inactive), including heightened locomotor activity during

laser sessions (18% of time locomoting on laser days versus 12% of time on non-laser days). However, unlike 9618, rat 9619 did not show eating bound discretely in time to laser exposure; during the three laser sessions of this six-day laserON/laserOFF cycle (L1, L2, and L3), 48% of eating occurred with laserON and 52% with laserOFF (Fig. 4.1).

After this initial six-day cycle, we conducted a first block of extinction sessions (TD7-9) to determine if inhibitory laser-enhanced eating could be extinguished. 9619's consumption declined gradually, eating 5.9g on the first extinction session, followed by 2.9g on the second and 0g on the third and final session. Inhibition-induced eating was then reinstated with two consecutive laser days. On TD10, 9619 underwent standard laser protocol (15minOFF/ 15min_constantON/15minOFF/15min_constantON) and ate 5.9g of rat chow, surpassing the amount consumed during the initial three laser sessions. On TD11, laser exposure was decreased to only one 15min block of laser exposure during the 1hr session (30minOFF/ 15min_constantON/15minOFF), and food intake decreased to 4.9g.

As with 9618, 9619 then underwent a second six-session extinction period spanning TD12-17 to ensure that the rat's pattern of decreased consumption during the first extinction period resulted from lack of inhibitory laser exposure. Consumption declined by 0.5g per day during this second extinction. 9619 ate an average of 3.4g per day during this six-day laserOFF period, with a low of 1.4g on TD17, as compared to 4.3 on average per L1, L2, and L3.

From TD18-24, 9619 underwent a series of probing, exploratory laser sessions involving gradually increasing laser exposure to determine if the lingering effect on food consumption was due to over-inhibition in prior sessions. During an initial probe session (TD18), inhibition was provided at pseudo-random intervals, resulting in 3.7g consumed. During a 15min period within TD19, laser was turned OFF as the rat began eating and ON as eating stopped to determine if

feeding could be discretely initiated by laser inhibition (15minOFF/15minON/OFF [with eating]/ 15minOFF). This resulted in only 0.7g consumed. On TD20, 9619 underwent a session totaling 45min, with a 15min constant laserON period between two periods of laserOFF (15minOFF/ 15min_constantON/15minOFF), and ate 1.8g.

ON TD21 and TD22, 9619 was put under standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON) to restore laser-heightened eating levels. 9619 ate 3.5g on TD21 and increased to 6g on TD22. This second standard laser day (TD22) is notable as the rat ate more and for longer than on any other day during this block of probing sessions, indicated that consistent and long-duration inhibition may produce the most dramatic increase in consummatory motivation.

Finally, on test days 23 and 24, 9619 underwent two non-standard, 15minOFF/15min_cycledON/15minOFF/15min_cycledON laser sessions. On TD23, the laser cycled 10secON/30secOFF during the two 15minON periods, and the rat ate 3.6g. On TD24, 9619 received bouts of laser cycling 3minON/3minOFF and ate 1.8g. Thus, since these session times were consistent with standard conditions (1hr), cycled, inconstant laser exposure seems to have a lesser effect on food intake.

Wave II (Fig. 4.2)

Rat 9788. Rat 9788 began testing with a habituation session involving no laser, during which it ate 5.0g of rat chow. Since Wave I rats showed a lingering effect of laser exposure during the initial six-day laserON/laserOFF cycle, inhibitory laser exposure was gradually *increased* in Wave II to determine if this gradually increasing laser exposure would correlate with increased food consumption. 9788 underwent six days of non-standard, increasing laser

exposure (TD2-7). Each of these, save for a non-laser day on TD3, comprised 15minOFF/15min_cycledON/15minOFF/15min_cycledON; rather than constant laser during ON periods, 9788 received laser pulses of varying duration. On TD2, the laser cycled 30sON/4.5minOFF, with the rat eating 2.1g. 9788 then ate 4.0g on the non-laser day that followed (TD3). The laser cycle on TD4 was the same as on TD2, and produced 4.4g of food intake. The duration of laser exposure was subsequently increased: on TD5, 9788 was exposed to 1minON/4minOFF laser cycles and ate 4.7g. ON TD6, laser increased to a 3minON/2minOFF cycle, producing 3.0g of eating, and on TD7 a 5minON/5minOFF cycle resulted in 4.6g consumed. Thus, 9788 did not show eating behavior that increased gradually with laser exposure periods of increasing length.

Following these step-wise increases in laser duration, 9788 underwent a six-day alternating laserON/OFF cycle identical to the TD1-6 cycle that produced heightened eating in Wave I (each 1hr; laser sessions L1, L2, and L3 comprising 15minOFF/15min_constantON/15minOFF/15min_constantON). On L1 and NL1 (TD8 and 9), 9788 ate 3.9g and 1.3g respectively. Next, 9788 ate 3.2g on L2 (TD10) and 5.6g on NL2 (TD11). Finally, 9788 ate 7.7g on L3 (TD12) and 8.0g on NL3 (TD13). Thus, rather than strictly eating more during laser sessions than non-laser sessions, 9788's pattern of eating increased over time irrespective of whether the session involved laser exposure (Fig. 4.2a). 9788 seemed to show a lingering, cumulative eating-induction effect of inhibitory laser exposure rather than a discrete, temporally specific one. This rat's behavioral data corroborate this trend: 9788 spent 24% of its time eating during both laser sessions and non-laser sessions. Percentages of time spent engaged in all other behaviors (e.g. drinking, grooming, locomotion, and inactivity) were nearly identical for laser and non-laser sessions (Fig. 4.2).

We observed no notable differences in time spent eating during laserON versus laserOFF periods within sessions: 49% of 9788's eating across L1, L2, and L3 occurred during laserON, with the other 51% occurring during laserOFF (Fig. 4.2). This corroborates the idea that, in 9788, laser exposure produced a cumulative rather than temporarily specific increase in appetitive behavior both within and across sessions.

Rat 9790. Rat 9790 first underwent a non-laser habituation day (TD1) followed by six days of increasing laser exposure (TD2-7) (TD2, TD4-7 comprising 15minOFF/ 15min_cycledON/15minOFF/15min_cycledON; TD3 no laser) to determine if we could gradually consumption. Like 9788, 9790 did not exhibit feeding behavior that increased gradually with incrementally longer periods of laser exposure. 9790 ate 5.3g on TD1 (habituation), 2.3g on TD2 (30sON/4.5mOFF cycle), and 1.0g during the non-laser session on TD3. 9790 ate 1.9g on TD4 (30sON/4.5minOFF cycle), 4.1g on TD5 (1minON/4minOFF cycle), 1.3g on TD6 (3minON/2minOFF cycle), and 3.9g on TD7 (5minON/5minOFF cycle), showing no consistent laser duration-to-food intake ratio.

9790 next underwent the standard six-day alternating laserON/laserOFF cycle used to directly compare all waves (TD8-13) (each 1hr; laser sessions L1, L2, and L3 comprising 15minOFF/ 15min_constantON/15minOFF/15min_constantON). During these sessions, 9790's patterns of food intake roughly matched expectations: it ate an average of 6.2g per each of the three laser sessions and 3.9g per non-laser session. This eating accounted for 28% of total time during laser sessions (L1, L2, and L3), and 21% of time during non-laser sessions (NL1, NL2, and NL3). However, examining the three laser test days, we see that 9790 did not eat more during laserON periods than during laserOFF periods: approximately 47% of 9790's eating

across L1, L2, and L3 occurred during laserON and 53% during laserOFF (Fig. 4.2). Additionally, 9790 spent more time inactive on non-laser days – 57% of total time – than on laser days – 44% of total time. All other behaviors were fairly similar between laser and non-laser test days (Fig. 4.2c).

Wave IV (Fig. 4.3)

Rat 10129. Rat 10129 began testing with a habituation session (TD1), during which it ate 2.9g. 10129 then underwent the six-day alternating laserON/laserOFF cycle used as a standard for all waves (TD2-7) (each 1hr; laser sessions L1, L2, and L3 comprising 15minOFF/ 15min_constantON/15minOFF/15min_constantON). The rat ate 3.8g, 4.5g, and 5.2g respectively on L1, L2, and L3 (TD2, TD4, and TD6), and ate 3.1g, 2.3g, and 1.9g respectively on NL1, NL2, and NL3 (TD3, TD5, TD7). On average, 10129 ate 4.5g per laser session and just 2.4g per non-laser session. This rat performed just as we would expect: consuming more during laser sessions and less during non-laser sessions.

Inactive-Viral Control Rats

Our inactive-viral control rats only expressing EYFP in the absence of NpHR-chloride ion channels, were 10098 and 10099 from Wave IV. They underwent the same testing conditions as did 10129: a habituation day (TD1) followed by a six-day alternating laserON/laserOFF cycle (each 1hr; laser sessions L1, L2, and L3 comprising15minOFF/15min_constantON/15minOFF/15min_constantON). On average, these control rats ate 1.5g per each L1, L2, and L3, and 2.1g per non-laser session Fig. 4.3).

Histological Placements

Rat 9618 histological analysis showed near optimal virus expression and fiber optic placement in this rat: halorhodopsin virus was expressed bilaterally in rostral NAc shell and core, with one fiber tip terminating in -medial shell and the other between medial core and shell (see Fig. 4.1). Rat 9619 expressed halorhodopsin virus bilaterally in accumbens core and shell. Both fiber optic tips terminated in the rostromedial NAc core, pointing towards shell, with moderate virus expression.

9788 histological analysis showed virus expression primarily in NAc shell. Both fiber tips terminated in the rostromedial portion of the shell (see Fig. 4.2). Rat 9790's placements were in rostromedial NAc shell/NAc core border, and showed moderate expression in the NAc shell.

Due to time constraints, we were unfortunately unable to complete behavioral or histological analyses for our final wave of rats, and these are continuing. The intended targets for 10129 were in rostral-medial NAc shell.

Comparison of NpHR Animals to Inactive-Viral Controls

Overall, we see that NpHR rats ate more during laser sessions than non-laser sessions – an average of 5.0g per laser day vs 3.5g per non-laser day. The same was not true of control rats, which ate an average of 1.5g during laser sessions and 2.1g during non-laser sessions. Further, food consumption was more variable within individual NpHR rats than controls. The average standard error within subjects for NpHR rats was 0.7g across laser sessions and 0.9g across non-laser sessions, while the same measure for inactive-viral controls was 0.1g across laser days and 0.5g across non-laser days. Overall, within-animal variation was higher for halorhodopsin rats than for inactive controls.

Discussion

Four of five NpHR rats classified as NAc inhibition eaters (9618, 9619, 9790, and 10129) ate more during laser sessions than non-laser sessions. A fifth rat (9788) showed a more generalized increase in food consumption across the six-day alternating laserON/laserOFF cycle. Of those displaying differences in laser session versus non-laser session eating, the effect of laser inhibition only showed temporal specificity in one rat (9618), with 80% of food intake occurring under laser illumination. That is, within laser sessions, rats generally did not eat more during laserON periods than during laserOFF periods, though they did eat more across the entire session than sessions where no laser was provided. Finally, we saw increased overall variability in the eating behavior of NpHR rats versus inactive-EYFP controls.

Only five of 18 total NpHR rats showed clear laser enhancement in food consumption – eating considerably more during laser sessions than during non-laser sessions – though we observed higher overall eating in NpHR animals than EYFP inactive viral controls independent of test day. We note that even classic stimulation-bound eating induced by lateral hypothalamic electrode stimulations also typically was displayed by only a small minority of tested rats (e.g., 10% to 30%). While variations in fiber placement and viral spread may partially explain these differences they may also be reminiscent of, the large individual differences in electrode-based stimulation-bound eating. Even among rats with accurate electrode placements, Wise (1971) noted "marked inter-individual differences" in responses to lateral hypothalamic stimulation (p. 569). Wise notes that electrode-responsive rats exhibited target behaviors, like increased eating and drinking, while others did not; some animals required more stimulation than average to display these behaviors while others required less. Many studies of this sort mention that only a

portion of animals responded at all to electrical stimulation of LH (Mendelson, 1967, 1970; Huston, 1971; Mogenson et al., 1971; Stephan et al., 1971; Bowden et al., 1975). These "responder" animals often numbered less than half of those tested, and sometimes only about 10% (Mendelson, 1970; Mogenson et al., 1971; Stephan et al., 1971). In fact, some researchers had such difficulty eliciting this effect that they "[abandoned] the problem entirely"p.426 (Olds, 1976).

Increased Eating During Sessions with Laser Exposure

Overall, our selected rats ate more during laser sessions than during non-laser sessions. Consistent with the inhibition hypothesis and studies including cued approach, eating, drinking, and drug self-administration (Cheer et al., 2005; Taha and Fields, 2006; Meredith et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010).

While our findings above and in Chapter 3 indicate that NAc inhibition is both necessary and sufficient to induce motivation for food, they do not negate the substantial evidence of enhanced motivation produced and encoded by NAc-excitation. For example, Janak et al. (1999) found that NAc cells showed increased firing prior to operant responses aimed at receiving ethanol rewards, but decreased firing during ethanol consumption itself. Many electrophysiology studies show that some NAc cells do increase their firing rates during instrumental responding for food or drug rewards, though others exhibit *decreased* firing during these same moments instrumental response (Carelli, 2000; Carelli et al., 2000; Hollander et al., 2002; Nicola et al., 2004a, b; Taha and Fields, 2005).

Laser-Induced Eating Lacks Temporal Specificity

While rats ate more during 1hr laser sessions, they generally did *not* eat more during the 15min laserON versus 15min laserOFF trials *within* these sessions. With Rat 9618 as an exception, our laser-induced eating effect was not temporally specific to within-session laser exposure; rather, laser exposure seemed to generate a lingering or longer-lasting increase in food consumption across the entire session.

One explanation stems from the nature of the NpHR chloride ion pump system. It could be that due to being a pump, rather than an ion channel, longer exposure time may be necessary to produce meaningful enhancement for food reward. It is possible that neuronal or synaptic plasticity, promoted by repeated optogenetic inhibition, is responsible for our laser-induced eating effect's lack of temporal specificity.

Direct hyperpolarization of the NAc may also result in elevated DA levels in accumbens. Key to this possibility are the reciprocal projections between the NAc and VTA: the accumbens innervates the VTA via its GABAergic MSNs, and the VTA sends dopaminergic projections back to the NAc (Humphries and Prescott, 2010). In theory, inhibiting the NAc's GABAergic projections should disinhibit the VTA, thereby increasing DA transmission back to the NAc. If inhibition of the NAc shell has the potential to increase extracellular DA in NAc, this could facilitate plasticity in accumbens MSNs and help to explain the longer-lasting effect on food intake we see following optogenetic NAc inhibition. Psychomotor stimulant use is associated with plasticity in the NAc's GABAergic MSNs, which make up over 95% of cells in the nucleus accumbens(Luscher and Malenka, 2011). Specifically, these MSNs show increased dendritic density following repeated exposure to either amphetamine (Li et al., 2003; Russo et al., 2010) or cocaine (Norrholm et al., 2003; Russo et al., 2010). At the same time, psychomotor stimulant use greatly increases levels of extracellular dopamine (DA) in the NAc; thus, it is possible that the

plasticity seen in NAc MSNs following amphetamine or cocaine use is produced in part by increased DA transmission to the accumbens (Church et al., 1987; Carboni et al., 1989).

It is also possible that NAc inhibition itself, regardless of involved DA transmission, may produce plasticity in this brain region, though no data I am aware of currently exists to support this possibility. However, we see a similar type of plasticity demonstrated in the electrophysiological LH stimulation literature, wherein researchers report that repeated and direct modulation of cell electrical potential via electrode stimulation "increased the proportion of hypothalamic electrode sites yielding elicited eating and drinking during electrical stimulation" (Cox and Kakolewski, 1971, p. 245). To the extent that modulation of neural potentials independent of exogenous receptor mechanisms can produce plasticity, this may hold true for the NAc.

Additionally, laser-enhanced eating may lack temporal specificity due to "leaky" halorhodopsin proteins. Halorhodopsins are green/yellow light-gated chloride pumps and theoretically should only bring chloride ions across the membrane – thereby hyperpolarizing the cell – when exposed to laser light of the appropriate wavelength (Kolbe et al., 2000). If these proteins were to leak, permitting non-laser-specific passage of chloride ions into the cell beyond the period of laser exposure, any resulting hyperpolarization may be temporally broad rather than produced exclusively in the presence of laser illumination. Further, general inhibition may produce persistent downstream recruitment of the VTA, independent of plasticity factors.

A similar phenomenon has been documented in other subtypes of light-driven proteins. For example, channelrhodopsin-2 has been noted to leak charged particles across the cell membrane (Feldbauer et al., 2009). Though the same potential has not been observed in

halorhodopsin channels, this remains a possible explanation for lack of temporal specificity seen in our laser-induced eating effect.

Together, these two possible mechanisms of temporally non-specific increases in food consumption following laser exposure – plasticity in NAc and "leaky" halorhodopsins – would provide particularly sound explanation for the behavior of Rat 9788. Rather than eating more during laser days than non-laser days, 9788 ate progressively more across the six-day alternating laserON/laserOFF cycle irrespective of whether the rat was or was not exposed to laser on a particular test day.

More Variable Eating in Experimental Versus Control Rats

Overall, NpHR rats ate more overall than did controls. They also showed more variability in their eating behavior. Variation in food intake during repeated 1hr laser sessions was considerably higher in NpHR rats than in controls. On average, each individual NpHR rat showed greater fluctuation in their own food intake from laser session to laser session than inactive-viral controls. Furthermore, on non-laser test days, NpHR animals still showed higher individual variation than inactive-viral rats. Thus, the addition of halorhodopsin chloride channels may create difficulties for an animal's ability to regulate ingestive behavior and food seeking, while potentiating food intake overall.

In conclusion, we found that five of 20 total animals displayed increases in food consumption following direct optogenetic inhibition of the nucleus accumbens medial shell. However, this effect was not temporally specific to discrete periods to laser exposure, potentially resulting from "leaky" halorhodopsin proteins or persistent activation of sites downstream of the NAc. Finally, our NpHR animals overall showed more variability in their consummatory

behavior than did inactive-viral controls, indicating that the addition of halorhodopsin ion channels may create difficulties in the regulation of motivational tendencies. These results seem to corroborate the hyperpolarization hypothesis of accumbens-generated motivation and show that neuronal inhibition in the NAc may be primarily responsible for generating motivated behaviors.

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Figures

Figure 4.1. Wave I. Initial Test Waves Suggest Laser Inhibition Induction of Food Intake **A)** Initial test days hinted that laser-hyperpolarization may produce enhancements of food intake. After 6 days of testing, animals went through extinction testing, in which it took several days to decrease food consumption. Upon the first laser probe, animals jumped to nearly 5-8 times the amount of food eaten on the previous day. We then extinguished animals for an additional 6 days, before attempting further laser probes. On days where rats received less than 15-minute blocks of laser inhibition no major trends for increased food intake were observed. Food Consumption amounts varied across test days, notably increasing on days involving inhibitory laser exposure. Standard laser sessions (i.e. 15OFF/15constant_ON/15OFF/15constant_ON) are marked with darker yellow bars, and non-standard/cycled laser sessions with lighter yellow bars. **B)** An example timeline of a typical 1-hour test day for 9618 or 9619 indicating periods of laser exposure (yellow blocks) and periods of eating (green blocks) within a single session. Appeared to be 9618's eating was bound to laser exposure, while 9619's eating was not. **C)** Average animal activity across 6-day "standard" test cycle (TD1-TD6). Both 9618 and 9619 spent more time eating during laser sessions than non-laser sessions, and both spent less time inactive on laser days versus non-laser days. **D)** Fiber optic placements for 9618 and 9619.Placements for 9618 were bordering on medial NAc shell and medial NAc core, with placements for 9619 in rostromedial core.

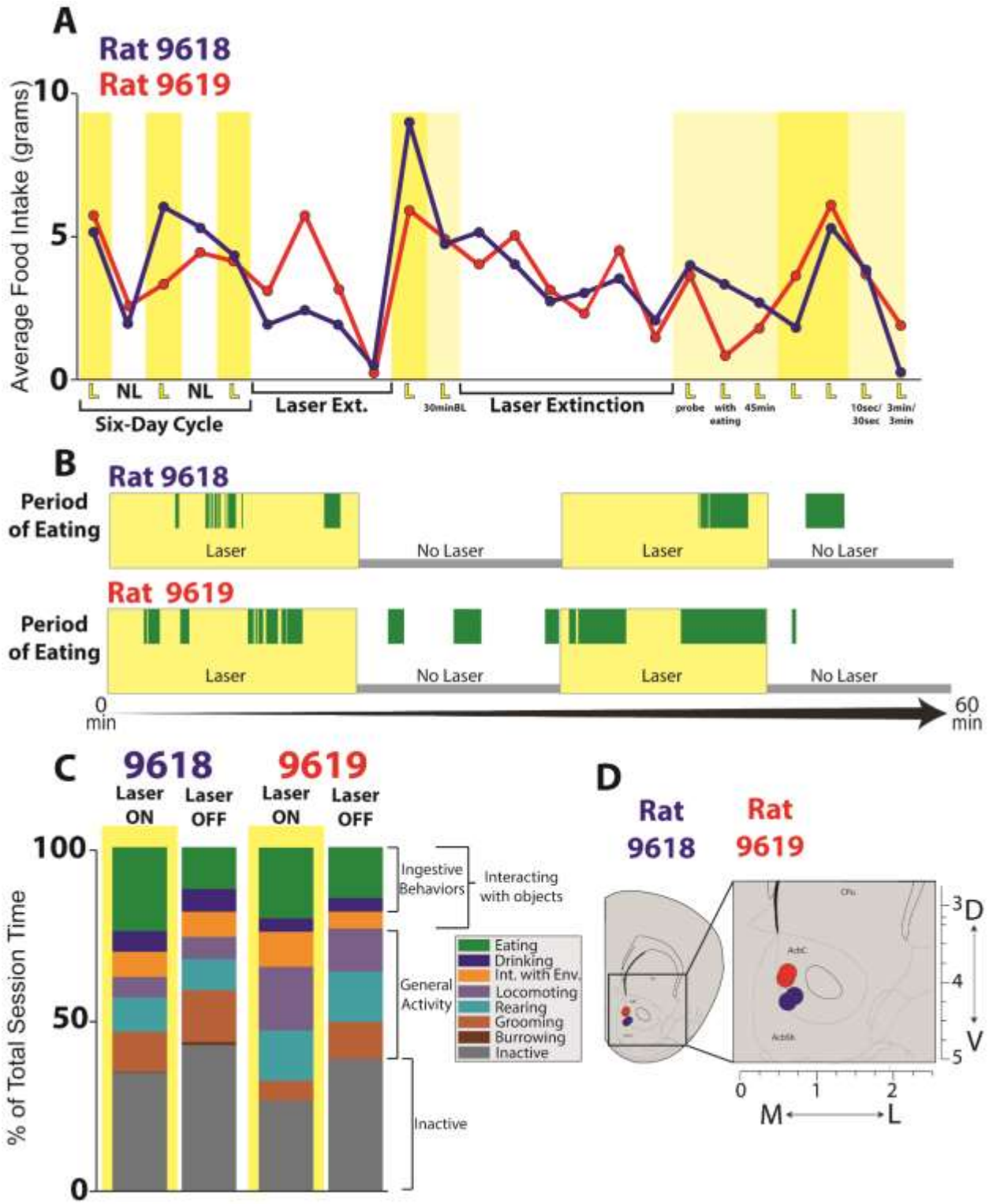
A
Rat 9618
Rat 9619
Average Food Intake (grams)
10
5
0
L NL L NL L
Six-Day Cycle
Laser Ext.
L L
30minBL
Laser Extinction
L L L L L L L
probe with eating 45min 10sec/ 30sec 3min/ 3min
B
Rat 9618
Period of Eating
Laser
No Laser
Laser
No Laser
Rat 9619
Period of Eating
Laser
No Laser
Laser
No Laser
0 min
60 min
C
9618
9619
Laser ON
Laser OFF
Laser ON
Laser OFF
100
50
0
% of Total Session Time
Ingestive Behaviors
Interacting with objects
General Activity
Inactive
Eating
Drinking
Int. with Env.
Locomoting
Rearing
Grooming
Burrowing
Inactive
D
Rat 9618
Rat 9619
CPu
AcbC
AcbSh
3
4
5
D
V
0
1
2
M
L

Figure 4.2. Wave II. Second test waves probed for minimal laser-induction of food intake. **A)** In our second wave, animals received minimal stimulations for the first several test periods to prevent lingering enhancement of laser-food intake. Following several days of variable lengths of laser exposure, rats were put through standard laser session conditions (i.e. 15OFF/15constant_ON/15OFF/15constant_ON) are marked with darker yellow bars, and non-standard/cycled laser sessions with lighter yellow bars. Within the six-day alternating laserON/laserOFF cycle, 9790's food intake increased on laser days and decreased on non-laser days, while 9788's eating increased across test days, regardless of laser exposure. **B)** A timeline indicating periods of laser exposure (yellow blocks) and periods of eating (green blocks) within a single session. Neither rat showed eating temporally bound to laser exposure. **C)** 9790 spent more time eating and less time inactive during laser sessions than non-laser sessions. 9788's eating time did not differ according to laser exposure, corroborating the ideal that this rat's food intake increased over time irrespective of laser. **D)** Placements for 9788 were in rostroventral shell and 9790 had placements in dorsal medial shell.

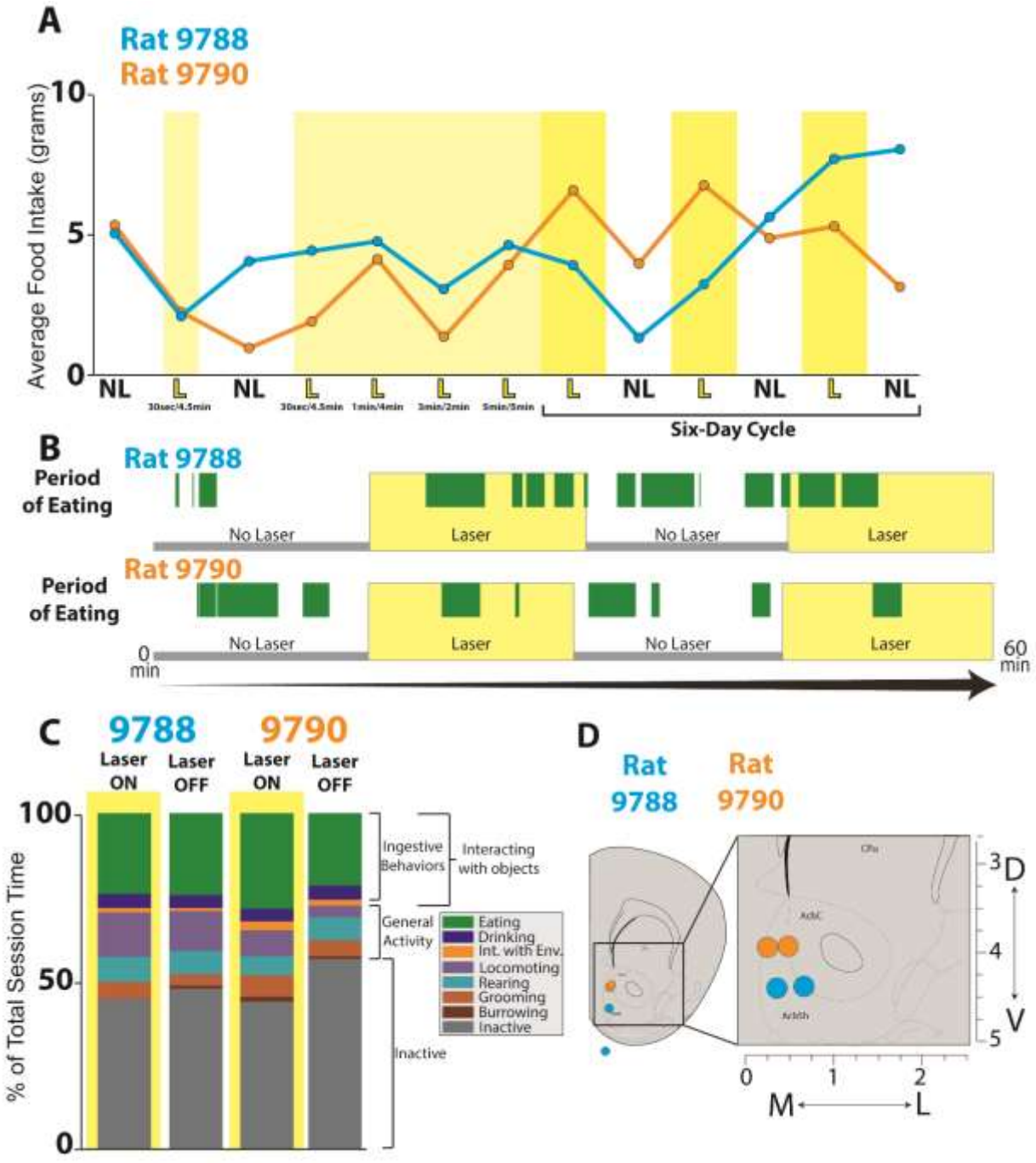
A
Rat 9788
Rat 9790
Average Food Intake (grams)
10
5
0
NL
L
NL
L
L
L
L
L
NL
L
NL
L
NL
30sec/4.5min
30sec/4.5min
1min/4min
3min/2min
5min/5min
Six-Day Cycle
B
Rat 9788
Period of Eating
No Laser
Laser
No Laser
Laser
Rat 9790
Period of Eating
No Laser
Laser
No Laser
Laser
0 min
60 min
C
9788
9790
Laser ON
Laser OFF
Laser ON
Laser OFF
100
50
0
% of Total Session Time
Ingestive Behaviors
Interacting with objects
General Activity
Eating
Drinking
Int. with Env.
Locomoting
Rearing
Grooming
Burrowing
Inactive
Inactive
D
Rat 9788
Rat 9790
3
4
5
D
V
0
1
2
M
L

Figure 4.3. Wave IV food intake. 10129 expressed NpHR while 10098 and 10099 were inactive-viral controls. 10129's food intake increased on laser days and decreased on non-laser days. Food intake for 10098 and 10099 did not follow this pattern, and remained steady across the seven test days. Due to time limitations, animal videos had not been scored, preventing detailed behavioral analysis on these days.

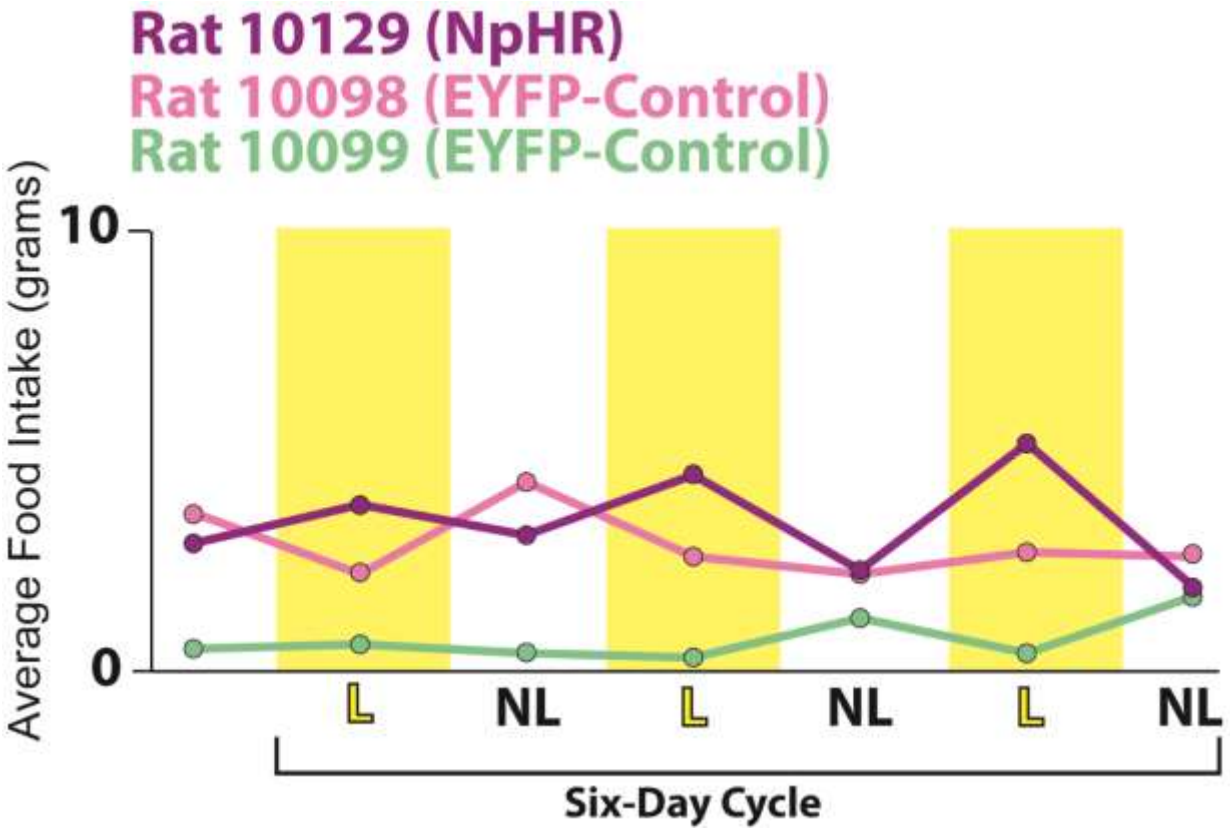

Figure 4.4. Average food intake and within-subject variation. A) Average food intake values for NpHR rats and inactive viral-control rats on laser and non-laser test days during 6-day "standard" condition. In general, rats that received NpHR stimulation ate more across days in which they received laser stimulation than on test days in which they did not receive laser stimulation with an average of about 5 grams on laser days vs 3 grams for non-laser days. Inactive-viral control showed roughly equal amounts of food intake, eating about 2.5 grams on either laser or non-laser days. This indicates that the presence of laser inhibition across the session encourages higher food intake, but not necessarily in a time locked manner. Additionally, NpHR rats ate more than controls across both laser and non-laser days, indicating that just the presence of NpHR proteins may encourage higher basal eating. **B)** Average standard deviation values for individual rats. NpHR animals showed individual variation than inactive-viral controls on both days in which they received laser inhibition and days in which they received no laser-inhibition. That is, individuals had more sporadic patterns of eating behavior in either condition. This may indicate that the presence of NpHR represents a difficulty in regulating eating behavior in these animals. Values calculated as individual rat standard deviation across 3 laser days or 3 non-laser days and averaged across NpHR or control groups.

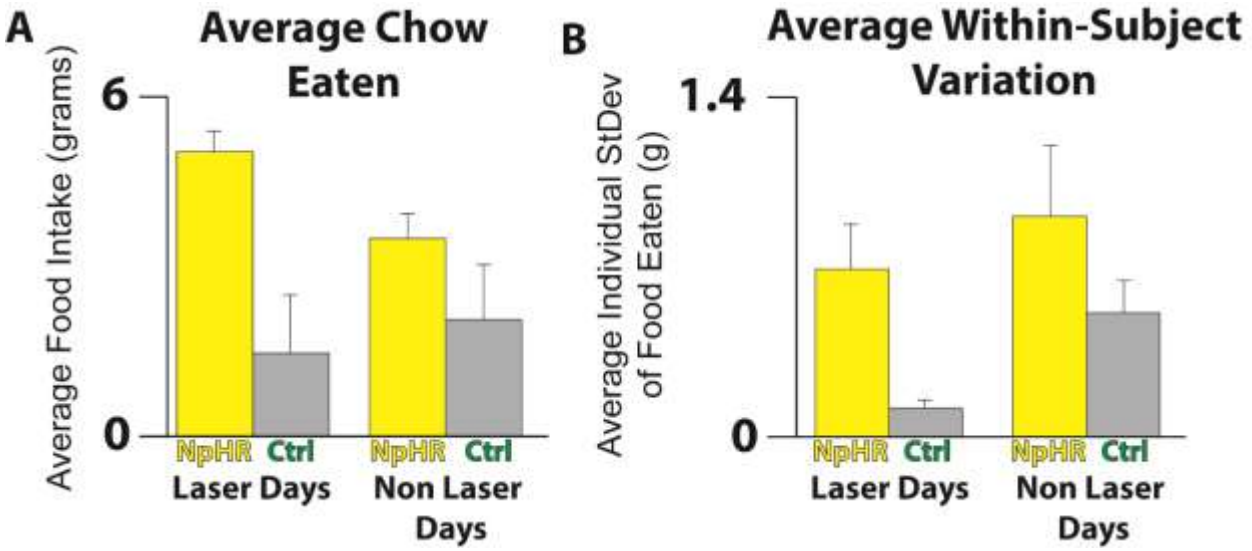

Table 1

Schedule of Test Days

Wave I. Rats 9618, 9619

TD1	Both rats run on standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON).
TD2	Both rats run without laser.
TD3	Both rats standard laser protocol.
TD4	Both rats no laser.
TD5	Both rats standard laser protocol.
TD6	Both rats no laser.
TD7	Both rats no laser.
TD8	Both rats no laser.
TD9	Both rats no laser.
TD10	Both rats standard laser protocol.
TD11	Both rats run 30minOFF/15min_constantON/15minOFF.
TD12	Both rats no laser.
TD13	Both rats no laser.
TD14	Both rats no laser.
TD15	Both rats no laser.
TD16	Both rats no laser.
TD17	Both rats no laser.
TD18	For both rats laser was turned on and off at pseudo-random intervals within session.

TD19	Both rats run 15minOFF/15minONOFF [with feeding]/15minOFF.
TD20	Both rats run on 45min laser protocol (15minOFF/15min_constantON/15minOFF)
TD21	Both rats standard laser protocol.
TD22	Both rats standard laser protocol.
TD23	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 10secON/30secOFF.
TD24	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 3minON/3minOFF.

Wave II. Rats 9788, 9790

TD1	Habituation (no laser).
TD2	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 30secON/4.5minOFF.
TD3	Both rats no laser.
TD4	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 30secON/4.5minOFF.
TD5	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 1minON/4minOFF.
TD6	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 3minON/2minOFF.
TD7	Both rats run 15minOFF/15min_cycledON/15minOFF/15min_cycledON; cycle 5minON/5minOFF.
TD8	9788 received laser unilateral left side from this point on; both rats run on standard laser protocol (15minOFF/15min_constantON/15minOFF/15min_constantON).

TD9	Both rats no laser.
TD10	Both rats standard laser protocol.
TD11	Both rats no laser.
TD12	9788 standard laser protocol; 9790 no laser.
TD13	9788 no laser; 9790 standard laser protocol.

Wave IV. Rat 10129

TD1	Habituation (no laser).
TD2	Standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON).
TD3	No laser.
TD4	Standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON).
TD5	No laser.
TD6	Standard laser protocol (15minOFF/15min_constantON/15minOFF/ 15min_constantON).
TD7	No laser.

Pilot Project II: Optogenetic Excitation of Dopamine Neurons Enhances Motivation for Social Partner

Introduction

Mesolimbic dopamine has long been implicated in reward seeking behaviors, such that changes in DA activity are thought to lead to altered responses for food, drug, and social rewards (Berridge and Robinson, 1998; Di Chiara, 2002; Aragona et al., 2003; Berridge, 2007; Berridge et al., 2009; Humphries and Prescott, 2010; Richard and Berridge, 2011b; Saunders et al., 2013; Ikemoto and Bonci, 2014; Yoest et al., 2014; Castro et al., 2015).

In looking more specifically at dopamine and social interaction, previous studies have shown positive correlations between the two. Looking generally at the relationship, reduction of dopamine in the brain is associated with decreases in social play (Vanderschuren et al., 1997; Trezza et al., 2010). However, different receptors lead to different responses in regards of interaction. For example, D3 receptors do not result in a significant effect on social play while D2 receptors will respond with increased play to low doses of a dopamine agonist and decreased social play at high doses of the agonist. Higher turnover rates of dopamine in the brain during social play also suggest that dopamine plays a role in social interactions and more specifically at certain dopamine receptors to increase interactions in rats (Vanderschuren, et al. 1997). By

allowing for widespread release of dopamine it is likely that some of these receptors could be activated and lead to a certain response in rats to increase socializing.

The connection between dopamine and interaction is further demonstrated by the varying dopamine levels in different species of rats and the overall effects this has on their social interaction. Fischer 344 rats are seen to have a deficient dopamine system in comparison to other rat species. In one study, Fischer 344s were directly compared in opposition of Sprague-Dawley rats (Siviy and Panksepp, 2011). Behaviorally, Sprague-Dawley rats are more social than the Fischer 344 rats. The Fischer 344 rats are less likely initiate playful contact and are less likely to maintain contact once it had been started. Physiologically, the Fischer 344 rats are seen to have less dopamine release specifically in the dorsal striatum and nucleus accumbens core, an area that is also important in the dopamine learning circuitry (Siviy and Panksepp, 2011). These differences between these rat species demonstrate the importance of dopamine in social interactions and although Long-Evans rats are used in this experiment the neurophysiological differences are important marker in behavior for this study. Furthermore, these researchers focus on deficits in dopamine and not increases in its levels compared to norm and offer more questions to be answered. Social play behavior has been shown to be highly rewarding (Vanderschuren, 2010; Trezza et al., 2011) and this social reward is thought to be mediated through motivational circuitry similar to other rewards, such as food, sex, and drugs (Trezza et al, 2010; Siviy and Panksepp, 2011).

The project below outlines preliminary data of a pilot project in which used transgenic rats to selectively target dopamine neurons in the ventral tegmental area (VTA), which project to the nucleus accumbens (NAc). In preliminary analysis, I found suggestion that stimulation of VTA dopamine increases motivation for a social partner, and that exposure to laser-associated

partners vs those not-associated with laser indicates a preference for a laser-paired rats. General activity and exploration also appear to be increased during sessions of laser excitation or even during exposure to social partners in the absence of laser stimulation

Methods

Subjects

17 female Th-Cre-positive Long-Evans rats (250-350 g and at least 3 months old) and 34 female Th-Cre-negative Long-Evans rats were housed in pairs or groups of three on a 12:12-hour reverse light/dark cycle at ~21°C with *ad libitum* access to food (Purina Rat Chow) and water and then run over the course of five waves (n = 3, n = 3, n = 4, n = 3, and n = 4, respectively). All animals were initially genotyped to check for expression of Cre under the Th promoter; those lacking the trait did not receive surgery and were used as compatriots (2 compatriots assigned to each test animal). Animals were taken from an in-house breeding colony and research model services (Envigo, Cambridgeshire, UK). The Committee on the Use and Care of Animals at the University of Michigan approved all experimental procedures.

Surgery

Virus infusion. All rats were anesthetized using isoflurane (5% induction, maintenance at ~2%) and pretreated with atropine (0.05 mg/kg) to aid respiration, cefazolin (0.05 mg/kg, i.p.) to prevent infection, and the analgesic carprofen (0.1 mg/kg, s.c.). Rats were secured on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and 1.0µl infusions of a Cre-targeting channelrhodopsin virus (AAV5-DIO-CHR2-EYFP) or an inactive control virus (AAV5-DIO-EYFP) at a rate of 0.1µl per minute were administered into the VTA (AP, -5.76;

ML, ±2.98; DV, -8.4). The virus was allowed to diffuse for 10 minutes immediately following the 10-minute injection. Data from 10 animals is described here, based from viral expression and fiber placement accuracy (7 expressing ChR2 and 3 expressing EYFP control virus).

Fiber implantation and skullcaps.

After diffusing, fiber optic implants were inserted 0.3mm above the injection site and at a 16.4° lateral angle to properly stimulate the virus target sites. Implants consisted of 230µm-diameter optic fibers inserted into 9mm-long ferrules and were secured to the skull with dental acrylic, leaving ~5mm of ferrule accessible above the skull cap. Following surgery, all rats were monitored for 7 days and given daily triple-antibiotic ointment around the skullcap. On the first and second days of the recovery period, rats received the same carprofen dosage that they received during surgery (0.1mg/kg, S.C.) for pain management. After the initial 7-day postoperative monitoring, animals were given three more weeks to recover and to allow for proper expression of the ChR2 virus before testing.

Handling and habituation

Rats were handled for ~15 minutes each for 2-3 days before starting habituation. During habituation, one animal was placed in the 1x2ft social testing chamber with cob bedding for 20 minutes to allow them to get familiar with the environment. Habituation lasted for three days and was done for all test animals and compatriot animals. Test animals had optogenetic fibers attached to their fiber implants throughout habituation, with no laser output. The laser power supply was turned on with no output for both test and compatriot animals to keep background noise consistent with the testing environment.

Social Interaction Testing

Social rat trials consisted of two separate twenty-minute sessions in the 1x2ft social testing chamber for each test animal, (I) laser-paired and (ii) non-laser-paired, unless otherwise noted. A laser-paired rat and a non-laser-paired conspecific were chosen randomly from the available Th-Cre-negative rats and assigned to each test animal; assignments remained constant throughout the experiment. One test animal and one if its conspecific animals were in the testing chamber at all times for social interaction testing. Test animals had optogenetic fibers attached to their fiber implants with a laser output of approximately 10mW for laser-paired sessions and no laser output for non-laser-paired sessions. To determine baseline activity for each animal without laser stimulation, the laser-specific sessions were split into four five-minute blocks of time: (I) no laser, (ii) laser, (iii) no laser, and (iv) laser. Laser stimulation was applied for ~10s starting upon initial contact between animals, followed by a brief (~30s) cooldown period with no laser. In the second session, interaction with a second and distinct, non-laser-paired partner (never paired with laser stimulation) was utilized to determine baseline social activity for the test animal when paired with an animal that is never associated with any stimulus. Each session was recorded and analyzed to determine if test animals showed increased social activity during stimulation as well as specific exploratory social behaviors such as anogenital sniffing, body contact, pinning, chasing, etc.

Partner Preference Testing

The partner preference test lasted 40 minutes and was conducted in the same social chamber used for social activity trials. Two small, clear receptacles were placed on opposite

sides of the social testing chamber: one contained the laser-paired rat paired with laser in previous sessions and d the other contained the non-laser paired animal for a given test subject. The test animal was then placed in the middle of the chamber and was able to roam free for the duration of the session. Animals were able to clearly see one another, as well as smell one another through small holes near the bottom of the receptacles containing the two paired rats, but they were unable to make direct contact. Primary data for these trials consists of behavior analysis as described above, as well as the total amount of time spent on each side of the chamber.

Histology

Following completion of partner preference testing, animals were euthanized by lethal injection of sodium pentobarbital and perfused using 4% paraformaldehyde to preserve brain tissue. Brains were stored in 4% paraformaldehyde for 1-2 days and then transferred to a 25% sodium solution for 2 days before being sliced into 40µm sections using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) to assess virus expression and fiber placement sites. Skullcaps were also removed during perfusions to examine general placements of fiber implants. Brain sections were then placed in well plates containing 4% paraformaldehyde and stored in a -20℃ freezer. Sections were individually mounted onto glass slides and cover slipped using paramount medium, then preserved in the freezer at -4 degrees. Using a microscope, sections were imaged to determine accuracy of fiber placements that occurred during surgery and observe virus expression in each individual test animal.

Results and Discussion

Comparisons of ChR2 stimulation vs EYFP controls in social exploration

In laser sessions, ChR2 or EYFP rats were given access to a rat that would be paired with laser illumination upon close proximity or contacted by the ChR2 or EYFP rat. In non-laser sessions, an alternative rat never paired with laser for any test session was introduced to the ChR2 or EYFP rat. Rats receiving DA ChR2 neuron stimulation spent about 280 seconds generally sniffing rats paired with laser-stimulation vs about 120 seconds with those that were never paired with laser stimulation (Wilcoxon, $Z=4.157$, $p<0.00001$, Fig. 4.5). More specifically, ChR2 rats showed about 130 seconds of anogenital sniffing for laser-paired rats vs about 75 seconds on non-laser rats (Wilcoxon, $Z=4.372$, $p<0.00001$, Fig. 4.5). For social partners paired with laser-illumination, ChR2 rats showed 30% higher general sniffing and 200% higher anogenital sniffing than EYFP controls (Kruskal-Wallis, $X^2=5.216$, $p=.022$; Kruskal-Wallis, $X^2=16.963$, $p<.001$, Fig. 4.5) and 600% more bouts of general sniffing and 400% more anogenital sniffing than EYFP controls (Kruskal-Wallis, $X^2=6.057$, $p=0.14$, Kruskal-Wallis, $X^2=17.206$, $p=0.0001$). No differences were seen in the amount of time burrowing, freezing, or grooming on laser sessions vs non-laser sessions (Wilcoxon, $Z=0.365$, $p=0.715$; Wilcoxon, $Z=0.943$, $p=0.345$; Wilcoxon, $Z=1.386$, $p=0.166$, Fig. 4.5). However, on laser sessions vs no laser sessions, ChR2 rats showed 25% decreases in rearing (Kruskal-Wallis, $X^2=2.731$, $p=0.006$) though no different than EYFP controls receiving laser-stimulation (Kruskal-Wallis, $X^2=0.19$, $p=.891$, Fig. 4.5). In other comparisons of general behavior between ChR2 rats and EYFP on laser-paired social partners, we observed no differences for grooming or burrowing (Kruskal-Wallis, $X^2=1.576$, $p=.209$; $X^2=1.576$, $p=.209$, Fig. 4.5). Further, ChR2 rats pinned conspecifics more (Kruskal-Wallis, $X^2=8.42$, $p=.004$), but showed no statistical differences in nuzzling (Kruskal-Wallis $X^2=.011$, $p=.917$) or mounting than EYFP rats with their laser-paired social

partner (Kruskal-Wallis X^2=1.395, p=.238, Fig. 4.5). Note: One rat in particular showed amounts of mounting time, whereas others were relatively low, and a different ChR2 rat in particular showed greatly elevated nuzzling, whereas almost all rats others showed almost no nuzzling in any sessions. ChR2 rats showed 30% higher general locomotion recorded as higher cage crosses than EYFP controls (Kruskal-WallisX^2=4.781, p=0.029, Fig. 4.5). During laser sessions, ChR2 rats showed total time active than in non-laser sessions (i.e., not immobile/inactive) (Wilcoxon, Z=2.731, p=0.006), and were more generally active than EYFP rats (Kruskal-Wallis=2.381, p=017).

In comparisons of ChR2 rats and EYFP controls on sessions with the never laser paired, ChR2 rats showed no difference from EYFP in the amount of general body sniffing (Kruskal-Wallis, X^2=.655, p=0.418, Fig. 4.5), but did show higher levels of anogenital sniffing at about a 2:1 ratio (Kruskal-Wallis, X^2=5.695, p=0.17). General behaviors, such as freezing, grooming, rearing, and burrowing were not different between ChR2 rats and controls on non-laser sessions (Kruskal-Wallis, X^2=1.647, p=0.199; Kruskal-Wallis, X^2=2.747, p=0.97; Kruskal-Wallis X^2=3.61, p=0.57; X^2=2.124, p=0.145). However, D2 ChR2 rats were generally more active than EYFP controls during sessions with no laser stimulation (800 seconds vs 500 second; Kruskal-Wallis, X^2=6.693, p=0.01), though not as active as on sessions in which they received laser-stimulation.

These findings suggest that laser induced DA enhancement may enhance social exploration of a partner paired with stimulation. Of social interaction behaviors, sniffing and anogenital sniffing showed the greatest consistent increase with laser stimulation in rats expressing ChR2, relative to both EYFP control rats or in sessions where ChR2 rats were given exposure to a separate conspecific with no laser stimulation. Further, ChR2 rats showed greater

general locomotion and activity, suggesting that DA stimulation is at the very least decreasing inactivity, which is manifest as heightened locomotion or some modes of social exploration, though not necessary play behavior.

Partner Preference Testing

Following social interaction testing with laser partners and non-laser partners, rats were given 3 days of testing in which they were presented with both the laser-paired rat and non-laser paired rat confined to opposite sides of the testing chamber by clear perforated calendars. Rats received no laser during this session and were able to freely about the chamber to see if they preferred a rat paired with VTA stimulation or no stimulation.

ChR2 rats did not show preferences for either the side paired with the laser rat (Wilcoxon, Z=.292, p=.767) or non-laser rat (Wilcoxon, Z=.292, p=.767, Fig. 4.6) (about 20 minutes each per side), and did not show a greater amount of time touching (Wilcoxon, Z=1.599, p=0.110, Fig. 4.6) or in the number of contacts with laser-paired rat chambers than non-paired chambers (Wilcoxon, Z=1.541, p=.123, Fig. 4.6). Further, in comparison to EYFP rats, ChR2 rats showed no differences in the time spent on either the side of laser-paired rats (Kruskal-Wallis, X^2=.990, p=0.32, Fig. 4.6) or non-laser paired rats, with both spending within 18-20 minutes on either side (Kruskal-Wallis, X^2=0.02, p=887, Fig. 4.6). However, relative to EYFP controls, two main differences were seen: 1) ChR2 rats made a greater number of contacts on to chambers containing either the laser-paired rat (about 38 vs 24) (Kruskal-Wallis, X^2=5.518, p=0.019, Fig. 4.6) or the non-laser rat (about 38 vs 22) (Kruskal-Wallis, X^2=1.640, p=0.20, Fig. 4.6), and 2) spent more time contacting the laser-paired rat chamber (about 450 vs 250 seconds) (Kruskal-Wallis, X^2=7.682, p=.006, Fig. 4.6). Further, laser rats showed more shifts between the

two chambers, as entries for both left (Kruskal-Wallis, X^2=8.30, p=004, Fig. 4.6) and right sides (Kruskal-Wallis, X^2=8.08, p=.004, Fig. 4.6) were higher than for EYFP controls.

Together, it appears that even in the absence of laser stimulation, rats who have received ChR2 stimulation in the presence of a social partner may show a slight preference for that same partner. Moreover, ChR2 rats showed higher levels of movement between regions where laser or non-laser paired rats were confined, and in general spent more time in contact with both chambers. This may indicate that ChR2 rats were more prone to investigating social partners, but they also showed a higher preference for laser-paired partners than ChR2. It may be due to the physical barrier between social partners that ChR2 rats spent more time determining each animals' identity. Alternatively, it may be that the presence of laser-partners served as a Pavlovian cue, serving as a predictor that the laser would arrive upon close proximity to the social partner and/or producing a craving state for laser induction. As there was no laser during partner preference testing, ChR2 rats may generally explore more in an attempt to acquire a ChR2 depolarization-UCS.

In conclusion, preliminary findings suggest that ChR2 stimulation of dopaminergic VTA neurons which project to the NAc may be able to enhance motivation for a social partner. While social play may not be enhanced *per se*, the seeking of a partner paired with dopamine stimulation hints at enhancement of social reward or enhancement of incentive motivation for a social partner. Furthermore, in comparison to EYFP-inactive viral controls, rats receiving ChR2 stimulation showed greater activity and moderate preference for laser-paired partner than non-paired partner. This provides evidence that even in the absence of laser stimulation, once a social partner has been paired with dopaminergic activity, they might serve as an incentive cue to the biological state of enhanced dopamine.

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Figures

Figure 4.5. Social Exploration is Enhanced by ChR2 Stimulation of Dopamine Neurons: Rats are tested in sessions where close contact to a social partner receives laser illumination or separate sessions where a different social partner is never paired with laser illumination. **(Top left)** General exploratory behaviors of sniffing and anogenital sniffing of a social partner are both enhanced by VTA stimulation, relative to interactions with rats not paired with laser stimulation or EYFP-controls in which laser illumination provides no stimulation. **(Bottom left)** specific social play behaviors and exploration behaviors are inconsistent between animals, though pinning appears to be enhanced with ChR2 stimulation. **(Top Right)** Non-social specific behaviors of grooming and rearing are lower for rats in session with ChR2 stimulation than in sessions with a non-laser social partner. **(Bottom Right)** Average cage crosses are higher for rats receiving ChR2 illumination vs EYFP controls, and ChR2 rats both receiving laser stimulation, or no stimulation show greater general activity than ChR2 controls. Days with laser illumination, ChR2 rats show higher levels of activity than on non-laser days. Blue bars represent data for sessions where ChR2 rats interacted with a rat that when contacted would produce laser stimulation. Purple bars represent data for EYFP animals on sessions with a laser-paired rat. Grey bars represent sessions where ChR2 or EYFP animals received no laser stimulation with a separate non-laser paired partner. * $p<0.05$

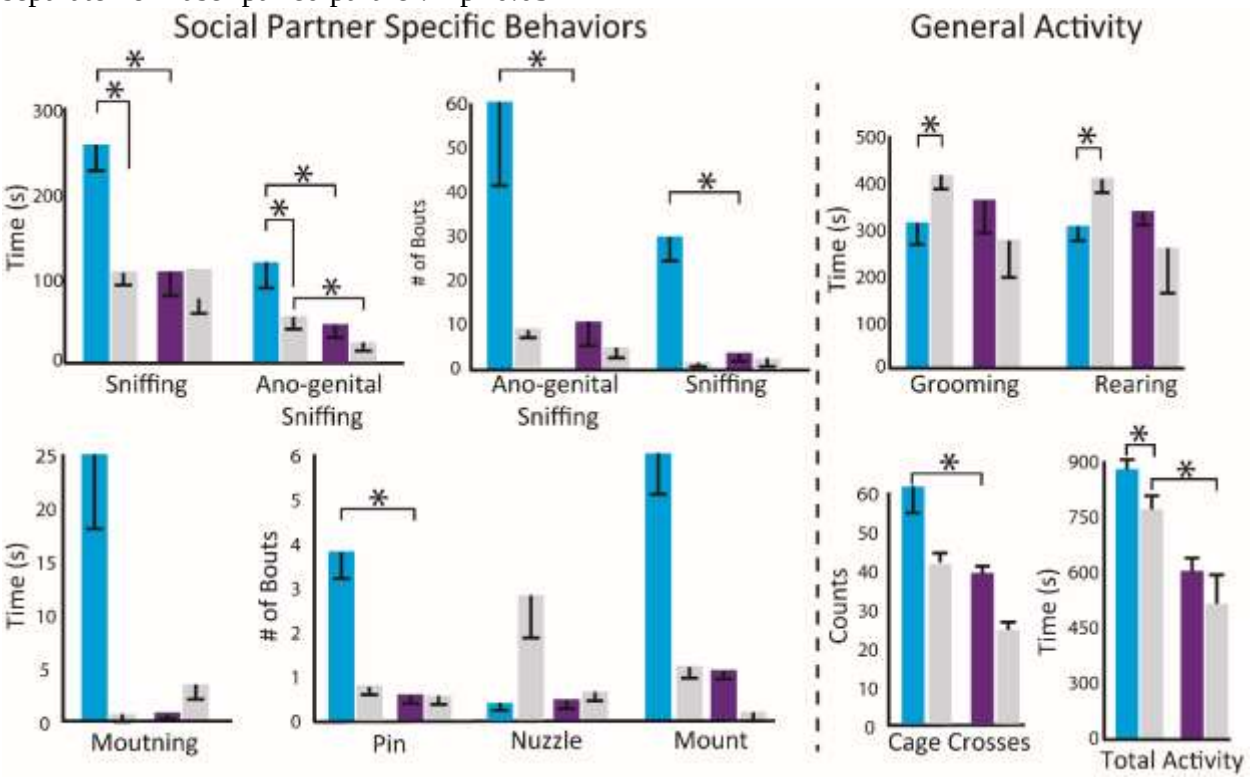
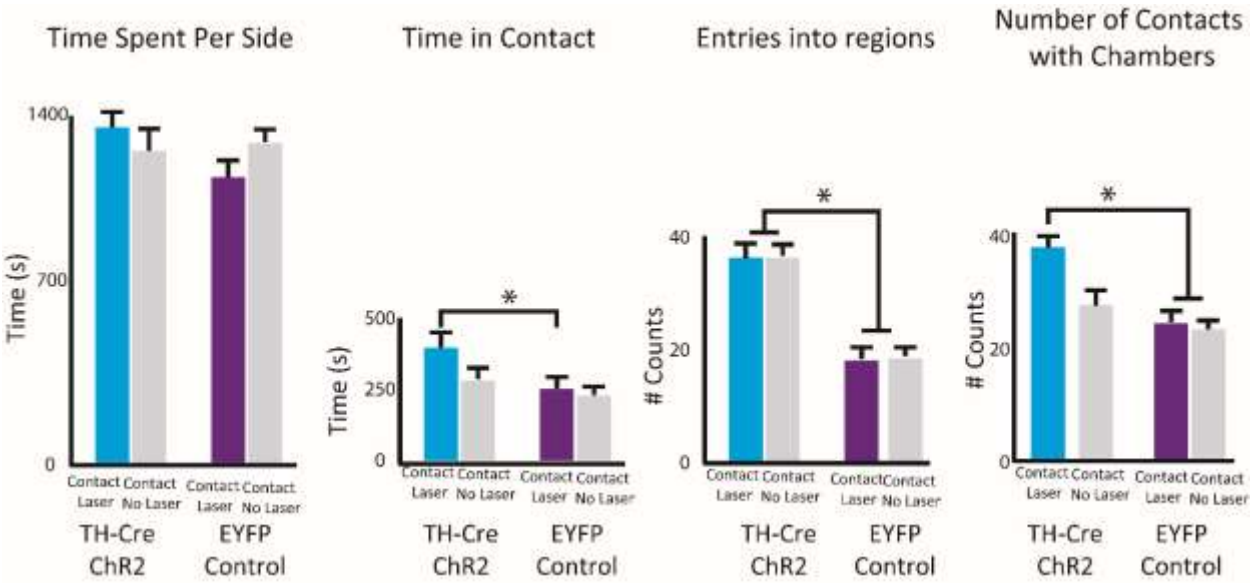


Figure 4.6. Partial Partner Preference for Laser-Paired Rats: Rats are given free access to both laser-paired and non-laser paired rats, which are confined by perforated clear cylinders. No laser is given during these test sessions, and the test rat can freely explore and interact with each rat. ChR2 and EYFP controls showed no preferences for either the side of the laser-paired rat or the no laser-paired rat. However, ChR2 rats showed more time in contact with the laser-paired rat's chamber. Interestingly, ChR2 rats showed a greater number of contacts on both the laser-rat chamber and non-laser rat chamber relative to EYFP controls, suggesting general greater investigation of both social partners. Finally, ChR2 rats showed greater movement across the chamber indicated by a greater number of entries. Blue bars represent data for ChR2 rats interacting with laser-paired rats. Purple bars represent data for EYFP rats interacting with laser-paired rat. Grey bars represent data for ChR2 or EYFP rats interacting with rats never paired with laser. * $p<0.05$



Chapter 5

General Discussion

The purpose of this dissertation was to identify mechanisms through which the nucleus accumbens (NAc) produces or gates motivation. Below, I address how my findings in the above chapters fit within two different literatures on motivation, each with substantial conflict and evidence on each side. First, I discuss how my findings of excitation-mediated reward or avoidance in D1 and D2-dopamine receptor expressing neurons correspond to traditional basal ganglia organization, which holds opposing roles for reward/go signaling mediated by a D1- "direct" projections pathway and punishment/avoidance/stop signaling mediated D2- "indirect" projection pathway. Second, I summarize my findings that reversal of DNQX-induced food intake and generation of food intake via optogenetic-inhibition, which provide evidence that inhibition within the NAc is both necessary and sufficient for certain motivational signals. Finally, I discuss how both excitation and inhibition of the NAc may be mechanisms creating motivated states.

D1 reward and D2 ambivalence in the nucleus accumbens

In Chapter 2, I sought to determine whether NAc optogenetic depolarization was sufficient to induce reward in NAc D1 neurons and whether D2 neuron stimulation could

produce avoidance/punishment in D2 neurons akin to in dorsal striatum (Kravitz et al., 2012). To determine if subpopulation-specific excitation would produce pattern of D1-reward/D2-avoidance, I optically stimulated transgenic mice expressing excitatory ChR2 receptors in either D1 or D2 neurons upon contact with a touch-sensitive bottle spout. I found that 1) D1 stimulation produces robust self-stimulation at 3000% of control animals with an inactive virus. 2) In contrast to predictions, D2 depolarization also produced mild self-stimulation, at ~500% of control animal rates of self-stimulation. Additionally, D1 animals readily tracked laser-delivering spouts as the location was changed every 3 days for a total of 9 test sessions, whereas D2 animals showed difficulty in tracking laser positions, but maintained moderate rates of self-stimulation. Furthermore, I demonstrated self-stimulation behavior was directly tied to laser delivery, as removal of laser abolished self-stimulation in both D1 and D2 mice.

To further establish that NAc D1 or D2 depolarization was rewarding, mice were run in a second paradigm similar to the original Olds and Milner (1954) self-stimulation experiments. Mice were exposed to an open field chamber where they were able to receive laser-stimulations based on location within a 4-corner chamber. One corner per session was paired with laser stimulation for three days, with laser-paired corners changing each day. Overall, D1 animals preferred laser-paired quadrants about 50% more than any other corner. However, in contrast to the spout-based stimulations, D2 ChR2 mice showed no general preference across all 3 test days, and a subgroup developed an avoidance to the laser-paired locations by the 3rd test session. The results of the location-based task are consistent with what Kravitz et al (2012) found with stimulations in dorsal striatum; that is, D1 mice will track locations paired with laser-depolarization, whereas D2 mice avoid laser-paired locations. However, in our study, D2 mice

did not demonstrate the freezing/pausing behavior seen by Kravitz et al. (2012) and both D1 and D2 mice were in motion for nearly 90% of the test sessions.

Stimulations for Reward and Avoidance

It is a bit perplexing that differences existed in D2 self-stimulation between the spout and location-based tasks. As described in Chapter 2, one explanation comes from the nature of stimulation duration, and that there may be relatively longer durations of laser stimulation in the location task than in the spout task. It may be that a greater depolarization of D2 neurons shifts from rewarding to undesirable due to longer exposure to periods of laser stimulation. Work by Eliot and Thelma Valenstein (1964) demonstrated in multiple limbic brain regions that almost all rats with control of electrode stimulation would eventually terminate stimulation, and that higher intensities of stimulation corresponded to earlier termination. A contemporary optogenetic study shows animals receiving stimulation of excitatory-glutamate neurons in VTA has also show a preference for shorter durations of shorter bins of stimulation, rather than preferring constant depolarization (Yoo et al., 2016). One simple explanation is that the series/longer durations of laser-depolarizations produces a buildup of negative/avoidant motivation, and is no longer desired. An alternative explanation to negative-build up and short-duration preference comes from the additional fact that rats who receive high-intensity electrical stimulation are quicker to again turn on electrode stimulation (Valenstein and Valenstein, 1964). Similarly, in our hands, it may be that these longer periods of D2 neuron depolarization may not reflect a shift from positive to negative, but that constant or rapid stimulation may decrease the rewarding nature of NAc depolarization and through longer periods of "off time" the laser-reward is renewed. It has been shown in electrode-based stimulation that fixed durations longer than those elected by the

test animal do not produce hesitation for subsequent operant stimulation (Valenstein and Valenstein, 1963) and can produce quicker response (Keesey, 1964) and increased willingness to work to obtain self-elected stimulation trains (Hodos, 1963). Extended to D2 neuron stimulation: although the mouse may elect to leave the laser-paired zone, if an optimum duration or intensity of laser-stimulation were identified for the individual mouse and stimulation beyond that preference imposed by the experimenter, a preference might be seen for laser-paired locations.

In our initial dose response and pulse parameter tests, we observed that the intensity of laser-stimulation enhanced self-stimulation in D1 and D2 mice, with 10mW intensities of laser-stimulation producing the highest rates of self-stimulation, 1mW produced moderate levels of self-stimulation, and 0.1mW producing the lowest rates of self-stimulation. Further, we also tested whether pulsed (25Hz) vs constant illumination would alter rates of self-stimulation, finding no statistical differences between 25Hz or constant stimulation. At both 25Hz and constant stimulation parameters, D1 and D2 mice showed increases in response to increases in laser intensity, and several D2 mice responded as high as our highly responding D1 mice, reaching up to just over 1000 stimulations per session. These findings are especially interesting considering the ambivalence in D2 mice: 1) this gives further indication that, at least for D2 mice, individuals may have optimal patterns of stimulation for the expression of certain actions. Longer durations of stimulation may not always be equivalent to a higher intensity to a D2 mouse, and they may produce opposite reactions to these stimulations. 2) An interaction between the duration of stimulation, intensity of stimulation, and nature of the stimulus/response differences between the spout and location tasks may create different "sweet spots" depending on the state of all tree variables. Borrowing from the electrode-stimulation findings described above: could it be that changing the contingencies of neuronal depolarization have revealed

thresholds by which certain D2 stimulations can be either “wanted” or “avoided”? That is, though a mouse may find the action and pattern of stimulations in the location task aversive, the shift in paradigm to spout may produce stimulation beyond an “avoided” threshold and into a “reward” range. If the time course and stimulation intensity of the spout task were somehow imposed upon the location-based task, it may be that the mouse finds locations paired with laser-stimulation rewarding. The dose-response data demonstrate that the stimulation of D2 neurons does not solely cause a bias towards laser-stimulation, but that the magnitude of stimulation can also enhance levels of self-stimulation and the degree of motivation made manifest. It would be interesting to know whether a shift from 1mW to 10mW stimulation is more readily avoided in the location task. As noted above, another element is the difference in stimulus/response nature between the two tasks. The fact that spout-stimulation can be instrumentally controlled by the discrete and localizable CS/UCS of the spout vs a diffuse context in the location task that may be the difference in avoidance or preference, which is perhaps a more parsimonious explanation for spout and location differences.

“Optimal” Optogenetic Stimulation: Pulse vs Constant Stimulation in Reward

Another point of interest in our pulse parameter test, is that *consistent differences* in the amount of self-stimulation were not found in comparisons of 25Hz vs constant stimulation in D2 mice. In D1-mice, 25 Hz and constant stimulation provided roughly equal levels of self-stimulation at 0.1mW, 1.0mW, or 10mW intensities. Some individual D2 ChR2 mice did show higher degrees of self-stimulation in the 25Hz condition, but both low animal count and high variation within condition make these findings somewhat difficult to interpret.

The logic to test at constant and low-intensity stimulations stemmed initially from the work of Lex Kravitz and Anotol Kreitzer (2011), who suggested that through low intensity, constant stimulation, neurons could be encouraged to fire while maintaining endogenous patterns of firing. This is in contrast in contrast to high-intensity, pulsed trains of stimulation which directly impose potentials upon neurons. In this paper, Kravitz and Kreitzer demonstrated a low intensity, dose response curve ranging from 0.1mW to 3.0mW, with two particularly interesting findings: 1) Individual neurons within striatum have optimum firing rates, and 2) even low intensity (0.1mW) constant stimulation can produce as high of a firing pattern a 3.0 mW in a separate neuron. In a separate experiment, Kravitz and colleagues (2010), showed that both the waveform characteristics during 1mW, constant stimulation in both striatal D1 and D2-expressing neurons are not altered between laser-stimulation periods and non-laser periods. That is, the wave forms of striatal neurons are nearly identical during ChR2-mediated depolarization and periods of no laser illumination, despite that firing occurs more frequently under laser illumination. This gives credence to the claim that low-intensity, constant stimulation may better mimic endogenous patterns of neuronal activity.

The Kreitzer group has made considerable revelations into the electrical nature of striatal neurons by utilizing selective D1 or D2-neuron stimulation in tandem with careful electrophysiological analysis. In addition to the details above, Kreitzer & Kravitz (2010) showed no differences between neurons expressing ChR2 sodium ion channels and neurons infected only with EYFP in the absence of illumination. Further, they showed that D1 neurons may normally have a lower firing rate than D2 neurons, and upon laser stimulation, D1 neurons have a greater net shift in firing than D2 neurons. Thus, each individual laser-depolarization has a greater net shift upon D1 neurons than D2 neurons, and may be partially responsible for the greater

behavioral impact observed in our study. This also fits with my finding that although D1 and D2 activation produced enhancements in Fos expression, D1 ChR2 stimulation produced higher densities of Fos expression, possibly as a result of greater electrical shift. It is interesting that D1 and D2 ChR2 mice show similar levels of distributed Fos, though D2 mice showed generally lower local recruitment of Fos plumes within the NAc as well as lower self-stimulation on the spout task and fewer excitations in the location-based task. An additional explanation for differences in local Fos and similar levels of downstream Fos is that individual D2 neuron excitations may have stronger effects than D1 neuron excitation, and so fewer neurons need to activated to produce the same degree of network activation. Though there may be a greater shift from neuron resting potential in D1 neurons, this may not necessarily equate to greater reward per excitation and doesn't appear to reflect greater downstream activity. In our case, a more psychologically and neurally potent signal would require fewer depolarizations to achieve similar network activation, such that an average of ~500 D1 neuron stimulations may be roughly equivalent to ~60 D2 neuron stimulations.

Kreitzer and colleagues (2013) also identified either various electrical phenotypes of neurons that fire quickly (within 15ms of laser onset) or those that take longer to spike (100ms or so). They purport that MSNs have low excitability and firing rates, and so many not fire reliably with quick, high-powered stimulation. Perhaps more interesting, MSNs have fluctuating membrane potentials between -50mV and -80mV, and the neurons identified through laser illumination only fire ~10-30% of the time. Further, by ramping laser intensity, there were a greater number of spike and higher spike fidelity. Taken together, I interpret this to indicate that MSNs have low activity, and through our 1mW, constant stimulation, we are rarely forcing action potentials unless the neuron is already close to firing. As power is increased, we induce

firing in a less "natural" pattern, though it may facilitate increase rates of self-stimulation. This may mirror the psychological consequences of experimenter-heightened stimulation in electrode-based studies described above.

An additional finding by Kreitzer and colleagues (2013) is that although neurons are generally depolarized following ChR2 stimulation, several are also inhibited. Excited or inhibited neurons have slightly different latencies to shifting potentials; neurons depolarize more quickly from the onset of laser illumination than the rate at which inhibited neurons become hyperpolarized. By 400ms from laser-stimulation, excited and inhibited neurons reach roughly equal population numbers. It may be that in studies using brief pulses at high intensities, the "inhibition" population of neurons is not permitted a voice to speak. In our use of 1s, constant stimulation, we may be allowing these neurons to influence behavior in ways not induced by trains low in pulse duration.

I interpret the meticulous analysis of the Kreitzer group to mean that with a low and constant stimulation, neurons fire due to an intrinsically depolarized state which is further potentiated by ChR2 depolarization. There appear to be multiple neuron response phenotypes to laser illumination. Some are excited, others are inhibited, and these occur at various time scales from 15ms-100ms+ after laser illumination, and to the extent that we are illuminating ChR2 expressing neurons we may be quieting other local neurons. In the context of D1 ChR2 stimulation producing virtually no differences between constant and 25Hz stimulation and D2 ChR2 stimulation producing higher variation between constant and 25 Hz (though, not statistically different), 1mW constant illumination may reflect endogenously relevant neuron activity, but this may not necessarily translate to the highest degree of motivation. Though not a novel concept in behavioral neuroscience, to the extent that we wish to understand the

psychological function of neural systems, it may be most prudent to encourage endogenous activity rather than merely impose states that induce robust shifts in behavior. Denoting a manipulation as "optimum" may depend upon the objective of the experimenter, and a robust behavioral response may confound identification of what a system can do with what it is adapted to do. Though, at least in our hands, 25Hz stimulations can produce the similar patterns of behavior to constant stimulation that fact does not in itself prove the induction of identical psychological state, which needs to be determined through converging evidence of additional tests. The same caution may be applied to low vs high intensity stimulation. Utilizing physiological feedback and tuning of artificial stimulation to promote physiologically relevant shifts in activity serves as a safeguard against incorrect or exaggerated interpretation of function.

D1/D2 neuron Schematics for Reward and Avoidance: Dorsal Striatum vs Nucleus Accumbens

Much of the hypothesized D1-positive vs D2-negative roles stems from original proposed organizations of basal ganglia (Albin et al., 1989) and supposed similarities in projection patterns and cell types between dorsal and ventral striatal neurons (Humphries and Prescott, 2010). While both dorsal striatum and NAc are predominantly comprised of GABAergic MSNs, which either express D1 or D2-dopamine receptors, D1-containing MSNs are the only population that project back to midbrain regions, which thereby encourage behavior, whereas D2-MSNs project first to pallidal regions and encode "stop" or avoidant signals. However, the NAc is not so absolute in its segregation of D1 vs D2 neurons. As described in earlier chapters, an explanation for why excitation of D2-expressing neurons in NAc might contribute to appetitive motivation similarly to D1 neurons is the anatomical overlap in their output projections. In dorsal striatum D1 and D2 receptors, only 5% are co-expressed on the same neuron, whereas up to 30% express both

receptors (Bertran-Gonzalez et al., 2008; Matemales et al., 2008; Perrault et al., 2011). As discussed in Chapter 2, NAc D2 MSNs and D1 MSNs both often send 'indirect' output projections targets to nearly the same sites in ventral pallidum and lateral hypothalamus (Humphries and Prescott, 2010; Kupchik et al., 2015; O'Connor et al., 2015). Work by Kupchik et al. (2015) showed that both D2 MSN axons and D1 MSN axons from NAc may even make synapses on the same individual neuron in VP. Further, we observed here that D1 stimulation and D2 stimulation in NAc produced quite similar patterns of functional connectivity, as reflected by an 85% overlapping recruitment of Fos activation in limbic structures, including similar levels in VP, LH, and VTA. Freeze et al. (2013) showed that D2 neuron stimulation in dorsal striatum enhances Fos expression in SNr, but D1 stimulation produces no differences from EYFP controls highlighting downstream differences from NAc. Thus, functional overlap in recruited circuitry may explain why D1 ChR2 and D2 ChR2 stimulations in NAc both produced positive motivated behavior in the spout-touch task here.

Kupchik et al. (2015) have argued against a traditional "direct" and "indirect" designation for the NAc. In this study, they show that NAc core D1 or D2-MSNs projecting to the VP have second order projections that do not fit with previous conceptions: 1) Nearly 50% of VP neurons receive direct input from D1 MSNs vs 83% from D2 MSNs. 2) while only D1 MSNs directly project to midbrain, about 42% of second order neurons receiving input from NAc->VP neurons also project to midbrain, which in turn project to midbrain regions (more akin to what would be expected of traditional D2 neurons). 3) D2 MSNs which project to VP have second order connection to thalamus. Kupchik and Kalivas (2015) argue that due to D1 neurons projecting first to pallidum then to midbrain and D2 neurons projecting outside of basal ganglia, the direct-indirect labels are inappropriate for ventral striatum.

Beyond stimulating types of neurons, with techniques such as optogenetic or chemogenetic manipulations, projection-specific roles can now be assessed. The location where subgroups of neurons communicate may further parse psychological function. One example comes from O'Connor and colleagues (2015) who used a systematic approach to identify which neuron subtype exerts control over NAc->LH-based feeding. Using retrograde identification of NAc->LH (peduncular LH) they determined that ~90% of LH projecting neurons contained D1 receptors, whereas only 5% of NAc shell->LH neurons contained D2 receptors. Upon ChR2 stimulation of NAc cell bodies, 56% of LH neurons showed inhibitions vs 17% upon D2 stimulation. During consumption of a fatty solution, D1-identified neurons in the NAc decreased activity, whereas D2 neurons showed no general trend during consumption. Upon somatic optogenetic inhibition, D1 mice decreased consumption of a fatty solution. Interestingly, activation of D1 NAc->LH terminals attenuated intake, whereas D2 activation had no effect. An additional study looking at neuron/projection specific features, Creed and colleagues (2016) found that ~93% of VP neurons responded to D1-MSN stimulation vs 73% for D2, and established that cocaine treatment potentiated transmission for D1 synapses, and depressed transmission at D2 synapses. The authors argue that this D1 connection is involved in behavioral sensitization to drug reward, whereas D2 is involved in "cocaine induced negative affective state". At a minimum, these findings challenge long-held positions on absolute neuron or projection roles, and demonstrate importance of point-to-point analyses. As an extension, it would be interesting to know if D1->VP, LH, or VTA stimulation or inhibition could support self-stimulation in the absence of external rewards, similar to our tasks.

Other Demonstrations of Both D1 and D2 MSN Reward

Other groups have also found positive roles for both striatal D1 and D2 neurons. Vicente and colleagues (2016) have recently shown that excitation of D2 neurons in ventrolateral striatum produces self-stimulation, similar to that observed with D1 MSN stimulation. Further, inhibition (Natsubori et al., 2017) or destruction of D2 neurons (Tsutsui-Kimura et al., 2017) impaired goal-directed behavior. Optogenetic activation of either D1 or D2 neurons has also been shown to increase willingness to work for food, and inhibition of D2 neurons decreases lever presses and break point for food (Soares-Cunha et al., 2016). Furthermore, the incongruent findings of my study above and that of D2 attenuation of drug reward (Lobo et al., 2010; Koo et al., 2014a) may be in part due to the activation of distant populations, as distinct drug vs natural rewards have been shown to activate different populations of NAc cells (Carelli et al., 2000).

Thus, it may be that through selective-targeting of these neuron subtypes and projections that we observe alteration in different psychological elements which do not necessarily fit with strict roles for neuron classes. Taken together, these studies and my findings demonstrate that with our advancements in ability to parse cell types and projections, we may find that 1) where we manipulate, 2) with which class of neuron, and 3) how we manipulate the neuron may provide new insights into mechanisms by which the brain does what it wants to do.

Inhibition as a Mechanism of Motivation

In Chapter 3, I tested whether *neuronal inhibition* of NAc neuron directly produces motivation or whether receptor-based mechanisms are responsible for inducing motivated behavior following glutamate-blockade. Over the last 20 years our lab has been able to induce eating and defensive behaviors via microinfusion of the AMPA-glutamate antagonist DNQX or activation of inhibitory GABA receptors via muscimol into the NAc shell (Reynolds and

Berridge, 2001, 2002, 2003, 2008; Richard and Berridge, 2011b; Richard et al., 2013). Hyperpolarization of NAc neurons had long been thought to be the primary mechanism by which intense food intake and defensive behaviors are produced through these microinjections, though there had yet to be a direct test of this hypothesis. I combated local DNQX-microinjections with localized optogenetic *depolarization* at the site of microinjection to determine whether cellular *inhibition* was *necessary* to produce these intensely motivated behaviors. I tested 4 conditions: 1) no laser+vehicle, 2) laser+vehicle, 3) DNQX alone, and 4) DNQX+laser stimulation.

I found no effect of general neuronal *excitation* on ingestive behavior, and under laser stimulation, food intake and time spent eating was nearly equal. Additionally, we replicated previous findings of DNQX-induced food intake, which enhanced both time eating and food intake by approximately 300% each. Finally, we found that laser depolarization at DNQX-injection sites lowered eating rates by approximately 50%, demonstrating that NAc *inhibition* is *necessary* for DNQX-induced motivation. Moreover, the effect of ChR2 stimulation was only found to decrease intake induced by glutamate blockade when fiber optic probes were within 1mm of microinjector tips, demonstrating that local depolarization is necessary to combat local microinjections within the NAc and that general stimulation of NAc is insufficient to nullify DNQX-induced motivation.

In Chapter 4, I tested whether direct hyperpolarization was *sufficient* to produce enhancements of food intake, as findings from Chapter 3 indicated that hyperpolarization was a necessary element of glutamate-blockade induced eating. Here, I found that halorhodopsin inhibition of the NAc shell is sufficient to produce increases in ingestive behaviors. Additionally, I found that halorhodopsin hyperpolarization can produce enhancement of food intake, though this effect is more moderate than that induced by DNQX. In a case study-style analysis, I found

that NAc inhibition does not entirely synchronize with NAc inhibition, though often producing enhancements in food intake. In comparing across time points on days in which animals receive laser inhibition, I found that laser-inhibition produces general, rather than just time locked increases. However, in one rat I found that approximately 80% of eating occurred under the presence of laser inhibition. Further, this individual ate at levels as high as animals under DNQX microinjections reaching over 9 grams within an hour. This demonstrates that intense inhibition-bound eating within the NAc is possible. These findings give suggest that inhibition is both a *necessary* and *sufficient* mechanism of NA-mediated motivation.

What is NAc inhibition doing?

Beyond modulation of food intake, NAc shell inactivation enhances instrumental responding, even in the absence of no reward and enhances lever pressing for non-rewarded levers, whereas inactivation of the NAc core reduces responding for the presentation of reward-cue (Di Ciano et al., 2008; Floresco et al., 2008). Suppression of shell activity during presentation of non-reward paired cues or in times when rewards are not available enhances both Pavlovian approach and lever pressing (Blaiss and Janak, 2009; Ambroggi et al., 2011). NAc lesions interfere with learning about which stimuli are important or not important (Weiner and Feldon, 1997; Gal et al., 2005), and lesions/chemical inactivation or removal of hippocampal inputs causes rats to return to unrewarded locations (Floresco et al., 1996, 1997; Floresco et al., 1999)

These findings indicate that the increases in food intake and fear may be representative of inappropriate, non-specific motivated responses to get away from or to remove the experimenter (defense) or to go toward and consume food (ingestion). That is, sated rats eat more and tame

rats show fear. In my hands, though rats are fed *ad libitum*, we see enhancement of food intake though food should not in theory be particularly salient or rewarding.

It could be questioned whether increases in the eating observed in inhibitions are truly appetitive, and mediated by incentive salience or 'wanting', at least when eating is evoked by microinjections of GABA agonist or glutamate antagonist in NAc shell. Alternatively, increased food intake could be viewed as pure motor activity or as due to an aversive state or drive (Solomon and Corbit, 1974; Koob, 1996). Do behaviors generated by NAc microinjections match the profile of incentive motivation? Incentive salience is posited to have signature features, when attributed to unconditioned reward stimuli such as food, or to related Pavlovian conditioned stimuli or cues (Robinson and Berridge, 1993). Many brain manipulations that increase incentive salience, such as dopamine or opioid stimulations in NAc, amygdala or neostriatum, amplify 'wanting' for both unconditioned rewards and for their learned CSs (Wyvell and Berridge, 2000; Mahler and Berridge, 2009; Smith et al., 2011; DiFeliceantonio et al., 2012; Pecina and Berridge, 2013). In brief, a Pavlovian conditioned stimulus is said to be imbued with incentive salience if it meets the following conditions 1) it is attractive or acts as a "motivational magnet" (e.g., elicits approach such as sign-tracking or goal-tracking) (DiFeliceantonio and Berridge, 2012; Robinson and Berridge, 2013; Yager and Robinson, 2013; Yager et al., 2014), 2) is 'wanted' itself, in the sense that an individual will work for it (typically measured in instrumental conditioned reinforcement tests as operant responding for CS+ alone), and 3) spurs pulses of higher motivation to obtain its unconditioned reward (typically measured in Pavlovian-Instrumental Transfer [PIT] tests, or in priming tests).

Yet, while GABA agonist and glutamate antagonist microinjections in NAc shell powerfully increase motivated behaviors toward unconditioned stimuli (e.g., sight and smell of

chow pellet; sight and touch of approaching human hand; sight of glittering light or external movement), those amino acid manipulations often fail to enhance learned appetitive motivation toward Pavlovian cues. For example, Kelley and colleagues reported that muscimol microinjections into the NAc shell failed to increase instrumental acquisition or breakpoint effort to earn food on a lever pressing task (Zhang et al., 2003; Hanlon et al., 2004). Similarly, muscimol microinjections in NAc fail to increase cue-triggered 'wanting' on a PIT task (Corbit and Balleine, 2011). These failures can be contrasted to opioid or dopamine manipulations in NAc shell, both of which positively enhance learned appetitive motivations (Pecina and Berridge, 2013). One reason why opioid or dopamine stimulation in NAc may be better able to enhance learned appetitive performance is that opioid/dopamine signals act as neuromodulators to alter complex endogenous signals that convey information about learned external stimuli and associated representations. By comparison, GABA and glutamate amino acid neurotransmitters often produce the signals themselves: definitively hyperpolarizing or depolarizing NAc itself. Therefore, drugs that act on GABA or glutamate receptors may actually disrupt endogenous signals (i.e., by either preventing or mimicking those signals), rather than amplifying endogenous signals, as opioid or dopamine agonists may. Learned Pavlovian cues may be especially vulnerable to signal disruption, since learning may recruit highly complex neurobiological signaling in brain circuits. By comparison, signals conveying the sight and smell of actual food as unconditioned stimuli may be more robust, and so resist disruption after NAc GABA or glutamate microinjections. This may be one reason why muscimol and DNQX microinjections can increase appetitive/defensive behavior elicited by unconditioned stimuli, yet not simultaneously increase related motivated behaviors elicited by learned cues. Still, the difference is not absolutely categorical: there are some reports that muscimol or DNQX microinjections in

NAc can sometimes succeed in enhancing learned behaviors for food reward, as well as unconditioned consumption. For example, Wirtshafter and Stratford reported that muscimol microinjections in NAc enhance responding for sucrose reward on an FR1 instrumental schedule (Wirtshafter and Stratford, 2010; Stratford and Wirtshafter, 2012), similar to amphetamine microinjections. Furthermore, muscimol or DNQX microinjections in rostral NAc sites have been shown to establish appetitive conditioned place preferences for an associated location (Reynolds and Berridge, 2002, 2003), similar to dopamine and opioid agonists (Liao et al., 2000; Castro and Berridge, 2014). Conversely, DNQX and muscimol microinjections into caudal NAc sites have been found to establish conditioned place avoidances (Reynolds and Berridge, 2002, 2003). Thus, while amino acid transmitter manipulations in NAc do not necessarily bear all the signature features of incentive salience, there are reasons to conclude that their incentive motivation effects overlap with some features of 'wanting'.

Nucleus Accumbens Inhibition…and Excitation?

The facts that NAc inhibition is both *necessary* and *sufficient* to produce intense food intake in the studies above is in line with the popular theory that hyperpolarization of MSNs in the NAc is *the* primary mechanism for generating appetitive motivation (Carlezon and Wise, 1996; Cheer et al., 2005; Roitman et al., 2005; Taha and Fields, 2006; Meredith et al., 2008; Roitman et al., 2008; Wheeler et al., 2008; Carlezon and Thomas, 2009; Krause et al., 2010). The inhibition of NAc projection neurons is viewed by this hypothesis to release downstream neurons in target structures from chronic GABAergic suppression, and consequently disinhibit those target neurons into states of excitation. This hypothesis is supported by findings that neural excitations in downstream targets, such as VP, LH, or VTA occur during reward events (Ljungberg et al.,

1991; Baldo et al., 2004; Stratford, 2005; Bromberg-Martin and Hikosaka, 2009; Tindell et al., 2009; Smith et al., 2011) For instance, muscimol inhibition of LH decreases food intake produced by DNQX infusion, indicating a requirement of LH activity in order for selective enhancement (Maldonado-Irizarry et al., 1995). Furthermore, the NAc inhibition hypothesis fits the desire-dread 'keyboard' effects of inhibitory drug microinjections, such as muscimol (a GABA agonist which should hyperpolarize NAc neurons) or DNQX (a glutamate AMPA antagonist which should induce relative NAc inhibition by preventing glutamatergic depolarization). It also has been suggested to apply to other drugs such as opioid agonists, on the presumption that those drugs have generally inhibitory effects (Kelley et al., 2005; Baldo and Kelley, 2007; Carlezon and Thomas, 2009).

Further support comes from electrophysiological reports which show that NAc neurons are proportionally more likely to show inhibitions of firing evoked by drug or sweet rewards (Peoples and West, 1996; Chang et al., 1997; Janak et al., 1999; Nicola et al., 2004a; Roitman et al., 2005; Roitman et al., 2010). Conversely, aversive tastes of bitter quinine evoke excitatory increases in firing (Roitman et al., 2005). Additionally, NAc neurons switch from reductions in firing to increases in response to a sweet taste that has become disgusting following acquisition of a Pavlovian taste aversion, and neuronal inhibition to the taste of food is augmented by physiological hunger that makes the taste more rewarding (Hollander et al., 2002; Wheeler et al., 2008; Roitman et al., 2010). Similarly, physiological states of salt depletion cause the normally aversive taste of hypertonic NaCl to become palatable, switching NAc neuronal responses from excitation to inhibition. Furthermore, thirst states are also seen to augment the inhibition of firing to the taste of water (Hollander et al., 2002; Loriaux et al., 2011). Further, pauses in firing of NAc neurons are important for initiation of sucrose consumption, and microstimulation at the

same sites actually suppresses consumption (Taha and Fields, 2006; Krause et al., 2010). In Pavlovian port-approach, 50% of NAc recorded neurons showed long lasting inhibition that corresponded to the onset of the cue and lasting as long as animals were in the reward port (Wan and Peoples, 2006).

Yet, beyond this evidence for NAc neuronal inhibition in reward, other evidence exists that rather paradoxically points toward an opposite conclusion: NAc neuronal *excitation* also may mediate motivation and reward. For example, electrophysiological studies by Roitman, Carelli, and colleagues reported that approximately 30% of NAc core and shell neurons increased in firing in response to sweet rewards (Roitman et al., 2005; Wheeler et al., 2008; Roitman et al., 2010). Taha and Fields (2005) reported that nearly 75% of shell and core neurons in NAc showed increases in firing elicited by sucrose rewards, with highest firing to the most concentrated sucrose solution. Additionally, several other electrophysiological studies report that approximately 30% to 50% of NAc shell and core neurons increase firing during anticipation or during instrumental actions aimed at obtaining food, water or cocaine rewards (Carelli, 2000; Carelli et al., 2000; Hollander et al., 2002; Nicola et al., 2004b).

A second line of evidence for NAc excitation in reward comes from several decades of studies on NAc electrode self-stimulation in rats. That is, rats will work to activate depolarizing electrodes in NAc sites, implying that excitation of some NAc neurons is sufficient as a reward (Rolls, 1971; Phillips and Fibiger, 1978; Mogenson et al., 1979; Van Ree and Otte, 1980; Phillips, 1984). Similarly, human deep brain self-stimulation has been reported for patients who have had electrode sites that likely included NAc (Rolls, 1971; Heath, 1972; Phillips, 1984; Heath, 1996). However, the exact effects of electrodes on nearby neurons is admittedly complex,

and has been suggested to involve neuronal disruption as well as neuronal stimulation (Ranck, 1975).

My optogenetic *depolarization* findings and that of others described above also gives evidence that neuronal excitation as well as neuronal inhibition can enhance motivation for drug reward (Lobo et al., 2010; Koo et al., 2014a) or food reward (Sohares-Cehuna 2016). Beyond direct excitation of intrinsic neurons of NAc, a final line of support for NAc excitation in reward is evidence that there are reward effects of stimulating excitatory glutamatergic inputs to NAc, especially from prefrontal cortex, (Britt et al., 2012) basolateral amygdala, and hippocampus (Will et al., 2004; Ambroggi et al., 2008; Britt et al., 2012). For example, Ambroggi and colleagues (2008) reported that glutamatergic inputs from the BLA to NAc were required for cue-triggered seeking of sucrose reward, but not reward consumption *per se*. Others have reported that optogenetic excitation of glutamatergic projections from prefrontal cortex, BLA, or ventral hippocampus to NAc produces self-stimulation conditioned place preference effects (Stuber et al., 2011; Britt et al., 2012). These observations suggest that glutamate release from those structures excites NAc neurons to contribute to reward processes.

Yet, experimenter-directed excitation is not always psychologically positive. Photo activation of BLA terminals has been shown to decrease licking for a sucrose solution (Prado et al., 2016). Interestingly, Prado and colleagues found that stimulation of PFC, BLA, or thalamic terminals in the NAc shell actually increased appetitive and consummatory behavior in mice licking when paired with licking during non-stimulation periods, but suppressed licking during the stimulation period itself. Further, the electrical stimulation of BLA terminals in the NAc shell has been shown to 1) decrease approach behavior and interrupted sucrose licking following electrical stimulation of the NAc shell (Krause et al., 2010). Additionally, in a recent study,

selective BLA terminal activation as found to decrease approach behavior, suppressed conditioned responding, and decreased intake for ethanol and food consumption (Millan et al., 2017). As such, there is clearly a complicated dynamic even among common excitatory mechanisms.

Can excitation and inhibition both produce enhancement of motivation?

Extrinsic Excitability of the NAc

In a comprehensive review of cortico-striatal literature, Floresco (2015) describes how NAc MSNs show low-general excitability, that 95% of all neurons within the NAc show low excitability and GABAergic, and that networks of cells require input from external regions as they do not have endogenous mechanisms which generate spontaneous firing (Uchimura et al., 1989; Pennartz et al., 1994). Thus, changes in NAc activity that lead to shifts in behavior are likely due to increasing or decreasing excitatory glutamate input. This is consistent with the profile of low MSN excitability outlined by the Kreitzer group described above, which suggests that it takes relatively strong external depolarizations to turn on MSNs.

The NAc receives inputs from cortical and allocortical regions, such as the hippocampus, basolateral amygdala, and prefrontal cortical areas (Humphries and Prescott, 2010). It is thought each of these inputs conveys different forms of information, which in turn guide the activity of the NAc to produce a motivated response. The hippocampus is thought to be necessary for spatial navigation and stimulus discrimination, relation, and novelty (Floresco et al., 1997; Ito et al., 2008; Mannella et al., 2013). BLA inputs are thought to play a role in stimulus valuation and associative learning (Shiflett and Balleine, 2010; Fernando et al., 2013). Prefrontal inputs are thought to be important for situations requiring focused attention and updating expectations

(Christakou et al., 2004). Further, corticolimbic inputs often converge upon the same cells within the NAc, and are thought to interact with each other both outside and within the NAc to influence motivated behavior (O'Donnell and Grace, 1994; Floresco et al., 2001; Britt et al., 2012).

Feed Forward Inhibition, Lateral Inhibition, and Glutamate

How can we get a holistic picture of excitation and inhibition act as dual mechanisms of motivation? One explanation is that excitatory inputs to the NAc, such as BLA glutamate, may actually be turning on inhibitory neurons within the NAc, which cause general inhibitory signals. For instance, it has been demonstrated that fast spiking interneuron (FSI) activation has striking effects on MSN excitability. When activating the same branch of the BLA inputs, FSI respond more quickly with greater amplitude, which then quiets adjacent MSNs. It may be that this serves to create a higher signal to noise ratio, such that any MSNs not inhibited have a greater voice, whereas others now have a much harder time becoming excited (Yu et al., 2017). Further, following long term potentiation of FSI and subsequent inhibition of surrounding neurons cocaine self-administration was enhanced. By contrast, Sun and colleagues (2014) reported that excitation of MSN and inhibition of FSIs in NAc produced positive reward effects of nicotine, whereas inhibition of MSN and excitation of FSIs produced negative avoidance effects in a place preference/avoidance task. So, the valence of effect may depend upon the area or specific FSIs of interest.

MSNs are also interconnected by local recurrent collateral synapses though MSN-MSN inhibition is much weaker than that of FSI-MSN (Wright et al., 2017), and findings in the last decade have shown some order to these connections. Taverna and colleagues (2007) found in

slice characterization that both D1 and D2 MSNs contained about 26% and 36% unidirectional synapses, respectively, but that D2 MSNs more commonly formed synapses on D1 MSNs (27%) rather than D1->D2 connections (6%). Further, D1 MSN connections tended to be weaker due to lower GABA receptor expression. Dobbs and colleagues (2016) gave some evidence that selective perturbation of D2 MSNs potently inhibits D1 MSNs and, and D2 *receptor* activation can actually disinhibit D1 MSNs, presumably by quieting D2 MSNs. By quieting D2 neurons, cocaine induced locomotion was observed, and D2 chemogenetic excitation returned D2 MSN inhibition and produced decreases in cocaine induced locomotion. It may be the case that these differences in cell type to laterally modulate neighboring neurons goes beyond individual neuron modulation and may influence ensemble groups. This is in line with the aforementioned work by the Kravitz group, showing ChR2 depolarization of MSNs results in activating or inhibiting groups of neurons. Thus, by turning "on", we may be also turning "off".

Neuronal Ensembles and Dynamic Roles for NAc Neurons

Pennartz and colleagues (1994) describe at great lengths the concept of neuronal ensembles in the NAc. Here, they argue that "behaviourally meaningful information in the nucleus accumbens is represented by fine-grained spatiotemporal firing patterns in spiny projection neurons rather than by massive waves of activity uniformly sweeping from Acb to the ventral pallidum and related fields." Pennartz and colleagues proposed that there may be many compartments with specific connections and behavioral functions, in part characterized by neurobiological or hodological analysis, which show dynamic patterns of excitation and inhibition. The fact that there is rarely a uniform response in every local neuron gives some credence to this position. Beyond anatomical ensembles which may be relatively sparse,

O’Donnell and colleagues (1999) discuss that electrophysiological overlap *in vivo* intracellular recordings revealed a much higher proportion of convergence (95%) for the same cortico-accumbens inputs, and suggested that ensembles may be categorized by electrical response rather than merely anatomical features (O'Donnell and Grace, 1995). This higher convergence observed is in part due to the ability of intracellular recordings to detect subthreshold responses that do not elicit action potential discharge and are not capable of observation in large-population extracellular recording arrays.

Using State Modes as a Classification of Ensembles

Accumbens neuronal ensembles, can be identified by coordinated firing patterns or shifts in excited state from a largely hyperpolarized downstate to a plateau “upstate”, which may then go on to be more likely to fire (Pennartz et al., 1994; O'Donnell and Grace, 1995; O'Donnell et al., 1999). In this way, ensembles have thought to be dynamic entities that determine the integration of information arriving into the accumbens, and immediate function of the NAc at any given time.

O’Donnell and colleagues (1999) outlined how NAc neurons show different characteristic activity patterns membrane potentials. Most NAc neurons exhibit a “bistable” membrane potential, with a normally negative resting potential or “down state”, which can shift to slightly depolarized “up states” about 100–1,000 ms in duration and 10–25 mV in amplitude (O'Donnell and Grace, 1995, 1998). It is through mechanisms such as inward rectifier K+ conductance that MSNs exhibit that these plateaus and relative states are maintained (Wilson, 1995). Although excitation via synaptic inputs are essential for the presence of up events, the relatively stable membrane potential and the long duration of these events may indicate that

certain membrane properties may limit the extent of depolarization, whereas others may contribute to its persistence. MSNs (which also show up and down states) have presence of slowly inactivating K+ currents (Gabel and Nisenbaum, 1998) that may limit the extent of depolarizations, such as those constituting the up state (Wilson and Kawaguchi, 1996), while maintaining the membrane potential during these depolarizations just below firing threshold. In addition, striatal and NAc neurons exhibit a slow voltage-dependent Na+ current (Cepeda et al., 1995) and slow Ca2+ conductances (O'Donnell and Grace, 1993; Hernandez-Lopez et al., 1997) that may contribute to the persistence of such lingering depolarizations. Because of the interaction between these forces both slowly driving and limiting depolarizations, up events may take the form of a stable plateau depolarization.

The significance of multimodal state suggests another reason to use low-intensity, constant stimulation. In maintaining the wave-form characteristics of neurons the complexities of signaling in these dynamic neurons are likewise maintained. There are clearly multiple electrical gradients and states, rather than simply "firing" or "not firing", which may also correspond to specific psychological states. The nuances of neuronal signaling may be lost via high-intensity pulsed stimulation may be lost and give inaccurate identification of neuronal roles, perhaps more so than would be expected by simple binary "on" or "off" signals.. Additionally, using intrinsic electrical state to identify and code for functional ensembles may be particularly useful to characterize the dynamic/plastic nature of a biological substrate for psychological plasticity.

As a result of resistance to down or upshifts, any change in membrane potential is effectively attenuated. However, with sufficient converging and synchronous arrival of glutamatergic excitatory inputs, a strong depolarization may occur. Additionally, up events are dependent on synaptic activation of NAc neurons, indicated by the fact that intracellular

recordings *in vitro* yield silent neurons with a very negative and stable membrane potentials, that lie within the range of the down states *in vivo* (Chang and Kitai, 1986; Uchimura et al., 1989; O'Donnell and Grace, 1993).

Could multiple kinds of ensembles have modes for various states? Specific ensembles can be selected by input from different cortical areas, which may provide different types of information. Ensembles may bias direction (e.g., approach or avoidance) or intensity of behaviors via projections to downstream regions (and perhaps particular downstream subunits) which have control over motor behavior. In the case of appetitive vs avoidant motivation, it may be that the ability of inhibition or excitation to generate both general "positive" or "negative" psychological phenotypes represents an interaction of clusters of neurons with flexible function. In my hands, D2 stimulation of the same neurons can produce both motivations. In previous studies from our lab, shifts in environmental settings were to re-tune whether appetitive or defensive behaviors were generated during DNQX-microinjections within the same injection site, and dopamine function upon these distinct behaviors mapped on to the psychological process generated instead of anatomical location (Reynolds and Berridge; Richard and Berridge, 2011). The psychological shift in these neurons suggests that "appetitive" and "defensive" ensembles in accumbens are dynamic and plastic entities that can be reorganized by circuit inputs to coordinate appropriate behavior.

Stronger hyperpolarization of shell via drug manipulation neuronal ensembles might conceivably produce sharper motivational valence gradients, as neurons are generally "muffled" though some neurons are still in an excited state. Further, there is evidence that some neurons retain some ability to function after a hyperpolarizing microinjection, and continue to generate action potential signals, though attenuated. For example, recordings demonstrate that infusion of

GABA directly onto accumbens neurons produces strong hyperpolarization, but does not completely stop all neuronal activity (Kiyatkin and Rebec, 1999). As neurons are still active following micro infusions of muscimol, it may be that broader, large-scale hyperpolarization produced with DNQX or muscimol microinjection increases signal-to-noise ratios by only permitting those with the most input to fire, and this must theoretically come from outside of the NAc. This indicates another mechanism by which a given microcircuit might can produce more than one motivational valence, depending on its intensity of hyperpolarization (or duration or constancy of hyperpolarization) and the states of those around it. One possibility is that a "bath" of hyperpolarization produced by microinjection of DNQX or muscimol exerts roughly even hyperpolarizing effects across its area of diffusion and may reorganize a functional ensemble by allowing only those neurons receiving the strongest external excitation to remain in relative "up states". Neurons in "upstates" have a higher likelihood to fire upon additional external input, increasing a given signal-to-noise ratio and defining the net output of a given ensemble as psychologically positive or negative. According to neural ensemble coding theory, dynamic and changing populations of neurons are organized mainly by glutaminergic input to accumbens shell from cortical areas. When these glutaminergic inputs, or other neurochemical inputs from different structures, are altered by changes in discrete stimuli, environmental contexts, or other types of information, ensemble activity is modulated (Pennartz et al., 1994; O'Donnell and Grace, 1995; O'Donnell, 1999; O'Donnell et al., 1999), and the psychological function of the ensemble is altered. If motivational valence produced by shell microinjection hyperpolarization is a product of such ensemble activity, it could be both affected by topographical location and by modulation of inputs to the shell that reflect factors such as environmental valence, which would

explain the environmental retuning of the rostrocaudal gradient observed in studies of DNQX (Reynolds and Berridge, 2008; Richard and Berridge 2011).

One interpretation of selective firing in the presence of general inhibition may be that the cells that are firing may be producing a minority coding for a set psychological state. While many downstream targets may be generally more excited via disinhibition, the few cells or ensembles left firing may maintain relative inhibition of a few cells or ensembles. This may provide its own code in the form of a "neuronal silhouette" or "punch card" that conveys information even though target neurons are inhibited. If both excitation and inhibition could serve as a particular element of a psychological state it would provide a greater freedom of function in the sense that more configurations are possible for communication, much like Morse code consists of variable lengths of "up states" and "gaps" produced by dashes or dots and the space between hammering a key.

Speculatively, it may be the case that one neuron at one excited state can encode a positively valenced behavior, and that depending on the activity of other neuronal ensembles around it/interacting with it, the same neuron may also produce positively valenced psychological signaling even while inhibited. In determining the role of a neuron, it may be that in addition to receiving excitatory input, the local field of neuronal ensembles serve as a gradient by which both excitatory and inhibitory shifts in electrical potentials convey or gate information. That is, both up states or down states may be psychologically positive or negative depending on the activity of surrounding neighbors, which may or may not share anatomical inputs or outputs. In discussing bistable or multi-modal neurons, I cautiously propose a theoretical system by which the same neuron or group of neurons is capable of psychological plasticity. Additionally,

this also presents an avenue by which excitation and inhibition are both viable mechanisms of motivation in the nucleus accumbens.

Conclusion

In this dissertation, I attempted to provide insight into several mechanisms by which the NAc is able to produce motivation. The NAc is responsible for processing many types of motivations, ranging from appetitive motivation for food, sex, and drugs, to fearful or avoidant motivation. As such, the NAc is likely comprised of several plastic units that can be tuned to meet multiple demands. Further, attempting to attach roles for certain cell types, neurotransmitters, or even states of relative excitation or inhibition may be difficult to do. In the future, the question of "what do these mechanisms do?" will have to be asked with greater specificity as to the nature of the transmitter, the cell type, the projection, and the relative state of neuronal ensembles.

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